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Immunomoddulatory activity of HBV vaccine and FOXp3 gene expression on purified human peripheral blood mononuclear cells treated with MRSA antigens

A Thesis
Submitted to the Council of the College of Medicine, University of Babylon, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in science/medical Microbiology

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من قبل

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بإشراف

الأستاذ الدكتور **الأستاذ الدكتور**

محـمـد عـــبـد كاظـــم الســـــــعدي **قيـــــــصر نعمـــــة مظلـــوم**

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بِسْمِ اللَّـهِ الرَّحْمَـٰنِ الرَّحِيمِ

(وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي
وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا)

صدقَ اللهُ العليُ العظيم

(.سورة الإسراء - الآية 85)

Dedication

To • • •

- *Who will fill the earth justice and equality :* **AL-Imam AL - Mahdi**
- *The SOUL OF my hero martyr father*
- *Greatest blessing and support in life my mother*
- *My flowers: Jana, Mohammed, ALI and Sedra*

I dedicate this work.

Entidhar 2023

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Entethar 2023

Decision of Examination Committee

We are the examiner committee, certify that we have read this thesis entitled (**Immunomoddulatory activity of HBV vaccine and FOXp3 gene expression on purified human peripheral blood mononuclear cells treated with MRSA antigens**) and have examined the student (**Entidhar Ali Al-Araji**) in its content, and that in our opinion; it is accepted as a thesis for degree of **Doctor of Philosophy** in medical Microbiology with **Excellent** estimation.

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In view of the available recommendation, I forward this thesis for debate by the examining committee.

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SUMMARY

This experimental analytical randomized controlled trail (parallel group) study aim to evaluate the immune modulatory activity of Hepatitis B virus vaccine antigen on purified human peripheral blood mononucle cells (PBMCs) treated with microbial antigens .

Blood samples were collected from 75 human apparently healthy males. Each sample was divided into five groups.Groups I was (Roswell Park Memorial Institute) PBMCs ($1*10^6$cells/ml) without the treatment cultured in medium RPMI (Roswell Park Memorial Institute) to serve as a control, and the group II of PBMCs (Peripheral Blood Mononuclear Cells) stimulated with somatic Methicillin Resistance *Staphylococcus aureus* (MRSA) antigen only , group III of PBMCs(Peripheral Blood Mononuclear Cells) stimulated with Hepatitis B virus vaccine antigen only , group IV of PBMCs (Peripheral Blood Mononuclear Cells) pretreated with Hepatitis B virus vaccine (HBV) for 48 hr , then with killed somatic MRSA Ag and group V of PBMCs (Peripheral Blood Mononuclear Cells) stimulated with killed somatic MRSA antigen plus Hepatitis B virus vaccine(HBV), PBMCs (Peripheral Blood Mononuclear Cells) were isolated by density gradient medium and all the groups were cultured with RPMI1640 medium under special condition at 37ºC with 5%CO_2 for 48hrs.

After 48 hrs. PBMCs(Peripheral Blood Mononuclear Cells) were collected from all groups to detect the PBMCs(Peripheral Blood Mononuclear Cells) viability by trypan blue dye and to estimate the concentration of cytokine profile Interferon gamma (IFN-γ), interleukin-4 (IL-4) and interleukin-5 (IL-5) by enzyme linked immunosorbent assay technique.Additionally, the genetic parameter was done to detect the level of Forkhead box P3 Protein (Foxp3) mRNA gene expression by real time PCR.

The results indicated a significant increase in the mean value of Interferon-gamma in group II 125.9995 pg/ml, group III is 133.6504 pg/ml, group IV 132.0271 pg/ml and group V 96.087 pg/ml as compared control group PBMCs (Peripheral Blood Mononuclear Cells) ($1*10^6$cells/ml) without the treatment cultured in medium RPMI Roswell Park Memorial Institute 49.6203 pg/ml. The differences between them were significant at $p<0.05$. The results also pointed to a significant in the concentration of this cytokine in group II and group IV as compared with (groupIII) (125.9995 pg/ml, 132.0271 pg/ml,133.6504 pg/ml, respectively).

Interleukin-4 concentration results revealed a significant increase in all groups (group V, group IV, group III and group II (8.1033 pg/ml, 7.735 p g/ml , 7.6809 p g/ml, 7.6170 p g/ml respectively) as compared with control group PBMCs (Peripheral Blood Mononuclear Cells) ($1*10^6$cells/ml) without treatment cultured in medium RPMI 1640 (Roswell Park Memorial Institute) 3.6568 pg/Ml in addition to a significantly in the mean value of Interleukin-4-4 levels in group IV, group III and group II with nearly the same effects as compared with group V 8.1033 pg/ml at $p< 0.05$.

Also the results pointed to a significant elevation in Interleukin-5 concentration in all group IV, III, II,V (10.3023 pg/ml, 9.9415 pg/ml, 9.8520pg/ml, 9.6087 pg/ml respectively) in comparison to control group PBMCs (Peripheral Blood Mononuclear Cells) ($1*10^6$cells/ml) without treatment components cultured in medium RPMI 1640 (Roswell Park Memorial Institute) 4.0469 pg/ml in addition to a significantly in the mean value of Interleukin-5 levels in group III and group II and group V with nearly the same effects as compared with groupIV 10.3023 pg/ml at $p < 0.05$.

Furthermore, the genetic parameter results of Forkhead box P3 Protein(Foxp3) gene expression , this study revealed a significant increasing in the (Forkhead box

P3 Protein) Foxp3 mRNA gene expression levels in V group, group IV, group III and group II(6.3178, 4.3950, 2.2468, 1.7980 respectively) as compared to control group PBMCs (Peripheral Blood Mononuclear Cells) ($1*10^6$cells/ml) without treatment cultured in medium RBMI 1640 (Roswell Park Memorial Institute) 0.4871. In addition the results pointed a significantly high elevation in Forkhead box P3 Protein(Foxp3) mRNA gene expression levels in groups V 6.3178 and IV 4.3950, effect as compared to III 2.2468 and group II 1.7980 at $p < 0.05$. Moreover, significant increasing in group V as compared to group IV group.

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List of Abbreviations	
Abbreviations	**Full Name**
Ag	Antigen
AMPs	Anti-microbial Peptides
BHI	Brain Heart Infusion
CDC	Center for Disease Control
CMI	Cell Mediated Immunity
CTC	Cytotoxic T-Cell
DCs	Dentric Cells
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
DW	Distilled Water
ELISA	Enzyme Linked Immune Sorbent Assay
FOXP3	Forkhead box p3
HRP	Horse Radish Peroxidase
IFN-γ	Interferon Gamma
IL-4	Interleukin-4
IL-5	Interleukin -5
IL-23	Interleukin-23
LPS	Lipopolysaccharide
MAb	Monoclonal Antibody
MAC	Membrane Attach Complex
MAMPs	Microbial associated molecular patterns
MDR	Multi-drug resistance
Mg/dl	Milligram Per Deciliter

MHC	Major Histocompatibility
NK	Natural Killer cell
NKT	Natural Killer T-Cell
OD	Optical Density
OMPs	Outer membrane proteins
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffer Solution
PG	Peptidoglycan
PRRs	Pathogen Recognition Receptors
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
SIgA	Secretory Immunoglobulin A
STAT-4	Signal Transducer and Activator of Transcription
T3SS	Type three secretion system
TCR	T-Cell Receptors
Th-1	T-helper 1
TLCs	Total Leucocytes Count
TLRs	Toll Like Receptors
TNF	Tumor necrosis factor
TNF-α	Tumor Necrosis Factor Alpha
T-reg	T- Regulator
WHO	World Health Organization

ألخلاصــة

تهدف هذه الدراسة التجريبية التحليلية المعشاة ذات الشواهد (المجموعة الموازية) إلى تقييم النشاط التعديلي المناعي لمستضد لقاح فيروس التهاب الكبد B على خلايا الدم الطرفية البشرية النقية (PBMCs) المعالجة بالمستضدات الميكروبية.

تم جمع عينات الدم من 75 من الذكور الذين يبدو أنهم أصحاء. تم تقسيم كل عينة إلى خمس مجموعات. كانت المجموعات الأولى (معهد روزويل بارك التذكاري) PBMCs (1 * 106 خلايا / مل) بدون علاج مزروع في RPMI المتوسط (معهد روزويل بارك التذكاري) ليكون بمثابة عنصر تحكم ، والمجموعة الثانية من PBMCs (خلايا الدم المحيطية أحادية النواة) محفزة بمستضد المكورات العنقودية الذهبية الجسدية المقاومة للميثيسيلين (MRSA) فقط ، المجموعة الثالثة منPBMCs (خلايا الدم أحادية النواة المحيطية) المحفزة بمستضد لقاح فيروس التهاب الكبد B فقط ، المجموعة الرابعة من PBMCs (خلايا الدم أحادية النواة المحيطية) المعالجة مسبقا بلقاح فيروس التهاب الكبد (HBV) B لمدة 48 ساعة ، ثم مع قتل MRSA Ag الجسدي والمجموعة الخامسة من PBMCs (خلايا الدم أحادية النواة المحيطية) المحفزة بمستضد MRSA الجسدي المقتول بالإضافة إلى لقاح فيروس التهاب الكبد (HBV) B ، تم عزل PBMCs (خلايا الدم المحيطية أحادية النواة) بواسطة وسط تدرج الكثافة وتم استزراع جميع المجموعات بوسط RPMI1640 في ظل ظروف خاصة عند 37 درجة مئوية مع 5٪ CO2 لمدة 48 ساعة.

بعد 48 ساعة. تم جمع PBMCs (خلايا الدم أحادية النواة المحيطية) من جميع المجموعات للكشف عن صلاحية PBMCs (خلايا الدم أحادية النواة المحيطية) بواسطة صبغة التريبان الزرقاء ولتقدير تركيز ملف تعريف السيتوكين إنترفيرون جاما (IFN-γ) ، إنترلوكين -4 (IL-4) وإنترلوكين -5 (IL-5) بواسطة تقنية مقايسة الممتز المناعي المرتبط بالإنزيم. بالإضافة إلى ذلك ، تم إجراء المعلمة الجينية للكشف عن مستوى التعبير الجيني لبروتين mRNA Forkhead box P3 (Foxp3) بواسطة تفاعل البوليميراز المتسلسل في الوقت الفعلي.

أشارت النتائج إلى زيادة معنوية في متوسط قيمة إنترفيرون جاما في المجموعة الثانية 125.9995 بيكوغرام/مل، والمجموعة الثالثة 133.6504 بيكوغرام/مل، والمجموعة الرابعة 132.0271 بيكوغرام/مل، والمجموعة الخامسة 96.087 بيكوغرام/مل مقارنة بمجموعة التحكم PBMCs (خلايا الدم المحيطية أحادية النواة) (1*106 خلايا/مل) بدون العلاج المزروع في متوسط RPMI معهد روزويل بارك

التذكاري 49.6203 بيكوغرام/مل. كانت الاختلافات بينهما كبيرة عند $p<0.05$. كما أشارت النتائج إلى وجود معنوية في تركيز هذا السيتوكين في المجموعة الثانية والمجموعة الرابعة مقارنة ب (المجموعة الثالثة) (125.9995 بيكوغرام/مل، 132.0271 بيكوغرام/مل، 133.6504 بيكوغرام/مل، على التوالي).

كشفت نتائج تركيز إنترلوكين -4 عن زيادة معنوية في جميع المجموعات (المجموعة الخامسة والمجموعة الرابعة والمجموعة الثالثة والمجموعة الثانية (8.1033 بيكوغرام / مل ، 7.735 جم / مل ، 7.6809 جم / مل ، 7.6170 جم / مل على التوالي) مقارنة بمجموعة التحكم PBMCs (خلايا الدم المحيطية أحادية النواة) (1 * 106 خلايا / مل) بدون علاج مزروع في متوسط RPMI 1640 (معهد روزويل بارك التذكاري) 3.6568 بيكوغرام / مل بالإضافة إلى قيمة معنوية في متوسط مستويات إنترلوكين -4-4 في المجموعة الرابعة والمجموعة الثالثة والمجموعة الثانية مع نفس التأثيرات تقريبا مقارنة بالمجموعة الخامسة 8.1033 بيكوغرام / مل عند $p< 0.05$.

كما أشارت النتائج إلى ارتفاع معنوي في تركيز إنترلوكين-5 في جميع المجموعات الرابعة، الثالثة، الثانية، الخامسة (10.3023 بيكوغرام/مل، 9.9415 بيكوغرام/مل، 9.8520 بيكوغرام/مل، 9.6087 بيكوغرام/مل على التوالي) مقارنة بمجموعة التحكم PBMCs (خلايا الدم المحيطية أحادية النواة) (1*106 خلايا/مل) بدون مكونات معالجة مستزرعة في متوسط RPMI 1640 (معهد روزويل بارك التذكاري) 4.0469 بيكوغرام/مل بالإضافة إلى معنوية في القيمة المتوسطة لمستويات إنترلوكين-5 في المجموعة الثالثة والمجموعة الثانية والمجموعة الخامسة مع نفس التأثيرات تقريبا مقارنة بالمجموعة الرابعة 10.3023 بيكوغرام / مل عند $p < 0.05$.

علاوة على ذلك ، كشفت نتائج المعلمة الجينية للتعبير الجيني لبروتين Forkhead box P3 (Foxp3) ، عن زيادة معنوية في مستويات التعبير الجيني Foxp3 mRNA (Forkhead box P3 Protein) في المجموعة V والمجموعة الرابعة والمجموعة الثالثة والمجموعة الثانية (6.3178 ، 4.3950 ، 2.2468 ، 1.7980 على التوالي) مقارنة بمجموعة التحكم PBMCs (خلايا الدم المحيطية أحادية النواة) (1 * 106 خلايا / مل) بدون علاج مزروع في وسط RBMI 1640 (معهد روزويل بارك التذكاري) 0.4871. بالإضافة إلى ذلك ، أشارت النتائج إلى ارتفاع معنوي في مستويات التعبير الجيني mRNA لبروتين P3 Forkhead box (Foxp3) في المجموعتين V 6.3178 و IV 4.3950 ، مقارنة ب III 2.2468

والمجموعة 1.7980 II عند 0.05> p. علاوة على ذلك ، زيادة كبيرة في المجموعة الخامسة مقارنة بمجموعة المجموعة الرابعة.

CHAPTER ONE

INTRODUCTION AND LITERATURES REVIEW

CHAPTER TWO

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1. Introduction:

Antibiotics are one of the outstanding discoveries in the medical field in treating infectious diseases caused by pathogenic bacteria. Before the antibiotic discovery era, the lethality and death rate caused by pathogenic microorganisms was high until the accidental rediscovery of penicillin in 1928 by Alexander Fleming (Hutchings *etal.*, 2019).This rediscovery grants the exploration of other types of antibiotics such as sulphonamides, lipopeptides, aminoglycosides, fluoroquinolones, and many more (Nicolaou and Rigol, 2018 ;Hutchings *et al.*, 2019) Antibiotics also allow modern medical technology to exist as it aids in preventing infection in chemotherapy and various surgical wounds.

Although antibiotics give significant advantages in treating diseases caused by pathogenic bacteria, Alexander Fleming warns of the danger of uncontrolled antibiotic usage where resistance can be developed. The warning appeared to be true as *Escherichia coli* started to exhibit antibiotic resistance (AR) towards penicillin in 1940 (Abraham and Chain,1940) Up until this day, antibiotic resistance has been a significant threat in the healthcare system as more bacteria developed resistance towards various classes of antibiotics. It is predicted that, by 2050, AR related death may reach 10 million per year (Dixit *et al*.,2019; Lv *et al.*, 2021).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an antibiotic-resistant type of *S. aureus* that is generally resistant towards beta-lactam antibiotics such as penicillin (methicillin and oxacillin) and cephalosporin (Nandhini *et al*., 2022) Beta-lactam inhibits the bacterial growth by halting the cell wall synthesis process (Pandey and Cascella, 2021). MRSA

generally overcomes the beta-lactam effects by producing beta-lactamase and altering the binding site for cell wall synthesis (Nandhini *et al*.,2022).

The current clinically approved method to treat MRSA infection involves different antibiotic classes such as vancomycin and teicoplanin , These glycopeptide antibiotics act on the bacterial cell wall similar to beta-lactam, but it utilises different target by binding to the peptidoglycan side chain, which prevents peptidoglycan crosslinking (Stogios and Savchenko,2020). However, the newer MRSA strain started to exhibit resistance towards glycopeptide antibiotics, which makes it difficult to treat the infection (Ahmed and Baptiste, 2018). Other types of antibiotics such as mupirocin, clindamycin, fusidic acid, and co-trimoxazole also used a second line option in treating MRSA (Brown *et al*.,2021) . However, these antibiotics can only be prescribed when there is no other alternative available due to the risk of resistance (Brown *et al*.,2021; Montravers, and Eckmann, 2021). Thus, alternatives to treat MRSA without the use of different classes of antibiotics are greatly needed.

The polymorphonuclear neutrophil leukocytes are an essential component of non-specific immunity and are the predominant phagocytic cells present in peripheral blood. Monocytes / macrophages are the other immune system effector cells that protect against microbial infection. Macrophages are phagocytic mononuclear cells derived from peripheral blood monocytes and reside in most tissues. They serve as a bridge between the innate and adaptive immune response by differentiating into cells in order to exert various functions after being stimulated by various stimuli, such as interferon-γ (IFN-γ). Monocytes/macrophages have three essential functions: phagocytosis, presentation of antigens to effective T cells and production of distinct inflammatory mediators (Orsatti *et al*.,2010).

Once bacteria enter the body as an antigen, neutrophils and monocytes/ macrophages can kill them. Phagocytosis is the principal mechanism in the innate immune system for the destruction of microorganisms within phagocytic cells. This complicated process has the following phases: chemotaxis of the phagocytes, adhesion of microorganisms to the phagocyte surface, endocytosis, and microorganism killing. Two pathways within phagocytic cells kill microbes, one is oxygen-dependent (reactive oxygen intermediates (ROI) and the other is oxygen-independent (including secretion of lysosomal enzymes including lysozyme and proteinases)(Tauffenberger and Magistretti, 2021).

Neutrophils are responsible as specialist phagocytes for destroying extracellular microorganisms, whereas monocytes / macrophages destroy pathogens-infected cells, including intracellular bacteria (e.g. Salmonella), parasites and yeasts.

T and B cells are another significant group of mononuclear peripheral blood cells (PBMCs), which play a key role in the adaptive immune response. The ability of these cells to distinguish self from non-self lets them clear exogenous antigens (Wolska *etal*.,2019).

Specific types of antigens bind and stimulate different T cells and/or B cell populations which have different immune functions, However, bacteria will be phagocytized within the macrophages and then recognized by the major histocompatibility complex II (MHC II) and identified as peptide antigen. MHC II binds to T lymphocytes (Li *etal*.,2021).

1.1. Aim of the study*:*

This study aimed to illustrate the immune response of purified human peripheral blood mononuclear cells against MRSA and to study the immunodulatory effect of HBV antigens on this immune response. This aim will be assessed through the following parameters:

1- Extraction of somatic antigen of *MRSA*.

2- Isolation of peripheral blood mononuclear cells(PBMCs) from healthy volunteers.

3- Evaluation of immune responses in PBMCs cell culture for Ag, and control by the following parameters:

A- Measuring the levels of interferon- gamma (IFN-γ), interleukin-4(IL-4) and interleukin-5(IL-5) in PBMC cell culture.

B-Measuring the Foxp3 gene expression level in PBMC cell culture by qRT-PCR.

1.2 Literatures Review:

1.2.1 Methicillin Resistance *Staphylococcus aureus* (MRSA):

The infections of bacteria is one of major causes of death in the world, and S. *aureus* considered a dangerous due to a wide range of these infections such as arthritis, pneumonia, endocarditis and abscesses of organs. The treatment of these infections has become more difficult as a result of multidrug resistant (Hegazi *et al*., 2014). *S. aureus* is Gram-positive, cocci, catalase-positive that happen in irregular "grape-like" clusters, non-spore forming and non-motile. Nearby, 20% of the population is always populated with *S. aureus*, 60% are intermittent transporters, and 20% not ever carry the organism (Foster, 2004). Methicillin Resistance *Staphylococcus aureus* was first informed in 1961 (Jevons *et al*., 1963), and it is a major cause of the infections that show a resistance to completely beta lactam antibiotics and their byproducts (Al-Hassnawi, 2012).

This resistance is due to existing *mec A* gene that encrypts for additional penicillin-binding protein 2a (PBP2a). These bacteria (MRSA) were tested for their sensitivity to cefoxitin (30 µg) that is used recently, where an inhibition zone with diameter (≤ 21mm) considers as an indication of MRSA, specificity and sensitivity of cefoxitin test were more than 90% (Rasheed *et al*., 2014). A good standard for MRSA detection is PCR to detect present of *mec A* gene or its products. In routine microbiological laboratory for screening the MRSA are oxacillin salt screening (OSS) test and cefoxitin disc agar diffusion (Abdulghany and Khairy, 2014), but the later screening considered to be better than OSS test because it is strong inducer of PBP2a (Rasheed *et al*., 2014). Usually β-Lactam antibiotics bind to PBPs in the cell wall, resulting in the disruption the synthesis of the peptidoglycan layer and death of the bacterium, since β-lactam antibiotics cannot bind to PBP2a, synthesis of the

peptidoglycan layer and cell wall are able to continue (Deurenberg *et al*., 2007). Staphylococcus aureus is Gram-positive bacteria with round shape morphology that commonly can be found in the body as a part of its microbiota.

Despite it acting commensally on the human body, it can be opportunistic bacteria since it can cause skin infections and food poisoning. Methicillin-resistant Staphylococcus aureus (MRSA) is an antibiotic-resistant strain of S. aureus that are mainly resistant to beta-lactam antibiotics. MRSA was first identified in 1961 in United Kingdom just a year after methicillin was introduced to treat S. aureus infection (Enright *et al.*,2022).Despite methicillin no longer being used clinically, the term methicillin-resistant is still used to reflect S. aureus resistance towards commercial antibiotics such as beta-lactams antibiotics including oxacillin. According to World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), MRSA has been a big and serious threat on the pathogenic bacteria watch list respectively (Centers for Disease Control and Prevention,2019).According to recent systematic analysis in the Lancet in 2019, MRSA alone caused more than 100,000 deaths (Murray *et al*.2022).

Originally, MRSA are common in the healthcare setting, and this type of MRSA is often dubbed as healthcare-associated or hospital-acquired MRSA (HA-MRSA) (Hibbitts and Leary,2018). The infection can be spread through direct contact with an infected wound or contaminated hands. Untreated infection can cause serious bloodstream infections, surgical site infections, sepsis and pneumonia,Other types of MRSA are community-associated (CA-MRSA) and livestock-associated MRSA (LA-MRSA) (Siddiqui and Koirala,2022).

Beta-lactam antibiotics act on the bacterial cell wall by binding to the penicillin binding protein (PBP), which is responsible for the cross linking of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc)

,this crosslinking will form a cell wall that protects the bacteria from external threats. MurNAc subunits have pentapeptide chains attached to it, typically with a sequence of l-Ala-γ-d-Glu-l-lysine (or -meso-diaminopimelic acid)-d-Ala-d-Ala (Pandey and Cascella,2021).

Beta-lactam antibiotics such as penicillin, cephalosporin, carbapenem and monobactams have a beta-lactam ring which shared similar structural homology to d-Ala-d-Ala of the pentapeptide chain (Kapoor *et al*.,2017; King *et al*.,2017).

The d-Ala-d-Ala substrate is responsible for the PBP binding site for crosslinking, and this similarity causes beta-lactam antibiotics bind to PBP, causing the crosslinking between the glycan stands to be halted (Pandey and Cascella,2021). The binding between beta-lactam and PBP causes the build-up of peptidoglycan precursors which trigger autolytic digestion of old peptidoglycan by hydrolase(King *et al*.,2017) . Without the production of new peptidoglycan, the structural integrity of the cell wall is significantly disrupted and led to cell damage due to high internal osmotic pressure (Pandey and Cascella,2021).

MRSA overcomes this detrimental effect by producing beta-lactamase, an enzyme to break down the antibacterial effect of beta-lactam antibiotics and production of the mecA gene, which changes the penicillin-binding protein (PBP) confirmation. Beta-lactamase is an enzyme produced by bacteria to counteract the effects of beta-lactam antibiotics. This enzyme hydrolyses beta-lactam in the periplasmic space, thus deactivating it before PBP interaction (Dixit *etal*.,2019). Beta-lactamase production in staphylococci is controlled by the repressor BlaI and the sensor protein BlaR1 (Figure 1.1a) (Kapoor *etal.*,2017). The genes encoding beta-

lactamase, the blaZ-blaR1-blaI genes, are repressed by BlaI is from transcribing beta-lactamase when beta-lactam is absent , Once beta-lactam is presented, the transmembrane sensor, BlaR1, covalently binds to it and irreversibly acylated at its active site serine. This will activate the intracellular zinc metalloprotease domain of BlaR1 and cause BlaI that are bound to blaI-blaRI operator to proteolytically cleave and dissociate from its binding site , The dissociation allows blaZ to be upregulated and transcribed beta-lactamase enzyme. The produced beta-lactamase enzyme later hydrolyses beta-lactam antibiotic by hindering it from binding with PBP , Thus, the peptidoglycan synthesis of the bacteria can be initiated as usual(Kırmusaoğlu *et al*.,2019).

In MRSA, the PBP responsible for the peptidoglycan cross-linking is altered to novel penicillin-binding protein 2a (PBP2a), which has a lower binding affinity to beta-lactam antibiotics(Peacock and Paterson, 2015).

The resistance arose from the mecA gene located in the staphylococcal cassette chromosome mec (SCCmec), and this resistance gene can be passed to other populations through horizontal gene transfer(Kırmusaoğlu *et al*.,2019). Upon acquiring the mecA gene, it will be localized in the S. aureus chromosome. The production of PBP2a is controlled by MecI repressor and transmembrane MecR1 sensor protein (Figure 1.1b) , In the absence of beta-lactam antibiotics, MecI represses mecA gene expression by binding to the promoter region of mec operon(King *etal*.,2017) .

In the presence of beta-lactam antibiotics, the antibiotic binds to the MecR1 sensor protein. It triggers autolytic activation of the metalloproteinase domain in the cytoplasm part of MecR1, causing signal transduction to be activated(Kırmusaoğlu *et al*.,2019). The latter caused the

MecI repressor to be proteolytically cleaved from its binding site, and this allows the expression mecA to produce PBP2a(King *etal*.,2017).The PBP2a production allows the peptidoglycan wall synthesis to continue without the interaction of beta-lactam antibiotics due to its low binding affinity to the antibiotic(Peacock and Paterson,2015).

Interestingly, the mec operon shared a similar structure and function with the bla operon, which produces beta-lactamase (Kırmusaoğlu *etal*.,2019). This similarity allows the Blal repressor to bind to the mec operon to repress mecA transcription (Figure 1.1) (King *et al*.,2017)

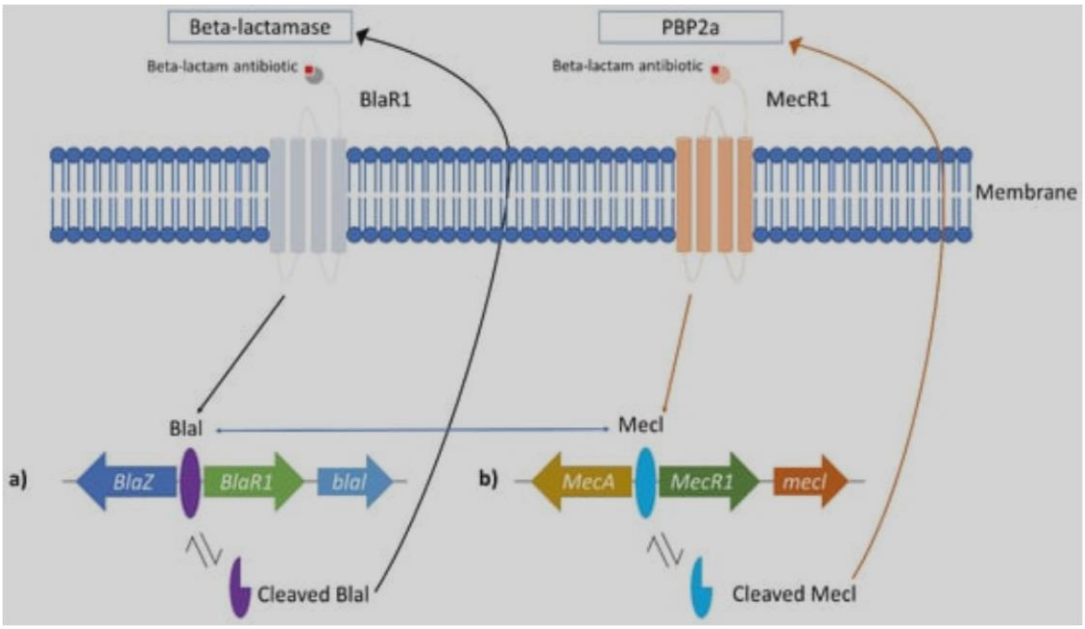


Figure 1-1 Correlation between Blal and MecA role in MRSA resistance. (**a**) *bla* operon responsible for beta-lactamase production and (**b**) *mec* operon responsible for the alteration of normal PBP to PBP2a. The blue arrows indicate that *bla* and *mec* operon shared similarities, which allows the repressor (Blal and Mecl) to bind to each operon.

1.2.2 Epidemiology of MRSA:

Epidemiology of MRSA is changing and their profile of antibiotics resistant differs from origin to another, where it has been become an epidemic in hospitals.

Center for disease control and prevention (CDC) in America reported the MRSA infections near 63% in 2007 as comparing in 1974 and 1995 were 2%, 22% respectively (Carlos *et al*., 2010), while Rossi *et al*. (2008) stated the overall MRSA prevalence was 48.3%. In Iraq, a study carried out by Mohammed *et al*., (2015) reported the frequency of MRSA among medical students were 20%, as well as Al-Dahbi and Al-Mathkhury in 2013 stated the MRSA distribution in healthcare workers and patients in Baghdad were 94.3% in nasal cavities.

The prevalence of MRSA infections, especially bacteremia, differs around the world. In 2014, the percentage of invasive MRSA isolates in Europe ranged from 0.9% in the Netherlands to 56% in Romania, with a population-weighted mean of 17.4% , MRSA prevalence exhibits a north–south variation in Europe, with a higher proportion of resistant isolates in southern countries compared with northern countries , Even though the proportion of MRSA isolates in Europe has decreased over time, 7 of the 29 European Union countries still report 25% or more of invasive *S.aureus* isolates as MRSA (European Centre for Disease Prevention and Control,2015).

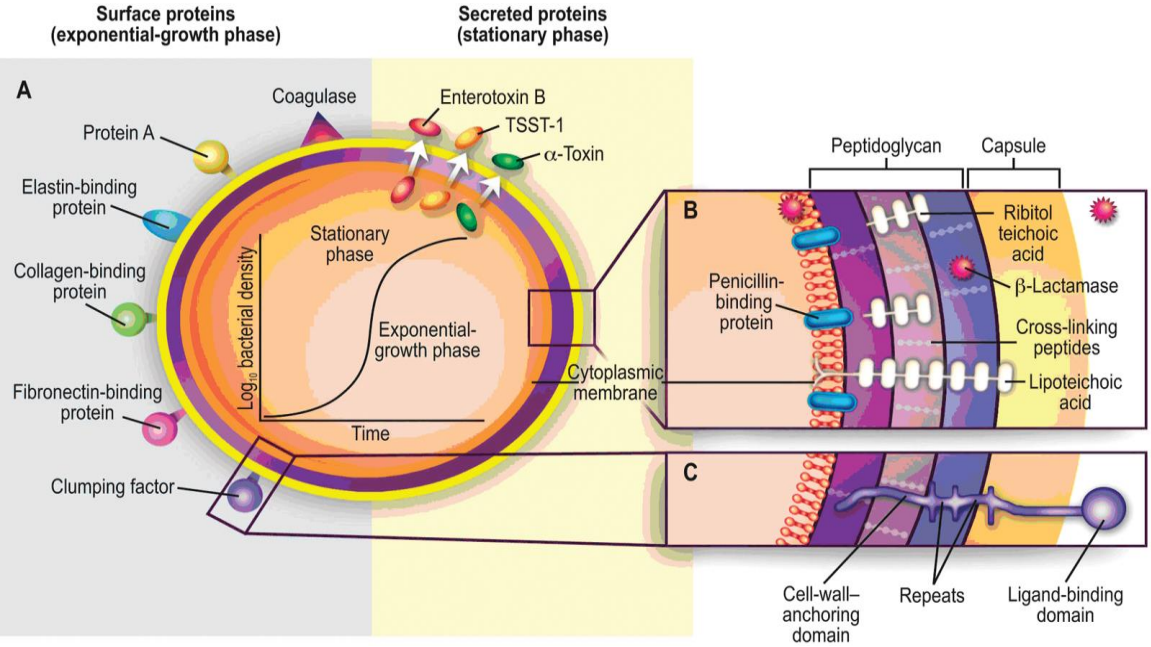
1.2.3 Virulence Factors of MRSA*:*

The high incidence of infections caused by *S. aureus* refers to a number of virulence factors that aid it to adhere to surface in order to avoid the immune system, and cause toxic effects to the host (Foster and Hook, 1998; Holmes *et al*., 2005). This organism expresses a number of membrane-damaging toxins and super antigen toxins that can cause tissue damage and the symptoms of septic shock, respectively.

There is a growing realization that *S. aureus* has multiple mechanisms for evading both innate immunity mediated by PMNs (Fedtke, *et. al.*, 2004) and induced immunity mediated by both B and T cells (Goodyear and Silverman, 2003; De Haas, 2004). These factors include surface structures (adhesions), exoprotiens, catalase that has ability to convert (H_2O_2) to water and oxygen and allow to survive the bacteria, the production of super-oxidize ions can also lead to tissue necrosis and the carotenoid pigment that has an antioxidant effect (Zetola *et al*., 2005). The range of virulence factors of *S. aureus* is wide, with both structural and secreted products playing a role in the pathogenesis of MRSA infections as in (figure1-2). In establishing an infection, *S. aureus* has several surface proteins, called "microbial surface components" that mediate adherence to host tissues. After *S. aureus* adheres to prosthetic materials or to host tissues, it is capable to grow and continue in different ways. *S. aureus* can form biofilms on prosthetic surfaces and host allowing it to persist by avoiding host resistances and antimicrobials (Donlan and Costerton, 2002). *In vitro*, *S. aureus* can also enter and persist inside epithelial cells, which may allow it to escape host lines, mainly in endocarditis (Hadi, 2007). It is also has ability to form small colony variants, which may contribute to persistent and periodic infection (Kahl *et al*., 1998).

Protein A bind to the Fc portion of immunoglobulin and, as a result, may stop opsonization. *S. aureus* secrete chemotaxis inhibitory protein, which inhibit neutrophil extravasation and chemotaxis to the place of infection (Cheung *et al*., 2002; Foster, 2005), as well as it produces many enzymes, such as lipases, proteases, and elastases that permit it to attack and destroy host tissues and metastasize to further sites. S. *aureus* expresses two adhesions that mediate binding to fibrinogen, clumping factor A (ClfA) and (CIfB). The factor ClfA enables S. *aureus* to adhere

to fibrinogen-containing substrates such as plasma clots and to clump in the presence of fibrinogen, so called clumping factor (O'Brien *et al*., 2002).



Figure(1-2) *Staphylococcus aureus* pathogenic factors. *A*, Surface and secreted proteins. *B* and *C*, Cross-sections of the cell envelope (Lowy, 1998).

Exoprotiens**,** nearly all strains of *S. aureus* produce exoprotiens such as enzymes and exotoxins that involve hemolysin (α, β, and γ,) nucleases, proteases, lipases, hyaluronidases, and collagenase.

The main function of these factors may be to convert local host tissue into nutrients that required for growth, as well as *S. aureus* is also capable of producing septic shock. It does this by interacting with and activating the host immune system and coagulation pathways (Timmerman *et al*., 1993; Lowy, 1998), and these bacteria produce a pyrogenic toxin and superantigens such as staphylococcal enterotoxins, and toxic shock syndrome toxin-1 (TSST-1) (Fey *et al*., 2003). The best character of this group is super antigenicity, ″e.i stimulate proliferation of T-lymphocytes without regard for antigen specificity″. Staphylococcal Enterotoxins (SEs), are a family of

major serological types of heat stable enterotoxins, they function both as potent gastrointestinal toxins as well as super antigens that stimulate non-specific T-cell proliferation (Balaban and Avraham, 2000). Exfoliative toxins**,** Staphylococcal scalded skin syndrome (SSSS), or Ritter's disease, a local infection releases exfoliative toxin A into the circulation, which leads to widespread skin blistering (Amagai *et al*., 2000).

1.2.4. Bi-component Toxins

Many types of staphylococcal toxins contain two synergistically acting protein designated S (slow) and F (fast), these includes Panton-valentine leucocidin (PVL), gamma hemolysin and other leukocidins such as LukE-LukD (Gillet *et al*., 2002), in addition, PVL is found in approximately 2% of clinical *S. aureus* isolates, but at very high percentages in community associated MRSA (CA-MRSA) isolates (Naimi *et al*., 2003; Shukla *et al*., 2004). Panton-valentine Leukocidin has ability to lyses leukocytes (Boyle-Vavra and Daum, 2007), and usually this toxin targets human and rabbit PMNs and mononuclear (Johnsson *et al*., 2004). Leukocidin LukE/LukD **t**oxins are a member of the staphylococcal bi-component Leucotoxin family; LukE is 58-68% identical with class S protein and LukD is 71-77% identical with class F protein of the leucotoxins family (Von Eiff *et al*., 2004). The production of this toxin is associated with infections resulting in furuncles, community pneumonia, and some antibiotic associated diarrhea (Bownik, 2006). The leucotoxins were shown to induce an important inflammatory response *in vivo* (Kaniko and Kamio, 2004).

1.2.5. Antimicrobial Peptides (AMPs):

Antimicrobial peptides (AMPs) are naturally occurring host defense mechanisms against infections. AMPs can be found in all living organisms

such as plants, microorganisms and animals(Baharin *etal.*,2021). Typical AMPs consist of 5–50 amino acid chains and have amphipathic or cationic structure. Despite AMPs being naturally occurring, synthetic AMPs have been developed by the scientist to overcome the naturally occurring AMP limitations ,While naturally occurring AMPs are susceptible to proteolytic degradation, synthetic AMPs have a longer half-life, and it is designed to improve their antimicrobial properties. AMPs then can be divided into four main groups based on its secondary structure including amphipathic alpha-helices, beta-sheets, a combination of both alpha and beta structure (mixed) and extended structure (without alpha and beta structure) (Patrulea *etal.*,2020).

In terms of AMP mechanism of actions, it can be divided into two main categories, membrane disruptive and non-membrane disruptive AMPs(Moravej *etal*.,2018; Benfield, and Henriques,2020; Zhang *etal.*,2021).

As the innate immune system, AMPs have broad spectrum antimicrobial properties which are said to be effective towards pathogenic microorganisms (Huan *etal*.,2020; Patrulea *etal*.,2020). These antimicrobial properties are greatly enhanced as AMPs can be found abundantly at the site of the infection, which makes it more time efficient since it can react faster to combat the infection((Huan *etal*.,2020; Zhang *etal*.,2021). Resistance towards AMPs is also said to be low, which makes it one of the suitable candidates to combat MRSA (Zhang *etal*.,2021). Besides that, AMPs also have good water solubility and thermal stability((Huan *etal*.,2020). However, AMPs do possess weakness as naturally occurring AMPs are susceptible towards proteolytic degradation, which limits their potential (Huan *etal*.,2020; Zhang *etal*.,2021). In addition, AMP

production and purification can be costly sometimes. Despite their broad antimicrobial spectrum, it can be a challenge to be used medically as some AMPs might induce hypersensitivity after application and might cause immunogenicity and toxicity when it is administered in humans(Huan *etal*.,2020; Patrulea *etal*.,2020).

1.2.6.Mechanism of antibacterial resistance for MRSA:

Antimicrobial resistance is a major global health concern, and, of the Gram-positive bacteria, drug-resistant *Staphylococcus aureus* is a serious threat (WHO,2014; Centers for Disease Control and Prevention,2013). *S. aureus* causes a wide range of infections commonly involving the skin, soft tissue, bone, joints, and infections associated with indwelling catheters or prosthetic devices (Tong *etal*.,2015). In addition, *S. aureus* is a leading cause of bacteremia in industrialized nations(Weiner *etal*.,2016).Although methicillin-resistant *S.aureus* (MRSA) bacteremia incidence has decreased over the past decade(Tong *etal*.,2015),MRSA remains associated with poorer clinical outcomes compared with methicillin-sensitive *S. aureus* (MSSA) (van Hal,2012). *S. aureus* bacteremia (SAB) often causes metastatic infections such as infective endocarditis (IE), septic arthritis, and osteomyelitis(Tong *etal*.,2015). Moreover, SAB can lead to complications such as sepsis and septic shock(van Hal *etal*.,2012). Taken together, these issues make SAB particularly challenging to treat.

Choice and timing of antibacterial therapy greatly affect treatment outcomes in SAB(van Hal *etal*.,2012). For SAB caused by MSSA, β-lactam therapy is considered the gold standard(van Hal *etal*.,2012; Keynan and Rubinstein ,2013) . For MRSA, the 2011 Infectious

Diseases Society of America guidelines recommend treatment with vancomycin or daptomycin (Liu *etal.*,2011;Tong *etal.*,2015). However, each antimicrobial agent has limitations. Several issues restrict the utility of vancomycin, including slow bactericidal activity, low tissue penetration, and increasing reports of resistance and failure (Han *etal.*,2012). While daptomycin is effective against MRSA bacteremia, treatment-emergent nonsusceptibility is concerning (Moore *etal.* ,2012; Kullar *etal.*,2013) , and evidence suggests prior vancomycin treatment may encourage daptomycin resistance in *S. aureus* (Moise *etal.*,2013). Given the substantial morbidity and mortality associated with SAB(van Hal *etal.*,2012). and the limitations of currently approved treatments, there is a need to identify alternative agents for the treatment of MRSA bacteremia. Time to effective treatment is largely dependent on pathogen identification (Nicolsen *etal.*,2013). Delays in diagnosing and treating SAB lead to poorer clinical outcomes(Lodise *etal.*,2003). Standard microbial identification techniques take between 48 and 72 h, while developed rapid diagnostic tests provide data within 3 h of collection(Palavecino,2014). By enabling optimized antimicrobial therapy, rapid diagnostic tests may lower mortality, hospitalization, and costs(Bauer *etal.*,2010).

Antimicrobial resistance among bacteria, viruses, parasites, and other disease-causing organisms is a serious threat to infectious disease management globally. Antibiotics were discovered in the middle of the nineteenth century and brought down the threat of infectious diseases which had devastated the human race.

However, soon after the discovery of penicillin in 1940, a number of treatment failures and occurrence of some bacteria such as staphylococci which were no longer sensitive to penicillin started being noticed. This marked the beginning of the error

of antimicrobial resistance. Scientific antibiotic discovery started in the early 1900s by Alexander Fleming, who observed inhibition of growth on his agar plate on which he was growing Staphylococcus spp. It was later found that a microorganism that was later to be called Penicillium notatum was the cause of the inhibition of the Staphylococcus around it as a result of excreting some chemical into the media. That marked the beginning of the discovery of penicillin which together with several other different antimicrobial agents was later to save millions of humans and animals from infectious disease-causing organisms (Centers for Disease Control and prevention,2013).

The observation of Staphylococci spp. that could still grow in the presence of penicillin was the beginning of the era of antimicrobial resistance and the realization that after all the drugs that were described as ‘‘magical bullets’’ were not to last for long due to the selective pressure that was being exerted by the use of these agents. However, the complacency between the 1940s and the 1970s that infectious microorganisms had been dealt a blow was later proved to be a misplaced belief that available antibiotics would always effectively treat all infections. Nevertheless, antimicrobial agents have improved the management of infectious diseases up to date. Increasing prevalence of resistance has been reported in many pathogens over the years in different regions of the world including developing countries. This has been attributed to changing microbial characteristics, selective pressures of antimicrobial use, and societal and technological changes that enhance the development and transmission of drug-resistant organisms .

Although antimicrobial resistance is a natural biological phenomenon, it often enhanced as a consequence of infectious agents’ adaptation to exposure to antimicrobials used in humans or agriculture and the widespread use of disinfectants at the farm and the household levels. It is now accepted that antimicrobial use is the

single most important factor responsible for increased antimicrobial resistance (WHO,2014).

1.2.7.Mechanisms of antimicrobial resistances:

Prior to the 1990s, the problem of antimicrobial resistance was never taken to be such a threat to the management of infectious diseases. But gradually treatment failures were increasingly being seen in health care settings against first-line drugs and second-line drugs or more. Microorganisms were increasingly becoming resistant to ensure their survival against the arsenal of antimicrobial agents to which they were being bombarded. They achieved this through different means but primarily based on the chemical structure of the antimicrobial agent and the mechanisms through which the agents acted. The resistance mechanisms therefore depend on which specific pathways are inhibited by the drugs and the alternative ways available for those pathways that the organisms can modify to get a way around in order to survive (WHO,2014).

Resistance can be described in two ways:

a) Intrinsic or natural or passive whereby microorganisms naturally do not posses target sites for the drugs and therefore the drug does not affect them or they naturally have low permeability to those agents because of the differences in the chemical nature of the drug and the microbial membrane structures especially for those that require entry into the microbial cell in order to effect their action.

b) Acquired or active resistance, the major mechanism of antimicrobial resistance, is the result of a specific evolutionary pressure to develop a counterattack mechanism against an antimicrobial or class of antimicrobials so that bacterial populations previously sensitive to antimicrobials become resistant. This type of

resistance results from changes in the bacterial genome. Resistance in bacteria may be acquired by a mutation and passed vertically by selection to daughter cells.

More commonly, resistance is acquired by horizontal transfer of resistance genes between strains and species. Exchange of genes is possible by transformation, transduction or conjugation (Yoneyama and Katsumata,2006). Acquired resistance mechanisms can occur through various ways.

Mechanisms for acquired resistance (Langton *etal*.,2005)

1-the presence of an enzyme that inactivates the antimicrobial agent

2-the presence of an alternative enzyme for the enzyme that is inhibited by the antimicrobial agent

3-a mutation in the antimicrobial agent's target, which reduces the binding of the antimicrobial agent

4-post-transcriptional or post-translational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent

5-reduced uptake of the antimicrobial agent

6-active efflux of the antimicrobial agent overproduction of the target of the antimicrobial agent

7-overproduction of the target of the antimicrobial agent

8-expression or suppression of a gene in vivo in contrast to the situation invitro

9-previously unrecognized mechanisms.

1.2.8.Resistance B-lactam antibiotics:

β-Lactam antibiotics are a group of antibiotics characterized by possession of a β-lactam ring (figure 1.3) and they include penicillins, cephalosporins, carbapenems, oxapenams, and cephamycins. The penicillins are one of the most commonly used antibiotics in developing countries because of their ready availability and relatively

low cost. The β-lactam ring is important for the activity of these antibiotics which results in the inactivation of a set of transpeptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis in bacteria. The effectiveness of these antibiotics relies on their ability to reach the penicillin-binding protein (PBP) intact and their ability to bind to the PBPs (Wilke *etal*.,2005).

Resistance β-lactams in many bacteria is usually due to the hydrolysis of the antibiotic by β - lactamase or the modification of PBPs or cellular permeability. β-Lactamases constitute a heterogenous group of enzymes which are classified according to different ways including their hydrolytic spectrum, susceptibility to inhibitors, genetic localization (plasmidic or chromosomal), and gene or amino acid protein sequence (He *etal*.,2013).

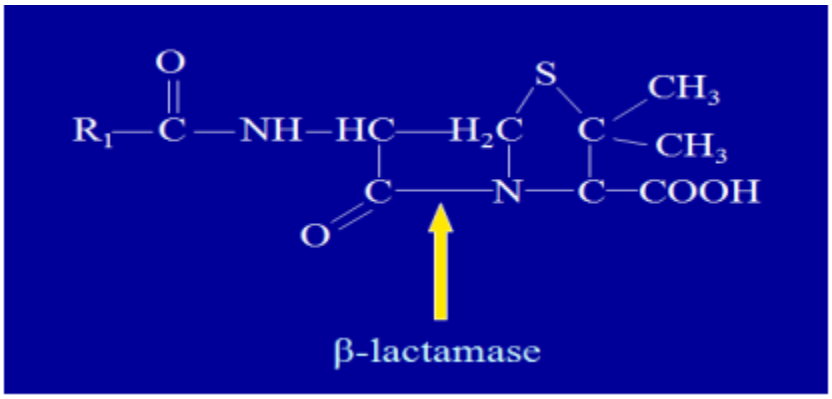


Fig1.3. Site of beta lactamase activity

Multidrug resistance among many organisms has become a big challenge to infectious disease management. It is increasingly being reported in bacteria and is often mediated by genetic mobile elements such as plasmids, transposons, and integrons. Integrons are mobile DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, site specific recombination, and they have an intergrase gene (int), a nearby recombination site (attI), and a promoter.

Integrons seem to have a major role in the spread of multidrug resistance in gram-negative bacteria but integrons in gram-positive bacteria have also been described. Class 1 integrons are often associated with the sulfonamide resistance gene sulI and are the most common integrons. Class 2 integrons are associated with Tn7. The majority of genes encode antibiotic disinfectant resistance, including resistance to aminoglycosides, penicillins, cephalosporins, trimethoprim, tetracycline, erythromycin, and chloramphenicol.

1.2.9. MRSA colonization :

About one-third of the general population is colonized with *S. aureus*, and the pooled prevalence of MRSA colonization is 1.3% (95% confidence interval [CI] 1.04–1.53%) , MRSA colonization varied between studies depending on the methodology used. For example, when cultures were taken at the time of hospital admission or outpatient assessment, prevalence of colonization with community-acquired MRSA was 1.8%, but when samples were taken from individuals outside of the healthcare environment, it was 0.76%(Salgado *etal.*,2003). While the percentage of the US population with *S. aureus* nasal colonization has decreased over time, the proportion of people colonized with MRSA has increased , Risk factors for colonization with MRSA in US females were age ≥60 years, diabetes, and poverty-level household income, whereas in US males the only significant risk factor was healthcare exposure (Gorwitz *etal.*,2008). Other studies identify chronic illness, injected drug use, recent hospitalization or outpatient visit, antibiotic use, and contact with an MRSA-infected person as risk factors.

While the most common site of MRSA colonization is the anterior nares (Kluytmans *etal.*,1997),*S. aureus* (including MRSA) may also be present in the throat, axilla, rectum, groin, or perineum, and frequently colonizes more than one site (Mermel *etal.*,2011; Albrecht *etal.*,2015) .

Studies suggest colonization of the throat is more prevalent than of the nose, and checking only the nose would fail to detect a significant portion of colonized persons (Albrecht *etal.*,2015; Kumar *etal.*,2015). Regarding nasal carriage of *S. aureus*, about 20% of the population are persistently colonized with one strain, about 60% are intermittent carriers of varying strains, and the rest of the population never exhibit nasal colonization(Williams ,1963). Hospital-acquired MRSA infections generally arise from persistent carriers undergoing antibiotic therapy or from intermittent carriers(Kluytmans *etal.*,1997) . Both intermittent and persistent MRSA nasal colonization significantly increase the risk of developing an MRSA-invasive infection, including bacteremia (hazard ratios of 22.8 and 36.8, respectively; *P* value for both compared to noncarriers is <0.001)(Vigil *etal.*,2015) .

To understand the relationship between colonizing and infecting strains, investigators analyzed patients admitted to the emergency department for closed skin abscesses , The majority (~90%) were colonized with the *S. aureus* strain isolated from the infection, and 31% of these patients were colonized with an additional strain. Having two MRSA strains was uncommon (4.1%), but MRSA + MSSA or two MSSA strains were present in 20.4 and 22.2% of patients, respectively(Albrecht etal.,2015).

Concordance of the colonizing and infecting strain was also seen in about 82% of SAB patients(von Eiff *etal.*,2001).

1.2.10. Diagnosis of MRSA

Identifying the causative organism can be challenging in SAB, especially for resistant strains. Traditional culture and susceptibility testing for MRSA takes between 48 and 72 h, including a 16- to 24-h incubation and another 16 to 24 h to complete the susceptibility tests , advances in molecular and nonmolecular testing methods greatly reduced the time required to detect MRSA(Palavecino,2014). These rapid and sensitive screening assays could help to improve infection control and decrease costs. With a rapid test, (Bauer *etal*.2010) . observed bacteremia patients diagnosed with MRSA had a shorter length of stay and lower overall hospital costs, and for patients with MSSA, the switch from empiric to targeted therapy was 1.6 days shorter. Use of rapid molecular diagnostic tests rather than conventional methods is also associated with a significantly lower mortality risk for patients with bloodstream infections (odds ratio (OR) [95% CI] 0.66 [0.54–0.80]), including those caused by Gram-positive organisms (OR [95% CI] 0.73 [0.55–0.97]) , Combining rapid molecular testing with an antibiotic stewardship program can further reduce the risk of mortality (Timbrook *etal.*,2017). Individual hospitals deciding which test to implement must consider the specificity, sensitivity, price, turnaround time, and expertise required for each test (Luteijn *etal.*,2011; Palavecino,2014).

An adaptation to the traditional culture method is the use of chromogenic agar, which produces a color reaction in the bacterial cultures , These media also contain antibiotics that only allow resistant bacteria to grow , Thus, MRSA can be detected in 20 to 26 h (Palavecino,2014).

1.2.11. prevention of MRSA transmition :

All healthcare personnel interacting with an MRSA-infected or -colonized person should use contact precautions to limit spread between patients , This means putting the MRSA-infected patient into a single or private room, and wearing gowns and gloves when entering the patient's room and removing them before exiting , Since MRSA colonization can be persistent, contact precautions should be used throughout an infected person's hospitalization (even after they have recovered from the MRSA infection) and with any person with a history of MRSA infection , Ideally, healthcare facilities should have a system in place to alert them to the readmission or transfer of an MRSA-infected patient, so appropriate controls can be put in place on their arrival (Calfee *etal*.,2014) .

Hospital-wide hand hygiene campaigns have also greatly contributed to reduction of MRSA infections (Marimuthu *etal*.,2014).

Because MRSA can contaminate the environment, the rooms of MRSA-infected patients require strict disinfection of furniture, overbed tables, handrails, sinks, floors, and any healthcare equipment used during patient care (e.g., stethoscopes, thermometers, blood pressure cuffs) (Calfee *etal*.,2014).

Xenon-UV light alone or in combination with normal cleaning decreases the presence of MRSA and other pathogens on surfaces by up to 99%(Jinadatha *etal.,* 2014). Use of certain materials such as copper alloys in building design can also reduce the environmental burden and transmission of MRSA and other hospital-acquired pathogens (Salgado *etal.*,2013).

Hospitals with high rates of MRSA infection should implement an active surveillance program to identify asymptomatic MRSA carriers and targeted MRSA decolonization programs to reduce infection rates (Calfee *etal*.,2014).

Surveillance combined with prophylactic treatment has been very effective in reducing surgical site infections , These protocols may combine intranasal antibiotics such as mupirocin with an antiseptic body wash or preoperative antibiotics , Surveillance is the key, though, to prevent misuse and overuse of antibiotics(Kavanagh *etal*.,2014).

1.2.12.Innate and adaptive immunity against MRSA:

Staphylococcus aureus is the most common cause of skin and skin structure infections (SSSI), including cellulitis and folliculitis(David and Daum,2010). Regardless of prior exposure or antibody status, patients with SSSI due to methicillin-resistant S. aureus (MRSA) exhibit 1-year recurrence rates as high as 27 to 45% (Shastry *etal*.,2007), and these infections often require surgical debridement (Wolk *etal*.,2004). Skin infection serves as a primary portal of entry for invasion and

subsequent hematogenous dissemination. For example, SSSI is a frequent prelude to bacteremia, endocarditis, and osteomyelitis (Tatteviu *etal.*,2012).

S. aureus is the second most common bloodstream isolate in health care settings, and it is the leading cause of infective endocarditis in developed countries (Leroy *etal*.,2015).

In humans, antibodies generated against many *S. aureus* antigens are prevalent and long-lasting (Stentzel *etal*.,2015). However, considerable discordance exists between the humoral response and protective immunity to *S. aureus*. For example, persistent carriers have significantly higher IgG levels for staphylococcal exotoxins than those of noncarriers (Verkaik *etal*.,2009). However, epidemiological studies have demonstrated that persistent carriers are at increased risk for recurrent MRSA infections (Kluytmas *etal*.,1997;Wertheim *etal.*,2005), suggesting that antibodies directed against *S. aureus* virulence factors are not sufficient for protection (Foweler and Proctor,2014). Consistent with this view, individuals with defects in humoral immunity are not necessarily at increased risk for *S. aureus* infections (Ballow,2002).

Moreover, approximately 5.7% of circulating memory T cells in both carriers and noncarriers are reactive to *S. aureus*, suggesting that these cells are not sufficient to mediate immune protection against MRSA infection by themselves (Kolata *etal.*,2015). Taken together, these data suggest that classical adaptive immunity alone is insufficient for optimal host defense against recurrent MRSA infections.

It is apparent that innate immunity plays a role in protection against recurring S. aureus SSSI. The findings of Montgomery et al. (Montgomery *etal*.,2014) suggested that immune mechanisms relying on antibody and IL-17A are important for protection against recurring skin infection .

Staphylococcus aureus is an important pathogen that causes a wide variety of infections, ranging from skin and soft tissue infections to severe invasive diseases, such as sepsis and endocarditis. Approximately 35% of skin and soft tissue infection cases occur in people over 65 years of age, and 25% of these subjects later present with invasive staphylococcal diseases (Demling and Waterhouse,2007).

Prior studies have demonstrated that immune cells from elderly subjects, compared to cells isolated from young subjects, exhibit reduced chemotaxis, phagocytosis, and respiratory burst activity in response to bacterial infection (Wenisch *etal*.,2000). An in vitro study of murine peritoneal macrophages attributes the decreased level of inflammation in elderly to lower levels of Toll-like receptors (TLR) expression on cell surfaces (Renshaw *etal.*,2002), suggesting that innate immune activation decreases with aging. Furthermore, decline in cellular and inflammatory responses has been shown to be associated with dysfunction of mitochondria in cells isolated from aged hosts (Csiszar *etal*.,2008). Therefore, decreased cellular functions and immune responses may result in ineffective clearance of bacteria and facilitate systemic infection.To prevent the systemic spread of pathogens, polymorphonuclear cells (PMNs) form neutrophil extracellular DNA traps (NETs) containing DNA, histone, granule enzymes, and antimicrobial components (Ermert *etal*.,2009). NETs promote killing of microorganisms and trap microbes locally (Wartha *etal*.,2007), thereby preventing dissemination of the pathogens. Several host factors have been shown to contribute to the formation of NETs, including TLR and interleukin (IL)-8 (Clark *etal*.,2007). Although NETs have been shown to interact with S. aureus and facilitate their killing (Brinkmann *etal*.,2004).

There is an increasing need to understand why elderly individuals are more susceptible to infection.

Age-dependent immune changes are likely to contribute to the increased incidence of severe infections (Klevens *etal*.,2007), and based on epidemiologic studies, the elderly population is more prone to invasive MRSA disease (Kuehnert *etal*.,2005). Aging impacts a number of innate immune functions, impairing the host's ability to clear invading pathogens (Niwa etal.,1989). Epidemiologic studies reported an increased incidence of soft-tissue infections in the elderly population (Young and Price,2008), and increased susceptibility to invasive disease after a cutaneous infection (Laupland etal.,2003).

Complex I function has been shown to play a role in lipopolysaccharide (LPS) induced inflammatory responses by modulating NF-κB activation (Zmijewski *etal*.,2008), suggesting that TLR4-dependent immune activation pathway is dependent on the mitochondrial function. Pertinently, Sweeney et. al. reported that the up-regulation of mitochondrial biogenesis transcription factors in S. aureus sepsis is TLR2- and TLR4-dependent, which further suggests an interplay between mitochondrial functions and TLR-dependent innate immune activation (Sweeney *etal*.,2010).

1.13. Immunoadjuvant :

Is a substance that stimulates, improves, potentiates or suppresses the main components of the immune system, including innate and adaptive immune responses. It increases the response to the vaccine and does not have any antigenic effects. Some of the adjuvants are endogenic (such as IL-1, histamine, and

interferon). Their mode of action is either antigen-specific, affecting a specific immune response to a narrow group of antigens or non-specific mode of action, leading to the increased immune response to a variety of antigens. Adjuvants are considered to be immune modulators since they enhance the body's immune response. It performs one or more of the three main functions. First, they enhance antigen depot for slow release, second, they enhance antigen targeting of immune cells and stimulate phagocytosis, and third, they enhance and modulate the type of antigen-induced immune response. Adjuvants may also provide a danger signals that the immune system needs to respond to the antigens as it does to an active infection.

As a result, immunoadjuvants have a significant role to play in every aspect of the immune response (Kostinov *etal*.,2020).

1.13.1. Blood cells:

Blood is a connective tissue made up of blood cells and plasma. The blood cells are made up of erythrocytes, leukocytes and thrombocytes. Peripheral blood mononuclear cells (PBMCs) are round-shaped blood cells, such as monocytes and lymphocytes. The lymphocyte population consists of CD4+ and CD8+ T-cells, B-cells and Natural Killer cells, CD14+monocytes, and basophils, neutrophils, eosinophils and dendritic cells (Al-Dulaimi *et al*.,2018).

1.13.2. Peripheral blood mononuclear cells (PBMCs):

PBMCs are a diverse mixture of highly specialized immune cells that play a critical role in keeping our bodies healthy (Sambor *et al*.,2014). On average, the majority of PBMCs are lymphocytes (70-90%). Lymphocytes play a key role in cell-mediated and humoral immune response, primarily associated with the T and B

cells activation. PBMCs are essential tools for research in new drug toxicity analysis that provide the potential toxicity of new compounds to the human immune system.

The effects of drug toxicity on PBMCs cause various serious toxic side effects, including suppression and toxicity of the immune system. PBMCs are therefore also explored for the determination of the dose limit for new drug compounds which are critical tools for predictive studies. Similarly, normal PBMCs and diseased PBMCs are analyzed to determine which pathways or molecules play an important role in the diseases. In addition, mRNA expression ratios or receptors expression in normal and diseased PBMCs are used for biomarker research (Ahangari *et al*., 2015;Sen *et al*.,2018).

PBMCs have also been studied in immunotoxicity or cytotoxicity studies for drug researches and chemotherapy compounds. Many scientists are studying PBMCs in the fields of immunology, including autoimmune diseases, transplantation immunology, hematological malignancies and infectious diseases. In addition, the PBMC-based vaccine strategy provides for a more marked and durable protective immune response (Kumar *et al.*,2017). Performing such experimentation in the PBMC require essential need for high recovery, viability and functionality of PBMCs for accurate assessments(Gill,2019).

1.13.3. Isolation of Peripheral Blood Mononuclear Cells:

Two primary techniques separate PMNCs from entire peripheral blood are the use of gradient density centrifugation or by leukapheresis. Because cells have a specific density, the use of a density gradient centrifugation separates the main cells population, including lymphocytes, monocytes, granulocytes, and RBC throughout the density. gradient medium, The medium will have had a density of 1,077 g/ml

allowing sufficient separation of PBMCs (density < 1,077 g/ml) from RBCs and granulocytes with density of (> 1,077 g/ml). The layering of whole blood under or over a density medium without mixing the two layers then samples were centrifuged and this will disperse the cells according to their density (Jia *et al*.,2018). After centrifugation, the PBMC fractions appear as a thin white layer at the interface between the density gradient medium and plasma, making it easy to isolate the PBMC fraction. Leukapheresis is an automated device that separates the inflow of the whole blood from the target PBMCs fractions using elevated speed centrifugation while returning the outflow content, such as red blood cells, plasma, and granulocytes back to the donor. PBMCs are an important component and a powerful tool for science and medical studies on human health and disease. Through efficient and effective processing and analysis of PBMCs, researchers and healthcare professionals can test immune responses, develop a greater understanding of the immune system, and apply their observations to treatments and medications for human diseases (Pourahmad and Salimi, 2015).

1.13.4 Primary cell culture:

Primary cell culture is the disassociation of cells from a parental animal or plant tissue through enzymatic or mechanical measures and maintaining the growth of cells in a suitable substrate in glass or plastic containers under controlled environmental conditions. Primary cell culture could be classified into two based on the kind of cells used in culture.

• Anchorage Dependent or Adherent Cell : These cells require an attachment for growth. Adherent cells are usually derived from tissues of organs, for instance from kidney where the cells are immobile and embedded in connective tissue (Freshney, 2006).

Anchorage Independent or Suspension Cells : These cells do not require an attachment for growth. In other words, these cells do not attach to the surface of culture vessel. All suspension cultures are derived from cells of the blood system, for instance, PBMCs is suspended cells. Cells derived from primary cultures have a limited life span. Cells cannot be held indefinitely due to several reasons. Increasing cell numbers in primary culture will lead to exhaustion of substrate and nutrients. Also, cellular activity will gradually increase the level of toxic metabolites in the culture inhibiting further cell growth.

At this stage, a secondary or a subculture has to be performed to ensure continuous cell growth (Ramos *et al*.,2014).

1.13.5. Secondary cell culture:

when cells in adherent cultures occupy all available substrate or when cells in suspension cultures surpass the capacity of the medium to support further growth, cell proliferation begins to reduce or to entirely cease. In order to maintain optimal cell density for continued growth and to stimulate further proliferation, primary culture has to be sub-cultured. This process is known as secondary cell culture.

During the secondary cell culture, cells from primary culture are transferred to a new vessel with fresh growth medium. The process involves removing the previous growth media and disassociating adhered cells in adherent primary cultures. Secondary cell culturing is periodically required to provide cells with growing space and fresh nutrients, thereby, prolonging the life of cells and expanding a number of cells in the culture.

Secondary culturing a certain volume of a primary culture into an equal volume of fresh growth medium allows long-term maintenance of cell lines. Secondary culturing into a larger volume of fresh growth medium is practiced to increase the

number of cells, for instance in industrial processes or scientific experiments (Luo,2019).

1.13.6.Characterization of Peripheral Blood Mononuclear Cells:

Most PBMCs are present as naive or resting cells with no effector function. In the absence of a continuous T-cell immune response, the largest fraction of isolated PBMCs are present mainly as naive or memory T-cells. Naive T-cells have never encountered their cognate antigen before and are commonly characterized by the lack of activation markers such as CD25, CD44 or CD69 and the absence of the CD45RO isoform memory marker. Recognition of the antigen by a naive T cell may result in activation of the cell (Ashraf and Khan, 2003), which will then enter a program of differentiation and develop T cell effector functions .

In peripheral blood, the frequency of lymphocytes with the specificity of a single antigen is low, therefore polyclonal activators are used for *in vitro* stimulation as they can activate a significant portion of lymphocytes independently of their antigen specificity. The most common activator are carbohydrate-binding proteins that bind to a number of glycoproteins expressed on the lymphocytes plasma membrane and the other activator was mitogenic lectins (Sen *et al*.,2017).

Polyclonal activation of T cells is also obtained by antibodies that bind to CD3 specifically, alone or in combination with CD28. Impacts on the immune function of PBMCs are generally monitored by studying changes in the characterization of cytokine secretion profiles, lymphocyte proliferation, or gene expression alterations (Kleiveland,2015). Upon activation with polyclonal activators, PBMCs produce cytokines and up-regulate stimulation markers. Characterization of cytokine profile and changes in the expression of activation marker particularly in T cells may provide relevant information as to whether the response is in the direction of Th1,

Th2, Th17 or regulatory T cells. Th1 cells have been identified as CD4+, CD69+ and CXCR3+ and Treg cells as CD4+, CD25+ and Foxp3+. Increased of IFN-γ and Il-10 production suggests increased levels of Th1 and Treg cells, while decreases in IL-17 support suppression of Th17 cells (Golubovskaya and Wu,2016).

1.13.7.Activation of PBMCs:

peripheral blood mononuclear cells can be activated *in vitro* by different methods, the most reliable one is phytohemagglutinin (PHA) activation that stimulates the (PBMCs) proliferation. Another methods including bacterial LPS, IL.2,Concanavalin A(Con A) and different cytokines(Wu *et al*.,2016).

The response of such a diverse group of cells to different stimuli provides insight into their role in the disease and the development of treatment options.

Activation of T-cell is normally initiated mostly by the interaction of the cell surface receptor with its specific ligand molecule along with a co-stimulatory molecule (This binding event triggers inositol phospholipids rapid hydrolysis by phospholipase C (PLC) to diacylglycerol and inositol phosphates. Diacylglycerol is an allosteric protein kinase C(PKC) activator . PKC activation and inositol phosphates which trigger Ca++ release and mobilization result in a chain reaction of supplemental cellular reactions mediating T cell activation.

1.14. HBV vaccine immunomodulation:

Chronic hepatitis B virus (HBV) infection remains a severe threat to global public health, leading to a high rate of morbidity and mortality(Razavi-Shearer *etal*.,2018). Especially in the Asian-Pacific region, it is the predominant etiology of liver cirrhosis and hepatocellular carcinoma (HCC)(Sarin *etal*.,2016;Omata *etal*.,2017;Wong *etal*.,2019). Functional cure, defined as hepatitis B surface antigen

(HBsAg) clearance with or without hepatitis B surface antibody (HBsAb) appearance, is regarded as the ideal endpoint of antiviral treatment in patients with chronic hepatitis B (CHB), which is associated with a favorable long-term prognosis(EASL,2017;Terrault *etal.*,2018;Kao *etal.*,2021). It is well known that the functional cure can be achieved spontaneously or by antiviral agents(Song *etal.*,2021). Interferon (IFN) treatment has remarkable advantages of higher rates of hepatitis B e antigen (HBeAg) seroconversion and HBsAg clearance and reduced risk of HCC development over NA (nucleoside/nucleotide analogues)(National Institute for Health and Care Excellence,2013;Marcellin,2016;Ren *etal.*,2018). However, HBsAg reversion occurs in a considerable proportion of patients after IFN-induced HBsAg clearance(Pan *etal*.,2021;Huang *etal.*,2022).

As we all know, HBV vaccination has made a great contribution to preventing maternal-neonatal transmission and eradicating HBV infection across the world(Sarin *etal.*,2016). It could clear the circulating HBsAg in the HBsAb-mediated way, providing long-lasting protection against HBV infection in a generally healthy population (Brunskole *etal*.,2016). Several previous studies have shown that the patients with HBsAb levels >100 mIU/ml at the end of IFN-based therapy were less likely to have HBsAg reversion than those with HBsAb levels ≤100 mIU/ml, (Li *etal.*,2019;Wu *etal.*,2020),suggesting HBsAb level is one of the influencing factors for off-treatment HBsAg reversion.

Hepatitis B Virus (HBV) infection is a global health problem that accounts for nearly one million deaths annually through complications of HBV-induced liver diseases, such as hepatocellular carcinoma and cirrhosis (WHO,2017). Despite the availability of a safe and highly effective vaccine for the past three decades, HBV remains endemic in many regions(Kramvis,2020).

The World Health Organization (WHO) has recommended universal vaccination of all infants against HBV, with a minimum of three doses of HepB administered at least four weeks apart (Centeras for Disease Control and Prevention,2003). Furthermore, the WHO recommends that an additional dose be administered to neonates within 24 h of birth (HepB-BD) as the risk of developing chronic HBV infection is greatest amongst infants infected during the first year of life (80–90%) – this risk decreases exponentially if infection is acquired in adulthood (1–5%) (Mcmahon,2009).

Optimal vaccine efficacy linked to producing systemic and mucosal antibodies and cellular immunological memory requires a complete set of immunological responses, as detailed. The extent of the primary immune response and the subsequent cellular memory response depends on several factors. Major factors are the type of vaccine and the added adjuvants, which can significantly improve antigen presentation and stimulation of lymphocytes (Zimmermann and Curtis,2019).

Vaccines containing the whole pathogen, for example, live attenuated vaccines (e.g., against smallpox), are often the most efficient. These vaccines generally consist of the antigen(s), in addition to a wide array of structural and secreted microbe-associated molecular patterns (MAMPs) of the pathogen, such as specific microbial DNA, RNA, proteins, and polysaccharide motifs or patterns that expose to immune cells (Loukov *etal*.,2015). These MAMPs specifically target their cognate pattern-recognition receptors (PRRs), including toll-like receptors (TLRs), as agonists on the host cells, resulting in natural adjuvant activity by stimulating innate immune responses and inducing the signals necessary for lymphocyte activation and differentiation (Medzhitov and Janeway,1997).

The antigens in parenteral or mucosal (oral, intranasal) vaccines are taken up by antigen-presenting cells such as dendritic cells (DCs) that are additionally stimulated by the vaccine adjuvants via PRRs (pattern recognition receptors) and activated. Activated DCs migrate to the draining lymph nodes where they release immunostimulatory cytokines and present the processed antigens to stimulate effector cells, including CD8+ and CD4+ T lymphocytes, resulting in B cell activation, proliferation and antibody production by the resulting plasma cells.

On the other hand, subunit vaccines (e.g., against hepatitis B) contain limited antigenic components of a pathogen without MAMPs. As a result, they are generally less immunogenic than live attenuated vaccines, thus needing additional adjuvants to boost immunity (Bashiri *etal*.,2020).

Besides vaccine composition, individual characteristics of the vaccine recipient, such as age and health status, have been shown to affect vaccination efficacy (Zimmermann and Curtis,2019). Immunosenescence is especially problematic, as age-related decline in immune function results in higher susceptibility to infectious diseases and simultaneously can facilitate less effective vaccine responses (Crooke *etal.*,2019).

This has led to the search for effective and safe adjuvant strategies in addition to the ones provided by the vaccine that can augment the acquired immune response in adult populations of different ages.

1.15.Immune response and immunization against MRSA:

Multiple-antibiotic-resistant *Staphylococcus aureus* is one of the leading worldwide causes of infections, causing signifcant mortality in societies (Gould,

2005). There are several helpful anti-staphylococcal antibiotics targeting bacterial cell walls, including methicillin (in addition to other β-lactams), vancomycin, and distamycin; however, *S. aureus* has developed resistance to all these agents, leading to methicillin-resistant *S. aureus* (MRSA). More importantly, the strains show decreased susceptibility to vancomycin (VISA) and daptomycin (Tiwari *et al.* ,2018), which can cause more problems in treating patients infected with this microorganism. Due to the antibiotic resistance of MRSA and the incidence of multidrug-resistant strains (Cascioferro *et al.*, 2021), Non-antimicrobial approaches to control MRSA have attracted potential interest in immunotherapy (Schafer and Lee, 2008). In this regard, vaccination, as an old and well-known approach, is one of the possible approaches to preventing resistant *S. aureus.*

Successful experience in controlling various infectious diseases in human beings showed that vaccination might be useful in handling this problem. A wide variety of vaccines against MRSA, exerted by targeting diferent proteins on the organism, have been developed and assessed in animal models or even in humans, none of which showed protection against MRSA. The surface proteins are considered crucial factors for *S. aureus* colonization and virulence.

Therefore, recombinant cell wall-anchored antigens have been recommended as potential *S. aureus* vaccine candidates (Kalali *et al*., 2019). Autolysin, a surface-associated protein, has both enzymatic (amidase and glucosaminidase) and adhesive functions (Heilmann *et al.*, 2005; Biswas *et al.*, 2006; Houston *et al*., 2011); it also makes the connection of the cells to a polymer surface (Heilmann *et al.*, 2005), excretion of cytoplasmic proteins (Pasztor *et al.*, 2010), bioflm formation (Heilmann *et al.*, 2003), and separation of daughter cells after cell division (Biswas *et al.*, 2006; Heilmann *et al.*, 2003, 2005; Houston *et al*., 2011). Autolysin can bind to vironectin

(Vn), suggesting its role in colonizing polymer surfaces and colonizing host factor-coated materials and host tissues. Recent studies have well reported the importance of autolysin in S. aureus pathogenicity and protective immunity to S. aureus infections. Active immunization with Autolysin protein could lead to the development of specifc antibodies and humoral immune responses, resulting in decreased bacterial loads and infammation response and an improved survival rate of experimental mice (Haghighat *et al.*, 2017a, b; Kalali *et al*., 2019). Adjuvants, another vaccine component, are critical in promoting the desired type of immune response and protection. While traditional adjuvants, such as Alum, have been exclusively employed clinically to stimulate immune responses.

S.aureus is considered the most common cause of nosocomial infections and one of the leading causes of death in hospitalized patients (Cascioferro *et al*., 2021). Because the MRSA epidemic is life-threatening and goes beyond antibiotic therapy, it is essential to develop an alternative approach, such as vaccines, to treat this challenge in an immunoprophylaxis setting (Søe *et al*., 2017). There are now several formulation platforms for producing a vaccine against S. aureus, but they sufer from some obstacles. Various reasons can be considered in the form of limitations and shortcomings in developing S. aureus vaccines. Pathogenicity factors are among antigen candidates for developing S. aureus vaccines; however, when used as a single component in the production of vaccines, they cannot provide an adequate immunity against S. aureus infections. The use of killed and attenuated live vaccines, although seeming to be a promising approach, sufers from problems such as the need for appropriate adjuvants in the killed vaccine and ensuring the patient's safety when attenuated live vaccines are used (Sandi *et al.*, 2015).

autolysin was allocated for vaccine structures. Autolysin is an essential protein in *S. aureus* with multiple functions, including cell isolation, cell lysis, hydrolysis of cell surface peptidoglycan, isolation of daughter cells after cell division and bioflm formation, making it a successful potential candidate for vaccine development with the remarkable ability to provide acceptable levels of protection (Foster, 1995; Singh, 2014; Haghighat *et al.*, 2017a, b; Kalali *et al.*, 2019).

Adjuvant selection for vaccine development is crucial to increasing immunogenicity and stimulating innate immunity, developing the appropriate protective response to combat the microorganisms (Marques Neto *et al*., 2017).

IFN-γ, one of the measured cytokines in this study efective in regulating Th1/Th2 balance, increasing antigen supply, and eliminating pathogenic bacteria by activating macrophages (Osugi *et al.*, 1997; Ahmadi *et al.*, 2021), is a crucial cytokine to determine the type of immune responses against foreign pathogens, which is the main cytokine to prevent and quench infection (Vahdani *et al.*, 2021).

IL-4, with various roles, including diferentiating B cells into plasma cells and stimulating activated B-cell and T-cell proliferation, is a critical regulator in humoral and adaptive immunity. IL-4 induces B-cell class switching to IgE and up-regulates MHC class II production (Sandova *et al.*, 2020; Silva-Filho *et al.*, 2014).

IL-12 and IL-4 are the polarizing cytokines of Th1 and Th2, respectively. An increase in the IL-12/IL-4 ratio indicated a Th1 response (Li *et al.*, 2018; Sirenko, 2018).

IFN-γ enhances Th1 responses, and TNF-α stimulates infammatory cells and causes infammation (Osugi *et al.*, 1997; Gough and Myles, 2020). An increased

IFN-γ/TNF-α ratio indicates that cellular immunity has increased without infammation, which can be considered a safe immune response.

1.15.1.Natural presence of anti *S. aureus* antibodies in the human population

Human antibody response to different types of pathogens is a crucial indicator of *in vivo* expression and immunogenicity of a particular pathogen. The healthy human immune system displays divergent antibody immune responses to a vast array of *S. aureus* antigens, which can increase sturdily during bacteremia. Potential antigen prediction for vaccine candidates may be done by comparing the antibody responses to *S. aureus* components (Romero Pastrana *et al*., 2018). In the case of *S. aureus*

1.15.2.Active immunization against *Staphylococcus aureus*

As MRSA is emerging, gradually there is a prevailing need for a vaccine that can counter the infections caused by this bacteria and ensure efficient protection. The development of vaccines primarily focuses on the immunogenicity of the vaccine, the level of antibody (Ab) response induced, and stimulation opsonization of the bacterial strain.

1.15.3.Nucleic acid vaccines

Nucleic acid or DNA/RNA vaccination is a rising technology with high potency results and can lead to dispensing antigens encoded like DNA or messenger RNA to cells which can transcribe or translate into an antigen/protein (Clegg *et al.*, 2021). Plasmids can be added by molecular adjuvants according to the need that directs

cellular immune responses. A multivalent polyprotein vaccine was evaluated for its efficacy against *S. aureus.* For this, a series of plasmids was used to immunize mice

1.15.4.Passive immunization against *Staphylococcus aureus*

Monoclonal antibody (mAb) is an alternate and effective system of passive immunization to manage *S. aureus* infections (Giersing *et al*., 2016). Anti-staphylococcal monoclonal antibodies are considered a novel anti-staphylococcal approach as they act as a strong defender to combat infection in patients at high risk.

2.1. Materials:

2.1.1. Laboratory instruments:

All instruments, glassware, and plasticware used in this study are listed in table (2-1):

Table (2-1): Laboratory instruments and tools used in this study

No.	Instruments	Company/ Origin
1	Autoclave	Memmert/Germany
2	Bensen burner	Cony/Germany
3	Cover slides	Citoglas/China
4	Centrifuge tubes	Cony/Germany
5	Conical flasks	Pyrex/ England
6	Cylinders	Technico/ England
7	Deep freezer	Revco/ USA
8	Disposable petri-dishes	Jordan
9	Distillate water	GFL/ Germany
10	ELISA washer	Italian
11	Epindorf tubes	AFCO/Jordan
12	Face mask	Broche/ China
13	Gloves	Sidra/ China
14	Heparin- tubes	AFCO/ Jordan
15	Incubator	Memmert /Germany
16	Laminar flow Hood	Labtech/Denemark
17	Light microscope	Olympus /Japan
18	Loop wire	China
19	Microcentrifuge	Fisons /Engeland
20	Micropipette	Dragon/China
21	Micro Plate Reader ELISA	PAPA MEDICAL/ Italian
22	Oven	Memmert / Germany
23	Plain tubes	AFCO/ Jordan
24	Plastic containers	AFCO/ Jordan
25	Plastic droppers	AFCO/ Jordan

26	Refrigerator	Concord/ Lebanon
27	Screw cap bottles	Pyrex/ England
28	Slides	Afco/ China
29	Swabs	Afco/ Jordan
30	Syringes 5ml, and 10ml	Sum bow/ China
31	Tips	Afco/ Jordan
32		
33	Water bath	Tafesa/ Germany

2.1.2.Biological and Chemical Materials:

All biological and chemical materials used in this study are listed in table (2-2):

Table (2-2) the biological and chemical materials used in this study:

No.	**Materials**	**Company/ Origin**
1	Blood agar base	Himedia / India
2	Gram stain set	Crescent/ KSA
3	HBV 20 mg/ml	Serum institute of india
4	Lymphoprep	Capricorn / Germany
5	Muller-Hinton agar	Oxoid / England
6	Normal saline	ADWIC/ Egypt
7	Nutrient agar	Himedia / India
8	Nutrient broth	Himedia / India
9	Phosphate buffer saline tablet	BDH/ England
10	RPMI 1640 medium	Capricorn / Germany

2.1.3.Antibiotic Disks:

All antibiotic discs that were used in this study are documented in the table (2-3).

Table (2-3) the antibiotic discs used through the study with their abbreviations and the content of each one

No.	Antibiotic name	Disc potency/ disc	Company/ Origin
1	Gentamicin	10µg	HiMedia/India
2	Methicillin		
3	Penicillin	10 unite	Bioanalyses, turkery
4	cefoxitin	30 µg	

2.2 Methods:

2.2.1 Preparation of Culture Media.

The culture media were prepared according to the method recommended by the manufacturing Companies mentioned in (table 2-2).

2.2.1.1 Nutrient Broth Medium.

This medium was prepared for primary isolation of bacteria by dissolving 1.3gm of a medium base in 100 ml of D.W., then sterilized by the autoclaved for 15 minutes at 121 ° C.

2.2.1.2 Muller-Hinton Agar .

This medium was prepared by dissolving 3.8 grams in 100 ml of D.W and autoclaved for 15 minutes at 121 ° C. This has been used for testing the resistance to antibiotics.

2.2.1.3 Blood agar.

Blood agar medium was prepared by dissolving 4 gm of blood agar base in 100 ml D.W. It was autoclaved for 15 minute at121ºC , and then cooled down to 50ºC, then 5% of fresh human blood was added. This medium was used for bacterial cultivation and to assess the capacity of bacteria to hemolysis blood cells.

2.2.1.4 Mannitol Salt Agar Medium:

It has been used as a selective medium for the isolation and identification of *Staphylococcus aureus*. This medium was prepared by dissolving 11.1 grams of mannitol salt agar base medium in 100 ml of D.W and autoclaved at 121°C for 15 minutes.

2.2.1.5 Brain-Heart Infusion (BHI) Broth:

The BHI medium was used for the primary isolation of *Staphylococcus aureus*. It was prepared by dissolving 3.7 grams in 100 ml of D.W and autoclaved at 121°C for 15 minutes, and stored in the refrigerator until used.

2.2.1.6 Preservative Medium Preparation :

This medium is used for bacterial isolate storage. It was prepared by mixing 15 ml of glycerol solution with 85ml of nutrient broth media that was prepared in 2.2.1.1 section and then autoclaved at 121°C/15 minutes. After cooling, the isolates were inculated and stored at -20°C for 6-8 months and re-subcultures monthly (Forbes, 2007).

2.2.2 Preparation of Solutions and Buffers:

2.2.2.1 Preparation of Phosphate Buffer Solution (PBS):

The phosphate buffer solution was prepared by dissolving one tablet in 100 mL of D.W. and sterilized by autoclave at 121°C for 15 minutes and kept at 4ºC. This solution was used for the preparation of the somatic bacterial antigen of MRSA.

2.2.2.2 Preparation of Oxidase Reagent:

Oxidase reagent was used for the detection of the ability of bacteria to produce oxidase enzyme. It was prepared by dissolving one gram of tetramethyl-paraphyenylen diamine dihydrochloride in 100 milliliters of D.W. This reagent was used immediately and kept in a dark bottle (MacFaddin, 2000).

2.2.2.3 Catalase Reagent Preparation:

Catalase reagent was used as a ready solution (3% H_2O_2), as an indicator for the production of bubbles through mixing with bacterial colonies to ensure the bacteria produced catalase enzyme.

2.2.3 Isolation and Identification of MRSA:

This bacterium (MRSA) was obtained from AL-Husseini Hospital in Karbala city that isolated from a burned person who has a skin lesion, and this isolate re-culture to ensure the diagnosis as below.

2.2.3.1 Morphological Study :

A morphological test was carried-out using Gram's stained smear to observe the color and shape of bacteria growing on blood agar media. The bacteria were observed as grape-shaped cocci, Grams positive, and no spore forms (Collee, 1996; Brooks *et al*., 2013; Hosseini Alfatemi *et al*., 2014).

2.2.3.2 Biochemical Tests:

2.2.3.2.1Catalase:

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

2.2.3.2.2.Oxidase:

A piece of filter paper was saturated in a petri dish with freshly prepared oxidase reagent (tetramethyl– P-phenylenediamine dihydrochloride), a small portion of the bacterial colonies was spread on the filter paper by a wooden stick. When the color of the smear turned to purple color, the oxidase test is positive (Forbes *et al*., 2007).

2.2.3.2.3. Coagulase:

Coagulase is an enzyme produced by *S. aureus* that converts fibrinogen into fibrin. There are two forms of coagulase enzyme, bound and free, and they are detecting as below (MacFaddin, 2000):

1-Slide method was used to detect bound coagulase by putting a drop of plasma and emulsifying with isolated colonies gently, the slide was rocked for 5-10 seconds to see clumping (coagulation).

2-Tube method was carried out by several isolated colonies emulsifying with 0.5ml of plasma with an equal volume of broth (0.5 ml). The tube was incubated at 37°C for 4 hours; the positive result was conducted by clot formation. This test was aided to different *S. aureus* from other species of staphylococci.

2.2.3.2.4.Mannitol-sugar fermentation:

Mannitol-sugar fermentation test was carried-out to differentiate S. *aureus* from other species of *Staphylococcus* by cultivation of tested colonies on mannitol-salt agar media by streaking out incubated at 37°C for 24 hours. The positive result (*S. aureus*) was change the purple color of media into yellow color that is reflects to mannitol-fermentation by *S. aureus*, the negative result was no change in color of media (Koneman *et al*., 1992).

2.2.3.3 Antibiotic Susceptibility Test :

This test was done by transferring 0.1ml of tested bacterial suspension onto surface of Muller-Hinton agar medium and spreading the suspension on the surface of media and left to dry, and then the antibiotic discs were placed and incubated at 37°C for 24 hours. The result detected by measuring the inhibition zone. The test helps to identify the methicillin-resistance S. *aureus* (MRSA). If the bacterial isolate has inhibition zone to penicillin ≤ 28mm and ≤ 21mm to cefoxitin this reflect to MRSA bacteria (CLSI, 2012).

2.2.4 Preparation of MRSA Suspension:

2.2.4.1 Preparation of Somatic Antigen of MRSA:

The killed somatic antigen of MRSA was prepared by streaking of S. *aureus* bacteria on nutrient agar media and incubated at 37°C for 24 hours. The bacterial growth was harvested and introduced into conical flasks that contain 100 ml of sterilized PBS and placed in a water bath at 100°C for two hours to kill the bacteria. The bacterial isolate was checked for sterility to ensure the killing of bacteria by re-cultivation on the nutrient agar medium. The flask that contains killed somatic MRSA Ag was Parafilmed and stored in refrigerator until use to challenge the immune system (Stanislavsky and Lam, 1997).

2.2.4.2 Preparation of Viable MRSA Suspension:

This bacterial suspension was prepared by harvested the bacterial growth of *S. aureus* that diagnosed through the (2.2.3) section into test tubes that contain PBS and incubated at 37°C for two hours, and this suspension was used in treatment . This suspension was 0.5 x10^8 cfu/ ml by using 0.5 McFarland tubes (Hegazi *et al*., 2014).

2.2.5. Study Design: Experimental analytical randomized controlled trail.

2.2.5.1. Subject Criteria- Inclusion & Exclusion Criteria: Apparently 15 healthy subjects with age range(20-35)years males. All subjects were not suffering from any health problems and did not receive any drugs. The excluded criteria included any person who had an infection or disease.

2.2.5.2.Ethical Approval:

Volunteer were verbally asked permission before taking any blood specimen. In addition, the study concept was accepted by the Research Ethical Committee at the College of Medicine / University of Babylon.

2.2.6. Collection of Blood Samples.

Blood samples (5 ml) were collected aseptically by vein puncture from apparently healthy male age range about (20-35 years), and collected in anticoagulant tubes containing heparin. Blood samples left for 15 minutes to cool to room temperature before the isolation of peripheral blood mononuclear cells from whole blood by density gradient centerfugation (Lewis, 2001).

2.2.7. Method of PBMCs separation by lymphoprep.

This method was applied according to (Bøyum,1968).

1-The lymphoprep medium bottle was firstly inverting gently to mixing thoroughly.

2- Five ml of lymphoprep was transferred aseptically to a 15 ml centrifuge tube.

3- The blood was diluted in 1:1 by mixing 5ml of blood with 5 ml of phosphate buffer solution.

4-The blood samples were kept at room temperature at15-20ºC prior to and during centrifugation.

5-Diluted blood layered carefully over the 5ml of lymphosep in a 15 ml centrifuge tube making defined blood-lymphosep interphase.

6-The tubes were centrifuged at 1800 rpm for 30 min. at room temperature this will precipitate erythrocyte and polynuclear leukocytes leaving mononuclear cells above lymphosep.

7-The top layer of clear plasma about 3cm above lymphoprep was discarded.

8-The lymphocyte layer and about half of the lymphoprep layer under it was aspirated and transferred to centrifuge tube containing about two times the volume of phosphate buffer saline.

9-The cells suspension was centrifuged at 800 rpm for 10 minutes at room temperature and the supernatant was aspirated and dsiscarded and the sedimented cells were washed to remove the lymphosep and reduce platelets.

10-The cells were washed again with phosphate buffer saline and resuspended in RPMI1640 complete medium at cell culture plate.

11-The cells number was determined by Neubauer chamber count under an light microscope. The cell concentration was adjusted to be $1x10^6$ cell/ml in RPMI complete medium.

2.2.8 PBMCs viability test.

Viability test was done as following:

a) Two handerd µl of PBMCs were added to 200 µl of 0.4% of trypan blue solution. Then 50 µl of the mixture was transferred to hemocytometer slide and examined under light microscope. Cells taking up a blue stain are dead cells.

b) Calculating viability percentage as follows: Cell Viability = total Viable cells/ total cells x 100%. Cell suspension having more than 95% viability should be used for culture (Strober, 2015).

2.2.9 Cell cultivation and antigenic induction.

Peripheral blood mononuclear cells that were isolated from whole blood were re-suspended with density of $1x10^6$ /ml in RPMI 1640 complete medium supplemented with 1% of penicillin/ streptomycin and 10% of fetal bovine serum. Cell suspension was divided into five groups, each group involve (15) samples with three replicates (500 µl per well). The five groups were as following; group I (control without treatment) was PBMCs suspended in culture medium without any other components to serve as a control. Group II (bacteria 10%) contains PBMCs stimulated with bacterial somatic antigen only, group III (virus 10%) contains PBMCs stimulated with HBVvaccine only, group IV (of PBMCs pretreatment with HBV vaccine for 48 hr , then treated with the MRSA Ag, group V contains PBMCs stimulated with bacterial antigen plus HBV vaccine. All these groups were incubated in a 48- cell culture plates at 37ºC with 5%CO_2 for 48hrs. Fig(2-1)(Flow chat).

2.2.10.Immunological Parameters:

2.2.10.1 .Estimation of IFN-γ, IL-4and IL-5 by ELISA:

2.2.10.1.1.Principle:

The microtiter plate provided in this kit has been pre-coated with the specific antibody. The standard, samples and HRP conjugated antibody were added to the wells. After incubation and washing to remove the uncombined enzyme, add chromogen solution A and B. The color of the liquid will change into blue. At the effect of acid, the color finally becomes yellow. The color change is measured

spectrophotometrically at a wavelength of 450 nm. The concentration of Interleukin in the samples is then determined by comparing the O.D. of the samples to the standard curve.

2.2.10.1.2 Components of Kits:

Each kit of that mention above contains the following items:

a-Microelisa stripplate

b-Standards (1 set)

c-Sample diluent

d-HRP-Conjugate reagent

e-20X Wash solution

f-Chromogen Solution A

j-Chromogen Solution B

h-Stop Solution

i-Closure plate membrane

j-User manual

k-Sealed bags

2.2.10.1.3.Reagent preparation :

20x wash solution: Diluted with Distilled or deionized water 1:20.

2.2.10.1.4. Assay Procedure:

1. All reagents were prepared before starting assay procedure. It is recommended that all Standards and Samples be added in duplicate to the Microelisa Stripplate.

2. Added standard: Set Standard wells, tested sample wells. Added standard 50µl to standard wells.

3. Added Sample: Added tested sample 10µl .Then added sample diluent 40µl to testing sample well; Blank well didn't added anything. (The sample is diluted 5-fold in this step.)

4. Added 100µl of HRP-conjugate reagent to each well, covered with an adhesive strip and incubated for 60 minutes at 37°C.

5. Aspirate each well and wash, repeated the process four times for a total of five washed. Washed by filled each well with Washed Solution (400µl) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last washed, remove any remained Washed Solution by aspirated . Invert the plate and blot it against cleaned paper towelled.

6. Added chromogen solution A 50µl and chromogen solution B 50µl to each well. Gently mix and incubated for 15 minutes at 37°C. Protected from light.

7. Added 50µl Stop Solution to each well. The color in the wells was change from blue to yellow. If the color in the wells is green or the color change did not appear uniform, gently tap the plate to ensure thorough mixing.

8. Read the Optical Density (O.D.) at 450 nm using a microtiter plate reader within 15 minutes.

2.2.11. Quantitative real time PCR methods

2.211.1. Total RNA extraction from serum.

I. A volume of 1ml of TRIzol reagent was added directly to liquid samples.
II. Pipetting up and down was done by micropipette until no visible precipitate appeared in lysate.
III. Incubation was for 5 minutes at room temperature.

IV. A volume of 200µl of chloroform was added and shaked well by hand for 30 seconds then incubation for 3 minutes at room temperature.

V. Centrifugation at 10000xg done for 15 minutes at 2-8 C. After centrifugation, the mixture was separated into three layers: lower organic pink layer which contain proteins, pale white middle layer contains DNA and colorless upper layer contain total RNA.

VI. The upper colorless layer was transferred to a new fresh tube and 500 µl of isopropanol were added and mixed well by hand then incubated at room temperature for 10 minutes.

VII. Centrifugation at 10000xg done for 10 minutes at 2-8 °C. Then the supernatant discarded and the RNA can be seen as colloidal precipitate at the walls and bottom of tube.

VIII. A volume of 1 ml of 75% ethanol was added to the vortexing tube.

IX. Centrifugation at 7500xg was for 5 minutes at 2-8 ° C.

X. The supernatant was discarded and air-dried RNA pellet was for 5 minutes.

XI. RNA pellet dissolved in 50 µl of RNA dissolving solution, and incubated at 55- 60̊ C for 10 minutes. Then the RNA solution was stored at (-80 ° C).

2.2.11.2. Estimation of Total RNA Concentration and Purity:

The purity of samples was measured by UV/Visible spectrophotometer instrument by adding extracted RNA in the instrument. A260/280 ratios of pure RNA would usually be at 2.0.

2.2.11.3. Reference Gene Selection:

The reference gene or housekeeping gene or endogenous control gene selected by finding the best and the more stable reference gene expressed in the serum samples. The best reference gene depends on three parameters: high expression level; stable

and expressed among all samples; and then showing of converge expression level among all samples (Sauer *et al*., 2014).

2.2.11.4. Determination of FOXp3 and U6 reference gene Expression in Samples by one step RT-qPCR:

GoTaq 1-Step RT-qPCR System combines GoScrip Reverse Transcriptase and GoTaq qPCR Master Mix in a single-step real-time amplification reaction. The system, optimized for RT-qPCR, contains a proprietary fluorescent DNA binding dye, SybrGreen Dye. The system enables the detection of RNA expression levels using a one-step RT-qPCR method: • GoTaq® 1-Step RT-qPCR component, total RNA, primers and Nuclease-free water wre all thawed on ice and each solution was mixed well. • GoTaq® 1-Step RT-qPCR reaction was prepared, as shown in table (2-4). • RT-qPCR reactions were performed using the cycling program shown in table (2-5).

Table 2-4: GoTaq® 1-Step RT-qPCR Reaction Mix.

Component	Volume Final	Concentration
GoTaq® qPCR Master Mix, 2X	10 µl	1X
GoScript™ RT Mix for 1-Step RT-qPCR (50X)	0.4 µl	1X
Forward Primer (20X)	0.6 µl	300 nM
Reverse Primer (20X)	0.6 µl	300 nM
MgCl2	1.6 µl	25 mM
RNA template	5 µl	100 ng
Nuclease-Free Water	1.8 µl	-

Table 2-5: One-step RT-qPCR programs.

Step	Temperature	Duration	Cycles
Reverse transcription	37℃	15 min	1
RT inactivation/Hot-start activation	95℃	10 min	1
Denaturation	95℃	10 sec	50
Annealing	58℃	30 sec	
Extension and data collection	72℃	30 sec	

2.2.11.5. Calculating Gene Expression (Gene Fold):

There are two strategies for analyzing qPCR data: absolute and relative quantification. The absolute quantification identifies the input gene amount based on a standard curve which created by Livak and Schmittgen. In contrast, the relative quantification determines changes in gene expression relative to a reference genes sample which is accomplished by Pfaffl (Pfaffl, 2001).

Errors caused by standard dilutions when creating a standard curve can also be avoided.

In addition, sometimes the relative gene amount between two treatment groups is of more interest than exact DNA/RNA molecular numbers. Therefore, the relative quantification is widely performed.

Gene expression or gene fold or RQ (Relative quantification) value were calculated by Pfaffl equation (Pfaffl, 2001):

$$RQ = 2^{-(\Delta\Delta CT)}$$

The gene fold was calculated firstly by collecting CT (CT - cycle threshold) average value from real time PC device for each triplicated sample then ΔCT value was calculated for each sample as follows:

Δ CT = CT (gene of interest) – CT (reference gene)

ΔCT is the difference in CT values for the gene of interest and reference gene for a given sample. This is essential to normalize the gene of interest to a gene, which is not affected by experiment. Calculating ΔΔCT value is found as follows:

ΔΔ CT = Δ CT (treated sample) – Δ CT (untreated sample (control))

After calculating ΔΔ CT for all samples, the final equation is taken to calculate the gene expression (fold change) as follows:

Fold gene expression RQ = 2-(ΔΔCT)

Table (2-6):RT-PCRCycling Program

Genes	Primer sequence (5-3)	Reference
Foxp3-F	TGTGCTAGGGCGGTATGAGA	Kleine *etal*.,2010
Foxp3-R	GCTGGGGTGCAACTATGGG	

2.2.12. Statistical analysis:

All statistical calculation were performed by the using of SPSS software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. USA) and Microsoft Excel (2010, Microsoft Corp. USA). A $P < 0.05$ was considered statistically significant according to (Solé *et al*., 2006).

3.1 Testing groups:

The 75 samples of apparently health males human were cultured in RPMI1640 medium and then each sample were divided in to five groups, table (3-1).

Table (3-1):The testing groups in this study :

Testing groups	No.Males	Treatment
Group I	15	PBMCs only
Group II	15	PBMCs+treated with killed MRSA antigen.
Group III	15	PBMCs+ treated with hepatitis B virus vaccine
Group IV	15	PBMCs+pretreatment with HBV vaccine for 48hrs,then killed MRSA Ag added .
Group V	15	PBMCs+mix(MRSA Ag and HBV vaccine).

3.2. Peripheral blood mononuclear cells (PBMCs) isolation:

Human blood samples were used in this study was replicons in the experimental model of the five groups instead of animal lab because the human blood samples give an immune response closer to reality than laboratory animals , as well as to avoid biological waste of laboratory animals.

The PBMCs were isolated by density gradient medium (lymphoprep). The principle of PBMC isolation protocols by the centrifugal separation of blood components against a high-density medium, PBMCs can be separated from other components of the blood, such as Granulocytes , erythrocytes and plasma due to their higher density, erythrocytes, granulocytes and dead cells will pass through the lymphosep layer, whereas lymphocytes and monocytes, based on their lower density, will accumulate at the plasma-gradient boundary. This approach is concordant with the method for isolation of PBMC, developed by (Boyum in 1968).

This method of isolation is fast and easy comparable to the other methods of PBMCs isolation, due to its uncomplicated and robust feasibility, density gradient centrifugation is now ubiquitously applied to isolate PBMCs.

To evaluate the quality of the separation, Turbidity of the liquid may indicate suboptimal separation of the PBMCs. The PBMCs composed of monocytes and lymphocytes were isolated from between the plasma and red blood cells were cultured after washing twice with phosphate buffer saline. Aliquots of $1*10^6$ cells were incubated without antigen in 24-well tissue culture plates at a final volume of 500µl per well at 37ºC in the presence of 5% CO2 after counting by hemocytometer by aseptic technique to prevent contamination of cell cultures according to (Absher,1973), the expected yield for mononuclear cells falls between 0.8 and $3.2*10^6$ cells/mL of blood, result falls with this expected range at 1and $2*10^6$ cells/mL in order to achieve the final concentration of $1*10^6$ for all samples in each well the samples were diluted using RPMI medium. In order to confirm the quality of the PBMC isolation using lymphosep, the PBMC samples were subjected to an analysis of their yield and viability. This techniques had been shown a cell viability rating of $\leq$ 95% immediately after isolation had taken place and this result was achieved by trypan blue test to estimate the availability of PBMCs before and after Ag addition .

3.3. Viability of Peripheral blood mononuclear cells (PBMCs) by trypan blue exclusion test:

The result of viability immediately after isolation was 95% and viability was within expected ranges that agree with (Chen *et al*.,2020) that had (96.6%) viability result.Trypan blue exclusion test for viability is based on the principle that living cells possess intact cell membranes that exclude certain dyes, such as trypan blue, or

eosin whereas dead cells do not. In this test, a cell suspension is mixed with dye and then visually examined to determine whether cells take up or exclude the dye (Strober,2015). In this test, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

3.4 .Immunological parameters:

3.4.1. Cytokines:

3.4.1.1. Interferon Gamma (IFN- γ) levels:

IFN-γ as a TH1 response marker was estimated by ELISA technigue. the IFN-γ concentration results pointed a significant elevation ($p<0.05$) the mean value of IFN-gamma in group III , IV ,II and V (133.6504 pg/ml, 132.0271 pg/ml,125.9995 pg/ml, 96.087 pg/ml respectively)as compared with control group (49.6203 pg/ml).

The highest concentration was estimated in group III which involves PBMCs treated with HBV antigenic vaccine . The level of this cytokine in Ag stimulated group was increased significant in comparison to control group as in table (3-2).

The findings of this study corroborated with (Ren *et al*., 2015), who found that levels of IFN- substantially increased at the MRSA Ag dose compared to the control group . The findings of this study were also consistent with those of Su *et al*., (2014) who revealed that oral administration of bacterial antigens caused IFN-γ to be produced, and that this was important for strengthening the immunological defenses against food and mouth illness. IFN-γ, which is, generated by immune cells that both influence innate immune responses and adaptive immunological responses. is a crucial cytokine for immunity.

Table (3-2) Concentrations of IFN-γ levels in study groups.

Study group*	Concentration (Pg/ ml)	P value * *
Group I	49.6023 ± 3.8366	< 0.05
Group II	125.9995 ± 8.3259	
Group III	133.6504 ± 5.7239	
Group IV	132.0271 ± 4.9892	
Group V	96.0878 ± 1.0341	

*Group I (without treatment),Group II treated with MRSA somatic antigens only , Group III treated with HBV antigen only , Group IV pretreated with HBV then treated with MRSA antigens , and group V treated with mixed of HBV antigen and MRSA somatic antigen

**significant differences between treated group with untreated (group I)

Cytokines are soluble glycoproteins formed by cells used for development and differentiation as well as regulators of host responses to immune responses, inflammation, infection and trauma. Furthermore, cytokines also well-known as a tool for message between cells and do not have a specific goal. Actions of cytokines can autocrine or paracrine. Production and activity of cytokines will affect other cytokines, so it will form a complex cytokine system (Prayitno *et al*., 2014). The IFN-γ is an essential cytokine for immunity that produced by cells that mediate both innate and adaptive immune responses. NK cells are the innate cells source of this factor and rabidly produce IFN-γ upon activation.

The innate cells that create this factor are NK cells, which, when stimulated, ferociously produce IFN. Only following Th1 differentiation, in which this cytokine released by Th1 cells triggers cell-mediated and immune-inflammatory reactions, is

IFN-expression by CD+4 T-cells significantly increased (Araújo-souza *et al.*, 2015). Additionally, IFN- promotes the expression of adhesion molecules and chemokines, which helps to guide leukocytes to the infection sites; some lactobacilli strains shifted the Th1/Th2 balance in favor of (Ghadimi *et al.*,2010) , and IFN- γ had activity in suppressing the B-cell differentiation process (Aden and Rifa, 2014).

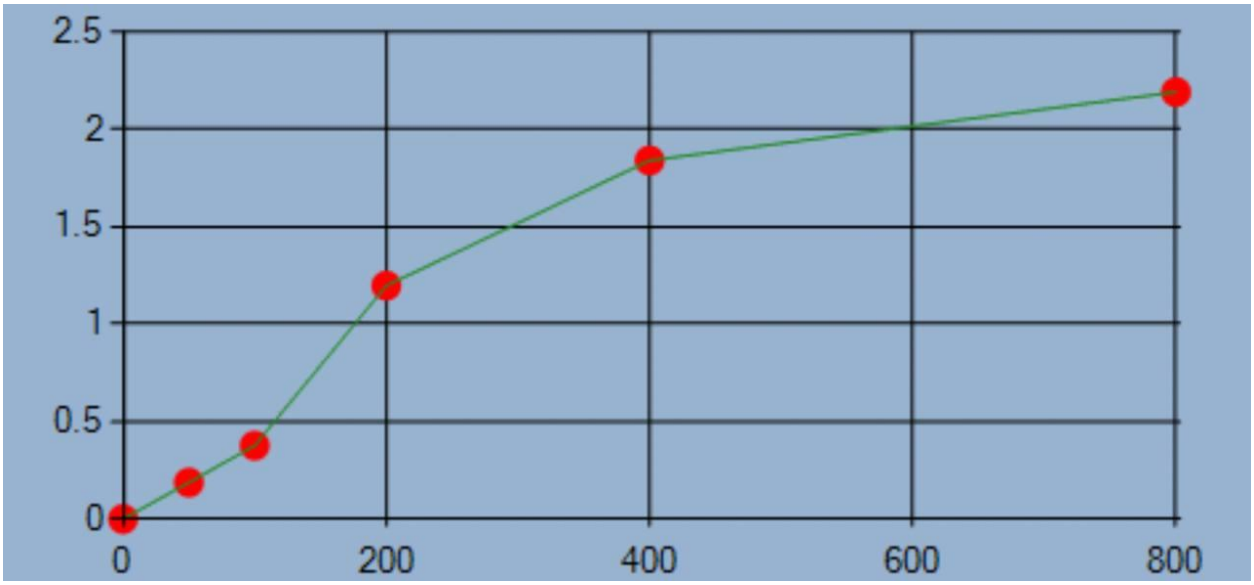
Most of the therapeutic HBV vaccines designed to date have used envelope proteins as the target antigen to develop both the prevention and treatment of hepatitis B. Th2 immune response, represented by IL-4 cytokine, just raises humoral immune response neutralizing new attacking and/or viruses and does not eradicate previously infected cells with HBV. In contrast, Th1 response, represented by IFN-γ raising cytotoxic response, targeting, and killing infected cells with HBV, is required for clearance of HBV infection (Zoulim and Locarnini,2009). IFN-γ cytokine is an important cytokine in the induction of immune responses against HBsAg. A number of healthy individuals, hemodialysis patients and hepatitis B carrier mothers' neonates could not respond to hepatitis B vaccine (Struve *etal*.,1994). The mechanism causing non-responsiveness to hepatitis B vaccines in humans is still unknown. However, another study revealed that the content of IFN-γ produced by PBMCs after PHA and HBsAg stimulation was obviously lower in non-responders compared with responder individuals. Therefore, Immunologic non-responsiveness to HBsAg after vaccination might be related to IFN-γ hypo-secretion in PBMCs (Li *etal*.,2011) and induction of IFN-γ response against HBsAg may abrogate the un-responsiveness in these populations.

In our result, the level of this cytokine in Ag stimulated group (III, II, IV and V) was increased significantly in comparison to control group with ($P<0.05$), this result was in agreement with Dezfuli *et al*.,(2014) who Administration of melittin and

HBV vaccine had no effect on lymphoproliferation and total antibody responses, but increased IFN-γ response and induced Th1 response.

IFN-γ is a type 1 cytokine that is involved in both innate and acquired antimicrobial immune regulation (Touma *et al*.,2021). A series of complex interactions between accessory cells including macrophages and dendritic cells, as well as T lymphocytes and natural killer (NK) cells, regulates the expression of IFN-γ. A complex interaction between immune cell activity and IFN- γ through organized integration of signals from other cytokines and Pattern Recognition Receptors (PRRs) such as Interleukin (IL-4, Lipopolysaccharide, TNF-α and Type-I Interferons) create a cascade of proinflammatory responses (Naglak *et al*., 2016).

This factor is produced by NK cells, which are innate cells that produce IFN-γ immediately upon stimulation (Kak *et al.*, 2018). IFN-γ also assists in the recruitment of leukocytes to infection sites by increasing the expression of adhesion molecules and chemokines and it was found to be effective in preventing B-cell differentiation (Day *et al*., 2017). (IFN-γ) has been shown to stimulate cells and potentiate the effect of LPS. When IFN-γ binds to its receptor, Janus kinases (Jak1 and Jak2) in the cytoplasm become activated, phosphorylating specific tyrosine residues on signal transducer and activator of transcription (STAT)-1 molecules, STAT1α forms homodimers, translocates to the nucleus, and binds unique STAT-binding DNA sequences when activated and phosphorylated leading to the transcription of a significant portion of IFN-γ induced genes.



Figure(3-1) standard curve of IFN-γ estimation .

3.4.1.2. Interleukin-5 (IL-5) levels:

IL-5 as a Th2 response marker was estimated by ELISA technique **.** the results indicated to a significant elevation of IL-5 level in all groups (IV,III,II and V), (10.3023 pg/mL, 9.9415 pg/mL,9.8520 pg/mL and 9.6087 pg/mL respectively) as compared with control group (I: is untreated group) (4.0469 pg/mL), that is illustrate in table(3-3).

The highest concentration of was IL-5recorded in the group IV 10.3023 Pg/ml which involves PBMCs pretreated with HBV then treated with MRSA antigens .

The obtaiend results in current study were in agreement with (Foreman *etal* .,2011) who stated a significantly higher in IL-5 in *S. aureus* in chronic rhinosinusitis (CRS) patient ,*S. aureus* biofilms and superantigens are significantly associated in CRS patients, suggesting the biofilm may be a nidus for superantigen-eluting bacteria.

Table (3-3) Concentrations of IL-5 levels in study groups.

Study group*	Concentration (Pg/ ml)	P value * *
Group I	4.0469 ± 0.88277	< 0.05
Group II	9.8520 ± 0.78714	
Group III	9.9415 ± 1.07763	
Group IV	10.3023 ± 2.26596	
Group V	9.6087 ± 1.03417	

*Group I (without treatment),Group II treated with MRSA somatic antigens only , Group III treated with HBV antigen only , Group IV pretreated with HBV then treated with MRSA antigens , and group V treated with mixed of HBV antigen and MRSA somatic antigen

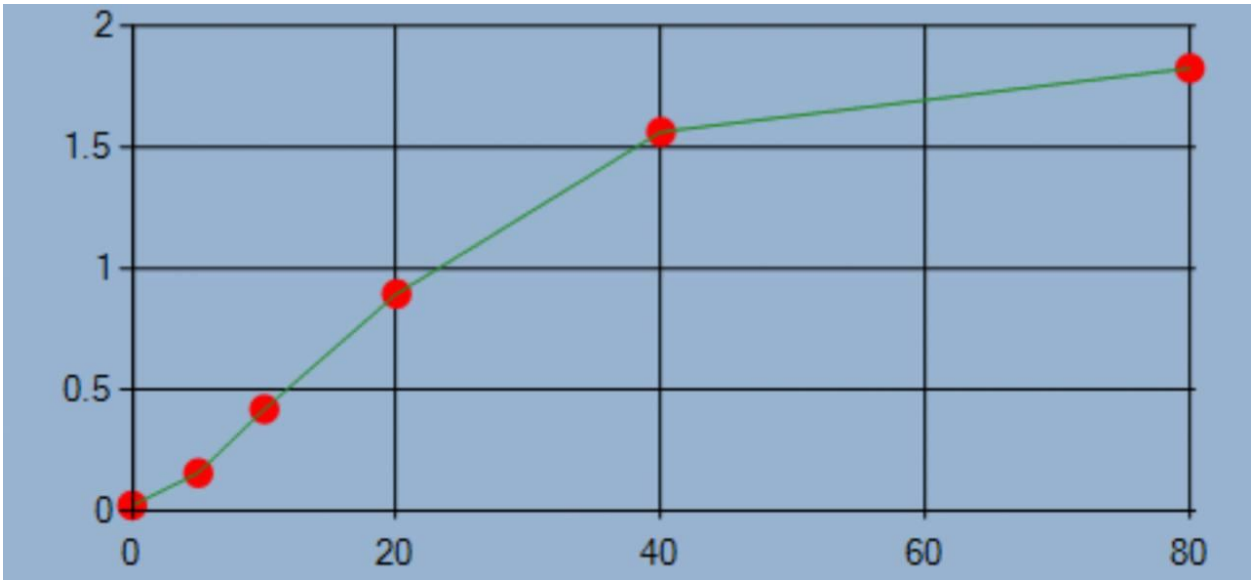
*significant differences between treated group with untreated (group I)

The presence of *S. aureus* biofilms is associated with eosinophilic inflammation, across the spectrum of CRS, on the back of a T-helper2 skewing of the host's adaptive immune response (elevated Eosinophilic Cationic Protein and IL-5). This can be distinguished from the superantigenic effect resulting in the induction of IgE (foreman *etal*.,2011) .On the other hand, the increasing of IL-5 by bacteria in the present study agreement with Heaton *etal.*, (2003) who mentioned Staphylococcal enterotoxin B selectively stimulates the production of interleukin-5 (IL)-5 in atopic eczema/dermatitis syndrome (AEDS) sufferers.

The obtaiend results in current study were in agreement with Wang *etal*., (2023) who stated a serum IL-5 levels increased remarkably in cured group throughout the course of treatment and had a positive correlation with HBsAg seroclarance. IL-5 was originally defined as a T-cell-derived cytokine, mainly produced by activated

Th2 cells, that triggers activated B-1 and B-2 cells for terminal differentiation into antibody-secreting plasma cells (Mita *etal*.,1989). IL-5 receptor (IL-5R) comprises α and βc chains. IL-5 specifically binds to IL-5Rα and induces the recruitment of βc to IL-5Rα (Ogata *etal*.,1998). B-1 cells constitutively express the IL-5Rα and give rise to Ab-producing cells in response to IL-5 stimulation (Kantor and Herzenberg,1993).

IL-5, mainly produced by T helper-2 (Th2) lymphocytes, can increase antibody secretion by promoting the differentiation and growth of B cells and enhancing the humoral immune response mediated by Th2 cells. It was also associated with viral response and HBeAg seroconversion after entecavir (ETV) therapy in CHB patients, suggesting serum levels of IL-5 may be an available marker to predict responses to anti-HBV therapy (Badary *etal.*, 2018).



(Figure 3-2) standard curve of IL-5 estimation .

3.4.1.3. Interleukin-4 (IL-4) concentration:

IL-4 as a TH2 response marker was estimated by ELISA technique **.**the results indicated to a significant increasing of IL-4 level in all study groups (V,IV,III and II), (8.1033 pg/mL, 7.7351 pg/mL,7.6809pg/mL and 7.6170 pg/mL respectively) as compared with control group (group I : is untreated group) (3.6568 pg/mL), that is illustrate in table (3-4).

The highest concentration of was IL-4 recorded in the groupV 8.1033 Pg/ml which involves PBMCs treated with mixed of HBV antigen and MRSA somatic antigen .

Table (3-4) Concentrations of IL-4 levels in study groups.

Studu group*	Concentration (Pg/ ml)	P value * *
Group I	3.6568 ± 0.79002	< 0.05
Group II	7.6170 ± 1.07618	
Group III	7.6809 ± 0.79862	
Group IV	7.7351 ± 0.73591	
Group V	8.1033 ± 1.46144	

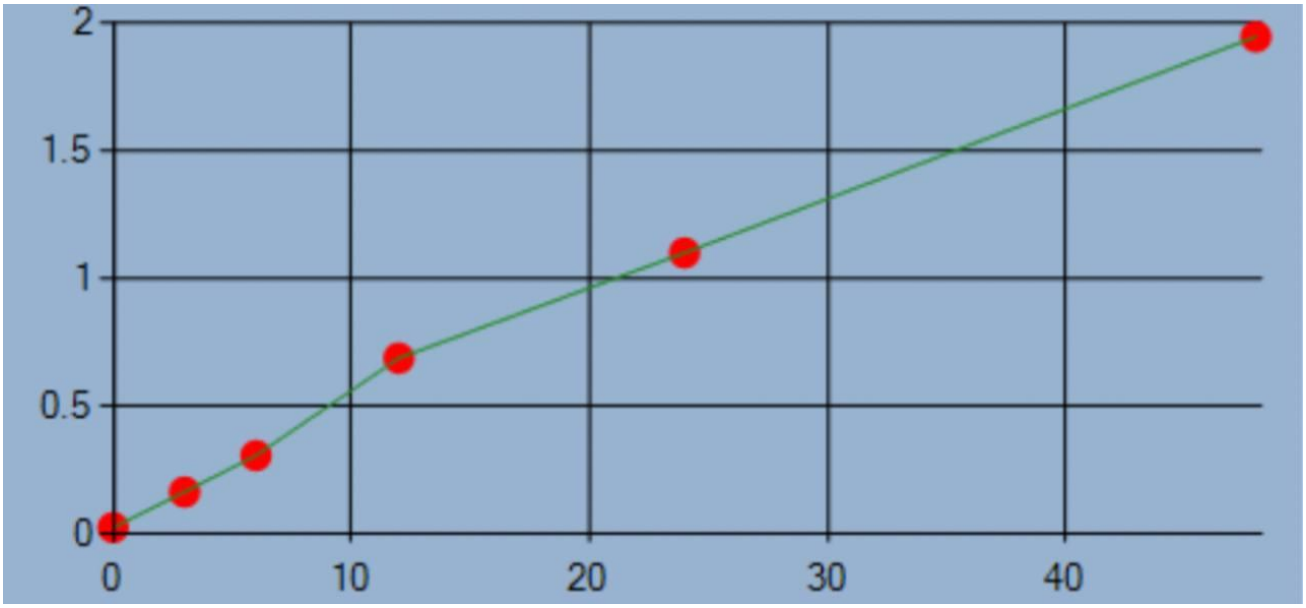
*Group I (without treatment),Group II treated with MRSA somatic antigens only , Group III treated with HBV antigen only , Group IV pretreated with HBV then treated with MRSA antigens , and group V treated with mixed of HBV antigen and MRSA somatic antigen

**significant differences between treated group with untreated (group I)

The obtained results in current study were in agreement with (Dezfuli *etal*.,2014) who stated a significant increase in IL-4 release was found in animals immunized with HBsAg vaccine or HBsAg vaccine plus 2.5µg, 5µg, or 10µg melittin in comparison to PBS and melittin control groups (P<0.045). In addition, IL-4 response in animals immunized with HBsAg vaccine plus 2.5µg, 5µg, or 10µg melittin was lower than the HBsAg vaccine-immunized animals. However, only the difference between HBsAg vaccine + 2.5µg melittin- and HBsAg vaccine-immunized mice was statistically significant (P=0.004).

Cellular immune response to a pathogen is mediated through the processing and presentation of antigen on the surface via major histocompatibility complex (MHC). The exogenous antigens are processed through lysosome and presented by MHC class II. The loaded peptide on MHC class II interacts with CD4+ T cells and a pattern of cytokine is synthesized and secreted. Depending upon the cytokines secreted, the T-helper cells polarize into diverse T-cell populations like Th1, Th2, Th17, or iTregs (Gutcher and Becher,2007) . In Th2 cell population, interleukin-4 is the major cytokine secreted. IL4 had been shown to play a critical role in diverse biological activities. This cytokine promotes the proliferation and differentiation of antigen presenting cells(Brown and Hural,1997) . IL4 also plays a pivotal role in antibody isotype switching and stimulates the production of IgE. This cytokine has been applied in the treatment of autoimmune disorder like multiple myeloma (Röcken *etal*.,1996), cancer (Kajiwara,2012), psoriasis (Ghoreschi *etal*.,2003) and arthritis(Lubberts *etal*.,2000) . IL4 has also been extensively applied to inhibit detrimental effect of Th1 (Biedermann *etal*.,2001).Hence, for the rational development of better immunotherapy/vaccines to provide protection against infection, it is pivotal to assess the immune response generated by these antigens.

The Th2 response is very important in vaccine or immunotherapy design against extracellular pathogen. IL4 is the principal cytokine that directs commitment of T cells to Th2 phenotype (Abbas *etal*.,1996).



(Figure 3-3) standard curve of IL-4 estimation . .

3.5. Genetic parameter :

3.5.1. Foxp3 gene expression:

The relative expression of the FoxP3 gene in volunteer PBMCs samples was estimated depending on Livak Method 2-(ΔΔCT) which is based on the normalization of RT-qPCR (CT values) of tested genes with (GAPDH) as reference gene in control and treatment groups, table (3-5) .

The results of relative gene expression in the FoxP3 gene showed that the mean concentration of Foxp3 gene expression level were significantly highly increased in groupV (6.23) as compared to control group(1.00), II (1.59), III(2.01)and IV(4.31),

and also the mean concentration level of Foxp3 gene expression was significantly increased in groups (II, III,IV,V) respectively as compared to control group .

Table (3-5): Relative Foxp3 gene expression and folding:

Samples	U6 average	Foxp3 average Ct	ΔcT	2-(ΔΔCT)	Average fold change
Control 1	15.85	31.47	15.62	0.81-	1.00
Control 2	16.04	30.04	14	2.43-	
Control 3	18.13	35.98	17.85	1.42	
Control 4	16.66	35.81	19.15	2.72	
Control 5	19.02	36.26	17.24	0.81	
Control 6	14.96	30.07	15.11	1.32-	
Control 7	16,06	32.24	16.18	0.25-	
Control 8	15.96	31.23	15.27	1.16-	
Control 9	16.97	33.22	16.25	0.18-	
Control 10	18.56	33.84	15.28	1.15-	
Control 11	18.54	35.36	16.82	0.39	
Control 12	16.66	35.81	19.15	2.72	
Control 13	19.22	36.11	16.89	0.46	
Control 14	15.24	30.69	15.45	0.98-	
Control 15	17.25	33.44	16.19	0.24-	
II	16.17	33.2	16.49	0.06	1.59
II	17.11	34.1	16.99	0.56	
II	16.4	32.89	16.49	0.06	
II	17.17	32.61	15.44	0.99-	

II	**17.3**	**32.73**	**15.43**	**1-**	
II	**22.57**	**37.63**	**15.06**	**1.37-**	
II	**17.01**	**32.6**	**15.59**	**0.84-**	
II	**19.32**	**33.99**	**14.67**	**1.76-**	
II	**15.57**	**30.96**	**15.39**	**1.04-**	
II	**17.71**	**32.45**	**14.74**	**1.69-**	
II	**24.57**	**40.44**	**15.87**	**0.56-**	
II	**20.07**	**35.64**	**15.57**	**0.86-**	
II	**21.26**	**37.27**	**16.01**	**0.42-**	
II	**17.8**	**33.08**	**15.28**	**1.15-**	
II	**21.34**	**38.78**	**17.44**	**1.01**	
III	**21.34**	**36.69**	**15.35**	**1.08-**	**2.01**
III	**19.65**	**35.55**	**15.9**	**0.53-**	
III	**17.87**	**34.12**	**16.25**	**0.18-**	
III	**18.06**	**33.69**	**15.63**	**0.8-**	
III	**20.15**	**34.63**	**14.48**	**1.95-**	
III	**18.68**	**33.46**	**14.78**	**1.65-**	
III	**21.02**	**36.91**	**15.89**	**0.54-**	
III	**16.98**	**32.72**	**15.74**	**0.69-**	
III	**18.08**	**33.89**	**15.81**	**0.62-**	
III	**17.98**	**32.88**	**14.9**	**1.53-**	
III	**18.99**	**34.87**	**15.88**	**0.55-**	
III	**21.58**	**35.49**	**13.91**	**2.52-**	
III	**21.54**	**36.54**	**15**	**1.43-**	
III	**23.04**	**38.74**	**15.7**	**0.73-**	

III	**22.23**	**38.37**	**16.14**	**0.29-**	
IV	**21.56**	**35.55**	**13.99**	**2.44-**	
IV	**21.24**	**35.65**	**14.41**	**2.02-**	**4.31**
IV	**18.61**	**33.21**	**14.6**	**1.83-**	
IV	**19.65**	**34.03**	**14.38**	**2.05-**	
IV	**21.74**	**36.32**	**14.58**	**1.85-**	
IV	**20.27**	**34.57**	**14.3**	**2.13-**	
IV	**21.21**	**35.12**	**13.91**	**2.52-**	
IV	**18.57**	**33.04**	**14.47**	**1.96-**	
IV	**19.67**	**34.04**	**14.37**	**2.06-**	
IV	**20.93**	**35.37**	**14.44**	**1.99-**	
IV	**19.24**	**33.11**	**13.87**	**2.56-**	
IV	**20.51**	**35.21**	**14.7**	**1.73-**	
IV	**22.25**	**36.06**	**13.81**	**2.62-**	
IV	**22.63**	**37.33**	**14.7**	**1.73-**	
IV	**23.82**	**38.14**	**14.32**	**2.11-**	
V	**22.56**	**36.55**	**13.99**	**2.44-**	**6.23**
V	**23.32**	**37.2**	**13.88**	**2.55-**	
V	**17.62**	**31.85**	**14.23**	**2.2-**	
V	**18.66**	**32.36**	**13.7**	**2.73-**	
V	**20.75**	**34.55**	**13.8**	**2.63-**	
V	**19.28**	**33.24**	**13.96**	**2.47-**	
V	**20.22**	**34.21**	**13.99**	**2.44-**	
V	**17.58**	**31.32**	**13.74**	**2.69-**	
V	**18.68**	**32.12**	**13.44**	**2.99-**	

V	**19.94**	**34.1**	**14.16**	**2.27-**
V	**18.25**	**32.01**	**13.76**	**2.67-**
V	**19.52**	**33.12**	**13.6**	**2.83-**
V	**21.26**	**34.99**	**13.73**	**2.7-**
V	**22.64**	**36.22**	**13.58**	**2.85-**
V	**21.91**	**35.22**	**13.31**	**3.12-**

*Group I (without treatment),Group II treated with MRSA somatic antigens only , Group III treated with HBV antigen only , Group IV pretreated with HBV then treated with MRSA antigens , and group V treated with mixed of HBV antigen and MRSA somatic antigen .

Forkhead box P3 Protein (Foxp3) is a regulatory T cell transcription factor that plays an important function in the body's immune system balance. Foxp3+ regulatory T (Treg) cells have pleiotropic immune-regulatory functions that are important for immunological homeostasis, autoimmunity prevention and the regulation of pathogen-induced inflammatory responses. The transcription factor Foxp3 controls Treg cell development, differentiation and function, T-cell receptor (TCR) signaling plays central roles in Treg differentiation and Foxp3-mediated gene regulation. Differentiating Treg will have recognized their cognate antigens and received TCR signals before initiating Foxp3 transcription, which is triggered by TCR-induced transcription factors including NFAT, AP-1 and NF-κB.

Once expressed, Foxp3 seizes TCR signal induced transcriptional and epigenetic mechanisms through interacting with AML1/Runx1 and NFAT. Thus, Foxp3 modifies gene expression dynamics of TCR induced genes, which constitute cardinal mechanisms for Treg-mediated immune suppression.

The deficiency in the Foxp3 gene results in hyper-activation of CD4+ T cells, overproduction of pro-inflammatory cytokines, and massive multi-organ

pathology. Regulatory T lymphocytes, (Tregs) that express, FOXP3 are involved in the beneficial attenuation of immune-pathology but are also implicated in the down-regulation of protective responses to infection (Sanz-Rubio *et al*.,2020). FOXP3 has been shown to have a direct role in inducing immune-suppression and has been identified as a good marker for cells with a suppressor function. In humans these cells were first thought to be specifically CD4+ CD25 high naturally occurring Tregs, but more studies have shown this not to be the case and FOXP3 is also expressed in other cells (such as CD8+) with a suppressor function (Morgan *et al*., 2005).

Via Foxp3 induction, TGF-1β priming was able to promote Treg cell differentiation from non-regulatory CD4+CD25–Tcells in a concentration-dependent manner. Following TCR activation, T cells never showed any regulatory functions or major Foxp3 expression.

Freshly isolated CD4+CD25–CD45RBLOW cells, on the other hand, were unable to suppress CD4+ effector T cell proliferation while expressing low levels of Foxp3 mRNA and protein, but acquired regulatory activity and de novo Foxp3 expression after TGF-1 exposure. TGF-β1 can also induce the conversion of CD4+ T cells into Treg cells by inducing FOXP3 expression (Zhu *et al*.,2020), as well as increasing the expression of other important Treg cell markers including CD25, CD122, IL-2 and CTLA-4 (Zheng *et al*., 2014).In autoimmune diseases, in vivo Treg expansion appears to be a good therap eutic option, and several studies have shown that treatments like IL2 administratio n are successful (Sakaguchi *et al*., 2020). As a result finding compounds, of natural resources that modulate Treg function is critical for preventing autoimmune or pathogen induced inflammatory diseases. In this study, The results of relative gene expression in the ($p<0.05$) FoxP3 gene showed that the mean concentration of Foxp3

gene expression level were significantly increased in Group II,Group III,Group IV,Group V respectively as compared to group I, that is illustrate in table(3-6).

Foxp3 (fork head box P3 protein) gene expression concentration expressed in table (3-6) showed a a significantly immunomodulation effect on Foxp3 mRNA gene expression in the mean value of the treated groups (II,IIl, IV ,V respectively) (1.7980, 2.2468, 4.3950, 6.3178) after 48 hours of induction in comparison to group I.

The high significantly immunomodulation effect on Foxp3 mRNA gene expression on PBMCs stimulated with group V by increasing its level after 48 hours of induction in comparison toGroup I.

The most precise molecular indicator of natural Tregs (nTregs), FOXP3, is associated with the immunosuppressive function of CD4+CD25+ Tregs. For CD4+CD25+ Tregs to mature and operate, FOXP3 expression is necessary. Peripheral Tregs, specifically CD4+CD25+ FOXP3+ Tregs, closely control B and T cell responses as well as auto-reactive responses.

The most well-known immune cell type, FOXP3+ Tregs have the most potent inhibitory mechanism and a wide range of inhibitory targets.

The prevalence of autoimmune and allergy illnesses is decreased, and FOXP3+ Tregs have anti-inflammatory capabilities (Agarwal *etal.*,2014). The most significant immunosuppressive cells are without a doubt regulatory T cells (Treg), which express the transcription factor FOXP (forkhead box protein) 3. For the preservation and proper operation of Treg, the transcription factor FOXP3 is essential. It determines lineage during development.(Hori *etal.*,2003) Their proper growth and operation are essential for building peripheral (self-)tolerance and,

consequently, for avoiding autoimmune disorders and immunological reactions to benign antigens or commensals.

Table (3-6) Concentrations of FOXP3 gene expression levels in study groups.

Study group*	Concentration (Pg/ ml)	P value * *
Group I	0.4871 ± 0.02828	< 0.05
Group II	1.7980 ± 0.35256	
Group III	2.2468 ± 1.25055	
Group IV	4.3950 ± 0.94532	
Group V	6.3178 ± 1.11790	

*Group I (without treatment),Group II treated with MRSA somatic antigens only , Group III treated with HBV antigen only , Group IV pretreated with HBV then treated with MRSA antigens , and group V treated with mixed of HBV antigen and MRSA somatic antigen . **significant differences between treated group with untreated (group I).

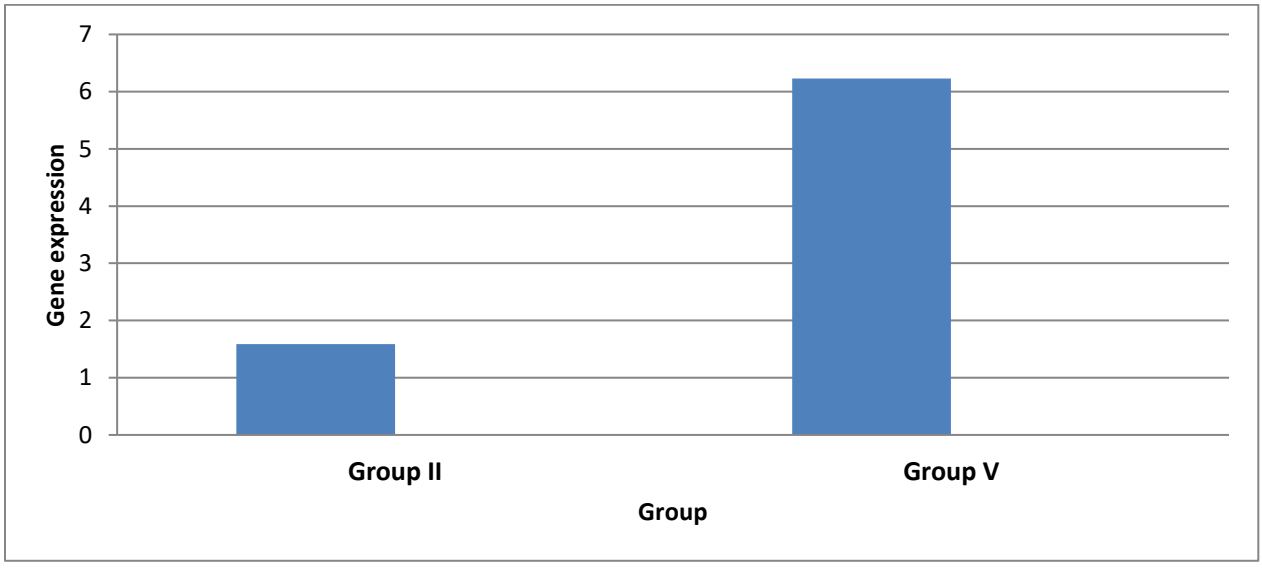
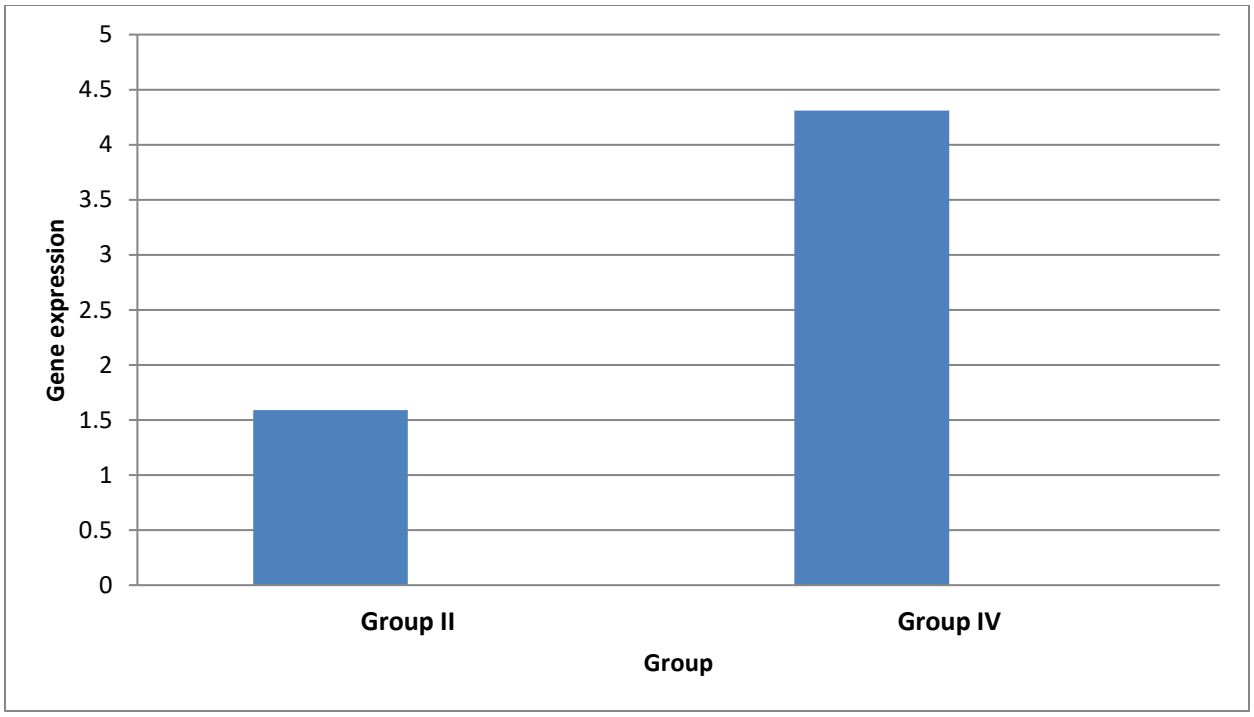
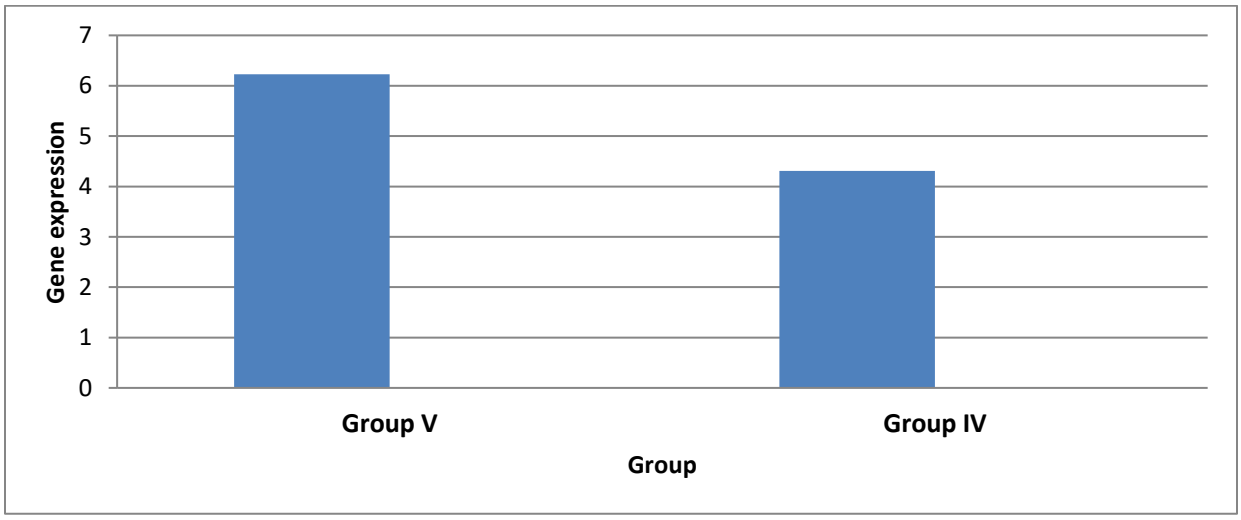


Figure (3-4) The correlation between Group II treated with MRSA somatic antigens only and group V treated with mixed of HBV antigen and MRSA somatic antigen.



Figure(3-5) The correlation between Group II treated with MRSA somatic antigens only and Group IV pretreated with HBV then treated with MRSA antigens.



Figure(3-6) The correlation between group V treated with mixed of (HBV antigen and MRSA somatic antigen).And Group IV pretreated with HBV then treated with MRSA antigens.

Table (3-7) The correlation between study groups

Correlations						
		Group II	**Group III**	**Group IV**	**Group V**	**Group I**
II	Pearson Correlation	1	.106	.289	.475	.05*
	Sig. (2-tailed)		.708	.297	.074	.804
	No.	15	15	15	15	15
III	Pearson Correlation	.106	1	.564*	-.112	-.03*
	Sig. (2-tailed)	.708		.029	.690	.520
	No.	15	15	15	15	15
IV	Pearson Correlation	.289	.564*	1	.085	-.042*
	Sig. (2-tailed)	.297	.029		.762	.483
	No.	15	15	15	15	15
V	Pearson Correlation	.475	-.112	.085	1	.003*
	Sig. (2-tailed)	.074	.690	.762		.325
	No.	15	15	15	15	15
I	Pearson Correlation	.05*	-0.03*	-.042*	.003*	1
	Sig. (2-tailed)	.804	.520	.483	.325	
	No.	15	15	15	15	15
*. Correlation is significant at the 0.05 level (2-tailed).						

*Group I (without treatment),Group II treated with MRSA somatic antigens only , Group III treated with HBV antigen only , Group IV pretreated with HBV then treated with MRSA antigens , and group V treated with mixed of HBV antigen and MRSA somatic antigen

Treg regulate immune responses to infections or sterile inflammatory illnesses to curb overreacting immune responses, (re-)establish immunological homeostasis, and guard against needless tissue damage. Treg can also aid in tissue healing.Because self-tolerance breaks broken, a variety of multiorgan autoinflammatory disorders are caused by Treg that are either under- or over-produced.(Ferreira *etal.*,2019) .

The helper T cell family include Th1, Th2, regulatory T (Treg) and Th17 cells. Developments in immunology and molecular biology have revealed that asthma is

not only associated with the imbalance of Th1/Th2 function (Deng *etal*.,2011) but also with Tregs, since imbalances in forkhead transcription factor P3 (FOXP3)+ Treg/Th17 and Th2/FOXP3+ Treg cells lead to asthma (Agarwal etal.,2014).Tregs are a subset of CD4+ T cells that play an essential role in maintaining peripheral immune tolerance and controlling allergic diseases. Tregs, together with effector T cells (Teffs), cytokines, immune antibodies and other cellular components, play an important role in maintaining immune balance (Swamy *etal.*,2012). As important immunosuppressive cells, CD4+CD25+ Tregs act in cell-cell contact-dependent inhibition patterns and ultimately inhibit immune diseases by inhibiting helper T cell activation and differentiation, and directly inhibiting B cell activation to produce antibodies (Yeh *etal*.,2013). FOXP3 expression is regulated by DNA methylation, histone modifications and posttranscriptional modifications (Liu *etal.*,2017). The epigenetic regulation and methylation of FOXP3 play an important role in its stable expression (Wieczorek *etal*.,2009). Changes in the methylation level of the FOXP3 gene may affect Treg differentiation and regulate the occurrence of an immune response. Thus, detecting the methylation statuses of upstream enhancers of FOXP3 may help in the diagnosis and subtype classifications of diseases (Yang *etal*.,2016).

Furthermore, down regulation of FOXP3 expression can impair Tregs' ability to protect against infection and tumors (Khan and Ghazanfar, 2018).

One of the aim of this study is to investigate the effect of a viral vaccine , which is commonly used in health institutions, on the immune response to other microbes such as bacteria based on the phenomenon of Ag competition , which implies that the immune response to a specific Ag may cause suppression enhancement , or ineffectiveness of the immune response to an other antigen . thus in this study, the effect of HBV Ag on immune response against MRSA is enhancement of this response and neither massive nor inhibit to my effect were detected.

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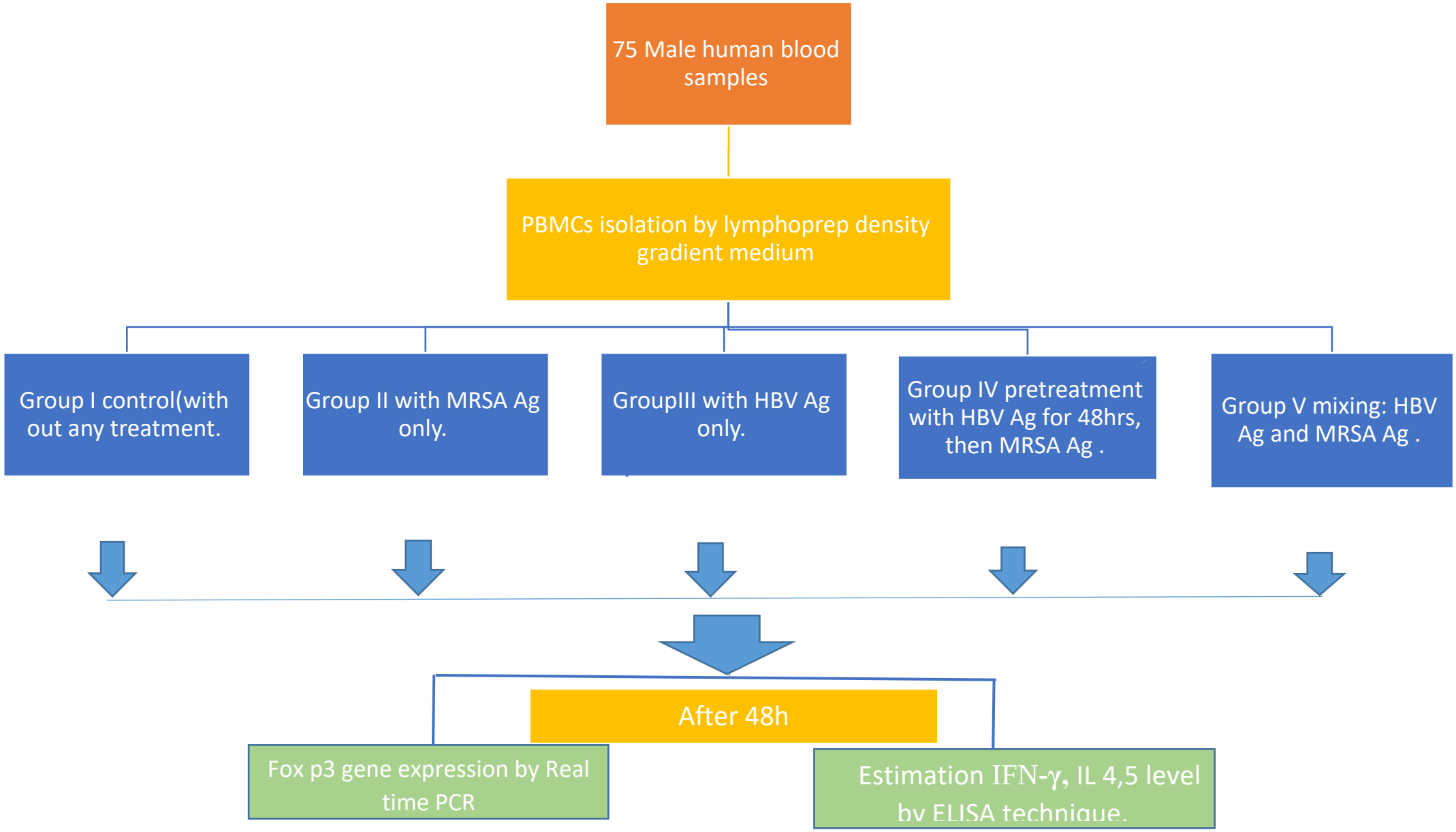

Figure (2-1) Schematic flow chat of the study procedures