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The Immune Protective Effect of Flagellin b as a Candidate Vaccine against *Pseudomonas aeruginosa* Respiratory Infections

A Thesis

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بسم الله الرحمن الرحيم

﴿وما توفيقي الا بالله عليه توكلت وإليه أنيب﴾

صَدَقَ ٱللهُ العَلِيُّ ٱلعَظِيمْ

هود / اية ٨٨

Dedication

I dedicate this work

To the Prophet of Mercy
***Mohammed** and **his household** (peace be upon them)*

To the Savior of Humanity
***Al-Imam Al-Mahdi** (Allah hasten his reappearance)*

To my first teachers
My father** and **My mother

To the source of my joy
My wife,
My son,** and **My daughter

To the dears
My brothers** and **sisters

To everyone I care

Mohammed Jaafar, 2022

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Summary

This case-control study aimed to investigate active immunization strategies using flagellin purified from *Pseudomonas aeruginosa* in order to provide complete protection against extensive drug resistance (XDR) *Pseudomonas aeruginosa* acute pulmonary infection in the rat model.

After *Pseudomonas aeruginosa* isolates identification was confirmed using standard tests, and VITEK 2 system, a Polymerase chain reaction (PCR) assay was used for detection of flagellin type by amplification of *flic* gene

The flagellin a and b were partially purified from *Pseudomonas aeruginosa* isolate no.23 and no.3 (respectively) using 70% ammonium sulfate and a special ultrafiltration tube (30KDa filter size) and the endotoxin (LPS) was removed using a special endotoxin removal spin column, and its concentration was measured by a LAL chromogenic assay.

The concentrations of flagellin-b and flagellin-a were measured after endotoxin removal by UV-visible spectrophotometer and the purified flagellin was detected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The animal groups were divided into flagellin-a immunized groups (A1 without infection and A2 with *Pseudomonas aeruginosa* MJ isolate infection), flagellin-b immunized groups (B1 without infection and B2 with *Pseudomonas aeruginosa* MJ isolate infection), and non-immunized groups (C1 without infection and C2 with *Pseudomonas aeruginosa* MJ isolate infection).

Blood samples were collected from all animals by heart puncture under anesthesia for hematological and immunological examination. The hematological examination was done to calculate the total lymphocytes by detection of complete blood count (CBC). The immunological examination was done for immune response tracking among animal groups by measuring total T cells, Th17 cells, Th1, Th2, total B cells, and IgM+ B cells, using specific markers by flow cytometry technique.

The results showed that the flagellin-b and flagellin-a concentrations were 4.8 mg/ml and 3.2 mg/ml respectively and the endotoxin levels were decreased to less than 2EU/ml (the acceptable level of endotoxin in the recombinant protein for preclinical use is less than 20EU/ml).

The molecular weight of flagellin-a and flagellin-b detected in SDS-PAGE were ~39KDa and ~45KDa respectively.

The results of complete blood count (CBC) showed a significant decrease of total blood lymphocytes from 0.65 in group C1 to 0.59 in group C2 while there was a non-significant decrease between B1 and B2 groups (from 0.69 to 0.67) and a non-significant difference between A1 and A2 groups (0.67 for both groups).

The immunological studies showed that the detection of lymphocytes using the CD3 marker was compatible with the results of CBC in the affection of flagellin-b and flagellin-a in the prevention of bacteria from declining the lymphocytes after the comparison between the immunized groups with flagellin-b or flagellin-a and the non-immunized groups after infection (P-value 0.038 and 0.039 respectively).

The detection of T helper cells revealed a significant elevation of T helper cells in the immunized groups with flagellin-b after infection more than before infection (P-value < 0.05) rather than in groups immunized by flagellin-a and the non-immunized group that showed a decline of T helper cells after infection.

The measurement of total Th17, Th17 subset from Th cells, and Th17 subset from CTL showed a good effect of flagellin-b in the elevation of total Th17 and Th17 subset from Th cells (P-value < 0.05) in the immunized group after infection (B2) more than before infection (group B1), while a decrease of these cells in the flagellin-a immunized group and non-immunized group.

The investigation of Th1 showed an elevation of Th1 cells in the flagellin-b immunized groups (B1 and B2) more than in flagellin-a immunized groups (A1 and A2) and more than in non-immunized groups (C1 and C2). While the investigation of

Th2 showed the elevation of these cells in the non-immunized groups C1 and C2 more than in the immunized groups A1, A2, B1, and B2 (before and after infection).

Flagellin-b showed a significant increase in IgM+ B cells in the immunized groups before and after infection (P-value < 0.05) in comparison with the non-immunized group. While there is no significant difference in these cells when compared to the flagellin-a immunized groups before and after infection when compared with the non-immunized group.

The results of the histopathological examination of the organs (heart, kidney, liver, spleen, and lung) of the immunized and non-immunized groups, showed that all organs (except the lung) were normal in all groups, while the examination of the lung showed a mild focal inflammatory cells aggregation in the immunized groups only.

The present study can conclude from the present study that the flagellin-b is considered a good candidate vaccine to eradicate XDR *Pseudomonas aeruginosa* pulmonary infections due to its improvement of the immune responses against this bacterium via enhancing the cellular immune response by elevating Th, Th17, and Th1 cells and shifting the immune response against pulmonary infection toward cure rather than chronic infection. It is activate humoral immune response by increasing IgM+ B cells, that necessary for antibodies production, as well as it didn’t show any histopathological changes in the vital organs, and shows an increase in mucosal immunity by recruiting the immune cells in the lung tissue.

The preparation of flagellin as a candidate vaccine by a modified purification method that can be used to protect rats against pulmonary infections caused by *Pseudomonas aeruginosa* is considered the first study conducted in Iraq.

List of Tables

List of Figures

List of abbreviations	
Code	**Meaning**
AIDS	Acquired Immuno-Deficiency Syndrome
AIs	Auto Inducers
AST	Antibiotics Susceptibility Testing
bp	Base Pair
CBC	Complete Blood Count
CD	Cluster of Differentiation
CF	Cystic Fibrosis
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
COPD	Chronic Obstructive Pulmonary Disease
CTL	Cytotoxic T Lymphocytes
DEAE	Diethylaminoethyl
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EPS	Extracellular Polymeric Substances
EU	Enzyme Unit
FC	Flow Cytometry
G-CSF	Granulocyte Colony Stimulating Factor
H&E	Hematoxylin and Eosin
HIV	Human Immunodeficiency Virus
hrs	Hours
I.M.	Intramuscular
I.N.	Intranasal
I.P.	Intrapretonial
IFN-γ	Interferon Gama
Ig	Immunoglobulin

IL	Interleukin
IL17	Interleukin-17
Kbp	Kilo Base Pair
KDa	Kilo Dalton
LPS	Lipopolysaccharides
LPS	Lipopolysaccharide
MDR	Multidrug Resistant
NAIP	Neuronal Apoptosis Inhibitory Protein
NLR	NOD-Like Receptor
NLR	Nucleotide Binding Domain and Leucine Rich Repeat Containing Proteins
OMPs	Outer Membrane Proteins
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDR	Pan Drug Resistant
PES	Polyethersulfone
QS	Quorum Sensing
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
T3SS	Type III Secretion System
TGF-β	Transforming Growth Factor Beta
Th	T helper Cells
Th1	T helper Type 1
Th2	T helper Type 2
TLR2	Toll Like Receptor-2
TLR4-MD2 complex	Toll Like Receptor 4 and Myeloid Differentiation Factor 2 Complex
TLR5	Toll Like Receptor-5
Treg cells	T Regulatory Cells

UTI	Urinary Tract Infection
VAP	Ventilator Associated Pneumonia
WBC	White Blood Cells
WHO	World Health Organization
XDR	Extensive Drug Resistance

Chapter One

Introduction

&

Literatures Review

1.1 Introduction

Bacterial resistance is the capability of bacterial cells to prevent antibiotic bacteriostatic or bactericidal effects (Miller *et al.*, 2014). The excessive and unintended usage of antibiotics contributes to resistance development in bacteria (Kraemer *et al.*, 2019). Because of the extensive antibiotics uptake, the evolvement of microorganisms resistant with the time and problems have arisen with these resistant microorganisms for the treatment of certain infections (Hasan and Aljanaby, 2019a). The resistance is determining as a big issue in the path of new drug synthesis, developing antibiotic resistance is a major public health problem worldwide (Hasan and Aljanaby, 2019b).

Pseudomonas and other critical group in MDR bacteria such as (*Acinetobacter* and some enterobacteriaceae) is consider a threat in hospital infection (nosocomial infection) and among the immunocompromised patients as those have a blood catheter. They can developed into severe septicemia or pneumonia leading to death ("WHO Publishes List of Bacteria for which New Antibiotics are Urgently Needed," 2017).

P. aeruginosa is consider one of the main opportunistic pathogen that cause infections in immunocompromised patients as those with malignant diseases or infected with Human immunodeficiency virus (HIV) (Christopher Adlbrecht *et al.*, 2020). Also increase the morbidity after infection by this bacteria in those suffer from chronic airway infection as chronic obstructive pulmonary disease (COPD), bronchiectasis or ventilator associated pneumonia (VAP) (Grimwood *et al.*, 2015; Tümmler, 2019).

The major threat of *P. aeruginosa* is its ability to adapt and acquire resistance to antibiotics in addition to its intrinsic mechanisms of resistance such as reduction of the inner and outer membrane permeability, Beta-lactamases Class C and Modifications in the membrane proteins (Hasan and Al-Harmoosh, 2020).

Many studies showed that the increase of multi drug resistance (MDR) *P. aeruginosa* infection especially among hospitalize patients (Centers for Disease Control Prevention and Others, 2020; Huang *et al.*, 2020) . Also the rapidly

development of antibiotic resistance to the last generations of a broad spectrum antibiotic were detected for many *P. aeruginosa* isolates (Bianconi *et al.*, 2019).

World Health Organization (WHO) include *P. aeruginosa* strain that resist carbapenems that consider one of "critical" group of pathogens that needed a new antibiotics become urgently (Tacconelli *et al.*, 2018; Horcajada *et al.*, 2019). Also a recent study performed in Iraq showed that 12.4% of isolates are resistant to Imipenem (Al-Khudhairy and Al-Shammari, 2020)

P. aeruginosa may develop several strategies to circumvent the host's immune response equipped with several virulence factors and antibiotic resistance machinery, which play important roles in the bacterium pathogenesis and drug resistance (Lister *et al.*, 2009; Moradali *et al.*, 2017).

P. aeruginosa possesses several virulence factors one of them is the flagella that composed from polymerization of small subunit called flagellin, its major structural protein, and attached to a trans membrane motor complex. Flagellin is classified into two distinct serotypes a and b and it consider a good pathogen-associated molecular pattern (Ramos *et al.*, 2004).

Due to that the flagellin is good pathogen associated molecular pattern that binds to the extracellular Toll like receptor-5 (TLR5) (Hayashi *et al.*, 2001) and intracellular NOD-like receptor (NLR) neuronal apoptosis inhibitory protein (NAIP) (Zhao *et al.*, 2011) in human (Ruiz *et al.*, 2017) That causes the MyD88 pathway (the pro-inflammatory pathway) to be activated, which subsequently activates the NLRC4-inflammasome (Vijay-Kumar *et al.*, 2010) make the flagellin is consider appropriate choice as a potential vaccine.

Administration flagellin proteins may direct the immune responses versus targets on the surface of bacteria. This makes it more vulnerable against immune responses and may be thereby increases the efficacy of vaccine candidate in a rat model.

The Aim of the Study

Aim and objectives

The aim of this study is to investigate active immunization that use flagellin to provide complete protection against acute lethal respiratory illness caused by XDR *P. aeruginosa* infection.

This aim was achieved using the following objectives:

1. Identification of *Pseudomonas aeruginosa* isolates and determine their antibiotic susceptibility by using Vitek2 system according to CLSI guidelines.
2. Molecular detection of the type of flagellin (a or b) in the bacteria.
3. Partial purification of *P. aeruginosa* flagellin.
4. Immunization the laboratory animals; Albino rats, with *P. aeruginosa* flagellin as an immunogen to evaluate the efficiency stimulation of immune response.
5. Investigate the immunological response by using CBC to determine the total lymphocytes and flow cytometry technique to Detect T lymphocytes, Th17 (total Th17, Th17 subset from Th1 or subset from CTL), T helper 1, T helper 2 and B lymphocytes.
6. Studying the histopathological changes in different spleen, liver, heart, kidney and lung tissues of laboratory animals.

1.2. Literature Review

1.2.1. *Pseudomonas aeruginosa* Taxonomy

Pseudomonas aeruginosa belong to Monera Kingdom, Proteobacteria phylum, Pseudomonadaceae order, *Pseudomonas* genus, *P. aeruginosa* species. The French drug pharmacist Carle Gessard was initially describe this bacteria in 1882 A.D. Carle Gessard was describe a blue-green Phenazine pigment that has a toxin properties and antimicrobial effect. The name this bacteria is gotten from two Greek words: Pseudo meaning 'false' and monas meaning 'single unit'; *aeruginosa* meaning 'greenish-blue' is from the latin aerūgō meaning 'rusted copper'(Diggle and Whiteley, 2020).

1.2.2. General Properties of *P. aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, heterotrophic, motile rod-shaped bacterium that is 1–5 µm in long and 0.5–1.0 µm in width. It's a strict aerobic (it can grow anaerobically in the presence of nitrate. *P. aeruginosa* may grow anaerobically with arginine but it has weak fermentative ability, therefore growth is sluggish or non-existent. As a prototroph, the organism can use over 100 organic compounds as a source of carbon and/or energy, and can thrive on a minimum basic growth media with a single source of carbon and energy. *P. aeruginosa* grows at 37°C, but may also thrive at temperatures ranging from 4 to 42°C. It is one of important soil bacterium capable of degrading aromatic hydrocarbons, although it is frequently found in water sources contaminated by animals and people, such as sewage and hospitals waste (Diggle and Whiteley, 2020). *P. aeruginosa* is frequently resist a wide range of antibiotics, which makes it difficult to treat during an infection. Because it rarely infects healthy people, it is known as a 'opportunistic' pathogen (Stover *et al.*, 2000).

The genome sequencing of *P. aeruginosa* was firstly performed to strain PAO1, that isolated originally from wound in 1950s and it used commonly as a laboratory standard strain. 6.3 Mbp is the genome size of PAO1, it was consider the largest bacterial genome sequenced in the year 2000. It is essential to note that there are a variety in the genomic and phenotypic among *P aeruginosa* strains in the world (Stover *et al.*, 2000).

The genome of *P. aeruginosa* carry a large number of genes and transcriptional regulators that mediate transport, degradation of many organic compounds. So it has flexibility in metabolism in addition to its genome that enable the bacteria for colonizing and thriving in a range of environments. (Freschi *et al.*, 2019).

1.2.3. Infections of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa virulence factors versatility lead to diversity of manifestations associated disease that caused by its infections. *P. aeruginosa* is one of important human pathogen, that cause wide range disease such as respiratory tract infection, UTI, skin and soft tissues infection, eyes and ears infection especially in the immunocompromised patients such as those who have a deficiency in their host defense mechanisms. *P. aeruginosa* infections constitute a heavy burden on the health-care system as a nosocomial pathogen, it is responsible on 70% of ventilator associated pneumonias (Sievert *et al.*, 2013), nine percent of other healthcare associated pneumonias, ten percent of catheter-associated UTIs, four percent of blood stream infections, and six percent of surgical site infections (Weiner *et al.*, 2016). Also, *P. aeruginosa* consider from the main causes of morbidity in those suffer from chronic airway infections such as chronic pulmonary disease, pneumonia and cystic fibrosis (CF*)* (Marshall *et al.*, 2018).

P. aeruginosa continues to pose a challenge in burn wound infections, due to the development of antibiotic resistance between this population (Bahemia *et al.*, 2015; Fournier *et al.*, 2016; Decraene *et al.*, 2018). Cancer patients also at high risk to *P. aeruginosa* infection as pneumonia and bacteremia because it suffer

from neutropenia result from chemotherapy (Carratalà *et al.*, 1998; Chatzinikolaou *et al.*, 2000; Thirumala *et al.*, 2010).

1.2.4. Virulence Factors

1.2.4.1. Flagella and Flagellin

Pseudomonas aeruginosa has a unipolar flagellum that is made up of polymerized flagellin, and is connected to the motor complex in the membrane. The flagellar-host interaction is critical in defining the immunological and inflammatory outcomes of *P. aeruginosa* infection because the flagellar component interacts with immune cells and other cells in addition to its motility function. Flagellin, the primary protein component of the flagella, is separated into two serotypes: a and b (Ramos *et al.*, 2004). Furthermore, flagellin's conserved domains are highly antigenic, and it is best known as a pathogen-associated molecular pattern that binds to the extracellular Toll-like receptor 5 (TLR-5) (Hayashi *et al.*, 2001) and intracellular NOD-like receptor (NLR) neuronal apoptosis inhibitory protein (NAIP) in human (Zhao *et al.*, 2011; Ruiz *et al.*, 2017) that lead to activate MyD88 pathway (the pro-inflammatory pathway) and then the NLRC4-inflammasome (Vijay-Kumar *et al.*, 2010). TLR-5 is a key mediator of the epithelial cytokine and chemokine responses that lead to recruitment of neutrophil in *P. aeruginosa* lung infection (Prince, 2006; Zhang *et al.*, 2007; Beaudoin *et al.*, 2013), as well as a it contribute to the production of pro-IL-1ß by monocytes and macrophages (Descamps *et al.*, 2012). The type-3 secretion system (T3SS) also transports flagellin into the cytoplasm of mammalian cells, that activate the NAIP-NLRC4-inflammasome and triggering mature IL-1ß production (Wei *et al.*, 2013; Ince *et al.*, 2015). IL-1ß, in particular, stimulates phagocytosis via autocrine and paracrine actions (Amiel *et al.*, 2010; Descamps *et al.*, 2012).

Both flagellin and a motile flagellum are necessary to activate the NAIP-NLRC4 inflammasome (Miao *et al.*, 2006; Lightfield *et al.*, 2011; Zhao *et al.*, 2011; Patankar *et al.*, 2013), although it is unclear how host cells detect flagellar motility. The flagellum promotes adherence and colonization to the host surfaces

in addition to activating host cell signaling pathways, and several specific targets have been identified, such as MUC1 mucin (Lillehoj *et al.*, 2002), heparin sulfate (Bucior *et al.*, 2012), surfactant protein A (Ketko *et al.*, 2013) and asialoGM1 (Adamo *et al.*, 2004). *P. aeruginosa* employs a variety of strategies to avoid flagellum-mediated host detection during chronic infection. Several global transcriptional regulators control flagellin expression (Starnbach and Lory, 1992; Arora *et al.*, 1997; Garrett *et al.*, 1999; Lo *et al.*, 2016). It is repressed in mucoid forms that overproduce the alginate (exopolysaccharide) (Tart *et al.*, 2006), during biofilm formation, and in response to the host environment (Guttenplan and Kearns, 2013). Neutrophil elastase generated at sites of inflammation(Jyot *et al.*, 2007), as well as flagellin is repressed in CF patients (Wolfgang *et al.*, 2004). *P. aeruginosa* also produce bacterial proteases, which degrade extracellular flagellin, indicating that flagellin-mediated immunological recognition is shut down via an internal mechanism (Casilag *et al.*, 2016).

Finally, flagellar motility loss is widespread in host-adapted *P. aeruginosa* strains in CF lung infections, and it's linked to higher bacterial load and disease severity (Luzar *et al.*, 1985). In fact, due to reduce the flagellin and T3SS expression, the *P. aeruginosa* isolates recovered from persistent CF lung infections fail to activate the inflammasome (Hauser, 2009; Huus *et al.*, 2016).

1.2.4.2. Type III Secretion System (T3SS)

The T3SS is a secretory apparatus (present in the gram negative bacteria) that allows bacterial translocate into the cytoplasm of host cells, that cause cytotoxicity or subversion of host defenses (Hauser, 2009). T3SS effectors disrupt the cytoskeleton of host cells and cleave phospholipases, resulting in cell death, a breach of epithelial and endothelial barriers, and phagocyte death (Wiener-Kronish *et al.*, 1993; Shaver and Hauser, 2004; Rangel *et al.*, 2015).

The T3SS effectors disrupt the cytoskeleton of the host cell and cleave phospholipases, resulting in cell death, a breach of epithelial and endothelial barriers, and phagocyte death (Dacheux *et al.*, 1999, 2000; Garrity-Ryan *et al.*,

2000). The T3SS enhances innate immune responses by secreting IL-1ß, in addition to cytotoxicity (Miao *et al.*, 2010). The T3SS apparatus may activate the NLRC4-inflammasome via NAIP recognition, resulting in apoptotic cell death and the production of mature IL-1ß and IL-18, independent of exotoxin (Sutterwala *et al.*, 2007; Miao *et al.*, 2010; Yang *et al.*, 2013).

1.2.4.3. Secreted Proteases

Several protease are secreted by *P. aeruginosa* such as LasA, LasB, AprA , and proteaseIV. *P. aeruginosa* proteases secreted into the environment interact with a wide spectrum of host molecules, resulting in a variety of effects ranging from structural component destruction to regulation of inflammatory responses. These proteases are largely recognized as virulence factors associated with the pathogenesis of acute infections, and they have received the most attention due to their capacity to induce direct tissue damage. LasB, a metalloprotease with a wide specificity, degrades elastin(Yang *et al.*, 2015), disrupts epithelial tight junctions (Nomura *et al.*, 2014), and decreases the integrity of endothelial barrier (Beaufort *et al.*, 2013; Golovkine *et al.*, 2014).

P. aeruginosa proteases also influence the immunological response of the host by degrading secreted mediators, that lead to lowering of inflammatory immune response, which make the pathogen to escape from host defenses. Some studies show that these proteases destroy the secreted cytokines (ex., INF-γ, IL-6), chemokine (ex., IL-8, MCP-1, CXCL-5, RANTES/CCL5) (Horvat *et al.*, 1989; Parmely *et al.*, 1990; Leidal *et al.*, 2003; Matheson *et al.*, 2006; Saint-Criq *et al.*, 2018), host immunological components such as antibodies (Heck *et al.*, 1990; Hong and Ghebrehiwet, 1992). LasB aids *P. aeruginosa* in subverting alveolar macrophage by suppressing oxidative burst and complement factor synthesis (Bastaert *et al.*, 2018). Phagocytosis resistance is also caused by LasB-mediated degradation of surfactant proteins SP-A and SP-D (Mun *et al.*, 2009; Kuang *et al.*, 2011). Finally, AprA and LasB degrade flagellin monomers, blunting TLR5-mediated responses (Casilag *et al.*, 2016) and inflammasome activation and P.

aeruginosa proteases inhibit inflammasome activation via proteolytic destruction of extracellular inflammasome components (J. Yang *et al.*, 2017).

1.2.4.4. Exopolysaccharides (EPS)

Pseudomonas aeruginosa manufactures three extracellular polysaccharides (or exopolysaccharides): alginate, Psl, and Pel. They have a variety of protective functions as well as surface adherence. They are biofilm matrix components, participate in surface colonization, and enhance bacterial evasion from the host immune system. The exopolysaccharide alginate is overproduced by mucoid *P. aeruginosa* , and these strains are usually linked with persistent lung infections (Pier *et al.*, 2001; Lyczak *et al.*, 2002; Mayer-Hamblett *et al.*, 2014). Through a variety of mechanisms, alginate overproduction impairs host defenses and enhances bacterial persistence. Overproduction of alginate restricts opsonophagocytosis and the activation of complement system, in addition to the inhibition of scavenges reactive oxygen species (ROS) and phagocytic killing (Learn *et al.*, 1987; Pier *et al.*, 2001; Leid *et al.*, 2005). It also provides resistance to antimicrobials found in the host, such as the reactive oxygen species (Malhotra *et al.*, 2018). This mucoidy inhibits flagellar production by co-regulating alginate and flagellin (Tart *et al.*, 2006), That lead to decreaseding in TLR5-dependent activation. Mucoidy, on the other hand, is related with the increase of bacterial lipoprotein expression (Firoved *et al.*, 2004), that lead to the activation of TLR2 in the epithelial cells host airway (Beaudoin *et al.*, 2012), and also is related to increased its resistance against the effects of anti-inflammatory corticosteroids (Mizutani *et al.*, 2017). Psl and Pel (type of exopolysaccharides) that contribute to biofilm resistance against antibiotic by providing structural and aggregative abilities to the biofilm matrix (Colvin *et al.*, 2012; Tseng *et al.*, 2018). Psl inhibits complement deposition and neutrophil opsonophagocytosis as well as oxidative death (Mishra *et al.*, 2013).

1.2.4.5. Lipopolysaccharides (LPS)

Lipopolysaccharides (sometimes referred as endotoxin) is a component of Gram-negative bacteria's outer membrane. The outer membrane of these bacteria is made up of three parts: lipid A and core oligosaccharides, as well as the O-antigen polysaccharide that interacts with the external environment. The TLR4-MD2 complex (TLR 4 and myeloid differentiation factor 2 complex) detects the LPS. The O antigen is made up of repeats of oligosaccharide that is highly variable which trigger a potent humoral reaction (Pier, 2007). Throughout the development of chronic infection, many structural change occur in the LPS as one of adaptation mechanisms of this bacteria to evade host immune response, one of them is the alteration in the structure of lipid A and loss of O antigen. other changes include addition of positively charged components or acylation of lipid A that make the outer membrane more resistant against antimicrobial peptide (Ernst *et al.*, 2006; Moskowitz and Ernst, 2010; Needham and Trent, 2013), that modulates the recognition by TLR4-MD2 receptor that reduce host inflammation (Di Lorenzo *et al.*, 2015).

1.2.5. Pathogenesis

Pseudomonas aeruginosa may evolve a variety of strategies to evade from the host's immune response by using a different virulence factors and antibiotic resistance machinery, that consider the important roles in bacterial pathogenicity and drug resistance (Lister *et al.*, 2009; Moradali *et al.*, 2017).

P. aeruginosa uses complex genotypic events to sustain a variety of phenotypes and molecular processes essential for survival throughout pathogenesis and antibiotic treatment. After infection, bacteria expose to many environmental stress such as the inflammatory responses, including oxidative stress, and the exposure to antibiotics (Furukawa *et al.*, 2006; Turner *et al.*, 2014). All that lead to the expression of many sets of genes, that allow the adaptation of bacteria to these stresses by many transition, but it become less virulent as in the form of biofilm formation (MacDougall *et al.*, 2005; Gellatly and Hancock, 2013).

1.2.5.1. Quorum Sensing and Bacterial Virulence

The communication between different single cells via specific chemical signals is known as quorum sensing. QS regulates bacterial social behavior via numerous interrelated signaling pathways (LaSarre and Federle, 2013). It enables bacterial communities to control a wide range of biological processes crucial to its adaptation and survival. This process is based on the regulation of particular gene expression in response to a crucial threshold of the signaling molecules called auto inducers (AIs). As a result, QS will regulate population density-dependent collective responses, which is important to community survival. Within a community, cell responses to QS signals and expression of the related gene are varied, resulting in increased fitness and survival chances (Grote *et al.*, 2015).

QS coordinates phenotypic modifications in the early stages of infection following attachment, which is crucial for survival and colonization in *P. aeruginosa* pathogenesis (González and Keshavan, 2006). QS-dependent gene expression has a significant impact on the progression of acute to chronic infection. QS regulates more than 10 percent of the genes in *P. aeruginosa* . These genes are primarily responsible on the synthesis of virulence factors, motility, the motility-sessility switch, and biofilm creation, as well as antibiotic resistance mechanisms and metabolic pathway modification for stress reactions (Venturi, 2006; Williams and Cámara, 2009; Barr *et al.*, 2015).

1.2.5.2. Biofilm formation

During acute infection, bacteria create a number of cytotoxic chemicals that impede host cellular activities, while bacteria encounter immune system responses that promote phagocytosis, such as the production of antimicrobial compounds and reactive oxygen species. *P. aeruginosa* that is motile has a more virulent phenotype. Virulent features are linked to several types of *P. aeruginosa* motility, including as swimming and swarming with flagella and twitching with type 4 pili (Winstanley *et al.*, 2016). By recognizing and inducing signaling pathways that drive inflammatory reactions and phagocytosis by macrophages, the flagellar

and/or other motility components of a motile cell are easily identified by the host immune system (Amiel *et al.*, 2010). Many harmful bacteria, such as *P. aeruginosa*, evade stressors and harsh circumstances by switching to a sessile lifestyle along with diminished virulence. They lose their mobility and adhere to surfaces, forming cellular aggregations or microcolonies that are protected from the environment by extracellular polymeric substances (EPS). Biofilms are formations that provide extraordinary resistance to phagocytosis, oxidative stresses, nutrient/oxygen limitation, metabolic waste accumulation, interspecies contests, and conventional antibacterial agents (Leid, 2009; Olsen, 2015).

1.2.6. Antibiotic Resistance

Infections caused by *P. aeruginosa* are becoming more difficult to treat since these bacteria is inherently resistant to several antibiotics, and the frequency of multidrug- and pan-drug-resistant bacteria is on the rise across the world. This strain has been shown to be resistant to almost all kinds of commonly used antibiotics, including aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems (Hancock and Speert, 2000; Poole, 2011).

1.2.6.1. Mechanisms of Resistance

The rise of antibiotic resistant microorganisms is, without a doubt, a worldwide health concern. *P. aeruginosa* has been highlighted as a key problem among notably MDR bacteria, posing a rising danger to world health and leading to a substantial increase in the frequency of nosocomial and chronic infections. This is owing to these bacteria's amazing ability to build resistance to a wide range of antimicrobials via a variety of molecular pathways, many of which are present in clinical isolates. Although each resistance mechanism is linked to a single class of antibiotics, resistance to each class of antibiotics is mediated by many mechanisms (Potron *et al.*, 2015).

Moreover, each mechanism's contribution differs from region to region. Loss or decreased transcriotion of OprD, as well as overexpresion of active efflux

pumps, AmpC β-lactamase, and extended-spectrum β-lactamases, have all been linked to multi-drug resistance in *P. aeruginosa* isolates. Many reviews have gone into great depth about the prevalence and significance of each resistance mechanism to each antibiotic class (Lister *et al.*, 2009; Strateva and Yordanov, 2009; Sun *et al.*, 2014; Hong *et al.*, 2015; Potron *et al.*, 2015). The resistance mechanisms are classified into intrinsic, acquired and adaptive mechanisms (Moradali *et al.*, 2017).

1.2.6.1.1. Intrinsic Resistance Mechanisms

Mechanisms of intrinsic resistance *P. aeruginosa*, like many Gram-negative bacteria, can be naturally resistant to some medications. The existence of genes in the bacterial genome encoding inherent features of cell structures and composition that provide protection against harmful chemicals and antimicrobials is the source of such intrinsic resistance mechanisms. It might also be due to the absence of susceptible parts seen naturally in antibiotic-sensitive organisms (Lambert *et al.*, 2011; Blair *et al.*, 2015). Hydrophilic antibiotics, on the other hand, can enter cells by diffusing non-specifically across membrane channels or porin proteins. *P. aeruginosa* restricts antibiotic entrance by lowering the number of non-specific porin proteins and replacing them with particular or more-selective channels for taking up necessary nutrients, resulting in reduced permeability to harmful substances as one of the intrinsic mechanisms (Tamber and Hancock, 2003). This adaptation is a common source of *P. aeruginosa* resistance to used broad-spectrum antibiotics including carbapenems and cephalosporins (Amin *et al.*, 2005; Baumgart *et al.*, 2010). Many clinical strains of *P. aeruginosa* that are resistant to carbapenems like imipenem are down regulation in the OprD porin, which aids in the diffusion of basic amino acids, short peptides, and carbapenems into the cell (Trias and Nikaido, 1990; Strateva and Yordanov, 2009; AL Charrakh *et al.*, 2016).

Antibiotic resistance in *P. aeruginosa* is mostly due to active multidrug efflux pumps. The genes involved are found on the chromosomes or plasmids of

Gram-negative bacteria. Multidrug efflux pumps are multi-protein complexes that cross the Gram-negative bacteria's envelope. They are in responsible of discharging a variety of harmful compounds as well as antimicrobials. Because of their diverse substrate specificities, they are resistant to a variety of antibiotic classes that are chemically unrelated (Blair *et al.*, 2015; Venter *et al.*, 2015).

The gene producing an inducible β-lactamase (AmpC) is another participant in intrinsic resistance and lower level antibiotic susceptibility in *P. aeruginosa* . Because these antibiotics substantially encourage the development of AmpC, which then hydrolyzes their substrates, chromosomal expression and production of AmpC provides low level resistance to aminopenicillins and most cephalosporins (Oliver *et al.*, 2015).

1.2.6.1.2. Acquired Resistance Mechanisms:

Pseudomonas aeruginosa can acquire antibiotic resistance by mutating intrinsic genes or by horizontally acquiring antibiotic resistance from other bacteria by transmitting plasmids containing genetic elements encoding for antibiotic resistance (Davies and Davies, 2010). Unlike intrinsic pathways, acquired resistance is associated with antibiotic selection, and this selective advantage occurs in the presence of antibiotic substances, resulting in an irreversible resistant population (Lee *et al.*, 2016). As a result, acquired resistance, like intrinsic resistance, is stable and may be passed down to progeny. However, because of over-expression of resistance genes and plasmid transmissibility, acquired resistance is a strong mechanism that gives resistance to a broad spectrum of antibiotics and leads to increasing predominance among clinical and environmental strains. Antibiotic resistance has increased as a result of mutations. Intrinsic resistance genes are negatively or positively controlled by one or more regulatory pathways, providing *P. aeruginosa* with a baseline-decreased sensitivity to a restricted range of antibiotics. However, mutations in the regulatory pathway may enhance promoter activity, leading in the activation of genes and the overproduction of protein products such as AmpC and multidrug

efflux pump systems. As a result, there is a higher level of antibiotic resistance (Blair *et al.*, 2015).

Bacterial plasmids have an important function as a powerful vehicle for obtaining resistance genes and delivering them to the recipient host. This is referred to as horizontal gene transfer, and it occurs when genetic elements transmitted across bacterial cells, most notably via conjugation. Some resistance plasmids have a large host range and can be transferred across species via bacterial conjugation, while others have a restricted host range and can only be transferred between a small numbers of cells from similar bacterial species (Kenward *et al.*, 1978).

Hong and his colleagues investigated the global epidemiology and features of *P. aeruginosa* producing metallo-β-lactamase (Hong *et al.*, 2015). They discovered that the prevalence of these genes differs by nation, but genes encoding carbapenemases, such as IMP, VIM, and NDM type metallo-β-lactamases, have been found on all continents (Johnson and Woodford, 2013; Hong *et al.*, 2015; Hussein, 2018). It is concerning because movable plasmids carrying some of the resistance genes throughout a diverse range of unrelated Gram-negative bacteria, increasing the incidence of antimicrobial resistance transmission and complicating treatment (Hong *et al.*, 2015). Some studies show that the resistance to polymyxins was achieved by chromosomal mutations (Moskowitz *et al.*, 2012; Gutu *et al.*, 2013).

1.2.6.1.3. Adaptive Resistance Mechanisms

Adaptive mechanisms, in comparison to other forms of resistance mechanisms, are little understood. Adaptive resistance is a transient and unstable kind of resistance that develops in the presence of certain antibiotics and other environmental stimuli. This form of resistance is mostly based on induced changes in gene expression and protein synthesis, as well as changes in antibiotic targets, and it is reversible upon removal of external stimuli, resulting in re-susceptibility (Barclay *et al.*, 1992; Xiong *et al.*, 1996; Fernández *et al.*, 2011). This pathway

has been seen to mediate *P. aeruginosa* isolates resistance to β-lactams, aminoglycosides, polymyxins, and fluoroquinolones (Zhang *et al.*, 2001; Poole, 2005; Fernández *et al.*, 2010; Khaledi *et al.*, 2016). It has been observed that after strains have been exposed to particular antibiotic concentrations, they may withstand larger doses in subsequent exposures, although cross-resistance to other antibiotics may also emerge (Mouneimné *et al.*, 1999; Fujimura *et al.*, 2009; Fernández *et al.*, 2011; Pagedar *et al.*, 2011). Also resistance to some polycationic antimicrobials, such as aminoglycosides, polymyxins, and cationic antimicrobial peptides, was also proven to be mediated by modifying the lipid A structure in LPS that was due to multiple mutations in cognate regulatory proteins (Barrow and Kwon, 2009; Fernández *et al.*, 2012).

1.2.7. Host Immune Responses to *P. aeruginosa*

Immune responses of the host to *P. aeruginosa* motility It has long been recognized that *P. aeruginosa* motility organelles are recognized by mammalian innate immune sensors. The external Toll-like receptor 5 (TLR-5) and the intracellular Naip5 protein bind flagellin and activate signaling pathways after the activation of NF-kB activation and NLRC4 inflammasome (Vijay-Kumar and Gewirtz, 2009; Amiel *et al.*, 2010). The additional ability to sense and respond to flagellar activity has recently been described by Berwin and his colleagues (Amiel *et al.*, 2010; Lovewell *et al.*, 2011; Patankar *et al.*, 2013). *P. aeruginosa* strains missing flagellar motor stator complexes (motAB motCD) form a non-motile flagellum and are about 100-fold more resistant to phagocytosis by neutrophils and macrophages than motile bacteria. Phagocytic cells respond to flagellar movement by activating the PI3K/AKT pathway through a TLR5-independent mechanism, which results in actin-dependent bacterial engulfment (Lovewell *et al.*, 2014). *P. aeruginosa* also induces flagellum-dependent signaling responses in epithelial cells (Tran *et al.*, 2014).

When bacterial aggregates bind to the apical surface of polarized epithelial monolayers, they create a protrusion enriched in the lipid PIP3, as well as polarity

and adherent junction proteins such Par3, a PKC, Rac1, and Par6a. Protrusion development is required for subsequent NF-kB activation, which also needs bacterial production of the Type 3 secretion system 'injectisome.' Infection models indicate that Type IV pili and flagella are detected during mammalian infection (Skurnik *et al.*, 2013). According to Lindestam Arlehamn and Evans, Type IVa pili may also stimulate pro-inflammatory host responses, leading to enhanced bacterial clearance (Arlehamn *et al.*, 2011). A human clinical research that looked at the relationships between virulence factor expression and *P. aeruginosa* acute infection discovered a link between flagellar motility and symptomatic infection in individuals with UTIs (Ledizet *et al.*, 2012).

Also, some studies show that the IL-17 that produced by Th-17 plays a protective role during acute pulmonary *P. aeruginosa* infection and they may contribute to the pathogenesis of acute pulmonary *P. aeruginosa* infections (Liu *et al.*, 2011) and the up-regulation of IL-17 may increase the bacterial clearance and survival rate through increasing neutrophil recruitment via IL-17's down-stream effectors, and playing protective effect in early phase of acute *P. aeruginosa* lung infection in mice (Xu *et al.*, 2014).

Furthermore, Th17 cells have stimulated extensive investigation, notably due to their participation in the mucosal immune response against pulmonary infections. (Ye, Garvey, *et al.*, 2001; Ye, Rodriguez, *et al.*, 2001; Kolls *et al.*, 2003). The many downstream effects of IL-17 suggest that the Th17 response achieves a difficult balance between preserving mucosal surfaces and promoting damaging tissue inflammation (Chan *et al.*, 2013). IL-17 controls granulopoesis by modulating G-CSF synthesis and actively attracts neutrophils to infection sites by inducing CXC cytokines at inflammatory sites (Laan *et al.*, 1999; Ye, Rodriguez, *et al.*, 2001; Jones and Chan, 2002; Kolls and Lindén, 2004).

In CF patients mount antibody responses to multiple *P. aeruginosa* antigens during bacterium colonization (Pier *et al.*, 1987). Antibodies to alginate were shown to mediate opsonophagocytosis in CF individuals who were not chronically infected with *P. aeruginosa* (Pier *et al.*, 1987), showing some

protective potential. Antibodies, on the other hand, are rarely able to stop infection from spreading, suggesting that infection-induced antibodies may not provide enough protection against future *P. aeruginosa* infections in these patients. Individuals with persistent *P. aeruginosa* lung infection exhibited a largely Th2 immune response, while patients who produced the most IFN-γ, a Th1 cytokine, had the greatest lung function, suggesting that Th1 T cells are important mediators of protection (Moser *et al.*, 2000). When compared to uninfected CF patients and healthy controls, another study of CF patients chronically infected with *P. aeruginosa* found increased levels of pulmonary Th2 cells and Th2 cytokines IL-4, IL-13, and activation-regulated chemokine (TARC, also known as CCL17) in bronchoalveolar lavage fluid, as well as lower levels of IFN-γ (Hartl *et al.*, 2006). The levels of these Th2 cytokines in bronchoalveolar lavage fluid were inversely related to pulmonary function (Hartl *et al.*, 2006). Th1 cells also play a protective effect in animal models of lung infection. In mice, resistance to re-infection with *P. aeruginosa* was linked to a Th1 response, as evidenced by a greater IFN-/IL-4 ratio (Moser *et al.*, 2002). In some studies show that passive transfer of pure IgG failed to protect mice against heterologous strain challenge in a vaccination research using a live attenuated *P. aeruginosa* strain, but active immunization proved protective (Priebe *et al.*, 2008; Priebe and Goldberg, 2014). Furthermore, mice with an obvious Th1 bias are better protected than mice with a clear Th2 bias (Moser *et al.*, 2000). These findings suggest that cellular immunity, particularly Th1 T cell immunity, may play a key role in *P. aeruginosa* infection resistance.

1.2.8. Vaccination

Antibiotic resistance necessitates a focus on other treatment techniques such as vaccines; hence, immuno-prophylaxis and immunotherapy for at-risk people may be a highly effective tool for addressing the difficult problem of severe *P. aeruginosa* infection (Dacoba *et al.*, 2017; Smith *et al.*, 2017; Pang *et al.*, 2019).

Despite the high morbidity and mortality associated with *P. aeruginosa*, no vaccine for infection prevention has yet been approved. Alexander and Fisher wrote a letter in 1970 claiming that a *P. aeruginosa* LPS-based vaccination

reduced burn patient mortality (Alexander and Fisher, 1970). Several attempts have been made since then to develop and push a *P. aeruginosa* vaccine toward approval. Historically, *P. aeruginosa* vaccine development has concentrated on identifying protective antigens and using a variety of vaccination platforms, including as live-attenuated or whole-cell inactivated strains, subunit, conjugate, and DNA vaccines. Several researchers from around the world have detailed reports on *P. aeruginosa* vaccine antigens and prior clinical studies (Sharma *et al.*, 2011; Worgall, 2012; Priebe and Goldberg, 2014; Grimwood *et al.*, 2015; Merakou *et al.*, 2018).

There are other vaccines based on *P. aeruginosa* antigens and virulence factors include toxins, outer membrane proteins, flagella, and pili. Protective antibodies are elicited after exposure to these immunogens, which can mediate opsonophagocytic death and/or virulence suppression (Sharma *et al.*, 2011; Grimwood *et al.*, 2015; F. Yang *et al.*, 2017). Despite significant research efforts, the vaccine that can be utilized for medical uses has yet to be developed due to a number of unsolved difficulties (Worgall, 2012).

One of the main reasons for the vaccine's failure is that *P. aeruginosa* infections in the airway have two separate stages: acute and chronic. Acute infection begins with a non-mucoid planktonic form that expresses a slew of virulence factors that failed to eliminate bacteria during the acute stage, leading to bacterial adaptation to the lung environment and the development of chronic infection (Moradali *et al.*, 2017). Since a result, mucoid biofilm-forming bacteria with various phenotypic traits are observed, as many virulence factors associated with acute infection may be incompatible with chronic infection, and their expression is suppressed (i.e. O-antigen, type III secretion systems, flagella, exotoxin, pili) (Gellatly and Hancock, 2013; Sousa and Pereira, 2014). In the chronic stage, the absence of highly immunogenic virulence factors and the inability of immune receptors such as TLR5 and TLR4 to identify them limit effective immunity (Sousa and Pereira, 2014; Grimwood *et al.*, 2015; Grishin *et al.*, 2015).

The vaccine's other defect is that it confuses the immune response against *P. aeruginosa*. Despite the fact that this bacterium is an extracellular pathogen, Th2 response (type 2 immunity) immunity, which produces IL-4 and IL-10 and suppresses phagocytosis, is not only effective against *P. aeruginosa* , but also has negative consequences for the host, such as inflammation and tissue damage (Spellberg and Edwards, 2001). However, evidence suggests that the Th1 response (type 1 immunity), which produces interferon-gamma (IFN-γ) and interleukin-12 (IL-12), can stimulate antibody production and phagocytic activity, leading to better outcomes such as inflammation control, clearance promotion, and eradication of *P. aeruginosa* infection (Spellberg and Edwards, 2001; Moser *et al.*, 2002).

Type 1 and type 2 immunity are not exactly identical with cell mediated and humoral immunity; yet, they can establish a balance between cellular and humoral immunity in any situation, and coordinating Th1/Th2 immune responses is a crucial problem for *P. aeruginosa* vaccine development. *P. aeruginosa* possesses an outstanding arsenal of weapons to evade the immune system, releasing many virulence factors (type III secretion system, N-acyl- homoserine lactone, and Cif Protein) that can impair or alter defenses by downregulating the Th1 and possibly the Th2 T-cell response (Maman *et al.*, 2011; Bomberger *et al.*, 2014; Song *et al.*, 2019). As a result, bacterial escape from CTL monitoring has substantial consequences for *P. aeruginosa* vaccines (Jiang *et al.*, 2003).

Immunoinformatics can help in the rational design of new vaccines, such as B and T cell epitope-based vaccines, chimeric vaccines, and improving vaccine efficiency and efficacy via in silico adjuvant prediction. An immunoinformatics-driven method was used to build a chimeric vaccination that can solve existing difficulties in the current investigation (Dhanda *et al.*, 2017; Kazi *et al.*, 2018).

In addition, other researchers developed a new multivalent chimeric vaccination based on *P. aeruginosa* protective antigens (LecB, PE and OprF) utilizing an immunoinformatics method. Due to the presence of multiple B and T cell epitopes as well as IFN-γ inducing epitopes, the rationally designed

vaccine was predicted to efficiently boost humoral and cellular immunity. With the immunoreceptor TLR4, they demonstrated high binding affinity and stability (Aminnezhad *et al.*, 2020).

1.2.8.1. The Roles of Vaccine-Induced Cellular Immunity

Two branches of the immune system that operate together to defend against *P. aeruginosa* infection, since increasing data shows that an effective *P. aeruginosa* vaccination should elicit both opsonizing and neutralizing antibodies (Drake and Montie, 1987; Dunkley *et al.*, 2003) in addition to CD4+ T cells provide whole protection against infection (Priebe *et al.*, 2008; Kamei *et al.*, 2013). The use of live, attenuated vaccinations revealed that vaccine-induced protection against *P. aeruginosa* intranasal infection in mice was dependent on Th17 cells, as antibodies -mediated IL-17 depletion prior to challenge, or the lack of the IL-17 receptor, abolished vaccine-induced protection against bacterial challenge (Priebe *et al.*, 2008).

Further investigation found that Th17 T cells were required for protection against *P. aeruginosa* pulmonary infection in neutropenic mice, and that pulmonary GM-CSF was important and linked to IL-17 production (Kamei *et al.*, 2013). The synthesis of IL-17 by CD4+ T cells following vaccination with an X-ray irradiation vaccine was linked to fast recruitment of neutrophils to the lungs in an immune-competent mouse model, resulting in protection against *P. aeruginosa* (Li *et al.*, 2016). A Th17-based reverse vaccinology technique employing a collection of *P. aeruginosa* outer membrane and secreted proteins found numerous proteins that triggered protective memory responses, including outer membrane protein L (OprL), PopB, PcrH, and PilQ. PopB and PcrH, in particular, when paired with the adjuvant curdlan, provided IL-17-dependent and antibody-independent protection against *P. aeruginosa* in an acute pneumonia model. (Wu *et al.*, 2012).

Further studies of PopB, PcrH, OprL, and PilQ in a variety of vaccine formulations revealed that vaccination-induced protection was linked to pulmonary CD4+ T cells and an increase in IL-17 (Gao *et al.*, 2017; Bakht azad *et*

al., 2018; Schaefers *et al.*, 2018). Over the last decade, a variety of different protein-based and multicomponent vaccines have been investigated such as pili, flagellin (Korpi *et al.*, 2016; Zare *et al.*, 2016; Behrouz *et al.*, 2017), outer membrane proteins (Krause *et al.*, 2013; Baker *et al.*, 2019) and some whole cell vaccines (Meynet *et al.*, 2018). All show the capacity to elicit both Th1 and Th17 immune responses, whether systemically or in the lungs.

1.2.8.2. Strategies of Vaccine to Target Cellular Immunity

Successful vaccinations against a variety of mucosal infections have been created, and some studies showed that immune responses were apparent at mucosal locations, including respiratory tissue, following systemic vaccine administration (Suzich *et al.*, 1995; de Haan *et al.*, 2001; Lin *et al.*, 2007; Belyakov and Ahlers, 2009). This collection of evidence suggests that systemic immunization is sufficient for protection against some mucosal pathogens, including the influenza virus and the human papillomavirus (Belyakov and Ahlers, 2009). Conversely, the failure to create vaccines against some mucosal diseases, such as *P. aeruginosa*, may be due to systemic immunization's incapacity to effectively engage various arms of the innate and adaptive immune systems and to direct those responses to the diseased mucosa. The main *P. aeruginosa* virulence factors involved in pathogenesis during pulmonary infections shown in Figure (1-1). *P. aeruginosa* vaccine research has replied to this problem by including new vaccination methods that have been found to generate mucosal immune responses, such as intranasal and intradermal immunization (Table 1-1). Evidence showed that the route of vaccination influences distinct T cell effector functions in tissues close to the immunization site, as well as variable systemic and mucosal antibody responses (Belyakov and Ahlers, 2009; Tozuka *et al.*, 2016). Thus, the immunological responses elicited by intradermal or intranasal vaccination with the same antigen/adjuvant formulation may not be recapitulated following systemic vaccination, such as intramuscular or subcutaneous immunization (Belyakov and Ahlers, 2009). Independent of adjuvant choice, intranasal vaccination has been found to result in elevation of Th17 cellular immunity in the lungs (Zygmunt *et*

al., 2009) In a mouse model of *P. aeruginosa*, intradermal vaccination has been demonstrated to promote the production of both IFN-γ and IL-17 in the lungs (Baker *et al.*, 2019).

Many authors worldwide study the effect of different immunogen as a vaccine against *P. aeruginosa* such as outer membrane proteins (OMP) (Mutharia *et al.*, 1982), OMPs are the major porin F (OprF) and the lipoprotein I (OprI) (Loots *et al.*, 2008; Westritschnig *et al.*, 2014; Cassin *et al.*, 2019; C. Adlbrecht *et al.*, 2020), OprF/I, and the OprF/I vaccine combination with a Th17-stimulating antigen (e.g., exotoxin PopB) (Westritschnig *et al.*, 2014) in addition to studies on whole organisms, such as killed whole-cell and live attenuated *P. aeruginosa*, are also being tested for vaccine development (Cripps *et al.*, 2006; Kamei *et al.*, 2011) and many other studies reviewed in Sousa *et al.* (2021).

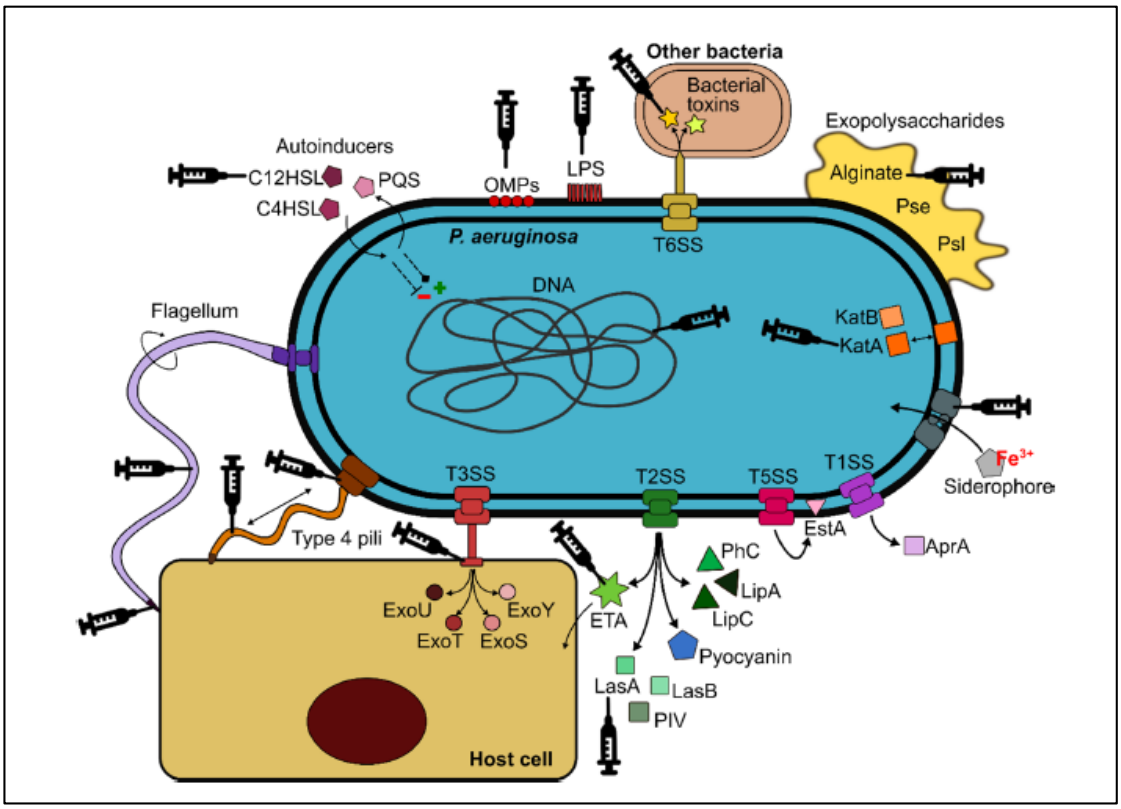

Figure (1-1): The main *P. aeruginosa* virulence factors involved in pathogenesis during pulmonary infections. The components highlighted with syringes have already been evaluated as vaccine antigen (Sainz-Mejías *et al.*, 2020).

Table (1-1): The experimental vaccine that used in many studies against respiratory *P. aeruginosa* infection

Study	Antigen/adjuvant	Route	Model	Cellular Immune Response
(Priebe *et al.*, 2008)	PA14ΔaroA	Intranasal	Murine model of intranasally induced acute pneumonia	Protection was dependent upon T cell-secreted IL-17
(Wu *et al.*, 2012)	PopB and PcrH/curdlan	Intranasal	Murinemodel of intranasally induced acute pneumonia	Protection wasantibody independent and correlated with enhanced mucosal IL-17 and Th17 responses
(Kamei *et al.*, 2013)	PA01ΔaroA	Intranasal	Neutropenic murine model of intranasally induced acute pneumonia	Vaccine efficacy was CD4+ T cell dependent and pulmonary GM- CSF was critical and associated with an increase in IL-17
(Krause *et al.*, 2013)	Adenoviral vector expressing OprF with RGD capsid modification	Intratracheal	Murine intratracheal Challenge with *P. aeruginosa* encapsulated in agar beads	Vaccination-induced reduction in pulmonary bacterial load was associated with an increase in IL- 17, IL-4, and IL-5 production by stimulated lung CD4+ T cells
(Zare *et al.*, 2016)	PilA/alum + naloxone ***a***	Subcutaneous	Murinemodel of intranasally induced acute pneumonia	Vaccine-induced protection was associated with an increase in IL- 17, IFN-γ, and IL-4 production by stimulated splenocytes
(Korpi *et al.*, 2016)	PilA and Type B flagellin	Subcutaneous	Murine burn wound sepsis model	Vaccine-induced protection was associated with an increase in IL- 17, IFN-γ, and IL-4 production by stimulated splenocytes
(Behrouz *et al.*, 2016)	Type B flagellin/alum	Subcutaneous	Murine burn wound sepsis model	Vaccine-induced protection was associated with an increase in IL- 17, IFN- γ , and IL-4 production by stimulated splenocytes

Table (1-1): Continued

(Li *et al.*, 2016)`	X-ray irradiated *P. aeruginosa*	Intranasal	Murinemodel of intranasally induced acute pneumonia	Vaccine-induced protection was dependent on CD4+ T cells and IL- 17 production
(Gao y*et al.*, 2017)	Recombinant OprL/curdlan	Intranasal	Murineintratracheal induction of acute pneumonia	Vaccine-induced protection was associated with an increase in CD4+ IL17+ T cells in the lungs of mice after *P. aeruginosa* infection
(Behrouz *et al.*, 2017)	Bilvalent flagellin	Intranasal	Murinemodel of intranasally induced acute pneumonia	Vaccine-induced protection was dependent on IL-17
(Schaefers *et al.*, 2018)	PopB and PcrH encapsulated into PLGA* nanoparticles	Intranasal	Murinemodel of intranasally induced acute pneumonia	Vaccine-induced protection was associated with an increase in CD4+ IL17+ T cells in the lungs and increased IL-17 production by stimulated splenocytes
(Bakht azad *et al.*, 2018)	PilQ and Type B-flagellin/alum	Subcutaneous	Burned mouse model	Vaccine-induced protection was associated with an increase in IL- 17 and IL-4 production by stimulated splenocytes
(Meynet *et al.*, 2018)	Killed But Metabolically Active (KBMA) *P. aeruginosa*	Subcutaneous	Murinemodel of intranasally induced acute pneumonia	Vaccine-induced protection was associated with a mixed Th1/Th17- type CD4+ T cell response
(Baker *et al.*, 2019)	Outer membrane proteins/dmLT	Intradermal	Murineoropharyngeal aspiration leading to acute pneumonia	Vaccine-induced protection was associated with a mixed Th1/Th17- type CD4+ T cell response and an increase in IFN- γ and IL-17 production in the lungs after *P. aeruginosa* infection

1.2.9. Protein purification

1.2.9.1. History

In the eighteenth century, proteins were known as a distinct class of biological molecules by Antoine Fourcroy and others. They distinguished these molecules by their ability to coagulate under treatment with heat or acid (e.g., albumin from egg whites, blood serum albumin). However, techniques for protein isolation and purification were developed by Edwin Joseph Cohn during World War II (Bailey, 1945). He carried out pioneering work on fractionation of plasma proteins. The solubility properties, precipitation, and crystallization dominated the design of early purification studies. The next major milestone in protein purification was chromatography (Lucy, 2003). Ion exchangers began to be used and have become indispensible in protein purification. Whatman introduced cellulose-based ion exchangers followed by introduction of dextran-based ion exchangers by Pharmacia. This was an important milestone. He studied the separation of a macromolecule (liver α-amylase) via its interactions with an immobilized substrate (starch). The term affinity chromatography introduced in 1968 by Pedro Cuatrecasas, Chris Anfinsen, and Meir Wilchek in an article that briefly described the technique of enzyme purification via immobilized substrates and inhibitors (Cuatrecasas et al., 1968). This progress was some kind of a mini-revolution in protein purification. For a couple of decades, a typical protein purification protocol invariably consisted of precipitation by ammonium sulfate, one or two ion-exchange steps, gel filtration, and finally an affinity chromatography step (Labrou, 2014).

Biotechnology seeks to develop new protein-based applications and their commercial exploitation. The first requirement for achieving these goals is the development of efficient and effective purification methods and materials (Marichal-Gallardo and Alvarez, 2012).

The various steps in the downstream processing protocol separate the protein and non-protein parts of the mixture and finally separate the desired protein from all

other proteins while retaining the biological activity and chemical integrity of the polypeptide. This last step is typically the most laborious and difficult aspect of protein purification. The purified protein should be free not only of contaminants (e.g., nucleic acids, viruses, pyrogens, residual host cell proteins, cell culture media, and leachates from the separation media) but also of the presence of various isoforms, originating from posttranslational modifications (Kalyanpur, 2002).

Separation steps may exploit differences in chemical/structural/ functional properties between the target protein and other proteins in the crude mixture (Table 1-2). These properties include size, shape, charge, isoelectric point, charge distribution, hydrophobicity, solubility, density, ligand-binding affinity, metal binding, reversible association, posttranslational modifications, and specific sequences or structures. By exploiting these tremendous variations in physical and chemical properties among proteins, several different fractionation and chromatographic steps can usually be exploited to design a workable purification scheme (Kallberg et al., 2012).

1.2.9.2. Protein Purification Methods

Downstream processing has been challenged with demands of high yields, resolving power, and cost efficiency. This has triggered remarkable developments in improvising process tools and innovative strategies for protein separation. A wide variety of protein purification methods that can be combined to generate a suitable purification scheme are available. Usually, one executes a series of purification steps, and only rarely proteins can be purified in a single step, even when this step is based on an exquisitely specific biological characteristic (Butler and Meneses-Acosta, 2012; Wilken and Nikolov, 2012). Early steps combine low-resolution and high-capacity methods (when large amounts of protein is present) with higher-resolution and lower-capacity ones (when less protein is present) at later stages of purification scheme. For low-resolution protein purification, methods such as fractional precipitation and two-phase partition systems usually

employed (Rosa et al., 2011). For applications requiring the highest purity and relatively small amounts of protein, chromatography can be chosen to selectively purify the target protein (Chon and Zarbis-Papastoitsis, 2011).

Table (1-2): Physicochemical basis of common bio-separation methods

Separation	Basis of separation
Precipitation	
Ammonium sulfate	Solubility
Organic solvents	Solubility
Polyethyleneimine	Charge, size
Polyethylene glycol	Solubility
Isoelectric	Solubility
Affinity precipitation	Molecular recognition, solubility
Phase partitioning	
Aqueous two-phase partition	Solubility/hydrophobicity
Three phase partitioning	Solubility/hydrophobicity
Chromatography	
Ion exchange	Charge, charge distribution
Hydrophobic interaction	Hydrophobicity
Reverse-phase HPLC	Hydrophobicity, size
Affinity chromatography	Molecular recognition
Gel filtration/size exclusion	Size, shape

Chromatography is certainly the principal and commonly used operation in downstream processing. This can be explained by certain advantages of chromatography over other unit operations. For example, chromatography displays high-resolution efficiencies which allow the resolution of complex crude mixtures with very similar molecular properties. In addition, chromatography is ideal for capturing molecules from the dilute solutions encountered in bioprocessing (Labrou, 2014).

Among all chromatographic techniques, affinity chromatography plays a major role (Roque et al., 2007). In fact, affinity chromatography is the most

specific and effective protein purification technique, providing a rational basis for the purification of target proteins. It exploits the principle of biomolecular recognition, that is, the ability of biologically active macromolecules to form specific and reversible complexes with affinity ligands. As conventional purification protocols for high-value proteins are replaced by more sophisticated procedures based on affinity chromatography, the focus is shifted toward designing and selecting ligands of high affinity and specificity (Labrou, 2003; Clonis, 2006).

The accumulated knowledge of structures obtained from X-ray crystallography, NMR and homology studies, the impressive growth of bioinformatics and molecular docking techniques, the defined and combinatorial chemical synthesis, the display techniques based on biological/genetic packages, and the technological advances in high-throughput screening has made the design and selection of high-affinity synthetic ligands faster and more effective (Labrou, 2003; Clonis, 2006; Roque et al., 2007).

1.2.9.3. Current Purification Methods

Recombinant DNA technology impacts the development of protein purification methods in two ways: Firstly, the development and availability of several different protein expression systems meant that sources of proteins are not limited to naturally occurring animals, plants, and microbes (Chen, 2012; Huang et al., 2012). Protein expression systems are used to produce wild-type proteins in biotechnology and industry and more recently to produce novel engineered variants of proteins that display improved properties. Commonly used protein expression systems include those derived from bacteria, yeast, baculovirus/insect, mammalian cells, and transgenic plants and animals. Second, the use of “affinity tags” and production of proteins in the form of fusion proteins became possible (Young et al., 2012).

1.2.9.4. Flagellin Purification Methods

Flagellin important in immune response by activation TLR5 as explained above in addition to its antigenicity make it as a predictable vaccine against many bacteria as *P. aeruginosa* (Lee *et al.*, 1999; Bruxelle *et al.*, 2017). Also the use of flagellin as adjuvant in some studies (Bates *et al.*, 2008) or in combination with some particle of virus to produce a good immune response in other (Liu *et al.*, 2016; Zhu *et al.*, 2016). Flagellin inter in diagnosis of bacteria subspecies because it consider a conserved compartment of bacteria (Mirhosseini *et al.*, 2017).

As a result, there are a variety of flagellin purification processes available, each with its own purity, amount of flagellin, and cost. Flagellin purification from native bacteria has been accomplished for decades, but most approaches are limited by various disadvantage, such as the need for an ultracentrifuge and multiple purification processes (Simon *et al.*, 2014).

Khani & Bagheri introduce a simple and cheap method to purify this protein from many gram negative bacteria, as *Salmonella typhimurium Escherichia coli* and other, were purified by a modified simple method. Depending on centrifugal precipitation and ammonium sulfate saturation. then dialyzed applied to diethylaminoethyl (DEAE)-Sephadex A-50 (Khani and Bagheri, 2018).

Chapter Two

Materials & Methods

2. Materials and Methods

2.1. Study design

This case-control study was achieved to investigate active immunization strategies using flagellin in order to provide complete protection against XDR *P. aeruginosa* clinical isolates in the acute fatal pulmonary infection.

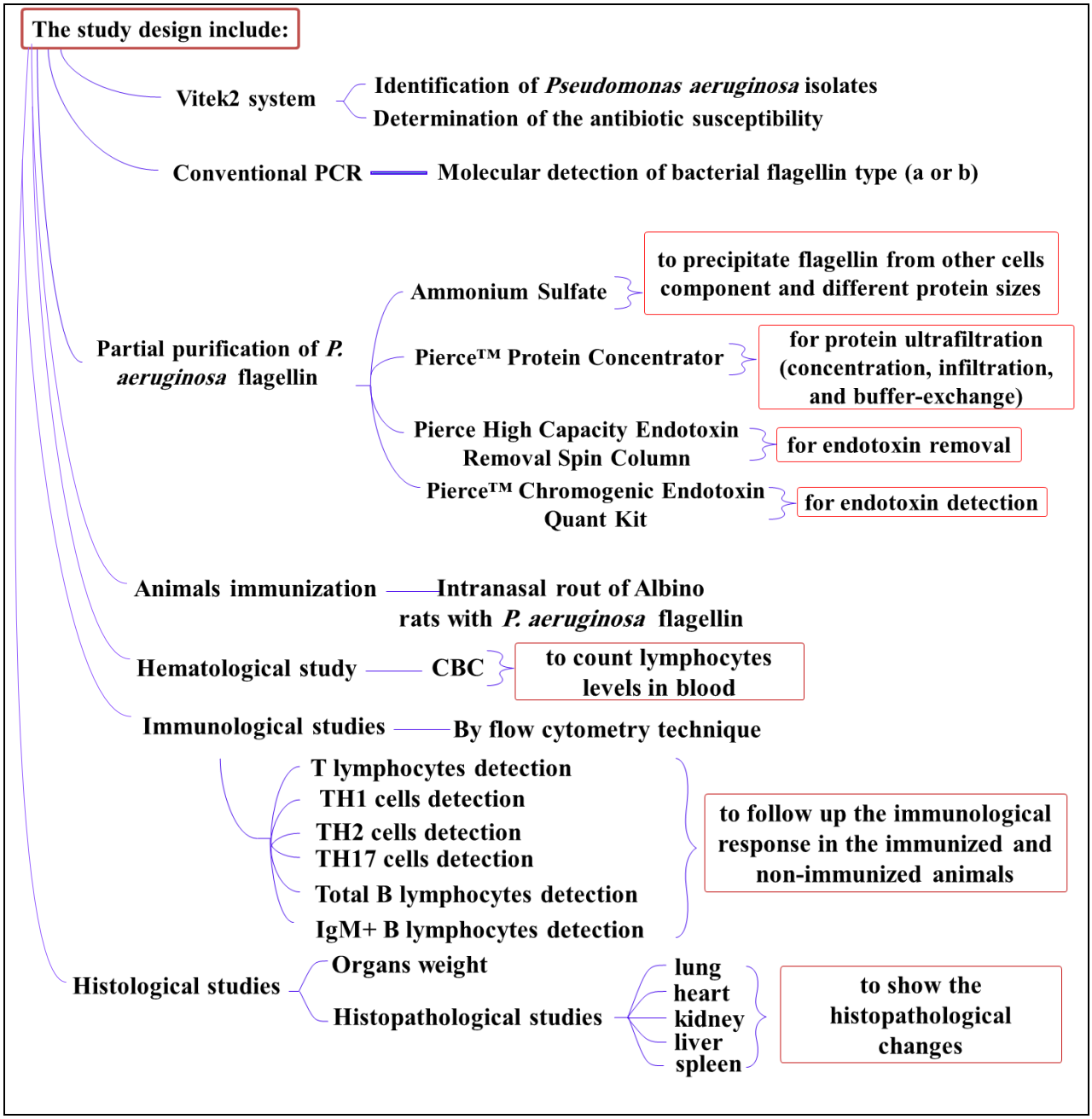

Figure (2-1): Diagram showing the study design

2.2. Ethical Approval

The study protocol forms were reviewed and approved by the committee on publication ethics at the College of Medicine, University of Babylon, under reference No. BMS/0259/016. The ethics committee of the Department of Biology (DOB) approved all experimental protocols and procedures for animal handling at the Faculty of Science, University of Kufa.

2.3. Laboratory Animals

Fifty Albino female adult rats were conducted at an animal house in the faculty of the Science\ University of Kufa in the province of Al-Najaf Al-Ashraf, their ages were 12-16 weeks, and their weight 130-185 g were used to carry out the investigations of the present study in the animal house of the same College, in which the temperature was 30 ±5°C. The animals were housed in plastic cages with hardwood chips for bedding in an air-conditioned room and maintained on special pellets diet with free access to water during all experiments.

2.4 Materials

2.4.1. Instruments and Equipment

The equipment and instruments that were used in this study were listed in Table (2-1).

Table (2-1): The instruments and equipment

Instruments and equipment	Manufacture's / State
Autoclave	Sturdy / Taiwan
BD FACSCanto II Flow Cytometry	BD Biosciences / Belgium
Centrifuge	Hermle / Germany
Cooling Centrifuge	Hettich/Germany
Deep freezer (- 40ºC)	GFL / Germany
Digital camera	Sanyo / Japan

Dissection set	Pakistan
Distillator	Lab Tech / Korea
Drying and sterilizing oven	Hermlse / Germany
EDTA tube (anticoagulant tube)	Sun / H.K.J
ELISA reader	Organon Teknika / Beelchum
Eppendorf tubes	Germany
Gel electrophoresis apparatus	Bioneer / Korea
Gel tube	AFCO / Jordan
Glassware	Duran / Germany
Incubator	Jarad / Syria
Light microscope	Genx / USA
Microcentrifuge	Lab Tech / Korea
Micropipette (different sizes)	/ Germany
Microscope slides	Shenzhen BDJK Technology Industry / China
Microtome	HEOTION / Australia
Microwave	Samsung / Malaysia
PCR tubes	Sterilin Ltd. / UK
Refrigerator	Hetticl / Germany
Sensitive balance	Japan
Thermal cycler	Analytic Jena/Germany
UV visible	England
UV-VIS Spectrophotometer (UV-1900I)	Shimadzu
VITEK 2 system	Biomerieux/ France
Vortex Mixer	Stuart / UK
Water bath	Kottermann / Germany

2.4.2. Chemicals and Biological Materials

The chemicals and biological substances used in this study were listed below.

Table (2-2): Chemicals and biological materials with their origins

Chemicals & biological materials	Manufacture's / State
Agarose	Promega / USA
Ammonium sulfate 70%	Sigma / USA
Bromophenol blue dye	BDH / UK
Deionized water	Bioneer /Korea
Ethanol (96%)	Lobachemie / India
Ethanol absolute	BDH / UK
Ethidium bromide dye	BDH / UK
Formalin 40%	Sigma / USA
Ketamine	BDH / UK
loading Dye	Promega / USA
PCR water	Promega / USA
Phosphate buffer saline (PBS)	Sigma / USA
Tri-Borate EDTA Buffer (TBE buffer) 10X	Promega / USA
wax	Italy
Xylazine	Himedia / India
xylene	Promega / USA

2.4.3. Molecular Kits and DNA Ladder

The PCR kits used in this study are clarified in the table below.

Table (2-3): PCR kits with their remarks

Type of kit	Manufacturer / State
Master mix **This kit contains the followings:** **1- dNTPs(dATP, dCTP, dGTP, and dTTP) 250µM** **2- KCl (30 µM)** **3- MgCI2 (1.5 µM)** **4- Top DNA polymerase (1U)** **5- Tris-HCl -pH 9.0 (10 µM)** **6- Stabilizer and Tracking dye**	Bioneer / Korea

The primers that used to amplify *fliC* gene

	Primer sequence	product size	Reference
CW45-F	5‘ GGCAGCTGGTTNGCCTG 3‘	1020 bp type a & 1250 bp type b	(Ertugrul *et al.*, 2017)
CW45-R	5‘ GGCCTGCAGATCNCCAA 3‘		

The molecular weight marker (Ladder) used in this work is clarified in the table below

Table (2-4): Molecular weight marker

DNA Ladder	Description	Source
Ladder (KAPA Universal Ladder)	This ladder kit is designed to determine the approximate size of dsDNA on an agarose gel. It contain many DNA fragments (in base pairs) these include: 100, 200, 300, 400, **500**, 600, 700, 800, 900, **1000**, 1500.	KAPA/ Biosystems

2.4.4. Endotoxin Removal Ultrafiltration Tube

Special ultrafiltration tubes called Pierce™ Protein Concentrator 30K MWCO,5–20 mL Thermo Fisher Scientific, Inc. / USA were used in this study, its details are listed in Appendix (1).

2.4.5. Endotoxin Detection Kit

Pierce High Capacity Endotoxin Removal Spin Column, 1 ml Thermo Fisher Scientific, Inc. / USA were used to remove the endotoxin from the sample, the details are listed in Appendix (2).

2.4.6. Endotoxin Detection Kit

The amount of endotoxin was detected by LAL Chromogenic assay using a special Endotoxin detection kit called Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific, Inc. / USA), the details are listed in Appendix (3).

2.4.7. SDS-PAGE

The materials used in the SDS-PAGE technique are listed in Appendix (4).

2.4.8. Flow-Cytometry

The monoclonal antibodies used in the flow cytometry technique are listed in Table (2-5), and other components are clarified in Appendix (5 and 6)

Table (2-5): The monoclonal antibodies used in the flow cytometry technique and their manufacture

monoclonal antibodies	Manufacture's / State
APC anti-rat CD3 Antibody	Biolegend / US
APC/Cyanine7 anti-rat CD4 Antibody	
FITC anti-rat IgG1 Antibody	
PECyanine7 anti-rat CD161 Antibody	
PerCPCyanine55 anti-rat IgM Antibody	
Purified anti-rat IgG2a Antibody	

2.5. Methods

2.5.1. Isolation of Bacterial Isolates

Twenty six *Pseudomonas aeruginosa* isolates were collected from the main hospital in Al-Najaf province (Al-Sadr Medical City) as proposed identified isolates. The Identification of bacterial isolates was confirmed as *P aeruginosa* using standard culturing and biochemical tests according to (Forbes *et al.*, 2007) and VITEK 2 system. Isolate no.23 and no.3 were used to purify flagellin-a and b (respectively), while the isolate no.26 that isolated from burn infection was named MJ isolate and used for animal dosing (infection).

2.5.2. Detection the type of flagellin (*fliC* Gene) of *P. aeruginosa* by Conventional PCR

The detection of type-a or b flagellin by PCR technique was carried out in a thermocycler device

2.5.2.1. Genomic DNA Profiling

After DNA extraction by a manual heat-shock method from *P. aeruginosa* isolates, the amount and purity of DNA were checkup using a special UV-spectrophotometer at 260nm and 280nm.

2.5.2.2. Primer Preparation

The primer preparation was occur according to manufacturer instructions by liquefying the lyophilized primers with deionized distill water (ddH2O) to produce a stock solution (100 pmol/µl concentration), primer working solution was prepared by dilution the stock solution with deionized water, according to this equation "C1V1=C2V2".

2.5.2.3. Preparation the PCR reaction

The PCR mix reaction was prepared by using Accu Power PCR Premix kit (Bioneer / Korea), and achieved according to company instruction as in table (2-6).

Table (2-6): The components of PCR reaction

Components	Volume (µl)
Master Mix	8
Forward Primer (10µm)	2
Reverse Primer (10µm)	2
Deionized Water	3
DNA	5
Final volume	20

2.5.2.4. The Conditions of Thermo Cycler (PCR)

Thermocycler conditions for amplification of *flic* gene were carried out by using conventional PCR device. The condition of this reaction was identified by Shehab and Jassim (2020) with some modifications as in table (2-7).

Table (2-7): The program of PCR that used for *fliC* gene amplification in this study

	Step	Temperature (°C)	Time in min.	Cycles No.
fliC gene	Initial Denaturation	95	5	1
	Denaturation	95	0.30	30
	Annealing	56.2	0.30	
	Extension	72	1	
	Final Extension	72	7	1
	Storage	∞	4	_

2.5.2.5. PCR Product Analysis

The PCR product analyzes by agarose gel electrophoresis technique to detect the *flic* gene. Seven µl of the PCR product was loaded in agarose gel (1.5% conc.) that was mixed with 0.5µl ethidium bromide previously and electrophoresed at 70 volt for 60 minutes with the use of 100 bp ladder as a standard and then were visualized by UV transilluminator system.

2.5.3. Extraction and Purification of Flagellin

Flagellin purified from *P. aeruginosa* according to the procedure described by (Khani and Bagheri, 2018) with some modification as follows:

2.5.3.1. Protein Precipitation

The flagellin was precipitated by using ammonium sulfate according to the following procedure:

1. Previously activated *P. aeruginosa* cultured on 1000 ml brain heart infusion for overnight incubation in shaking incubator at 37°C until the absorption at OD600 reach to 0.6.
2. Bacterial cultures precipitated for 10 minutes by centrifugation at 5000 xg.
3. Washing: The pellet then resuspended in 200 mL phosphate buffer saline (PBS) (PH is 7.4) and then centrifuged again under the same conditions.
4. Pellet was resuspended in PBS (50 ml) after two times.
5. Bacterial suspensions were stirred for 45 minutes at 1000 rpm on a magnetic stirrer at 4°C.
6. The remaining bacteria were then precipitated at 4500 xg for 30 minutes, and the supernatant, which included bacterial flagellin, was collected.
7. Flagellin proteins then precipitated from the aqueous phase by slowly saturating the supernatant with 70% ammonium sulfate for 18 hrs. at 4°C.
8. Afterwards, pellets formed by centrifugation in 4500 ×g at 4° C for 20 minutes.
9. Finally, pellets suspended in PBS.

   Note: All steps except step 1 were performed at -20°C.

2.5.3.2. Protein Ultrafiltration

Proteins concentrating Pierce™ Protein Concentrator, infiltrating, and buffer exchanging were done using ultrafiltration centrifugal devices called Protein concentrator 30 KD. It contained a vertical low protein-binding, high-flux polyethersulfone (PES) membrane, which allowed for processing of volumes between 5 mL and 20 ml. It was done according to the Thermo Fisher Scientific Procedure guidelines (Appendix 1).

Then measure the protein absorption by using UV spectrophotometer at 280 nm wavelength.

2.5.3.3. Endotoxin Removal

Endotoxin (LPS) removal from partially purified flagellin well had done using a special endotoxin removal spin column according to the Thermo Fisher Scientific procedural guidelines (Appendix 2).

The Thermo Scientific™ Pierce™ High Capacity Endotoxin Removal Resin that made up of porous cellulose beads with modified ε-poly-L-lysine covalently linked to the surface. The affinity ligand in modified polylysine has a high affinity for endotoxins, which eliminates the toxicity associated with other methods that use polymixin B ligands and sodium deoxycholate buffers. Endotoxin levels can be decreased by 99% in samples containing 10,000EU/mL due to the resin's binding capability of 2,000,000 Endotoxin Units (EU)/mL; typical protein samples treated with the resin have a final endotoxin concentration below 5EU/ml.

Then protein absorption was measured again by using a special spectrophotometer at 270 nm wavelength.

2.5.3.4. Endotoxin Detection

The amount of the remaining endotoxin was detected by LAL Chromogenic assay using a special Endotoxin detection kit according to the Thermo Fisher Scientific Procedural guidelines (Appendix 3).

The Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant Kit is an efficient, quantitative endpoint assay that uses amebocyte lysates derived from the blood of the horseshoe crab to quantitate endotoxin in protein, peptides, antibodies, or nucleic acid samples. Amebocyte lysates are widely used as a simple and sensitive assay for the detection of endotoxin LPS, the membrane component of Gram-negative bacteria.

When endotoxin encounters the amebocyte lysate, a series of enzymatic reactions results in the activation of Factor C, Factor B, and the pro-clotting enzyme. The activated enzyme catalyzes the release of p-nitroaniline (pNA) from the colorless chromogenic substrate, Ac-Ile-Glu-Ala-Arg-pNA, producing a yellow

color. After stopping the reaction, the released pNA is photometrically measured at 405 nm. The correlation between absorbance and endotoxin concentration is linear in the 0.1-1.0 EU/mL and in 0.01-0.1 EU/mL range. The developed color intensity is proportional to the amount of endotoxin present in the sample and can be calculated using a standard curve.

2.5.3.5. Protein Concentration Measurement

The Concentration of protein in the sample was followed up after each step of purification by measuring its absorption at 280nm (Chang and Zhang, 2017) according to the following equation (Stephenson, 2003; Noble and Bailey, 2009):

$$Protein\ concentration = Sample\ absorption\ at\ 280\ nm \times 1\ {}^{mg}/_{ml} \times dilution\ factor$$

2.5.3.6. Detection the Presence of Flagellin by SDS-PAGE

After flagellin purification and endotoxin removal, the presence of protein and its purity level was confirmed by electrophoresed in polyacrylamide gel using the SDS-PAGE technique in 10% separating gel. The procedure in details were shown in Appendix (4).

2.5.4. Laboratory Animals study

In this study, ten groups of albino female adult rats were conducted at an animal house in the faculty of the Science \ University of Kufa \ province of Al-Najaf Al-Ashraf. Each group contained five animals that were immunized with flagellin-a, flagellin-b, or combined flagellin-a & b against *P. aeruginosa*. The study groups were distributed as follows:

1st group (A1): it included treated animals with (4.8μg/10μl) flagellin-a only.

2nd group (A2): it included treated animals with (4.8μg/10μl) flagellin-a and then infected with *P. aeruginosa* MJ isolate.

3rd group (B1): it included treated animals with (4.8μg/10μl) flagellin-b only.

4th group (B2): it included treated animals with (4.8μg/10μl) flagellin-b and then infected with *P. aeruginosa* MJ isolate.

5th group (C1): it included non-treated animals that were infected with *P. aeruginosa* MJ isolate.

6th group (C2): it included normal animals without immunization or infection.

7th group (D): it included treated animals with (4.8μg/10μl) combined flagellin-a & b.

8th 9th 10th (E1, E2, E3) groups: these groups were immunized with (4.8μg/10μl) flagellin-a, flagellin-b, or combined flagellin-a & b respectively, and let to survive in order to investigate the clinical symptoms that shown on the animals after immunization with these proteins.

2.5.4.1. Animals Immunization

The animals were immunized intranasally after sedative the rat intraperitoneal (I.P.) injection by a mixture of Ketamine (40 mg for each kg) and Diazepam (5 mg for each kg) (ULAM Veterinary Staff, 2021) and then immunized by applying 10 μl of mixed flagellins (2.4 μg of each flagellin), or 10 μl of flagellin-a (4.8 μg), or 10 μl of flagellin-b (4.8 μg) on each nostril for 100mg weight of rat at weekly intervals. After 42 days from the beginning of infection, the rats were exposed to $2*10^7$ CFU of *P. aeruginosa* MJ isolate in each nostril. The rats from each group were sacrificed 24 h after infection, then blood was taken for hematological and immunological studies, and the organs (lung, liver, kidney, and spleen) were harvested for histopathological investigation.

The dose of flagellin was calculated according to the equation C1V1=C2V2.

2.5.4.2. Animals Scarification

The animals were weighed by using an electrical balance, then anesthetized use in a mix of ketamine/xylazine (0.5ml/0.1ml) per 200-250 gm of animal weight. Then, vivisected the animals open the abdominal cavity and removed their organs (lung, liver, kidney, and spleen) and taking the weight of all organs mentioned early

with a sensitive balance, and calculating the absolute weight of each organ according to the following equation:

$$\text{Relative Organ Weight} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100$$

Then, the dissected animal organs were fixed with formaldehyde solution (10%) and left until tissue processing and the animals were destroyed in the crematorium.

2.5.4.3. Collection of Blood Sample

Disposable syringes and needles used for blood collection (5 ml) by heart puncture after opening the abdominal cavity before dying. The samples placed in labeled tubes contain EDTA as anticoagulants to prevent clotting of blood to be used for immunological and hematological studies (using flow cytometry techniques and CBC devise respectively) during 24 hrs.

2.5.5. Hematological Examination

Study the differential blood cells was estimated by reading the complete blood count (CBC) using the automated device to read the total WBC, lymphocytes, and other blood parameters.

2.5.6. Immunological Studies Using Flow-Cytometry Techniques

2.5.6.1. Principle

Flow cytometry is a powerful tool to analyze multiple parameters on an individual cell basis. Cell populations can be characterized using a combination of antigens both on the surface and intracellularly. There are a number of practical applications regularly used by immunologists including immunophenotyping, measuring intracellular cytokine production, cellular proliferation, assessing cell viability, and analysis of cell cycle, rare events, stem cells, and fluorescent proteins. Cell sorting based on flow cytometry is used to separate cells into populations of interest.

Flow cytometry technology is based on the measurement of fluorescence associated with cells, typically for immunology detection of monoclonal antibodies

coupled to fluorochromes e.g. FITC anti-CD3, or dilution of fluorescent dyes such as CFSE during proliferation.

Essentially flow Cytometry runs cells past a laser a single cell at a time, detect fluorescence and light scattered from the cell, and records this information for subsequent analysis. Several lasers are commonly used and are named after the emission wavelength or color: 488nm (Blue argon laser), 633nm (Red HeNe laser), 405nm (Violet laser), 532nm (Green laser), and 360nm (UV laser).

Fluorochromes which are preferentially excited on only one of the lasers are available with new fluorochromes and dual-conjugated i.e. 'tandem' dyes being commercially produced. Some common fluorochromes are FITC, PE, PerCP, APC, and Pacific Blue, commonly used tandem fluorochromes include PerCP-Cy5.5 and APC-Cy7.

2.5.6.2. The Markers that Used in Flow-Cytometry Techniques

The Flow Cytometry was performed to count the immunological cells differentially by using six-CD markers specific to each cell using 3 leasers 8 color FACSCanto^{TM} II flow Cytometry device.

Seven CD markers were used in this study, they include:

1. anti-rat IgM antibody as a marker for B cell (Male *et al.*, 2006).
2. anti-rat CD3 antibody as a marker for T cell (Male *et al.*, 2006).
3. anti-rat CD4 antibody as a marker for T helper cells (Male *et al.*, 2006).
4. anti-rat CD161 antibody as a marker for Th17(Fergusson *et al.*, 2011).
5. anti-rat IgG2a antibody as a marker for Th1 (William and Paul, 2003; Abbas and Lichtman, 2011):

Gamma interferon (IFN-γ) was often secreted by T helper type 1 (Thl) cells. It is now shown that IFN-γ stimulates the expression of immunoglobulin (Ig) of the IgG2a isotype and inhibits the production ofIgG3, IgG1, IgG2b, and IgE(Snapper and Paul, 1987).

6. anti-rat IgG1 antibody as a marker for Th2 (William and Paul, 2003; Abbas and Lichtman, 2011):

High doses of aqueous protein antigens induce a form of immunological tolerance in which interleukin 2 (IL-2) and interferon γ (IFN-γ)-secreting T helper type 1 (Thl) cells are inhibited, but IL-4-secreting (Th2) cells are not. This is manifested by reduced proliferation of antigen specific T cells upon *in vitro* restimulation, and marked suppression of specific antibody responses of the immunoglobulin (Ig)G2a, IgG2b, and IgG3 isotypes, but not of IgG1 and IgE(Burstein and Abbas, 1993).

Th1 cells – CD4+ T cells that produce IL-2 and interferon-γ (IFNγ), but not IL-4, are designated Th1 and are chiefly responsible for delayed-type hypersensitivity responses, but can also help B cells produce IgG2a (mouse), but not much IgG1 or IgE. Th2 cells – CD4+ T cells that produce IL-4, IL-5, IL-10, and IL-13, but not IL-2 or IFNγ, are designated Th2 and are very efficient helper cells for the production of antibodies, especially IgG1 and IgE (Male *et al.*, 2006).

The Markers that are used in this study are from BioLegend Company/ and their details are clarified in Appendix (5).

2.5.6.3. Flow Cytometry Procedures

The procedure of flow cytometry was performed according to company instructions as shown in Appendix (6).

2.5.7. Histopathological Examination

After keeping in 10% formaldehyde, the specimens of rat tissues (lung, spleen, liver, heart, and kidney) were dehydrated, then embedded in wax of paraffin followed by paraffin blocks sectioned into (4 μm thick). Hematoxylin and eosin (H&E) dyes have been used to stain the de-waxed sections on slides to study the pathological effect in each organ. (Bancroft and Gamble, 2008; Suvarna *et al.*, 2018) as shown in Appendix (7).

2.6. Statistical analysis

Statistical analysis was carried out using SPSS version 27 (SPSS, IBM Company, Chicago, IL 60606, USA) using a one-way ANOVA and Mann-Whitney tests for the normally and not-normally distributed value (respectively).

Chapter Three

Results & Discussion

3. Results and Discussion

3.1. Isolation of Bacterial Isolates

The identification of *P. aeruginosa* isolates was confirmed using VITEK2 system after using ordinary culturing and biochemical tests. Two bacterial isolates (number 3 and 23) were taken, isolate no.3 contain *fliC* gene encode for flagellin-a were used to purify flagellin-a protein and isolate no.23 that contain *fliC* gene encode for flagellin-b were used to purify flagellin-b protein.

For animal dosing (infection), another *P. aeruginosa* isolate (named as MJ isolate) recovered from burn infection was selected for this purpose. The AST of MJ isolate showed that it had extensive drug resistance (XDR) pattern (Table 3-1).

Table (3-1): Antibiotics susceptibility patterns of XDR *P. aeruginosa* recovered from burn wound infection (MJ isolate) using VITEK 2 system

Types of antibiotics	Sensitivity	Types of antibiotics	Sensitivity
Amoxicalve (AMC)	R	Tetracyclin (TE)	R
Amikacin (Ak)	R	Cefdinare (fed)	R
Cefotaxime (CTX)	R	Nalidixic acid (NA)	R
Ceftriaxon (CTR)	R	Norfloxacin(NOR)	R
Imipenem (IPM)	R	Gentamycin(cN)	R
Ciprofloxacin (Cip)	R	Colistin	H.S
Levofloxacin (Lev)	R	Ceftazidime (caz)	R
Lincomycin	R	Azthromycin (AZH)	R
Cefoxitine (fox)	R	Fosfomycin	R
Cefixime (cfm)	R	Doxycycline DXT	R
Trimethoprim/sulphamethoxazole	R	Pipracillin with tazobactam	R
Mropeneam (mem)	R	Azitronam (ATM)	R
Tobromycine (tob)	R	Pipracillin	R
Netimicine (Net)	R	Trimethoprim	R
Cefepime (Cpm)	R	Ticarcillin/Clavulanic acid	R
Nitrofurantion (NIT)	R	Refampine RA	R
Ampicillin/ Cloxacillin	R	Tigecycline	R
R: Resistance; H.S: High sensitive			

3.2. Flagellin Gene (*fliC*) Detection in *P. aeruginosa* by PCR

Several studies reported that there are two types of flagellin proteins have been identified in *P. aeruginosa,* type-a and type-b, which can be detected based on molecular sizes. *P. aeruginosa* flagellin type-a and b do not exhibit phase variation; a single strain produces single type of flagellin, and no switching between types a and b has been observed. Oligonucleotide primers specific for N-terminal (CW46) and C-terminal (CW45) conserved regions of flagellin gene used for PCR amplification of the flagellin gene of *P. aeruginosa* (Allison *et al.*, 1985).

In the present study after using the specific primers for *fliC* gene for identification the type of flagellin in *P. aeruginosa* isolates by conventional PCR, The results of PCR showed the two types of flagellin in different isolates; type-a at 1020 bp., and type-b at 1250 bp., as shown in Figure (3-1).

The amplification of *fliC* gene in *P. aeruginosa* isolates by using specific primer in conventional PCR was give variable product size for two types; type-a at 1020bp , type-b at 1250bp, and both band for flagellin-a and after the optimum condition for this primers in PCR reaction.

Shehab and Jassim who amplified *flic* gene using the same primers and they obtained two different bands (1250bp and 1020bp) (Shehab and Jassim, 2020).

3.3. Results of Flagellin Protein Purification

The cost-effectiveness of vaccination adjuvants is one of the most critical factors in their commercialization. Flagellin has been used in modest dosages (Honko *et al.*, 2006), but it should be cost-effective as a substitute for present adjuvants or in combination with future vaccines. Flagellin has been found to be an effective vaccination adjuvant (Lu and Swartz, 2016). According to various research, flagellin has a greater potential for use as an adjuvant in animal vaccinations than in human vaccines (Song *et al.*, 2011). The use of flagellin as a vaccine antigen has also showed promise. According to some studies, it can be a protective vaccine antigen that stimulates humoral and cellular immunity (Behrouz *et al.*, 2017). Because of flagellin sequence conservation, it can also protect individuals from other closely related species (Beatson *et al.*, 2006).

By modifying of several steps from the standard procedure, this study developed a method for isolating flagellin from *P. aeruginosa*. This procedure is cost-effective with an acceptable purity, making it suitable for use in most limited laboratories. This method, to purify flagellin from *P. aeruginosa*, aids in vaccine development.

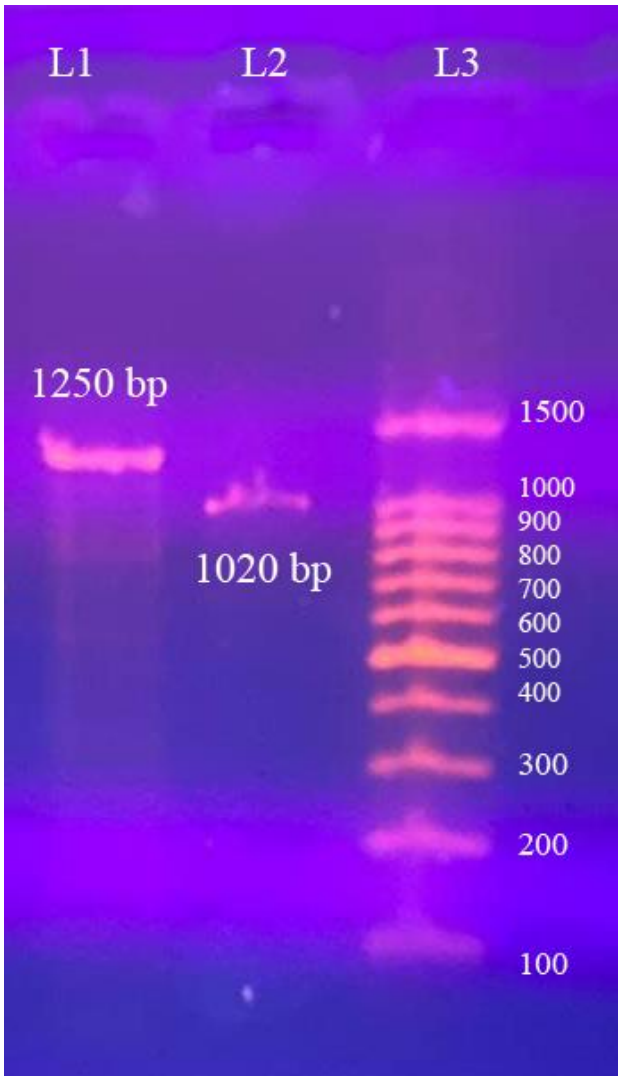

Figure (3-1): The gel of agarose (1.5% conc.) under UV light after electrophoresis of amplified *fliC* gene PCR products for 60 min. at 70 V. using Ethidium bromide stain. L1: band with size 1250 bp that represent type-b *fliC* gene amplified from isolate no.3 L2: band with size 1020 bp that represent *fliC* gene type-a amplified from isolate no.23. L3: DNA ladder 100 bp.

3.3.1. Flagellin Protein Precipitation

The flagellin-a and b were partially purified from *P. aeruginosa* isolate no. 23 and 3 respectively using 70% ammonium sulfate and special ultrafiltration tube called Pierce™ Protein Concentrator.

About 0.5 ml of sample contain the partially purified flagellin was obtained from 1000 ml of *P. aeruginosa* culture broth after using ultrafiltration tubes.

For purification of low molecular weight proteins by ammonium sulfate, like interleukin-1β, as a rule require higher salt concentration for precipitation than larger molecular proteins, for example, large multiprotein complexes can often be salted out with < 20% saturation (Wingfield, 1998). Salt precipitation has been widely used to fractionate membrane proteins (Schagger, 1994). During centrifugation, these precipitates will often float to the top of tube rather than pelleting. Crystallization is a traditional method of protein purification. Jakoby (1971) described a general method that involves extracting $(NH_4)_2SO_4$-precipitated protein with successively dilute $(NH_4)_2SO_4$ solutions at low temperature.

Ammonium sulfate and other neutral salts stabilize proteins by preferential solvation (Timasheff, 1997). Proteins are often stored in $(NH_4)_2SO_4$, which inhibits bacterial growth and contaminating protease activities. Protein unfolded by denaturants such as urea can be pushed into native conformations by the addition of $(NH_4)_2SO_4$ (Mitchinson and Pain, 1985). A practical application is the folding of recombinant proteins. For example, HIV-1 Rev expressed in *E. coli* was solubilized using urea, purified by ion-exchange chromatography in the presence of urea, then folded by the addition of 0.5 to 1.0 M $(NH_4)_2SO_4$ (Wingfield *et al.*, 1991).

The use of Pierce™ Protein Concentrator (a vertical low protein-binding, high-flux polyethersulfone (PES) membrane), which allows for processing of large sample volumes to 20 ml in a short time (1-2 hrs.).

This device has versatility in work that perform sample concentration, infiltration, or buffer-exchange that consider an excellent replacement to the dialysis to remove the salt because the dialysis consume time and dilute protein (*Pierce™ Protein Concentrator PES, 10K MWCO, 5-20 mL*, 2022).

3.3.2. Endotoxin Removal

Endotoxin (LPS) removal from the two purified protein was doing using special endotoxin removal Spin column according to the Thermo Fisher Scientific Procedural guidelines .

The Thermo ScientificTM PierceTM High Capacity Endotoxin Removal Resin was making up of porous cellulose beads with modified ε-poly-L-lysine covalently linked to the surface. The affinity ligand in modified polylysine has a high affinity for endotoxins, which eliminates the toxicity associated with other methods that use polymixin B ligands and sodium deoxycholate buffers. Endotoxin levels can be decreasing by 99% in samples containing 10,000 EU/ml due to the resin's binding capability of 2,000,000 Endotoxin Units (EU)/ml; typical protein samples treated with the resin have a final endotoxin concentration below 5 EU/ml.

The use of modified poly-L-lysine instead of polymixin B in this study was due to the possibility of polymyxin B to interact with immune response as reported by Zhong *et al.* (2008) that the capability polymyxin B is capable to activate murine NK cells.

This method also used by Faezi *et al.* (2017) for removal of enotoxin (lipopolysaccharide) from protein (PilQ and triple PilA-related disulfide loop peptides fusion protein) purified from *P. aeruginosa*.

3.3.3. The Results of UV-Spectrum

UV-spectrum of the flagellin-a and b (purified from *P. aeruginosa* isolate no.23 and 3 respectively) was detected before and after endotoxin removal using (UV-1900i uv-vis spectrophotometer) to show the presence of protein and other contaminants. Figure (3-2) shows the UV-spectrum (absorbance) of samples in different wavelengths (190-350 nm). The absorbance of protein detected at 280 nm while other contaminants detected at the other wavelengths. The results of absorbance showed that the protein absorbance at 280 nm in flagellin-a and b after endotoxin removal (marked as L3 and L4 respectively) were less than absorbance in flagellin-a and b before endotoxin removal (marked as L2 and L1 respectively).

The decreasing of protein was due to its subsequent lose in the steps of endotoxin removal.

On the other hand, the endotoxin (LPS) (at 190-250 nm) was decreased in flagellin-a and b after endotoxin removal (L3 and L4) to about zero in compared with same samples before endotoxin removal (L2 and L1) as shown in (Figure 3-3)

The final concentration of flagellin-a and flagellin-b after endotoxin removal were 3.2 mg/ml and 4.8 mg/ml respectively as shown in Table (3-2).

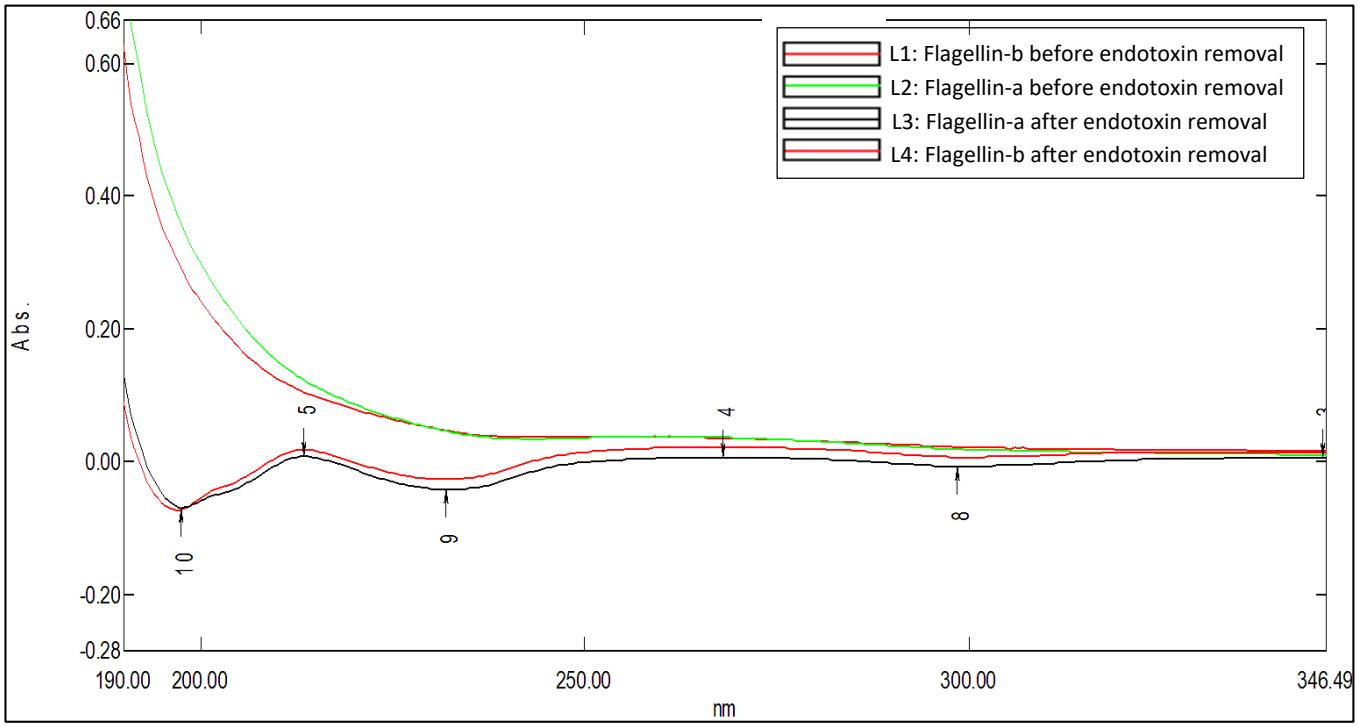

Figure (3-2): UV-spectrum for protein sample after purification using UV-1900i uv-vis spectrophotometer. L1 for flagellin-b purified from *P. aeruginosa* isolate no.3 and L2 for flagellin-a purified from *P. aeruginosa* isolate no.23 before endotoxin removal. L3 for flagellin-a purified from *P. aeruginosa* isolate no.23 and L4 for flagellin-b purified from *P. aeruginosa* isolate no.3 after endotoxin removal.

Table (3-2): Purification steps results from *P. aeruginosa* isolates no. 3 and 23

	Purification step	**volume**	**Flagellin-a conc.**	**Flagellin-b conc.**
1	Culture broth	1000ml	-	-
2	Centrifugation	20ml	-	-
3	(NH4)2SO4 70% saturation	0.5ml	12 mg/ml	6.4 mg/ml
4	Endotoxin removal	0.5ml	3.2 mg/ml	4.8 mg/ml

3.3.4. Endotoxin Detection

Endotoxins consist of LPS, which are biologically active, structural components of the outer cell membrane of all gram-negative bacteria. Small amounts of endotoxin in recombinant protein preparations can cause side effects, including endotoxin shock, tissue injury and death in host organisms; therefore, it is essential to remove endotoxins from drugs, injectable and other biological products (Huszczynski *et al.*, 2019).

After endotoxin removal from purified protein using (Endotoxin Removal Spin Column) and measuring the concentration of endotoxin by a LAL Chromogenic assay using Endotoxin detection kit (Pierce™ Chromogenic Endotoxin Quant Kit), according to the standard curve, the endotoxin levels in the two purified a and b flagellin samples were 1.3 and 1.85 EU/ml respectively, as shown in (Figure 3-3).

A recommendation levels of endotoxin in the preclinical use is <10EU/ml for genetic vectors was chosen due to the extensive purification needed for these types of vaccines. A level of <20EU/ml for recombinant subunits and poly- saccharide vaccines was recommended due to the high purity and highly controlled method of manufacture for these vaccine types. A recommendation of <200EU/ml for live attenuated vaccines is based on the complex nature of these vaccines containing multiple antigens. A similar rational goes into the recommendation of <500EU/ml for inactivated vaccines. Toxoid vaccines have the highest recommended values of <200,000EU/ml due to the complexity of these vaccines as mentioned by Brito and Singh (2011).

The levels of endotoxin in this study were indicated on the excellent of Endotoxin Removal Spin Column in decreasing of endotoxin to less than the 2EU/ml.

The results of present study showed that the amount of endotoxin remaining in the purified flagellin samples were less than amount of endotoxin in the study of Zhang *et al.*, (2003) who used two steps of chromatography to remove LPS from purified flagellin samples to reach 2.7 EU/mg of protein. This indicates that

endotoxin removal using Spin Column is more effected in decreasing of endotoxin in purified flagellin samples than chromatography.

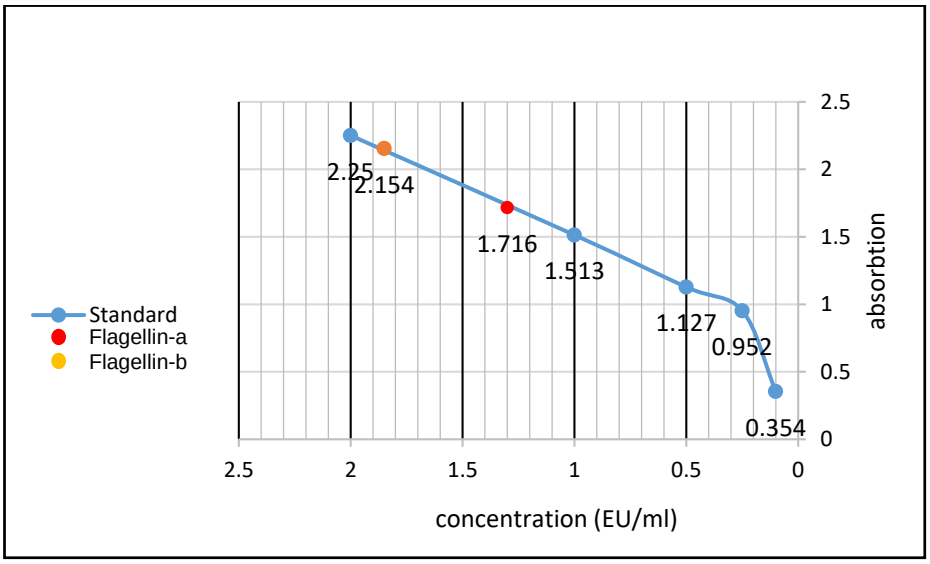

Figure (3-3): Endotoxin levels of the flagellin-a purified from *P. aeruginosa* isolate no.23 and flagellin-b purified from *P. aeruginosa* isolate no.3 after endotoxin removal using Pierce endotoxin quantitation kits

3.3.5. Detection of Purified Flagellin Using SDS-PAGE

To confirm the presence and to determine the size of partial purified flagellin-a and b in samples and to detect its purity, SDS-PAGE was performed.

The results of SDS-PAGE technique showed one band with size ~39KDa in the flagellin-a purified from *P. aeruginosa* no.23 and one band with size ~45KDa in the flagellin-b purified from *P. aeruginosa* no.3 as shown in Figure (3-4). The results of SDS-PAGE showed that there is only one band for each protein, which indicated the high purified flagellin obtained throughout the purification processes used in this study as shown in (Figure 3-4).

Many authors worldwide studied *P. aeruginosa* flagellin and they found that flagellin-a and b isolated from different *P. aeruginosa* strains had different sizes ranging from 39 to 53 KDa (Totten and Lory, 1990; Brimer and Montie, 1998; Arora *et al.*, 2004).

Many authors worldwide used SDS-PAGE in the detection of flagellin purified from *P. aeruginosa* and other bacteria (Schirm *et al.*, 2004; Oliveira *et al.*, 2011; Faezi *et al.*, 2016; Khani and Bagheri, 2018; Hashemi *et al.*, 2019).

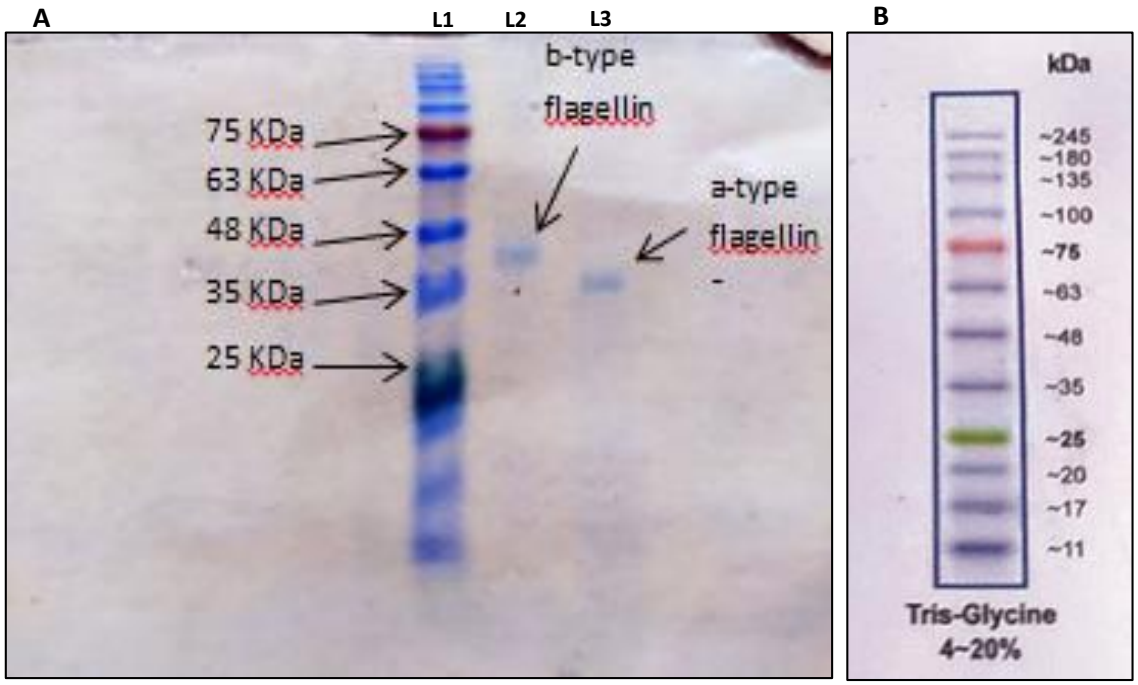

Figure (3-4): Polyacrylamide gel after electrophoresis of flagellin-a purified protein from *P. aeruginosa* isolate no.23 and flagellin-b purified protein from *P. aeruginosa* isolate no.3 by SDS-PAGE technique. A; L1: Protein Ladder; L2: flagellin type-b ~45kDa; L3: flagellin type-a ~39kDa. B; Standard ladder provided by manufacturer's instructions

3.4. Results of Animal Immunization

The animals were immunized intranasaly with the purified flagellin. (For six doses separated by 1 week interval) A1 and A2 immunized with flagellin-a, B1 and B2 immunized with flagellin-b, C1 and C2 non-immunized, and D immunized with bivalent flagellin-a and b.

However in in order to investigate the clinical symptoms that may be appeared on the animals after immunization groups other groups same as A1, B1 and D designated as E1, E2 and E3 (immunized with flagellin a, flagellin b, and combined flagellin respectively) were allowed to survive for three months after complete the all doses of immunization.

Results of this study found that group E2 didn't show any clinical respiratory symptoms (such as nasal secretion, sneezing, shortness of breath, etc.) or any other systemic symptoms after immunization for three months. While the three other groups D, E1 and E3 showed a severe respiratory symptoms such as nasal secretion,

sneezing, shortness of breath in addition to bleeding from eyes and blindness (Figure 3-5) and then they were all died.

On the other hand, the immunized animals of group A2 (with flagellin-a) and group B2 (with flagellin-b) were more active after infection when compared with non-immunized infected animals (group C2) that may be to the effect of flagellin in prevention of respiratory *P. aeruginosa* infection.

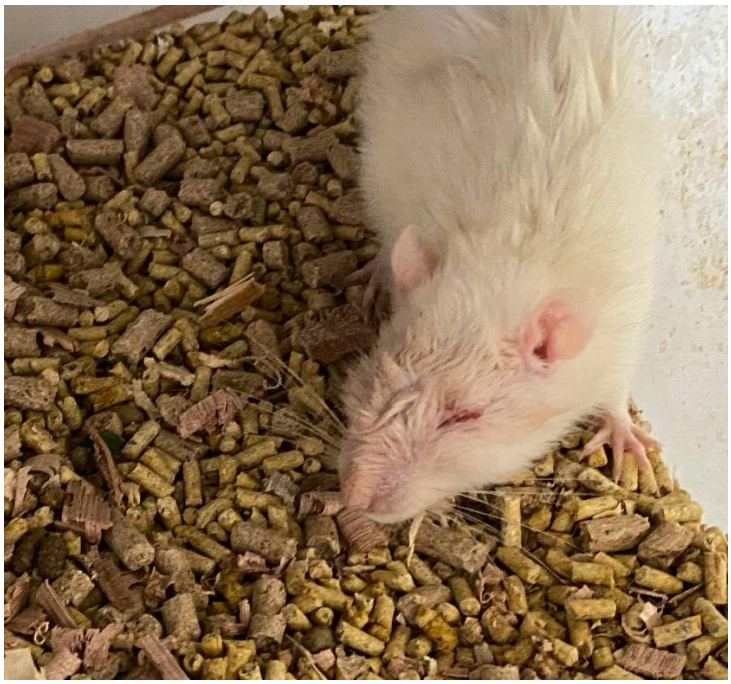

Figure (3-5): The clinical symptoms of Albino rat after immunization with bivalent (a & b) flagellin (group E3). The animal showing severe respiratory symptoms such as nasal secretion in addition to bleeding from eyes.

The flagellin is best known as a pathogen-associated molecular pattern that binds to the extracellular TLR5 (Hayashi *et al.*, 2001) leading to activation of the pro-inflammatory MyD88 pathway and the NLRC4-inflammasome, respectively (Vijay-Kumar *et al.*, 2010). TLR5 mediates a major component of the epithelial cytokine and chemokine responses leading to neutrophil recruitment in *P. aeruginosa* lung infection (Prince, 2006; Zhang *et al.*, 2007; Beaudoin *et al.*, 2013), and contributes to the production of pro-IL-1ß in monocytes and macrophages (Descamps *et al.*, 2012).

Behrouz and his colleagues showed that the immunization with bivalent flagellin-a and b increase the production of IL-17 from Th-17 cell that give effective cellular and humeral immune responses (Behrouz *et al.*, 2017).

Th17 cells and Treg cells share a common signaling pathway mediated by TGF-β. However, pro-inflammatory signals present during cell activation regulate the fate of these cells reciprocally (Ivanov *et al.*, 2007; Zhu and Paul, 2010). Th17 cells produce IL-17, IL-22, and IL-23, recruit neutrophils, and promote inflammation at the infection site. By contrast, Treg cells produce anti-inflammatory cytokines IL-10 and TGF-β, suppress activity of a variety of immune cells, and thereby inhibit immune responses. Thus, these two cell types play opposite roles during inflammatory and immune responses this maintain immune homeostasis (Littman and Rudensky, 2010).

Despite the important of Th17 in eradication of the bacteria but there have been many reports indicate the contribution of Th17 cells and IL-17 that cause imbalance of Th17/Treg cell homeostasis is an important factors in the developing of chronic autoimmune disease in human and mouse (Lee, 2018; Baker *et al.*, 2020).

Other authors showed the production of IFN-γ in lung tissue was associated with increased bacterial clearance, but its overproduction appeared to contribute to *P. aeruginosa* infections in the eye reach to blindness (Hazlett, 2002; Sadikot *et al.*, 2005).

Results of this study regarding the clinical symptoms appeared in group D , and E3 after immunization with bivalent flagellin (a and b) and appeared in group E2 after immunization with flagellin-a maght be due to the imbalance of Th17/Treg cell homeostasis and the overproduction IFN-γ from Th-1 (as shown in the section 3.6.4) that lead to autoimmune disease.

While group E2 didn't show any pathological symptoms that indicate that flagellin-b has no disturbing effect on the animals.

3.5. Hematological Examination

3.5.1. Analysis the Number of Lymphocytes in Blood

The survival immunized groups were further tested for hematological analysis. The complete blood count for all animal groups was investigated.

The results showed that the lymphocytes were decreased in all groups after infection, but this decrease was more in the non-immunized groups (C1 and C2) than immunized groups (A1, A2, B1, B2) as shown in Table (3-3). It was showed that a significant decrease between C1 and C2 groups (p-value= 0.018) while there was non-significant decrease between B1 and B2 groups (p-value= 0.175). Also, there was no significant difference between A1 and A2 groups (p-value= 0.638) (Table 3-3).

The decreasing of lymphocytes that occur after infection with *P. aeruginosa* in A2, B2, and C2 groups may be due to the effecting of virulence factors that produce by *P. aeruginosa* that inhibit lymphocytes proliferation (Theander *et al.*, 1988) or due to the homing of lymphocytes in poulmonary tissues (Häusler *et al.*, 2002)

Theander and his colleagues showed the effect of protease AP and elastase (ELA) in the inhibition of lymphocytes proliferation by the effect on IL-2 that inhibit growth factor binding to its receptors on lymphocytes (Theander *et al.*, 1988). Also, these results were also compatible with the results of Häusler and his colleagues who showed that a decrease in prephiral lymphocytes is due to the homing of these cells in poulmonary tissues after comparing its results with its histopathologic findings (Häusler *et al.*, 2002).

Table (3-3): The difference in lymphocytes among animals groups

	Mean of Lymphocytes $\times 10^9$	Std. Error	Mean Difference	p-value
Group A1	6.67 ±	1.18	0.77	0.638
Group A2	5.9 ±	1.46		
Group B1	7.17 ±	0.73	2.03	0.175
Group B2	5.14 ±	0.59		
Group C1	6.3 ±	1.07	3.24	0.018*
Group C2	3.06 ±	0.87		
***significant less than 0.05**				

3.6. Immunological Analysis

The immunological analysis performed using flow cytometry (FC) that consider a powerful tool to analyze multiple parameters on an individual cell basis. Cell populations can be characterized using a combination of antigens both on the surface and intracellularly**.** The using of FC technique to keep track of the cellular immune response is a very rapid, accurate, and highly sensitive method in counting the direct immune cells rather than other traditional immunological techniques (ELISA, etc.) (Moncrieffe, 2022).

The immunological response among immunized animal groups (A1, A2, B1, B2, C1 and C2) was analyzed by flow cytometery (FC) technique in order to measure the immune cells using specific markers (Paragraph 2.5.6.1)

3.6.1. Analysis of CD3+ Lymphocytes

Results of detection of CD3+ among different animal groups (before and after infection) showed that there was a non-significant decline in CD3+ cells percentage from 0.69 in group B1 to 0.67 in group B2 (P value 0.53). Also this decline was noted more clearly when compared between C1 and C2 groups (from 0.65 to 0.59). However, this value didn't reach the significance level (P value 0.12) as shown in Table (3-4).

However the results of detection of CD3+ between A1 and A2 groups revealed that there was no decline of CD3+ percentage in group A1 compared to group A2 (P value 1). Table (3-4) show the analysis of CD3+ among different animal groups.

The non-significant decrease of CD3+cells numbers in the immunized groups compare to non-immunized groups that may be due to the elevation of these cells in the immunized groups before infection as the effect of immunization by flagellin.

Table (3-4): The mean and mean difference of CD3+ lymphocytes among the experimental groups before and after infection.

	Mean of CD3+ Lymphocytes ± std. Error		**Mean Difference**	**P value**
Group A1	0.67 ±	0.01	0	1
Group A2	0.67 ±	0.01		
Group B1	0.69 ±	0.03	0.02	0.53
Group B2	0.67 ±	0.02		
Group C1	0.65 ±	0.04	0.06	0.12
Group C2	0.59 ±	0.03		
***significant P value is less than 0.05**				

The results also found that there was slight decrease of CD3+ lymphocytes between groups B1 and B2, however, there was no decrease of lymphocytes between groups A1 and A2 as shown in (Figure 3-6).

On the other hand, the comparison between immunized and non-immunized groups regarding the detection of CD3+cells among immunized groups after infection, results showed that there was a significant difference between group B2 and group C2 (P value ≤ 0.05) and between group A2 and group C2 (P value ≤ 0.05) as shown in Table (3-5).

These results are confirmed by the results of lymphocytes count by measuring CBC (Table 3-3) that indicated a significant decline of lymphocyte count after infection.

These results may be due to the effect of flagellin in the increasing lymphocytes proliferation (Shim *et al.*, 2016) in the immunized groups in converse with the decreasing of lymphocytes as a results of *P. aeruginosa* infection in the non-immunized groups (Theander *et al.*, 1988; Häusler *et al.*, 2002).

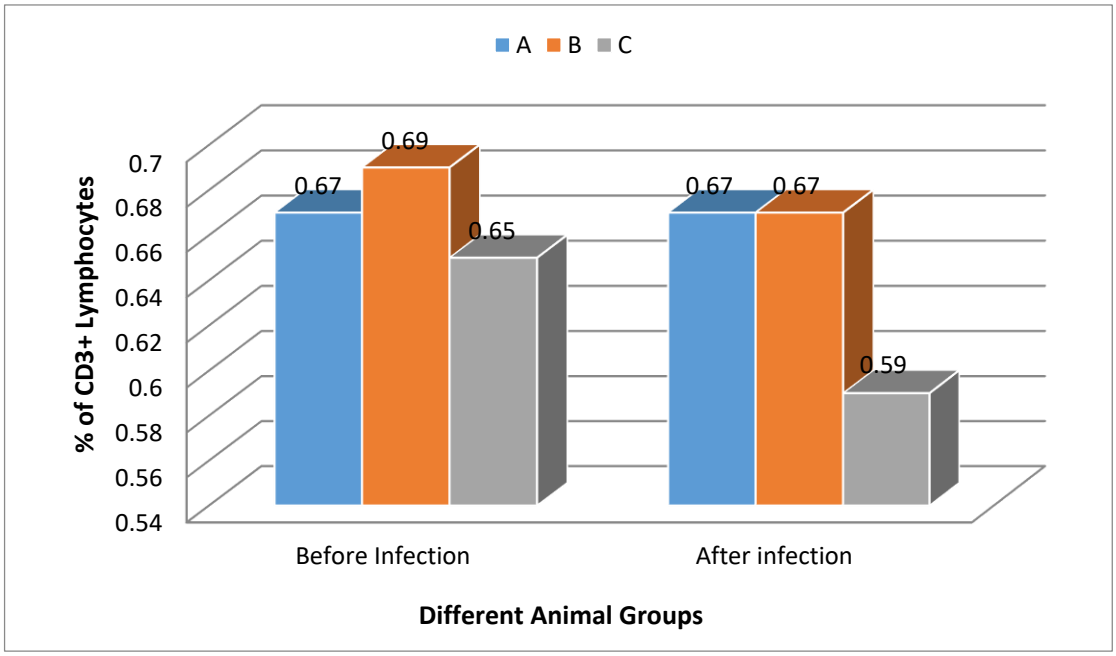

Figure (3-6): The CD3+ lymphocytes count among immunized with flagellin and non-immunized groups before and after infection with *P aeruginosa*. Significant (P value ≤ 0.05) for (B2 & C2) and (A2 & C2). A before infection represent group A1; A after infection represent group A2; B before infection represent group B1; B after infection represent group B2; C before infection represent group C1; C after infection represent group C2.

Table (3-5): The mean difference of CD3+ lymphocytes among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups			
Experimental groups		**Mean Difference**	**P value**
Before infection	B1 & A1	0.01	0.78
	B1 & C1	0.037	0.31
	A1 & C1	0.026	0.5
After infection	B2 & A2	- 0.01	0.7
	B2 & C2	0.07	0.038*
	A2 & C2	0.08	0.039*
***significant P value is less than 0.05**			

3.6.2. Analysis of T Helper (CD3+CD4+) Lymphocytes

In this study the detection of T helper cells by CD3 and CD4 markers using the FC technique and the comparison among groups before and after infection showed a significant increase CD3+CD4+ cells after infection in the flagellin-b immunized groups (Group B2) (P value ≤ 0.05), while the comparison between the flagellin-a-immunized groups before and after infection showed a significant decrease of CD3+CD4+ cells after infection (Group A2) (P value ≤ 0.05). While when comparing between non-immunized groups C1 and C2, the results showed a non-significant decrease between them (P value 0.97) as shown in Table (3-6).

Table (3-6): The mean and mean difference of CD3+CD4+ lymphocytes (T helper cells) among the experimental groups

	Mean of (CD3+CD4+) Lymphocytes ± std. Error		Mean Difference	P value
Group A1	0.98 ±	0.006	0.045	0.029*
Group A2	0.93 ±	0.049		
Group B1	0.94 ±	0.014	- 0.04	0.008*
Group B2	0.98 ±	0.005		
Group C1	0.98 ±	0.0051	0.0006	0.97
Group C2	0.97 ±	0.0058		
***significant P value is less than 0.05**				

Regarding to comparison between immunized and non-immunized groups before or after infection separately, the results showed a significant elevation of T helper cells in group A1 compared with group B1. On the other hand, elevation of T helper cells in group C1 compared with group B1 (P value ≤ 0.05) (Table 3-7).

On the other hand, when comparing among these groups after infection the results showed a significant elevation of T helper cells in group B2 greater than group A2 (P value ≤ 0.05) and non-significant elevation of T helper cells in group B2 compared with group C2. These results indicate that the flagellin-b had a significant role in the elevation of T helper cells more than flagellin-a. This

elevation is very important in the eradication of *P. aeruginosa* infection among immunized groups (Table 3-7; Figure 3-7).

The decrease of CD3+CD4+ cells after infection in group A2 and C2 in this study were compatible with the results of Dzik *et al.,* (2022) who showed a low level of CD3+CD4+ cells after *in vitro* incubation with lysates of *P. aeruginosa* strains that associated with a higher concentration of alkaline protease (AP) or elastase in the lysates. These results were also compatible with the results of Häusler and his colleagues, they revealed CD4 and CD8 cell counts were decreased or normal after infection with *P. aeruginosa* (Häusler *et al.,* 2002).

However, flagellin-b immunized groups showed a contrast results with the studies of Dzik *et al.* (2022) and Häusler *et al.,* (2002) relevant to significant elevation of CD3+CD4+ cells after infection (group B2) which indicated that flagellin-b is better than flagellin-a in the elevation of T helper cells which necessary in the eradication of this bacteria.

Table (3-7): The mean difference of CD3+CD4+ lymphocytes (T helper cells) among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups			
Experimental groups		**Mean Difference**	**P value**
Before infection	B1 & A1	- 0.04	0.02*
	B1 & C1	- 0.04	0.009*
	A1 & C1	- 0.006	0.69
After infection	B2 & A2	0.047	0.014*
	B2 & C2	- 0.004	0.76
	A2 & C2	- 0.05	0.009*
***significant P value is less than 0.05**			

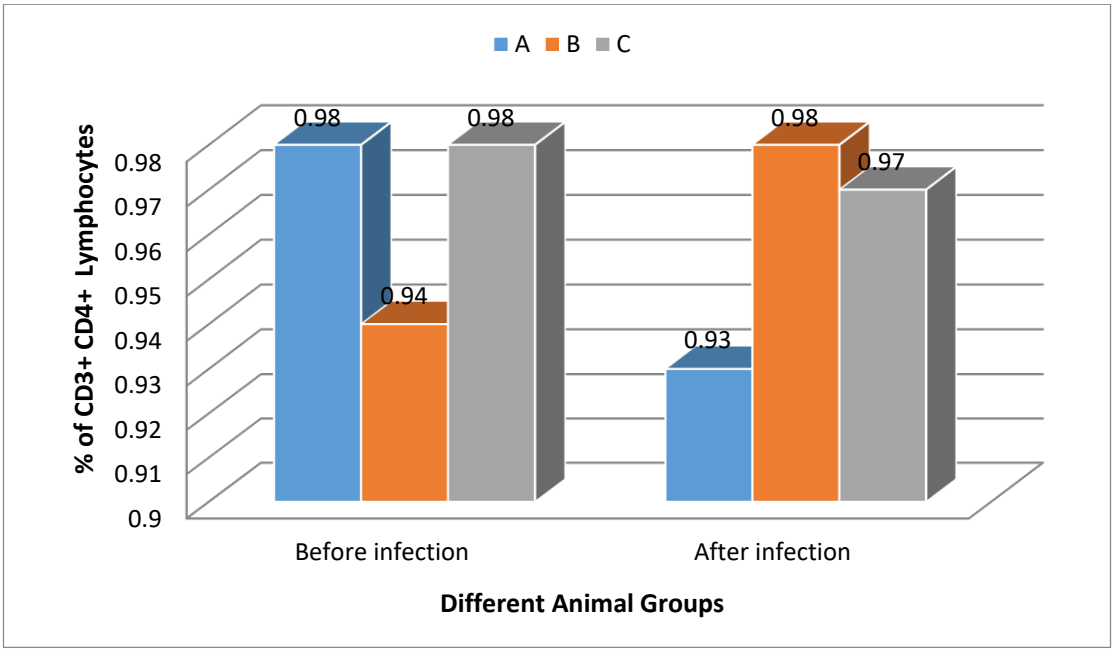

Figure (3-7): The difference of (CD3+CD4+) Lymphocytes among immunized and non-immunized groups before and after infection. A before infection represent group A1; A after infection represent group A2; B before infection represent group B1; B after infection represent group B2; C before infection represent group C1; C after infection represent group C2.

3.6.3. Analysis of Th17 Lymphocytes

Several studies revealed that many cell subsets from T lymphocytes produce IL-17 cytokine that play an important role in the regulation of immune response through the promoting of epithelial cells in the lung to produce antimicrobial peptide or by mediating as a pro-inflammatory cytokine that recruit the phagocytic cells (neutrophil and microphage) to the lung to enhance its bacterial phagocytosis and clearance (Priebe *et al.*, 2008; Shahrara *et al.*, 2010; Wu *et al.*, 2012).

In the peripheral blood, the CD4+ cells secret IL-17 which have CD161 marker on its surface that consider a hallmark to Th17 cells. These cells secrete two type of IL-17 (IL-17 A and F) and consider a novel subtype from the T helper cells (Ivanov *et al.*, 2006). Also there are other cells that secret IL-17 and have CD161 on their surface but they are subtype from CD8+ T cells (Billerbeck *et al.*, 2010). So that CD161 marker was used as indicator for detection of Th17 in this study.

3.6.3.1 Analysis of Total Th17 (CD3+CD161+) Lymphocytes

The investigation of CD3+CD161+ cells comprise the total Th17 regardless it subtype from T helper or cytotoxic T cells. When comparing among study groups, results found that a significant increase of these cells from 0.42 in group B1 to 0.71 in group B2 (P value ≤ 0.05). While there were no significant differences between immunized groups (A1 and A2) on the one hand, and non-immunized groups (C1 and C2) on the other hand, respectively (Table 3-8).

However, the comparison among infected groups between group B2 and C2 was showed a significant difference between them (P value ≤ 0.05). Also when comparing between the group B2 and the group A2 showed a significant difference between them (P value ≤ 0.05). While there was no significant difference found between group A2 and group C2 (Table 3-9 ; Figure 3-8).

These results indicate that the flagellin-b had a significant role in the elevation of Th-17 more than flagellin-a. This elevation is very important in the eradication of *P. aeruginosa* infection among immunized groups and even among non-immunized groups.

Table (3-8): The mean and mean difference of CD3+CD161+ lymphocytes (Total Th17) among the experimental groups

	Mean of (CD3+CD161+) Lymphocytes ± std. Error		**Mean Difference**	**P value**
Group A1	0.48 ±	0.129	0.13	0.28
Group A2	0.35 ±	0.1		
Group B1	0.42 ±	0.017	- 0.29	0.008*
Group B2	0.71 ±	0.081		
Group C1	0.45 ±	0.06	0.13	0.21
Group C2	0.32 ±	0.04		
***significant P value is less than 0.05**				

Table (3-9): The mean difference of CD3+CD161+ lymphocytes (Total Th17) among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups			
Experimental groups		**Mean Difference**	**P value**
Before infection	B1 & A1	- 0.06	0.58
	B1 & C1	- 0.03	0.75
	A1 & C1	0.03	0.78
After infection	B2 & A2	0.36	0.003*
	B2 & C2	0.39	0.001*
	A2 & C2	- 0.03	0.798
***significant P value is less than 0.05**			

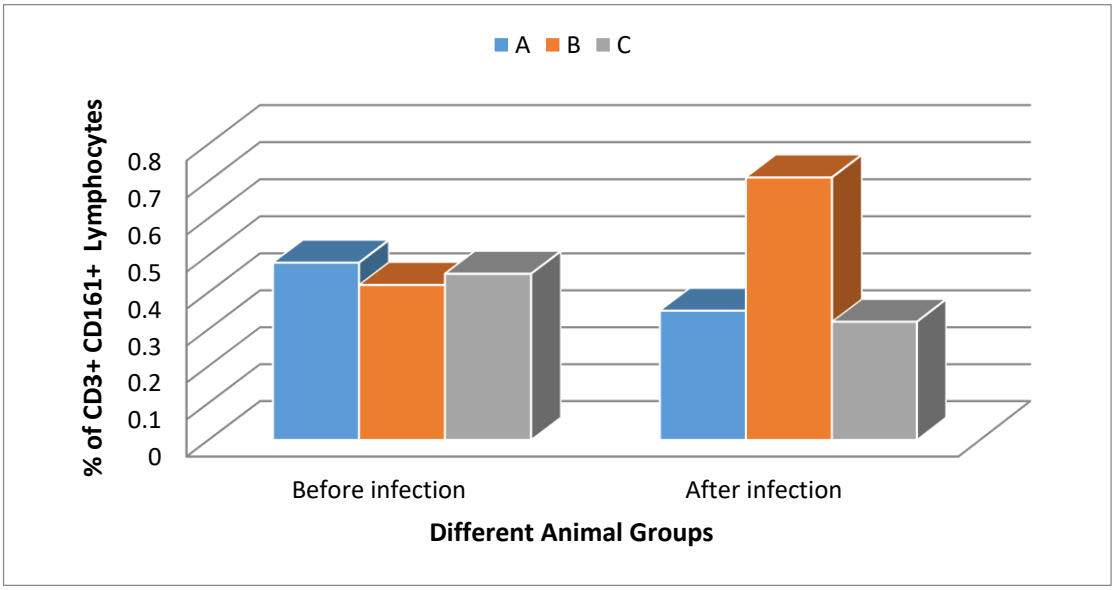

Figure (3-8): The difference of (CD3+CD161+) lymphocytes among immunized and non-immunized groups before and after infection. A before infection represent group A1; A after infection represent group A2; B before infection represent group B1; B after infection represent group B2; C before infection represent group C1; C after infection represent group C2.

3.6.3.2. Analysis of Th17 Subset of Th (CD3+CD4+CD161+) Lymphocytes

The analysis of Th17 subset from T helper cells was achieved by the investigation of cells that carry CD3+CD4+CD161+ markers on their surface.

After the comparison among study groups, the results showed a significant difference between group B1 and group B2 (P value ≤ 0.05) (Table 3-10). The comparing between immunized group by flagellin-b (B2) and the non-immunized group (C2) after infection, the results found that there was a highly significant difference between them (P value ≤ 0.001) a shown in Table (3-11).

On the other hand, when compare among the groups immunized by flagellin-a, the results showed there was no significant difference between A1 (before infection) and A2 (after infection) (Table 3-10) as well as when comparing between A2 and the non-immunized group (C2) after infection (Table 3-9). However the comparing between non-immunized groups before and after infection (C1 and C2), the results revealed a non-significant difference between them (Table 3-10).

These results in combination with the results of total Th17 confirm that flagellin-b was considered a good immunogen rather than flagellin-a and enhanced the elevation of Th17 especially the subset from T helper cell (Figure 3-9 and 3-12).

Table (3-10): The mean and mean difference of CD3+ CD4+CD161+ lymphocytes (Th17 subset from Th) among the experimental groups.

	Mean of (CD3+ CD4+CD161+) Lymphocytes ± std. Error		**Mean Difference**	**P value**
Group A1	0.48	0.13	0.18	0.136
Group A2	0.3	0.08		
Group B1	0.38	0.01	- 0.32	0.003*
Group B2	0.7	0.08		
Group C1	0.37	0.05	0.05	0.619
Group C2	0.32	0.04		
***significant P value is less than 0.05**				

Table (3-11): The mean difference of CD3+ CD4+CD161+ lymphocytes (Th17 subset from Th) among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups			
Experimental groups		**Mean Difference**	**P value**
Before infection	B1 & A1	- 0.09	0.37
	B1 & C1	0.012	0.9
	A1 & C1	0.11	0.3
After infection	B2 & A2	0.4	0.001*
	B2 & C2	0.38	< 0.001*
	A2 & C2	- 0.02	0.834
***significant P value is less than 0.05**			

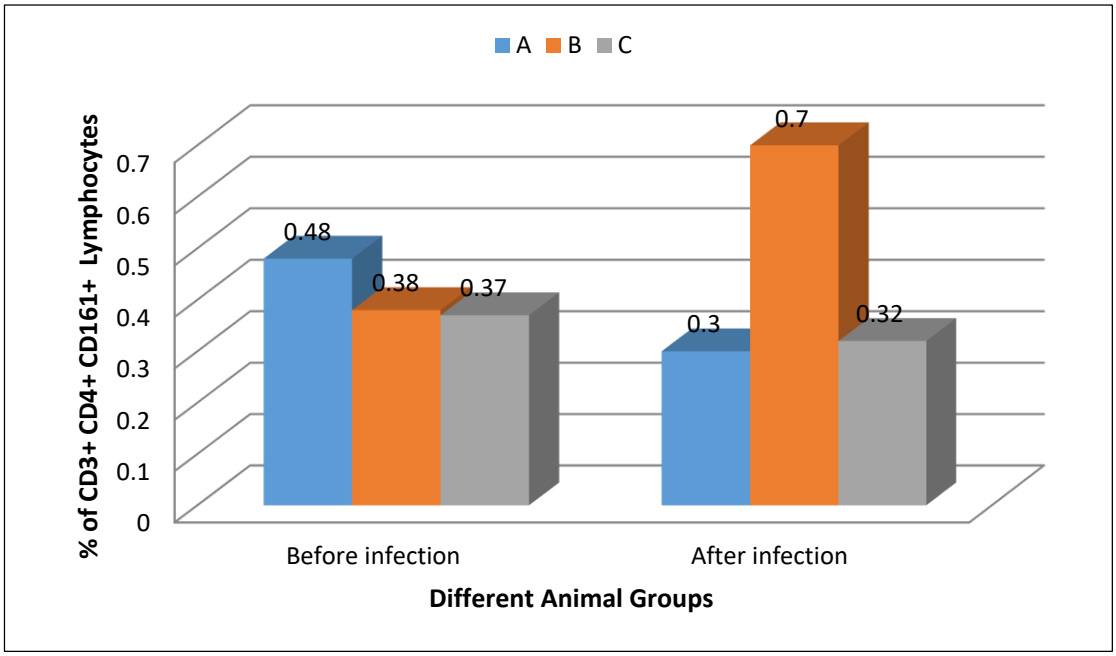

Figure (3-9): The difference of (CD3+CD4+CD161+) Lymphocytes among immunized and non-immunized (C) groups before and after infection. A before infection represent group A1; A after infection represent group A2; B before infection represent group B1; B after infection represent group B2; C before infection represent group C1; C after infection represent group C2.

3.6.3.3. Analysis of Th17 subset of CTL (CD3+CD4-CD161+) Lymphocytes

To complete the investigation of Th17, Th17 subset from CTL was calculated by subtracting CD3+CD4+CD161+ cells from the CD3+CD161+ cells (total Th17).

When comparing among the immunized groups before and after infection the results showed that there was no significant differences between flagellin-b immunized groups (B1 and B2) as well as between flagellin-a-immunized groups (A1 and A2) (P value 0.174 and 0.376) respectively. The results also found that there was no significant difference between the non-immunized group before and after infection (C1 and C2) (P value 0.447) as shown in Table (3-12).

The results of comparison among immunized and non-immunized groups before or after infection revealed that there was no significant difference among groups that clear in Table (3-13) and Figure (3-10).

As clear from the results above, the elevation of total Th17(CD3+ CD161+), due to the effect of flagellin-b, was attributed to the elevation of Th17 subset from T helper cells (CD3+ CD4+CD161+) rather than Th17 subset from CTL (CD3+ CD4-CD161+).

Table (3-12): The mean rank of CD3+ CD4-CD161+ lymphocytes (Th17 subset from CTL) among the experimental groups

	Mean Rank of (CD3+CD4-CD161+) Lymphocytes**	**P value***
Group A1	2.83	0.376
Group A2	4.17	
Group B1	6.38	0.174
Group B2	3.90	
Group C1	5.75	0.447
Group C2	4.40	
***significant P value is less than 0.05 **The mean rank estimated according to Mann-Whitney Test**		

Table (3-13): The difference of CD3+ CD4─ CD161+ lymphocytes (Th17 subset from CTL) among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups		
Experimental groups		**P value***
Before infection	B1 & A1	0.212
	B1 & C1	0.663
	A1 & C1	0.471
After infection	B2 & A2	0.365
	B2 & C2	0.237
	C2 & A2	0.273
***significant P value is less than 0.05**		

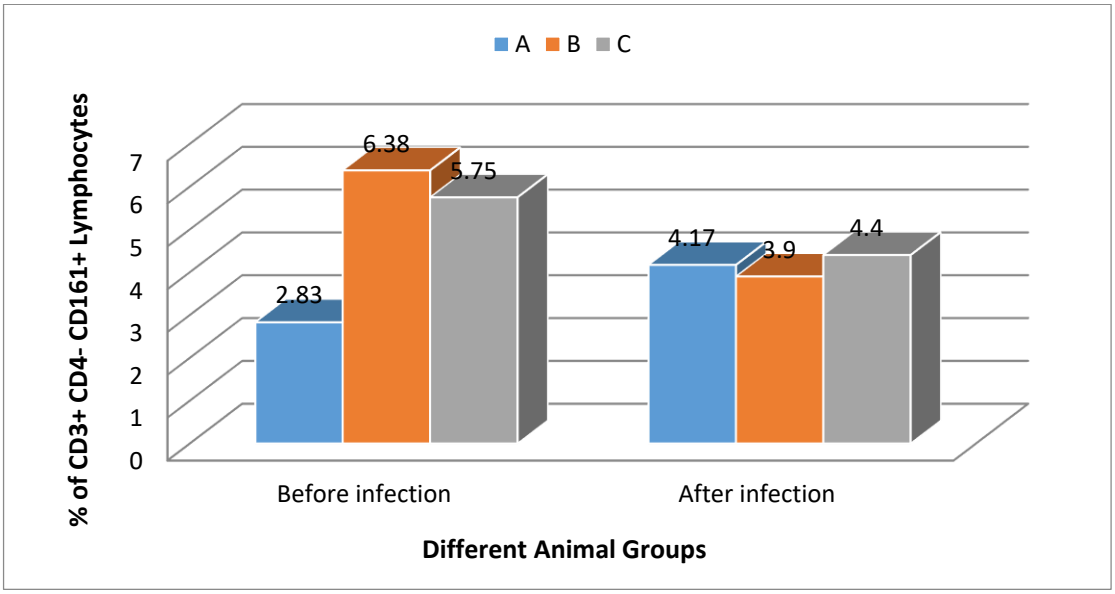

Figure (3-10): The difference of (CD3+CD4-CD161+) lymphocytes among immunized and non-immunized groups before and after infection. A before infection represent group A1; A after infection represent group A2; B before infection represent group B1; B after infection represent group B2; C before infection represent group C1; C after infection represent group C2.

3.6.3.4. Interpretation the Results of Total Th17, Th17 Subset from Th Cells, and Th17 Subset from CTL

Th17 cells play a critical role in the specific and non-specific immune response against bacteria (Ye, *et al.*, 2001a; Ye, *et al.*, 2001b; Kolls *et al.*, 2003; Chen *et al.*, 2011). The indirect role of Th17 cells to protect against infection is the recruitment of phagocytic cells to the site of infection (Li *et al.*, 2012). These cells were shown to increase in 8 hour post-acute respiratory infection with *P. aeruginosa* and elevate the production of IL-23 production from these cells. This cytokine promotes memory CD4+ cells differentiation to Th17 cells (Liu *et al.*, 2011). Also, the neutrophil is considered the major cells in the lung in *P. aeruginosa* infection (Sadikot *et al.*, 2005; Wu *et al.*, 2012). So, the protection of Th17 against this bacteria was achieved by neutrophil recruitment to the lung (Buret *et al.*, 1994).

As clear from the results above (section 3.6.3.1 ; 3.6.3.2 ; 3.6.3.3) in the analysis of total Th17, Th17 subset from T helper and Th17 subset from CTL, flagellin-b is a good in activation of Th17 in the immunized groups rather than flagellin-a especially Th17 cells subset from Th cells rather than Th17 subset from CTL. These results were compatible with the results of several authors worldwide (Sadikot *et al.*, 2005; Wu *et al.*, 2012) who found that the elevation of Th17 due to the immunization against *P. aeruginosa* has important role in eradication of this bacterial infection.

Th17 cells especially those subset from Th cells rather than those subset from CTL is necessary in the eradication of *P. aeruginosa* infection in the lungs, it was achieved by secretion of IL-17 that promoting the release of IL-8 (Laan *et al.*, 1999) lead to rapid recruitment of neutrophil in the lung and then may benefit the treatment of *P. aeruginosa* lung infection (Ivanov and Lindén, 2007).

Xu and his colleagues supported this hypothesis. They showed that the up-regulation of IL-17 may increase the bacterial clearance and survival rate through increasing neutrophil recruitment via IL-17's downstream effectors, and playing protective effect in early phase of acute *P. aeruginosa* lung infection in mice (Xu *et al.*, 2014).

The results of present study are aligned with the study of Behrouz and his colleagues who showed that the intranasal administration of bivalent flagellin-a and b of murine model induced protection was associated with an increase in CD4+ IL17+ T cells in the lungs (Behrouz *et al.*, 2017).

The result regarding rising of IL-17 were compatible with study of Korpi and his colleagues who found that a splenocytes immunized by recombinant flagellin-b combined with recombinant pilin A lead to rise in IL-17 production which can act as an inducer of Th17 responses (Korpi *et al.*, 2016).

3.6.4. Analysis the T helper (Th) Cells Subtypes:

In this study, to investigate the immunological pathway of the immune response to *P. aeruginosa* respiratory infection if it directs the infection to cure or it develops into chronic infection, it must investigate the Th1 and Th2. So, this study analyzes IgG2a as an indicator for Th1 cells and IgG1 as an indicator for Th2 cells by flow cytometry technique as mentioned previously.

3.6.4.1. Analysis of Th1 (CD3+CD4+IgG2a+) Lymphocytes

The investigation of IgG2a among immunized and non-immunized groups showed that the group B2 was significant elevated than the group B1 (from 0.05 to 0.61 ; P value < 0.001) (Table 3-14). On the other hand, the comparison between the group B2 and group C2 the results showed a highly significant difference between them (P value ≤ 0.05). The mean of Th1 lymphocytes of immunized group B2 after infection was more than the non-immunized group C2, 0.61 and 0.32 respectively (Table 3-14, Table 3-15).

While there was a non-significant decrease in IgG2a levels when comparing between the non-immunized group before and after infection (C1 and C2) from 0.51 to 0.32 respectively (P value > 0.05) as shown in Table (3-14). On the other hand, when comparing between the groups that immunized by flagellin-a before and after infection (A1 and A2) the results showed a significant decrease of IgG2a from 0.56 to 0.31 respectively (P value ≤ 0.05) (Table 3-14). However the results found that there was no significant difference among flagellin-a-immunized groups and the

non-immunized groups before infection (A1 and C1 / P value > 0.05) and after infection (A2 and C2 / P value 0.88) as shown in Table (3-15) and Figure (3-11).

Table (3-14): The mean and mean difference of CD3+CD4+IgG2a+ lymphocytes (Th1) among the experimental groups.

	Mean of (CD3+CD4+IgG2a+) Lymphocytes ± std. Error		**Mean Difference**	**P value**
Group A1	0.56	0.02	0.26	0.037*
Group A2	0.31	0.03		
Group B1	0.05	0.013	- 0.56	< 0.001*
Group B2	0.61	0.04		
Group C1	0.51	0.1	0.18	0.069
Group C2	0.32	0.09		
***significant P value is less than 0.05**				

Table (3-15): The mean difference of CD3+CD4+IgG2a+ lymphocytes (Th1) among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups			
Experimental groups		**Mean Difference**	**P value**
Before infection	B1 & A1	- 0.52	< 0.001*
	B1 & C1	- 0.46	< 0.001*
	A1 & C1	0.06	0.58
After infection	B2 & A2	0.3	0.009*
	B2 & C2	0.29	0.005*
	C2 & A2	0.01	0.88
***significant P value is less than 0.05**			

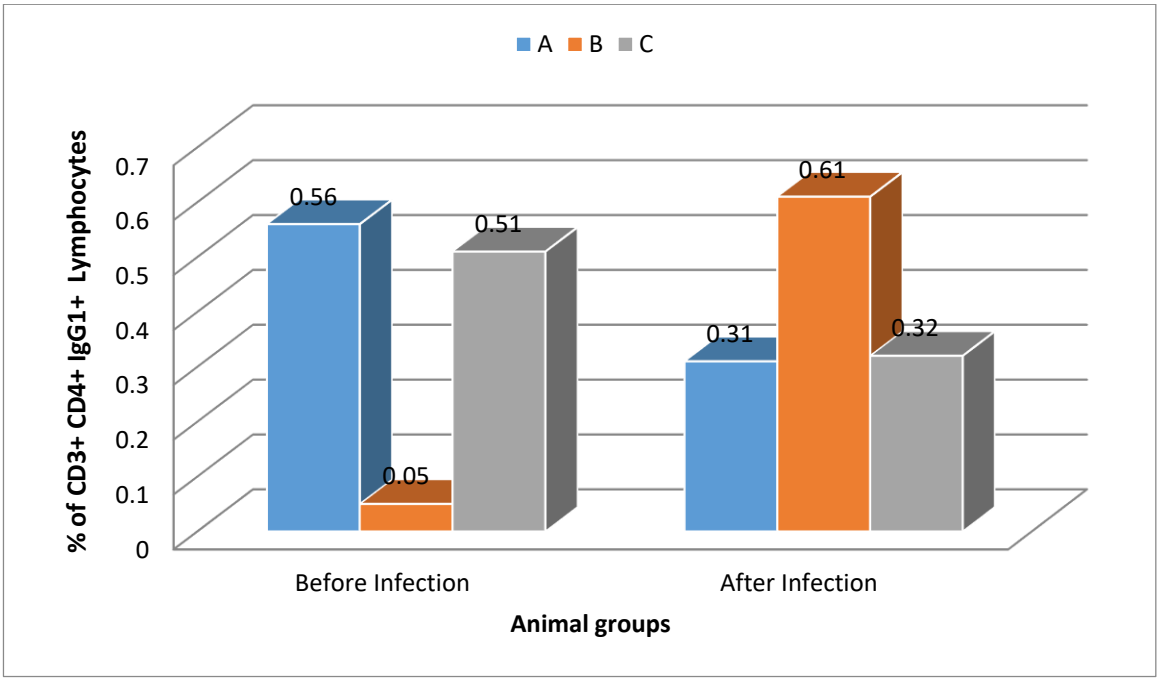

Figure (3-11): The difference of (CD3+CD4+IgG2a+) lymphocytes among immunized and non-immunized groups before and after infection. A before infection represent group A1; A after infection represent group A2; B before infection represent group B1; B after infection represent group B2; C before infection represent group C1; C after infection represent group C2.

3.6.4.2. Analysis of Th2 (CD3+CD4+IgG1+) Lymphocytes

The investigation of Th2 cells among the immunized groups with flagellin-b when compare between these groups before infection B1 and after infection B2, the results showed a highly significant increase (from 0.001 to 0.06 / P value < 0.001). While there was no significant difference in flagellin-a immunized groups between group A1 and group A2 (P value > 0.05). However, the results showed that there were no significant differences in the non-immunized groups between the C1and C2 groups (P value > 0.05) as shown in Table (3-16).

On the other hand, when comparing among the groups before infection the results found that non-immunized group (group C1) showed a highly significant elevation than flagellin-a (group A1) and flagellin-b (group B1) immunized groups separately (P value ≤ 0.05). However, when compare among the groups after infection the results found that the non-immunized group (C2) showed a highly significant elevation than the immunized group with flagellin-a (group A2) (P value

< 0.001) but it showed non-significant elevation than immunized group with flagellin-b (group B2) (P value > 0.05) as shown in Table (3-17) and Figure (3-12).

Table (3-16): The mean and mean difference of CD3+CD4+IgG1+ lymphocytes (Th2) among the experimental groups.

	Mean of (CD3+CD4+IgG1+) Lymphocytes * 10^{-2} ± std. Error		**Mean Difference**	**P value**
Group A1	0	0	- 0.06	0.957
Group A2	0.067	0.067		
Group B1	0.25	0.05	- 0.07	<0.001*
Group B2	7.40	0.40		
Group C1	٦.0	٢.0	- 0.02	0.141
Group C2	٨.1	1.0		
***significant P value is less than 0.05**				

Table (3-17): The mean difference of CD3+CD4+IgG1+ lymphocytes (Th2) among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups			
Experimental groups		**Mean Difference * 10^{-2}**	**P value**
Before infection	B1 & A1	0.2	0.858
	B1 & C1	- 5.8	0.003*
	A1 & C1	- 6.0	0.001*
After infection	B2 & A2	7.3	<0.001*
	B2 & C2	- 0.8	0.520
	C2 & A2	8.1	<0.001*
***significant P value is less than 0.05**			

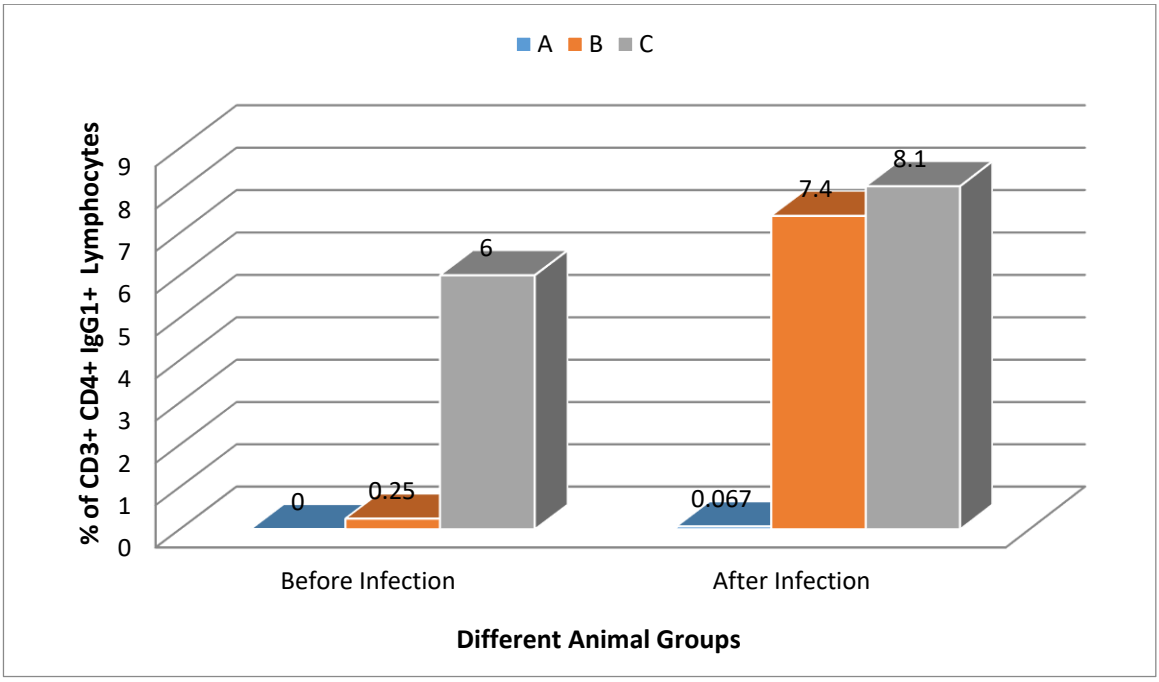

Figure (3-12): The difference of (CD3+CD4+IgG1+) lymphocytes among immunized and non-immunized groups before and after infection. A before infection represent group A1; A after infection represent group A2; B before infection represent group B1; B after infection represent group B2; C before infection represent group C1; C after infection represent group C2.

3.6.4.3. Results of Th2/Th1 Ratio

In this study, the calculation of IgG1/IgG2a ratio were determined in the animal groups to find out the direction of immune responses toward Th1 or Th2 against *P. aeruginosa* respiratory infection. The results of comparison between the flagellin-b-immunized group (B1) and the non-immunized group before infection (C1), showed that this ratio was lower in group B1 (20.2%) than group C1 (84.3%). while the comparison among groups after infection, the results also showed reduction of this ratio in group B2 (25.8%) than group C2 (63.6%). On the other hand, this ratio in the groups immunized by flagellin-a, calculated according to mean only because the median of IgG1 is zero, was highly elevated after infection (group A2 / 173.2%) while it couldn't be calculated in group A1 due to the IgG1 is zero detected in most cases of these groups (Table 3-18 ; Figure 3-13).

Table (3-18): Ratio statistics for IgG1/IgG2a among the experimental groups.

Ratio Statistics for IgG1 / IgG2a				
Group	**Mean**	**Median**	**Coefficient of Variation**	
			Mean Centered	**Median Centered**
Group A1	0	0	-	-
Group A2	0.002	0	173.2%	-
Group B1	0.044	0.044	20.2%	20.2%
Group B2	0.131	0.128	25.8%	26.5%
Group C1	0.131	0.131	84.3%	84.3%
Group C2	0.378	0.420	63.6%	58.5%

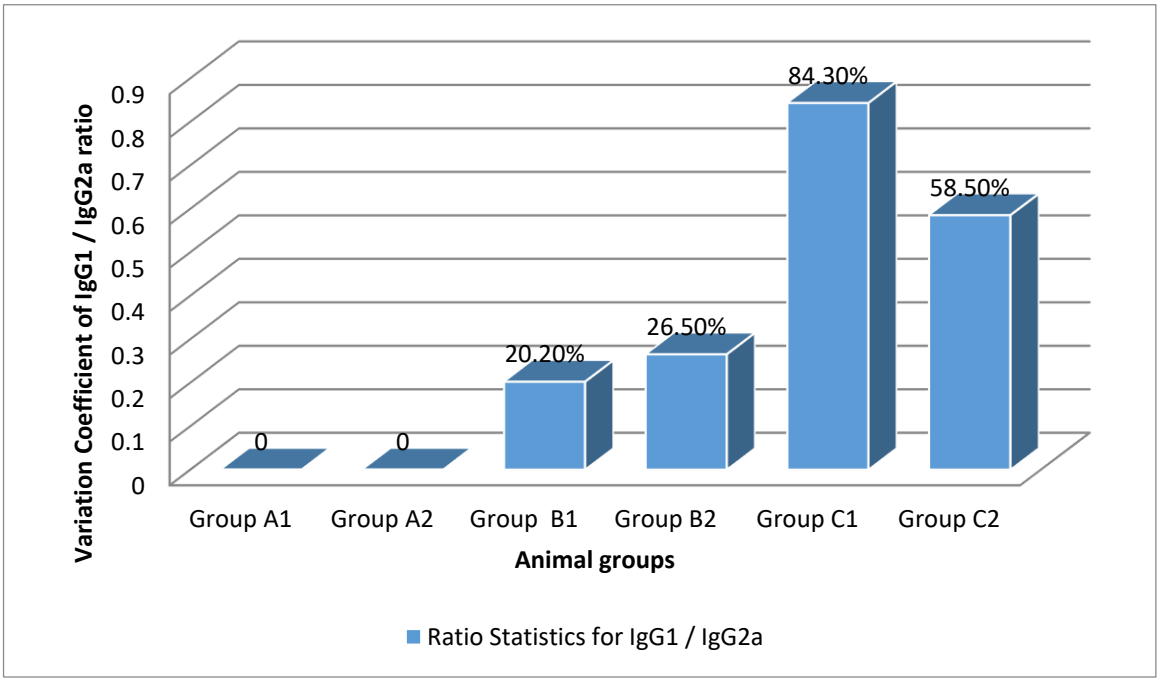

Figure (3-13): IgG1 / IgG2a Ratio among immunized and non-immunized groups before and after infection with *P. aeruginosa*. Variation ratio calculated according to median

3.6.4.4. Interpretation the Results of Th1, Th2, and Th2/Th1 Ratio

Many authors worldwide reported that immune response in the chronic respiratory infection with *P. aeruginosa* showed an elevation of Th2 cytokines in blood (Moser *et al.*, 2000; Brazova *et al.*, 2005). Other researcher showed that the increasing of Th2 cytokines in BAL of *P. aeruginosa* infected CF patients lead to increasing the severity of the disease (Hartl *et al.*, 2006).

Sen-Kilic and his colleagues identified the presence of IgG2a and IgG1 as surrogate markers to study the Th1 and Th2 immune responses respectively against *P. aeruginosa* acute murine pneumonia, who observed FpvA peptide-specific IgG2b antibodies were detected in sera of all mice vaccinated by FpvA-KLH while IgG2a was detected only in some of these mice (Sen-Kilic *et al.*, 2019).

Many studies on the other hand, the patients who had a predominance of Th1 subtype showed a good function of lung which lead patients undergoing to cure (Moser *et al.*, 2000; Bisgaard *et al.*, 2007; Upritchard *et al.*, 2008; Hector *et al.*, 2016).

Another studies revealed that the elevation of Th2 cytokines (as IL-4 IL-13) as well as the decrease of Th1 cytokine (IFN-γ) in the bronchoaleveolar lavage fluid were noted in cystic fibrosis patients rather than in healthy controls (Hartl *et al.*, 2006).

Johansen and his colleagues used IFN-γ to treat chronically infected rats with *P aeruginosa* and they found a significantly reduced severity of macroscopic lung inflammation and a complete shift immune response from Th2 to Th1response. That offer a possible new strategy of modifying the inflammatory response of chronically infected CF patients (Johansen *et al.*, 1996).

As clear from the results of Th1, Th2 and Th2/Th1 ratio (section 3.6.4.1 ; 3.6.4.2 ; 3.6.4.3), the investigation of Th1 (by IgG2a) showed an elevation of Th1 cells in the flagellin-b immunized groups (B1 and B2) more than the flagellin-a immunized groups (A1 and A2) and more than the non-immunized groups (C1 and C2) as shown in Table (3-15). These results indicate that flagellin-b is more efficient than flagellin-a in the activation of Th1 cells after respiratory infection with *P.*

aeruginosa and revealed that flagellin-b showed a significant elevation of Th1 in the immunized groups while they decreased in the non-immunized group after infection (Figure 3-16).

Several authors worldwide reported that Th1 response resulted in effective protection against *P. aeruginosa* infections (Sawa *et al.*, 1999; Moser *et al.*, 2002; Chen *et al.*, 2011).

The results of present study are in contrast with Korpi and his colleagues, who studied the effect of recombinant b flagellin combined with recombinant pillin A on burn infection in mice model and they found the r-b-flagellin-alone didn't has a significant differences in the IgG2a subtype titer among immunized and non-immunized groups (Korpi *et al.*, 2016).

The investigation of Th2 (by detection of IgG1) showed elevation of these cells in the non-immunized groups C1 and C2 more than the immunized groups A1, A2, B1 and B2 (before and after infection) indicate that the flagellin decreases the opportunity of immune response to be directed toward chronic infection to *P. aeruginosa* in the immunized groups rather than non-immunized groups. Moser and his colleagues showed that the elevation of Th2 shifted the infection to side chronic infection (Moser *et al.*, 2000).

Regarding the decrease of IgG1/IgG2a ratios among flagellin-b-immunized groups (compared to the non-immunized groups) indicated that flagellin-b tendency to direct immune responses toward Th1 responses (elevation of Th1 cells more than Th2 and scale down of Th2 cells in the immunized groups). These results were in contrast with the results obtained by Korpi and his colleagues who found a high of IgG1/IgG2a ratio in burn infection with *P. aeruginosa* in mice model after immunization with r-b-flagellin-alone (Korpi *et al.*, 2016). The results of present study also compatible with Moser and his colleagues who focused on the protective role Th1 to Th2 ratio against *P. aeruginosa* re-infection in mice model (Moser *et al.*, 2002).

There is evidence that an effective *P. aeruginosa* vaccine may require elicitation of both opsonizing antibodies, CD4+ T cells and IL-17 production to

prevent infections. Thus, more recently, several preclinical studies leading to the induction of Th17-type cellular immunity are being pursued (Kamei *et al.*, 2011; Merakou *et al.*, 2018).

As clear from the results of this study, intranasal administration of flagellin-b in rat model enhanced the cellular immune response against *P. aeruginosa* respiratory infection by increasing the levels of Th cells especially Th1 subset at the expense of Th2 subset that lead to shift the immune response toward cure rather than development the infection to the chronic infection.

In addition, the flagellin-b leads to an increase Th17, which is essential in the eradication of bacterial infection especially *P. aeruginosa* infection.

All that makes flagellin-b a good candidate vaccine to eradicate *P. aeruginosa* respiratory infection. On the other hand, the flagellin-a showed improper enhancement of the immune response because it lead to an imbalance in the cellular immune response that lead to many side effects in the immunized rats.

3.6.5. Analysis of B Lymphocytes

3.6.5.1. Analysis of Total B Lymphocytes

The results of present study didn't shown significant differences in total B lymphocytes levels in blood among flagellin-a immunized groups (A1 and A2), flagellin-b immunized groups (B1 and B2), and non-immunized groups (C1 and C2) before and after infection (P value < 0.05) as shown in Table (3-19).

On the other hand, when comparing the total lymphocytes between immunized and non-immunized groups before infection didn't shown a significant deference between groups (B1 and C1) and between groups (A1 and C1). While there was a significant elevation of total lymphocytes in the non-immunized group after infection (group C2) than immunized groups (groups A2 and B2) (P value < 0.05) as shown in Table (3-20).

As it should be noted that there was no different between flagellin-a and flagellin-b immunized groups before (groups A1 and B1) or after infection (A2 and C2) as shown in Table (3-20).

Table (3-19): The mean and mean difference of total B lymphocytes among the experimental groups.

	Mean of B Lymphocytes ± std. Error		Mean Difference	P value
Group A1	0.32	0.015	0	1
Group A2	0.32	0.015		
Group B1	0.31	0.026	-0.021	0.54
Group B2	0.33	0.019		
Group C1	0.35	0.035	-0.056	0.118
Group C2	0.41	0.026		
***significant P value is less than 0.05**				

Table (3-20): The mean difference of total B lymphocytes among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups			
Experimental groups		**Mean Difference**	**P value**
Before infection	B1 & A1	-0.011	0.78
	B1 & C1	-0.037	0.31
	A1 & C1	-0.026	0.50
After infection	B2 & A2	-0.011	0.77
	B2 & C2	-0.083	0.039*
	A2 & C2	0.072	0.038*
***significant P value is less than 0.05**			

3.6.5.2. Analysis of CD3+IgM+ B Lymphocytes

In this study, the B lymphocytes that express IgM on its surface investigated using FC technique by screening CD3+IgM+ B lymphocytes among all tested animals.

The comparison of CD3+IgM+ B lymphocytes among groups before and after infection showed there are no significant differences between groups A1 and A2, and between groups C1 and C2 (P value > 0.05). While the flagellin-b immunized groups showed a significant elevation in IgM+ B lymphocytes after

infection (group B2) than before infection (group B2) (P value < 0.05) as shown in Table (3-21) and Figure (3-14).

On the other hand, the comparison of CD3+IgM+ B lymphocytes among before infection showed a significant decrease of these cells in group B1 than group C1 (P value < 0.05), while there is no significant difference between groups A1 and C1 and between groups A1 and B1 (P value > 0.05) as shown in Table (3-22).

However the comparing among groups after infection, the results revealed a non-significant difference between (A2 and C2), between (B2 and C2), and between (A2 and B2) as shown in Table (3-22).

Table (3-21): The mean and mean difference of CD3+IgM+ B lymphocytes among the experimental groups

	Mean of CD3+IgM+ B Lymphocytes ± std. Error		**Mean Difference**	**P value**
Group A1	0.47	0.17	0.057	0.722
Group A2	0.41	0.086		
Group B1	0.2	0.043	-0.4	0.006*
Group B2	0.6	0.081		
Group C1	0.53	0.135	-0.15	0.264
Group C2	0.68	0.059		
***significant P value is less than 0.05**				

Table (3-22): The mean difference of CD3+IgM+ B lymphocytes among the immunized and non-immunized experimental groups

Comparisons among Experimental Groups			
Experimental groups		**Mean Difference**	**P value**
Before infection	B1 & A1	-0.26	0.089
	B1 & C1	-0.33	0.028*
	A1 & C1	-0.06	0.683
After infection	B2 & A2	0.19	0.184
	B2 & C2	-0.07	0.561
	C2 & A2	0.27	0.074
***significant P value is less than 0.05**			

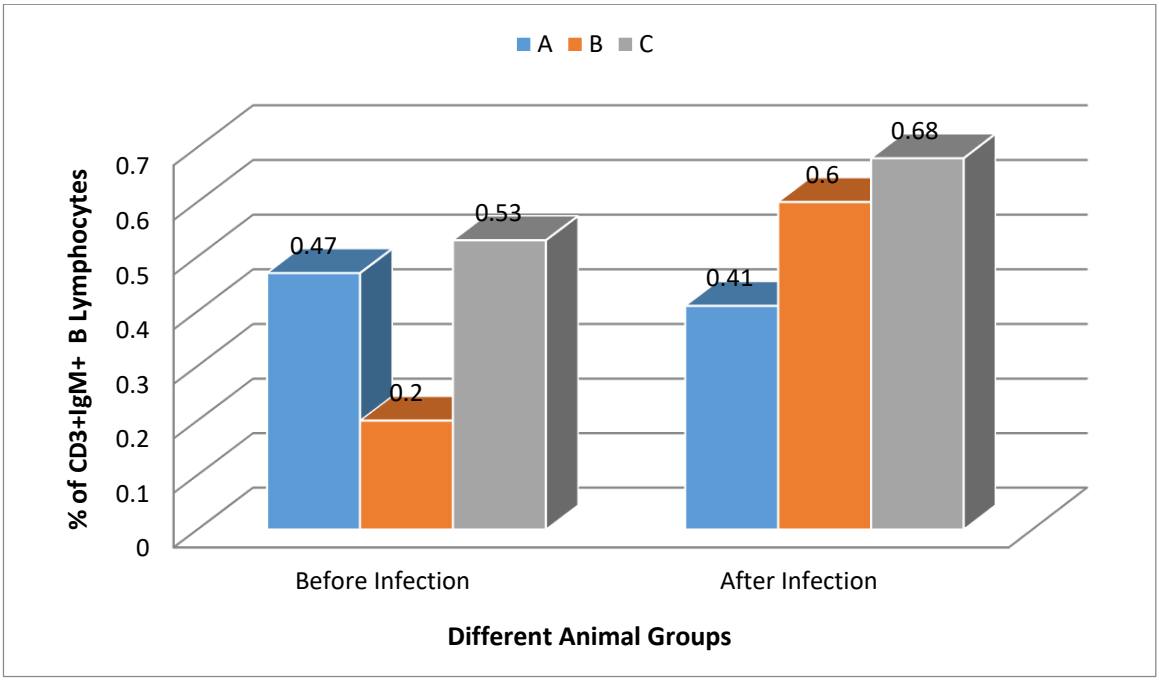

Figure (3-14): The difference of CD3+IgM+ B lymphocytes among immunized and non-immunized groups before and after infection. A before infection represent group A1; A after infection represent group A2; B before infection represent group B1; B after infection represent group B2; C before infection represent group C1; C after infection represent group C2.

3.6.5.3. Interpretation the Results of Total B lymphocytes and CD3+IgM+ B Lymphocytes

The results of present study didn't shown a significant differences before and after infection in the immunized and non-immunized groups that due to that B lymphocytes responses and recruitments require many days of infection and the animal scarified within 24 hours. Neill and his colleagues reported that B cell influx into the lung progressively increased in 7 days post *P. aeruginosa* infection (Neill *et al.*, 2014).

On the other hand, the significant elevation of CD3+IgM+ B lymphocytes in flagellin-b immunized group after infection (B2) than before infection (B1) within 24 hours of infection indicate on the presence of memory B cells formed during the period of vaccination. Because the antigen-driven activation of memory B cells results in their rapid proliferation and differentiation into plasma cells that produce very large amounts of higher-affinity antibodies (Siegrist, 2008).

The results of FC for all animal groups are shown in Figures (3-15 to 3-20) which indicate the graphics and reports of FC apparatus BD FACSCanto II Flow Cytometer.

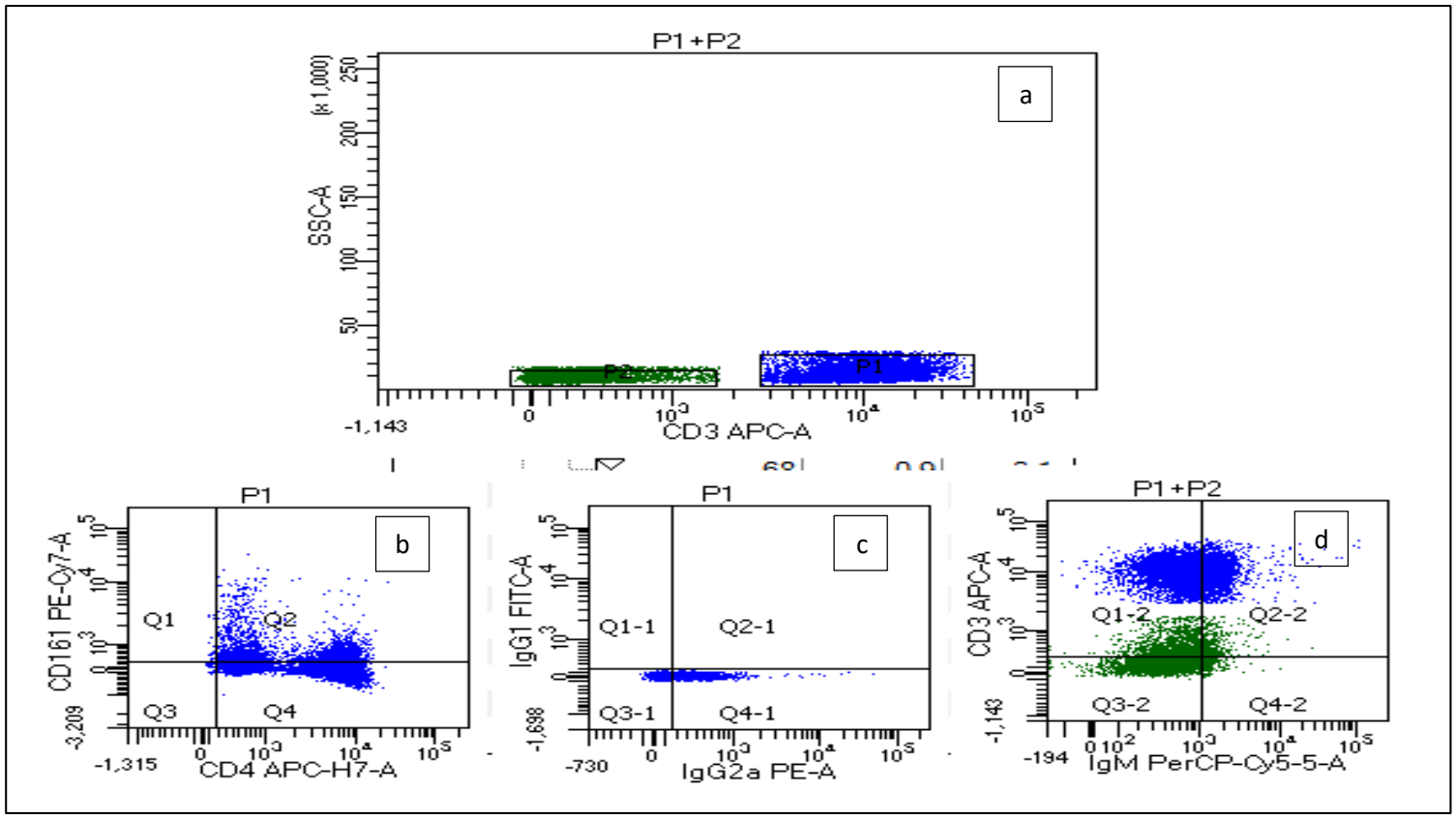

Figure (3-15): The results of flow cytometry for animal immunized by flagellin-a before infection (group A1). a. CD3 positive and negative cells (blue for T lymphocytes and green for B lymphocytes). b. CD4+ cells (Th cells) and CD161+ cells (Th17) that subset from Th cells ; CD161 negative cells. c. IgG2a (indicator for Th1) to IgG1 (indicator for Th2). d. IgM positive cells (indicates active B cells). BD FACSCanto II Flow Cytometer.

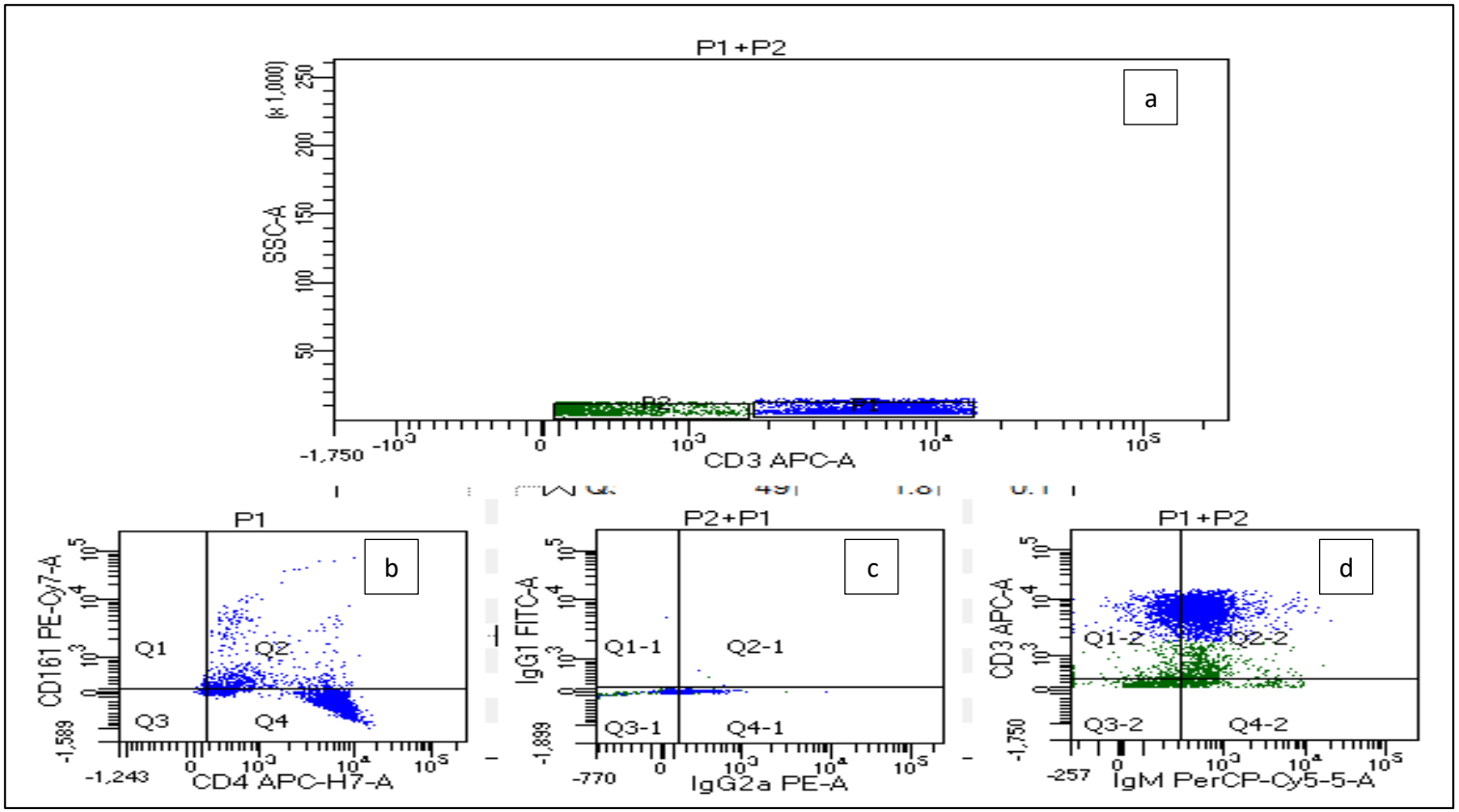

Figure (3-16): The results of flow cytometry for animal immunized by flagellin-a after infection (group A2). a. CD3 positive and negative cells (blue for T lymphocytes and green for B lymphocytes). b. CD4+ cells (Th cells) and CD161+ cells (Th17) that subset from Th cells ; CD161 negative cells. c. IgG2a (indicator for Th1) to IgG1 (indicator for Th2). d. IgM positive cells (indicates active B cells). BD FACSCanto II Flow Cytomete.

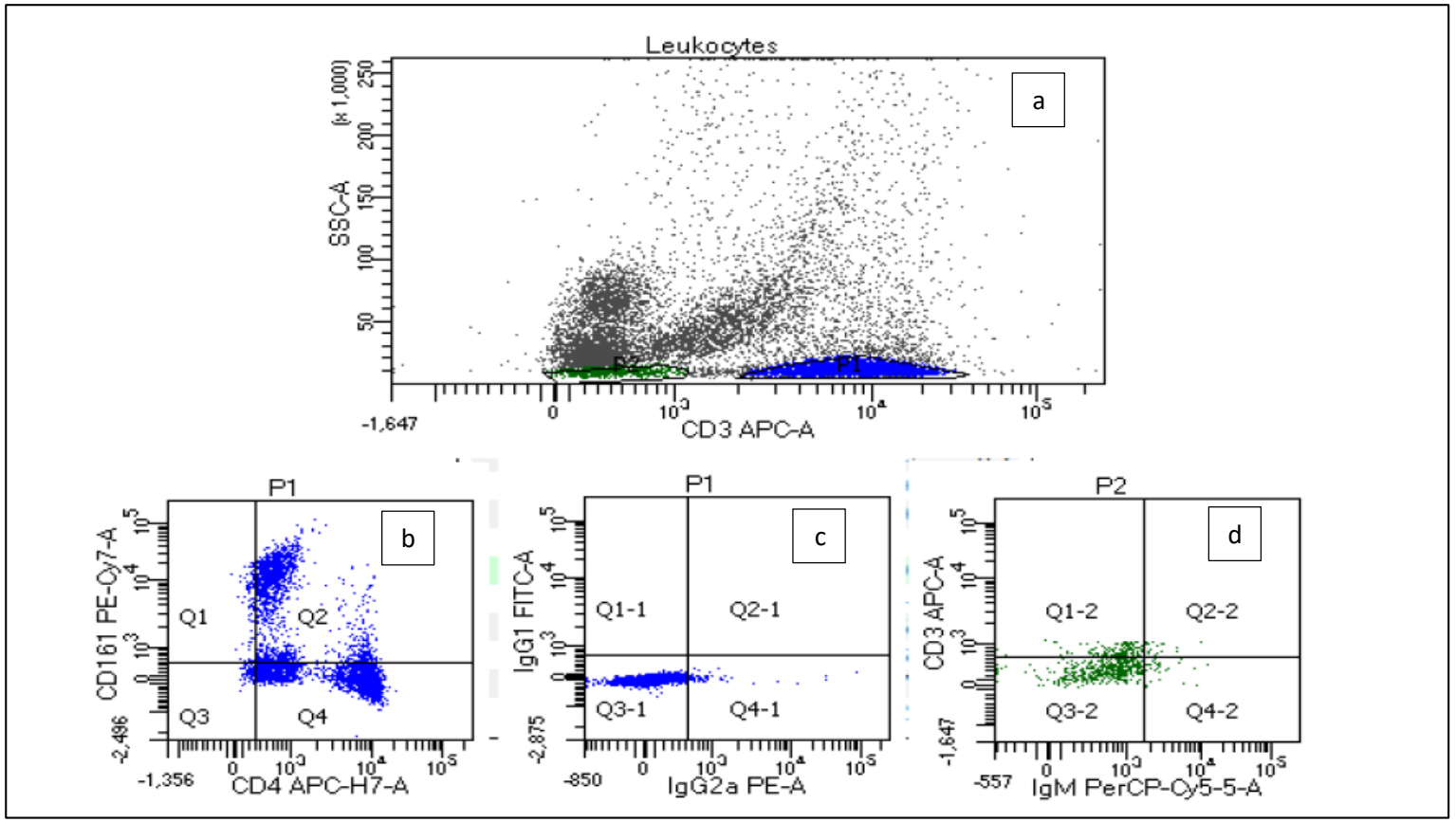

Figure (3-17): The results of flow cytometry for animal immunized by flagellin-b before infection (group B1). a. CD3 positive and negative cells (blue for T lymphocytes and green for B lymphocytes). b. CD4+ cells (Th cells) and CD161+ cells (Th17) that subset from Th cells; CD161 negative cells. c. IgG2a (indicator for Th1) to IgG1 (indicator for Th2). d. IgM positive cells (indicates active B cells). BD FACSCanto II Flow Cytometer

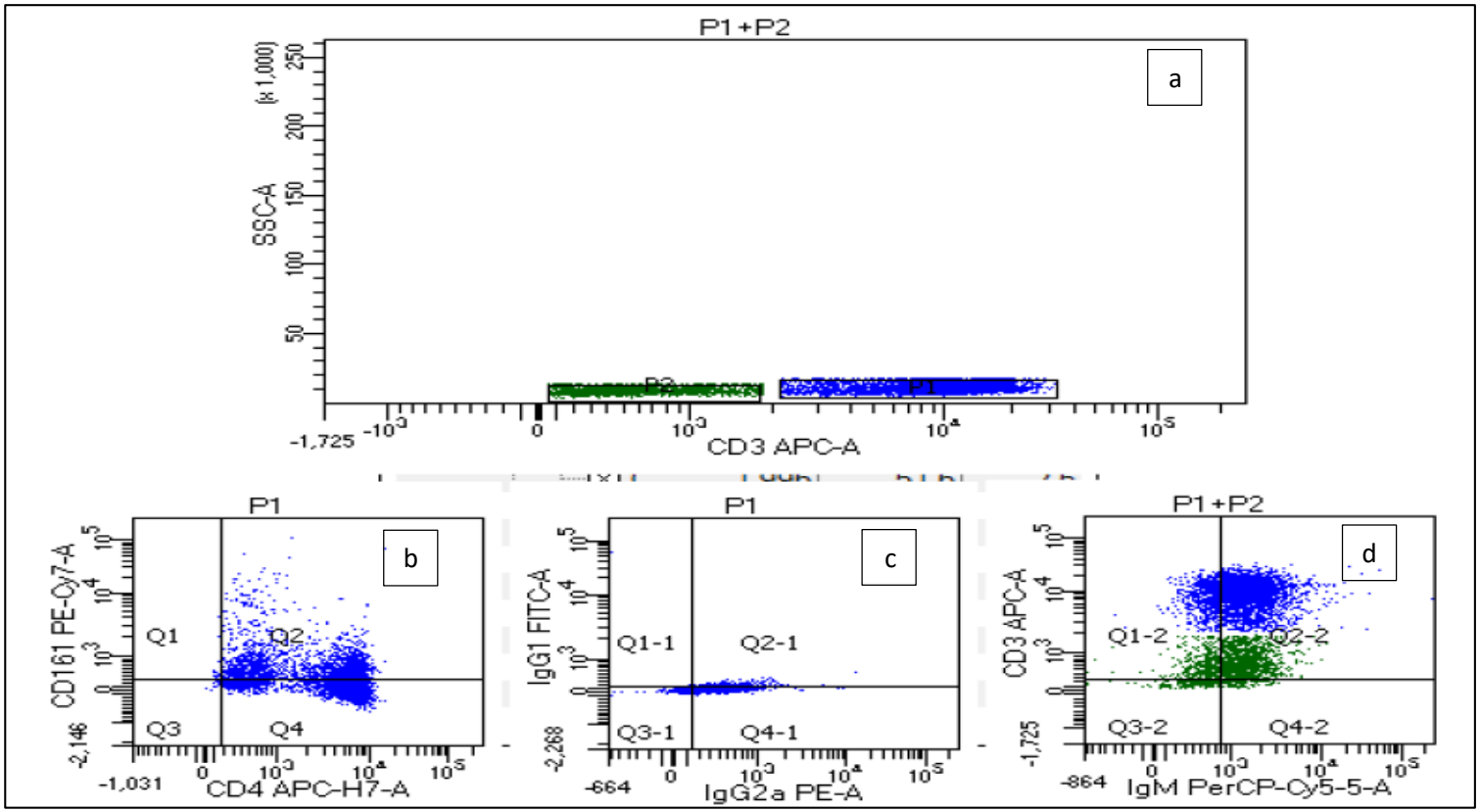

Figure (3-18): The results of flow cytometry for animal immunized by flagellin-b after infection (group B2). a. CD3 positive and negative cells (blue for T lymphocytes and green for B lymphocytes). b. CD4+ cells (Th cells) and CD161+ cells (Th17) that subset from Th cells ; CD161 negative cells. c. IgG2a (indicator for Th1) to IgG1 (indicator for Th2). d. IgM positive cells (indicates active B cells). BD FACSCanto II Flow Cytometer

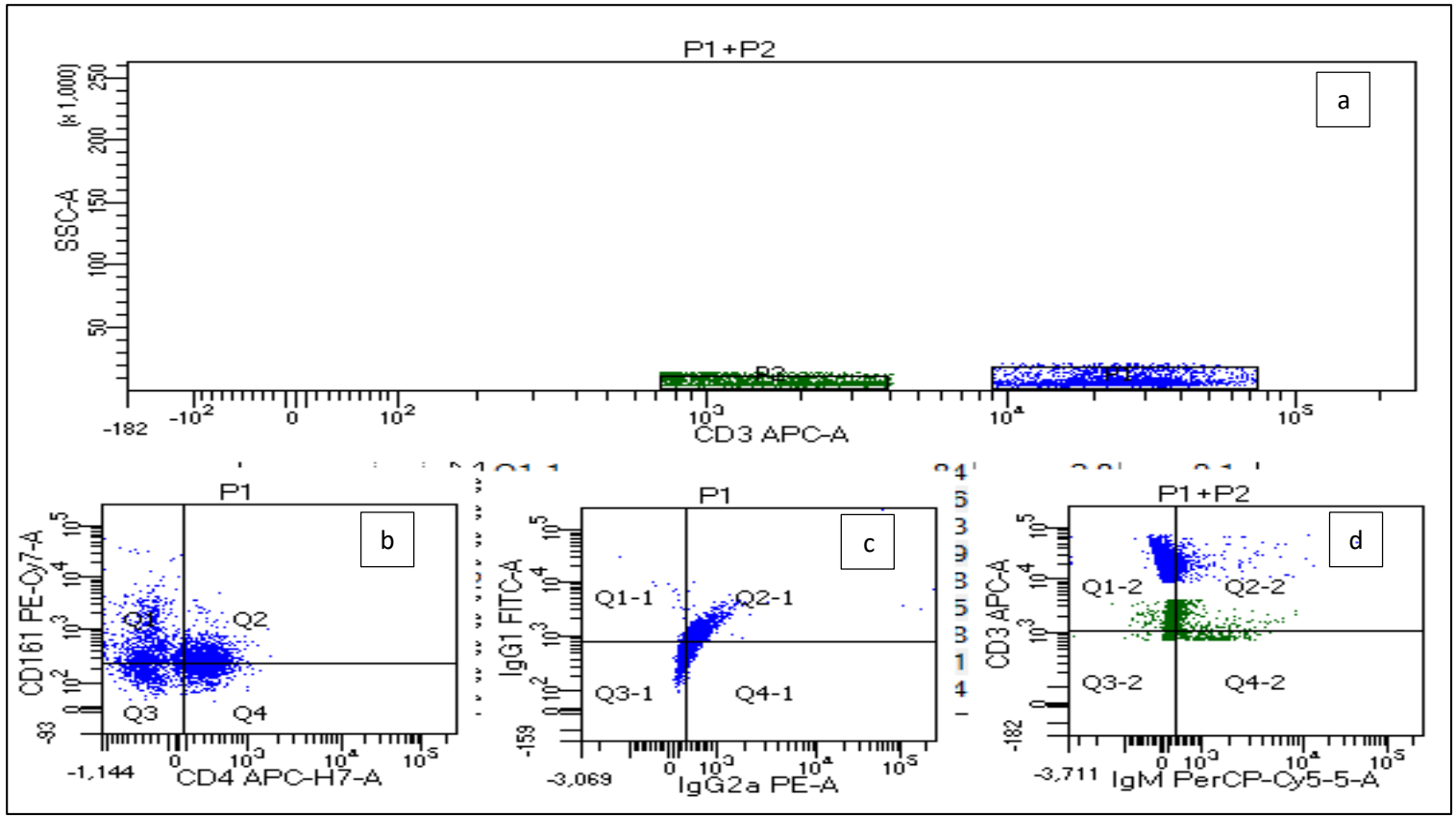

Figure (3-19): The results of flow cytometry for non-immunized animal before infection (group C1). a. CD3 positive and negative cells (blue for T lymphocytes and green for B lymphocytes). b. CD4+ cells (Th cells) and CD161+ cells (Th17) that subset from Th cells ; CD161 negative cells. c. IgG2a (indicator for Th1) to IgG1 (indicator for Th2). d. IgM positive cells (indicates active B cells). BD FACSCanto II Flow Cytometer.

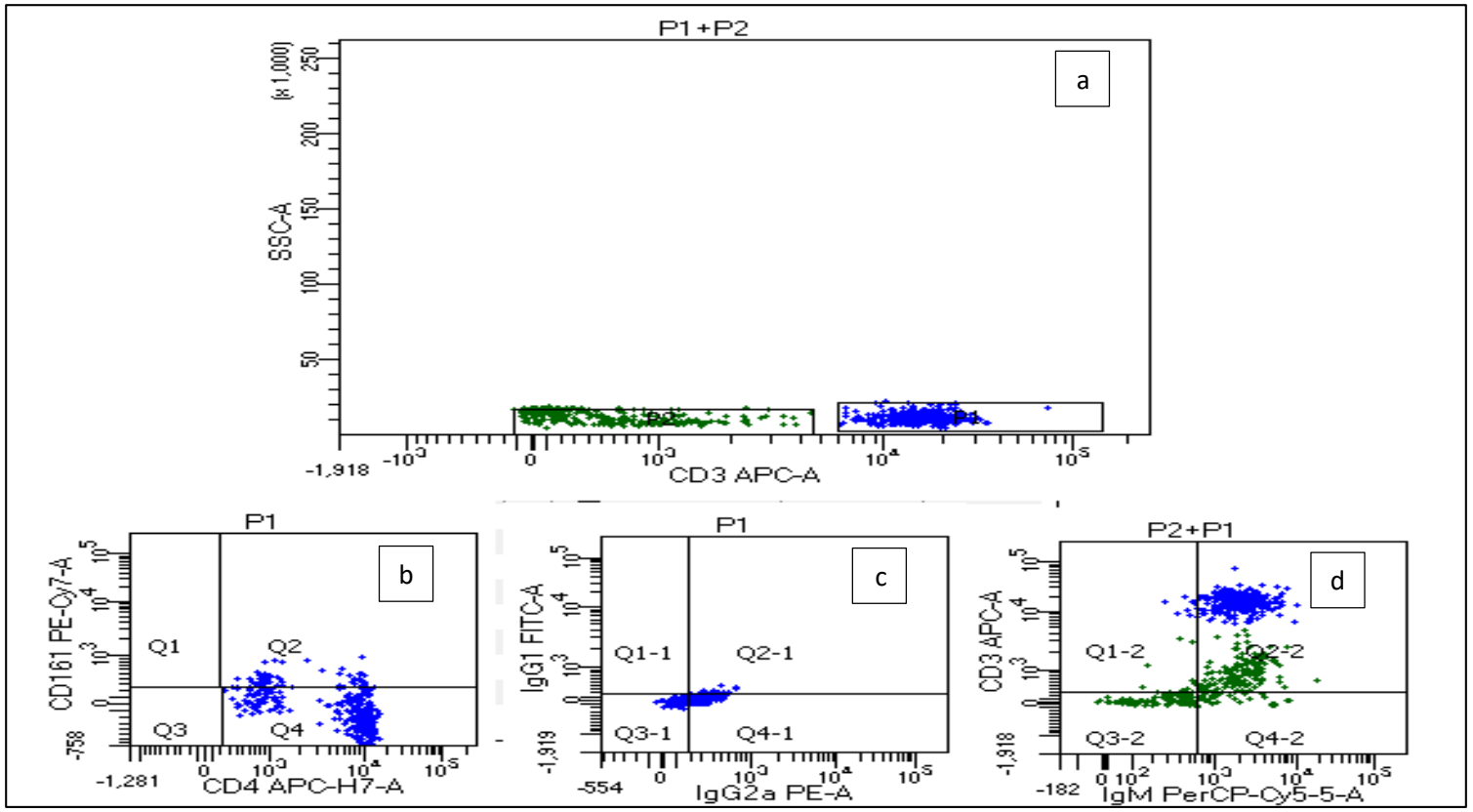

Figure (3-20): The results of flow cytometry for non-immunized animal after infection (group C2). a. CD3 positive and negative cells (blue for T lymphocytes and green for B lymphocytes). b. CD4+ cells (Th cells) and CD161+ cells (Th17) that subset from Th cells ; CD161 negative cells. c. IgG2a (indicator for Th1) to IgG1 (indicator for Th2). d. IgM positive cells (indicates active B cells). BD FACSCanto II Flow Cytometer.

3.7. Histological examination of animals

In addition to the hematological and immunological analysis, the animals (immunized with flagellin-a or flagellin-b, and non-immunized) were scarified and the organs (heart, kidney, liver, spleen and lung) were taken in order to estimate the relative organ weight in all animals which may indicate the side effect of flagellin immunization on the animals.

The results of the present study showed a significant elevation in weight of all studied organs (except spleen) in flagellin-a immunized group (A2) than non-immunized group (C2) when comparing between these groups after infection (P value <0.05). While the comparison between groups A1 and C1 (before infection) showed no significant differences among weights of the studied organs (Table 3-23).

The comparison among flagellin-b-immunized groups and non-immunized groups showed that there were non-significance differences between groups B1 and C1 (before infection) in weight of all organs except liver and spleen, also there were no significant differences between B2 and C2 (after infection) among weight of all organs except spleen. The liver showed a significant lowering of weight in group B1 than group C1, and the spleen showed a significant elevation in the weights of groups B1 and B2 than in groups C1 and C2 respectively (Table 3-24).

On the other hand the comparison among flagellin-a immunized groups and flagellin-b immunized groups showed a significant decrease in the weight of all organs after infection in group A2 as compared with group B2 (P value < 0.05). While the comparison among these groups before infection (A1 and B1) showed a significant differences in the weight of lung and spleen (decrease) and liver (increase) as shown in Table (3-25).

Table (3-23): The mean difference of relative organs weight (lung, liver, heart, kidney and spleen) among flagellin-a-immunized groups compared with non-immunized groups.

Animal groups			lung	liver	heart	kidney	spleen
Group A1	**Group C1**	**Mean Difference**	-0.18	0.13	-0.02	-0.02	-0.05
		P-value	0.2	0.58	0.44	0.395	0.541
Group A2	**Group C2**	**Mean Difference**	-0.44	-1.6	-0.2	-0.2	-0.11
		P-value	**0.004***	**<0.001***	**<0.001***	**<0.001***	0.192
***The mean difference is significant at P value ≤ 0.05 level.**							

Table (3-24): The mean difference of relative organs weight (lung, liver, heart, kidney and spleen) among flagellin-b-immunized groups compared with non-immunized groups.

Animal groups			lung	liver	heart	kidney	spleen
Group B1	**Group C1**	**Mean Difference**	0.2	-0.46	-0.04	-0.04	0.2
		P-value	0.117	**0.046***	0.151	0.14	**0.01***
Group B2	**Group C2**	**Mean Difference**	0.08	-0.32	0.03	0.03	0.15
		P-value	0.522	0.128	0.282	0.268	**0.044***
***The mean difference is significant at P value ≤ 0.05 level.**							

Table (3-25): The mean difference of relative organs weight (lung, liver, heart, kidney and spleen) among flagellin-a-immunized groups compared with flagellin-b-immunized groups

Animal groups			lung	liver	heart	kidney	spleen
Group A1	**Group B1**	**Mean Difference**	-0.38	0.59	0.02	0.02	-0.25
		P-value	**0.014***	**0.026***	0.581	0.588	**0.01***
Group A2	**Group B2**	**Mean Difference**	-0.51	-1.28	-0.23	-0.22	-0.26
		P-value	**0.001***	**<0.001***	**<0.001***	**<0.001***	**0.004***
***The mean difference is significant at P value ≤ 0.05 level.**							

Regarding these results, the animals immunized with flagellin-a showed a lowering of the weight of most of their vital organs especially after infection, while this effect didn't show in animals immunized with flagellin-b or non-immunized animals that making this a disadvantage of flagellin-a to use as a candidate vaccine rather than low effect of flagellin-b on the organs weight compared with the non-immunized animals. This makes flagellin-b considered as advantages as a candidate vaccine.

The results of this study regarding the lung weight in animals immunized with flagellin-b and the non-immunized animals are compatible with Sen-Kilic *et al.* (2019) who found that there were no significant differences in the lung weight among the vaccinated (FpvA-KLH or WCV) and non-vaccinated (NVNC) groups, while in-contrast with flagellin-a-immunized animals in this study that shown a decrease of lung relative weight in the present study.

3.8. Histopathological Examination of animals

After tissue processing of the organs (heart, kidney, liver, spleen, and lung) of the immunized and non-immunized groups, the examination of the slides was done. The examination of all organs (except lung) were normal in all groups (C1, C2, A1, A2, B1, and B2) as shown in Figures (3-21 to 3-23).

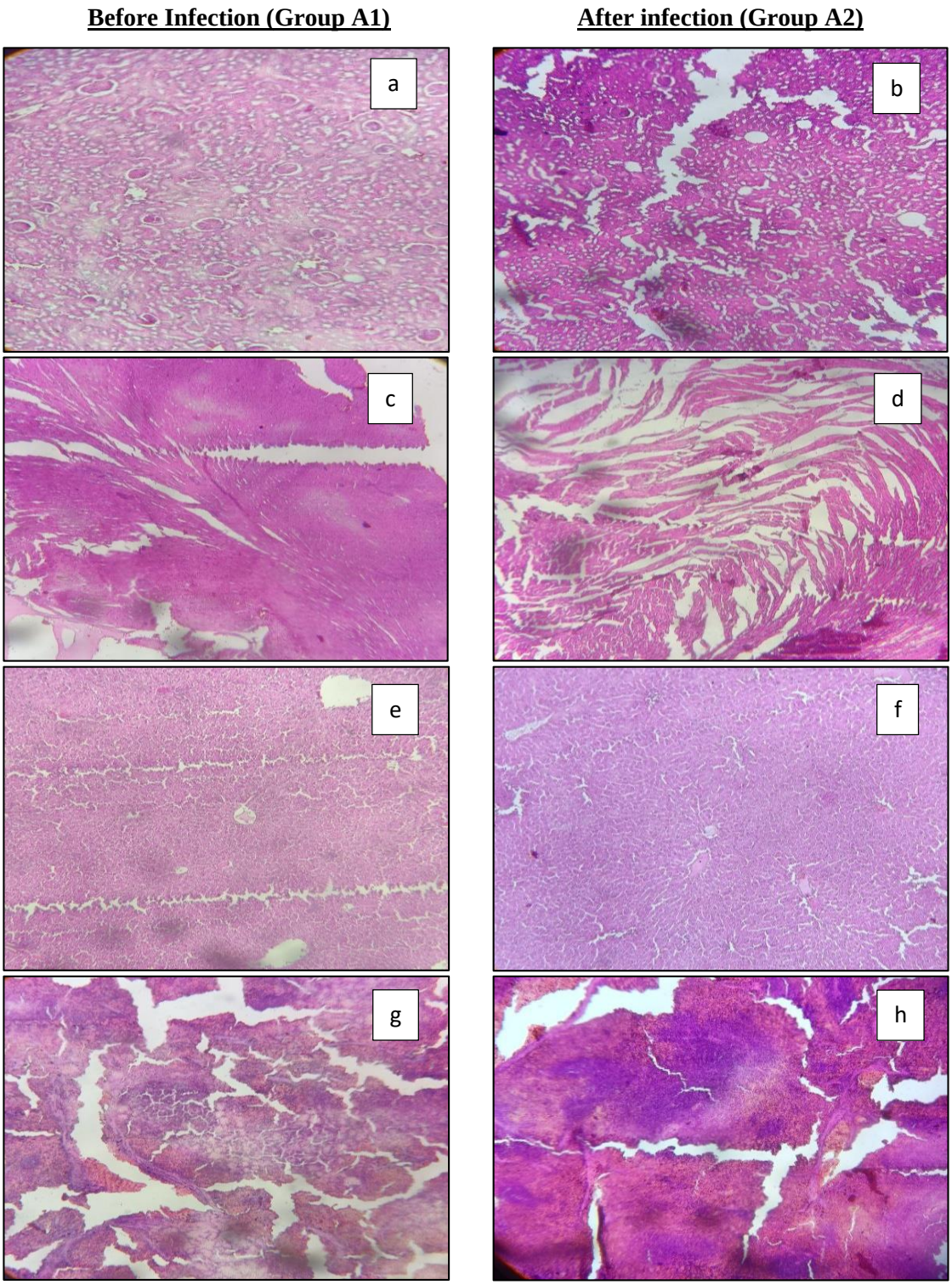

Figure (3-21): The histopathological examination of organs (Kidney, heart, liver and spleen) taken from animals immunized with flagellin-a before (group A1) & after (group A2) infection with *P. aeruginosa* MJ isolate. The tissues stained with hematoxylin and eosin stain [H&E], magnification ×10. a & b show normal kidney (intact renal tissue). c & d show normal heart (intct cardiac tissue). e & f show normal liver (intact liver tissue). g & h. show normal spleen (intact spleen tissue).

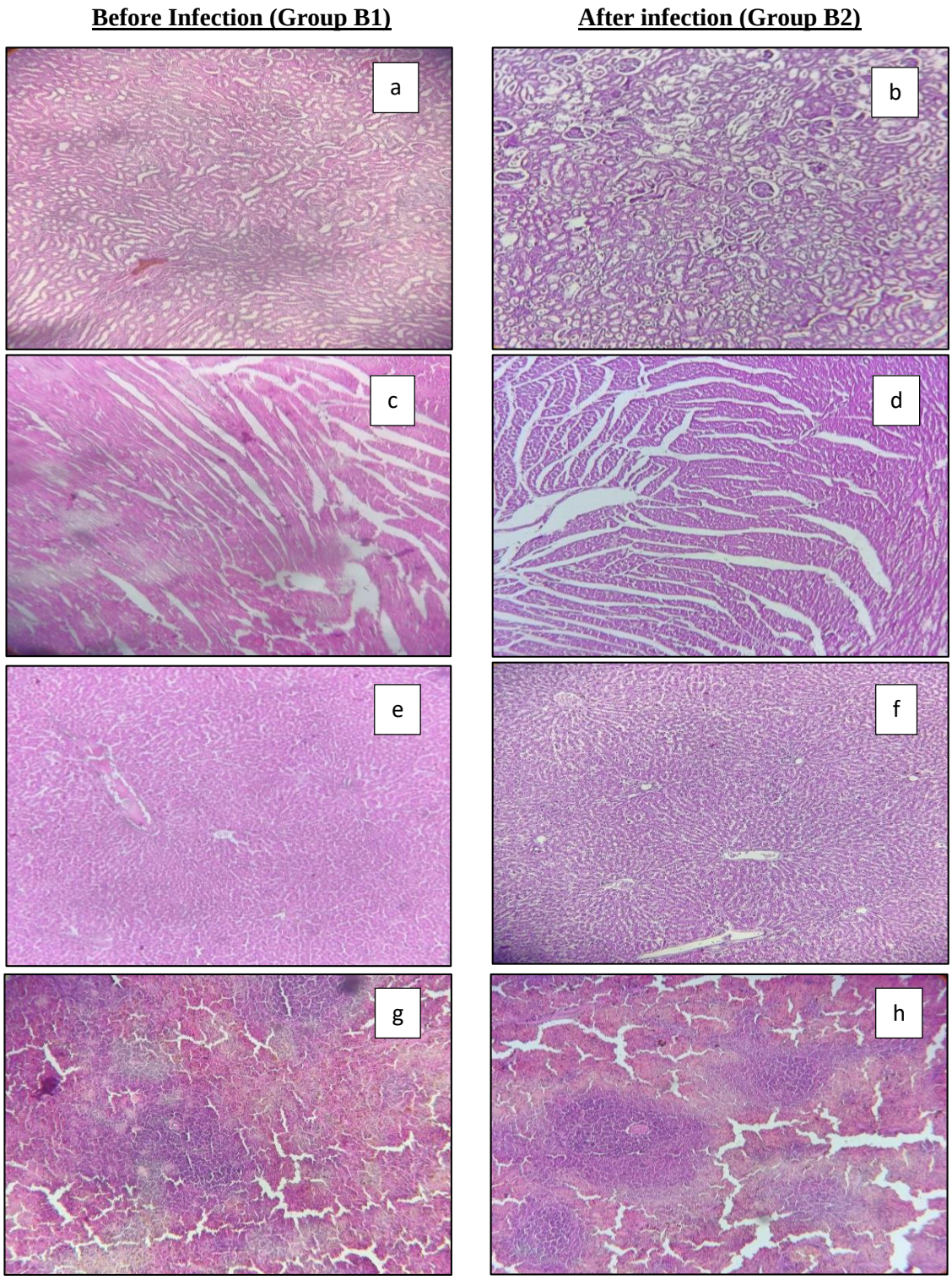

Figure (3-22): The histopathological examination of organs (Kidney, heart, liver and spleen) taken from animals immunized with flagellin-b before (group B1) & after (group B2) infection with *P. aeruginosa* MJ isolate. The tissues stained with hematoxylin and eosin stain [H&E], magnification ×10. a & b show normal kidney (intact renal tissue). c & d show normal heart (intact cardiac tissue). e & f show normal liver (intact liver tissue). g & h show normal spleen (intact spleen tissue).

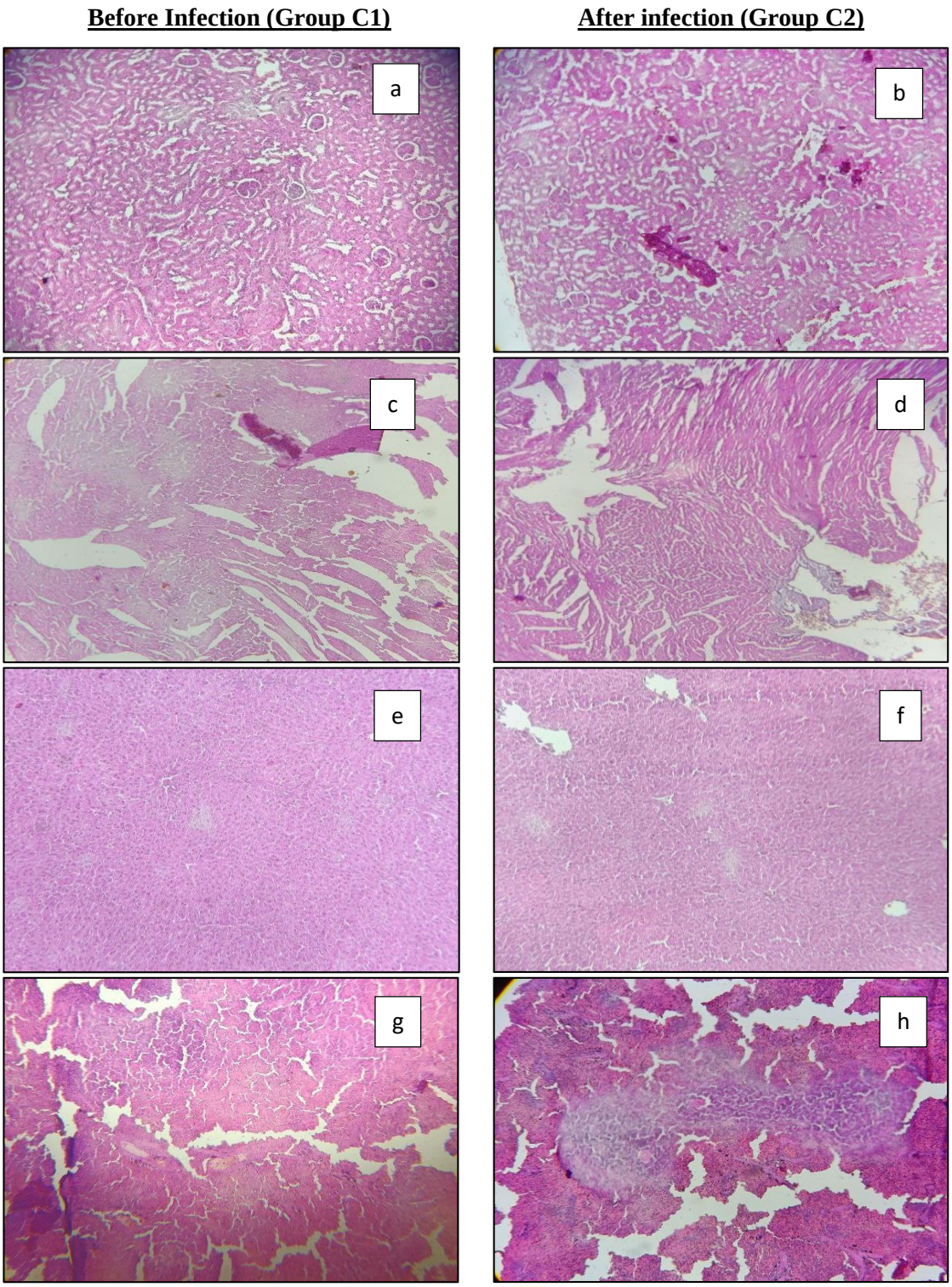

Figure (3-23): The histopathological examination of organs (Kidney, heart, liver and spleen) taken from non-immunized animals before (group C1) & after (group C2) infection with *P. aeruginosa* MJ isolate. The tissues stained with hematoxylin and eosin stain [H&E], magnification ×10. a & b Show normal kidney (intact renal tissue). c & d Show normal heart (intact cardiac tissue). e & f Show normal liver (intact liver tissue). g & h Show normal spleen (intact spleen tissue).

On the other hand, the histopathological examination of the lung in the immunized groups with flagellin-b showed a mild focal inflammatory cells aggregation before infection (group B1) and after infection (group B2), however the thickening of alveolar wall, the presence of necrotizing cell, and other inflammatory conditions were not clear in these groups (Figure 3-25). Also, the same results were appeared when comparing between flagellin-a immunized groups before (group A1) and after infection (group A2) where a mild focal inflammatory cells aggregation in the lung without any inflammatory conditions were seen (Figure 3-24).

Flagellin are highly antigenic, and it is best known as a pathogen-associated molecular pattern that binds to the extracellular (TLR-5) (Hayashi *et al.*, 2001) and intracellular NOD-like receptor (NLR) neuronal apoptosis inhibitory protein (NAIP) (Zhao *et al.*, 2011), in human (Ruiz *et al.*, 2017), that lead to activate MyD88 pathway (the pro-inflammatory pathway) and then the NLRC4-inflammasome (Vijay-Kumar *et al.*, 2010). TLR-5 is a key mediator of the epithelial cytokine and chemokine responses that lead to recruitment of neutrophil in *P. aeruginosa* lung infection (Prince, 2006; Zhang *et al.*, 2007; Beaudoin *et al.*, 2013).

While the examining of non-immunized groups before (group C1) and after infection (group C2) shows normal lungs without any inflammatory cells aggregating as shown in (Figure 3-26). The absence of inflammatory conditions in the lung of the non-immunized groups after respiratory infection with *P. aeruginosa* is may be due to the histological examination done within 24 hours of infection before tissue damage occurs.

This result was in contrast with Sen-Kilic who found strong recruitment of immune cells (especially neutrophils) to the lung of non-vaccinated mice after infection with *P. aeruginosa* accompanied by an increase in lung weight (Sen-Kilic *et al.*, 2019).

Wilson and his colleagues described the histopathology of immune versus non-immune rats with acute *P. aeruginosa* pulmonary infection, found that the histologically early PMN infiltration correlates with protective immunity and

eventual bacterial clearance in the immunized host in contrast with the non-immunized host, the histopathology associated with acute infection in showed a slower inflammatory response containing mostly mononuclear cells (Wilson *et al.*, 1995).

Sen-Kilic and his colleagueas evaluated the effectivety and immunogenicity of the FpvA peptides conjugated to keyhole limpet hemocyanin (KLH) in compared with whole-cell vaccine (WCV) (prepared from *P. aeruginosa* PAO1 strain). They showed the vaccinated groups (with FpvA-KLH or WCV) had a lower recruitment of polymorphonuclear cells and reduce lung damage and inflammation compared to non-vaccinated group after infection with *P. aeruginosa* (Sen-Kilic *et al.*, 2019).

Present study can conclude from the results above that although there was an aggregate of inflammatory cells in the lung tissues, the absents of any inflammatory conditions is a result of the effect of flagellin alone which is considered a good indicator of the effect of flagellin in enhancing of mucosal immunity in the immunized animals.

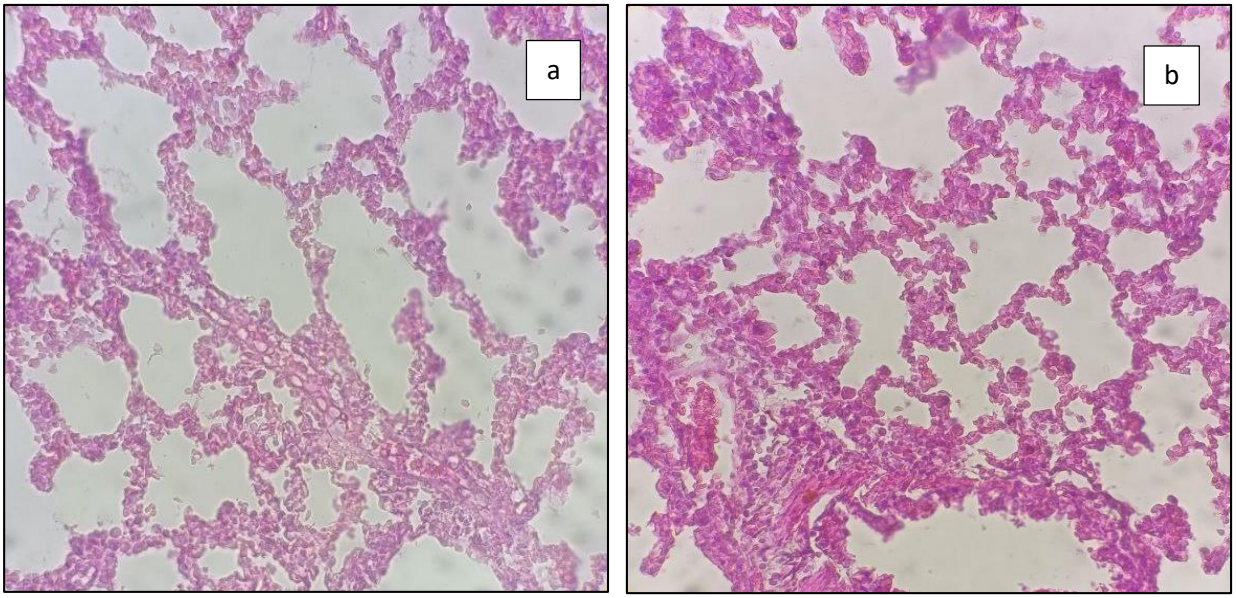

Figure (3-24): The histopathological examination of lung taken from flagellin-a immunized groups after staining the tissues with hematoxylin and eosin stain [H&E], magnification ×40. **a.** Before infection (A1) mild focal inflammatory aggregate seen. **b.** After infection (A2) mild focal inflammatory aggregate seen.

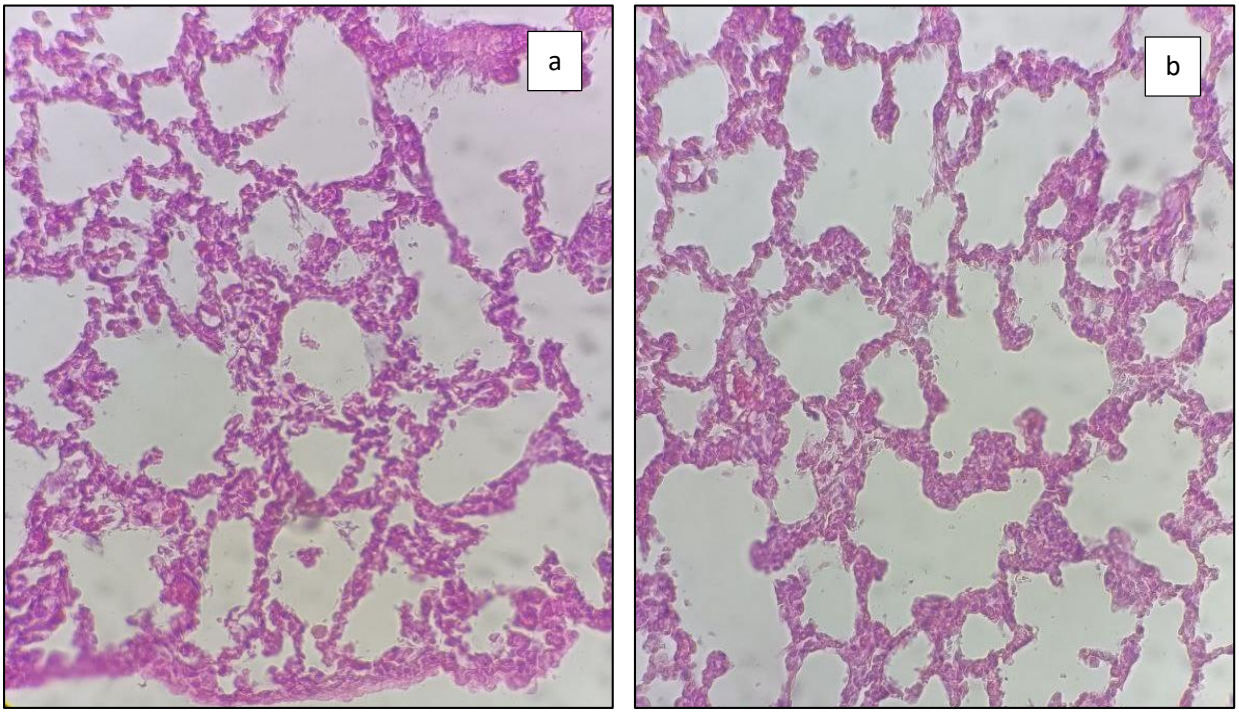

Figure (3-25): The histopathological examination of lung taken from flagellin-b immunized groups after staining the tissues with hematoxylin and eosin stain [H&E], magnification ×40. **a.** Before infection (B1) very few inflammatory cells aggregate seen. **b.** After infection (B2) mild focal inflammatory cells aggregate seen.

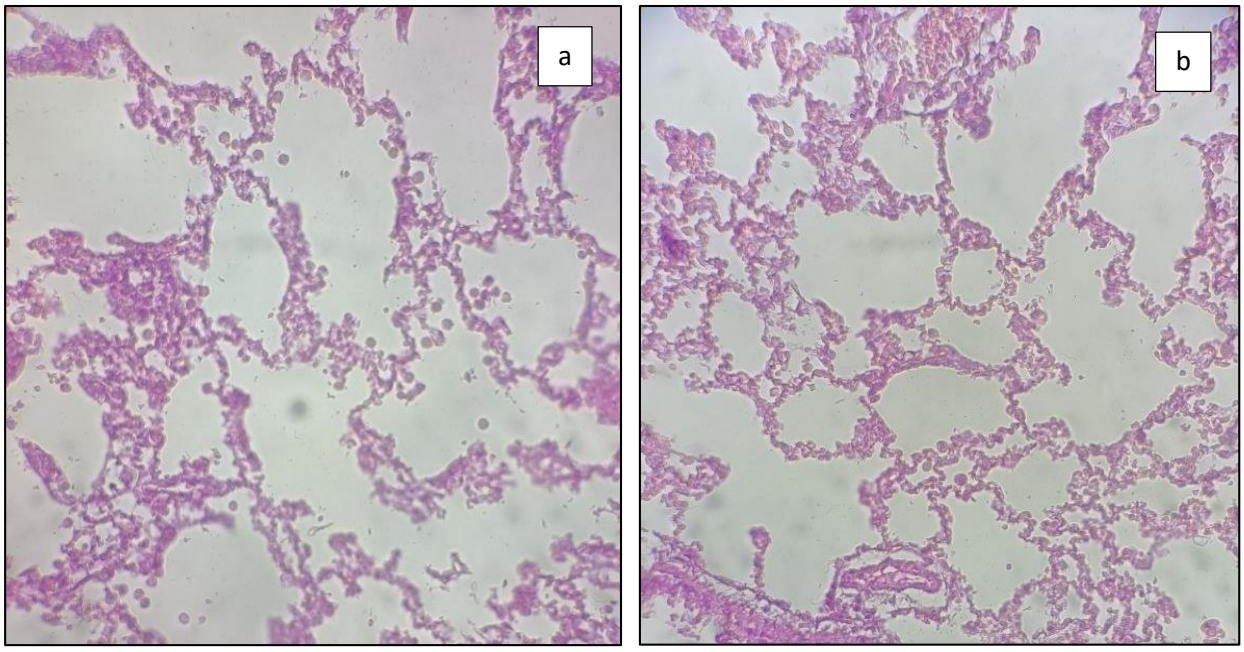

Figure (3-26): The histopathological examination of lung taken from non-immunized rat after staining the tissues with hematoxylin and eosin stain [H&E], magnification ×40. **a.** Before infection (C1) Normal. **b.** After infection (C2) Normal.

Conclusions

&

Recommendations

Conclusions

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

1. The partial purification of *P. aeruginosa* flagellin was considered a successful modification method with a high concentration of protein, fewer contaminants, and is cost-effective.
2. The ultrafiltration concentrator tube and the endotoxin removal column used in this study give highly concentrated flagellin, consume less time in the purification and give flagellin with highly acceptable levels of endotoxin for preclinical use.
3. The purified flagellin-b is more efficient than flagellin-a in the protection against *P. aeruginosa* respiratory infections in the rat model which make it a good candidate vaccine to eradicate XDR *P. aeruginosa* respiratory infection.
4. The purified flagellin-b is efficient in the elevation of IgM+ B cells and T helper cells especially Th17 and Th1 rather than T helper 2 cells after *P. aeruginosa* respiratory infection in the immunized groups, which consider essential in the eradication of these bacterial infections shifting the immune response toward a cured state.
5. The purified flagellin-a and flagellin-b have no histopathological effect on immunized animals' organs (liver, heart, kidney, and spleen).
6. Bivalent flagellin (a and b) is improper to use as a vaccine due to its pathological effects on the immunized animals which lead to their death.
7. The preparation of the flagellin candidate vaccine by a modified purification method that can be used to protect rats against respiratory infections caused by *Pseudomonas aeruginosa* is considered the first study in Iraq.

Recommendations

1. Investigation of the effect of the flagellin-b purified from *P. aeruginosa* in the eradication of other MDR bacterial pathogens.
2. Study the physical and chemical characterization of the purified flagellin-b from the isolated *P. aeruginosa*.
3. Investigation the activation effect of purified flagellin on memory T and B cells.
4. Study the correlation between flagellin-b as a vaccine and the development of autoimmune disease.
5. Study the purified flagellin-b as a candidate vaccine in clinical trials.
6. Study the bacterial load in the immunized animals' organs after infection.

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Appendices

thermo scientific USER GUIDE

Pierce™ Protein Concentrators, PES

For 3K, 10K, 30K, 50K, and 100K MWCO: 5–20 mL

Catalog Number 88525, 88526, 88527, 88528, 88529, 88531, 88540, 88541, 88532, and 88533

Doc. Part No. 2162597 **Pub. No.** MAN0015694 **Rev.** B.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Thermo Scientific™ Pierce™ Protein Concentrators are disposable ultrafiltration centrifugal devices that provide reliable and consistent results for concentrating, diafiltrating, and buffer exchanging of biological samples such as proteins and nucleic acids. The 5–20 mL Concentrators have a built-in dead stop and contain a vertical low protein-binding, high-flux polyethersulfone (PES) membrane, which allows for processing of volumes between 5 mL and 20 mL. The PES membrane is available in 5 distinct molecular-weight cutoffs (MWCOs) of 5K, 10K, 30K, 50K, and 100K. The MWCOs are etched on the sides of the Concentrators for easy identification, and a clear window with graduations on the side of each device allows for visual determination of the retentate volume.

Typical protein recovery is > 90% for proteins that are 2-fold greater than the membrane MWCO (see Table 2). Sample concentration of 10- to 30-fold can typically be achieved in 15 minutes or less (see Figure 1).

The 5–20 mL Concentrators are compatible with most swinging-bucket and fixed-angle centrifuges that accommodate 50-mL conical tubes.

Contents and storage

Table 1 Pierce™ Protein Concentrators

Product	Cat. No.	Capacity	Amount	Storage
Pierce™ Protein Concentrator, 3K MWCO[1]	88525	5–20 mL	10 per pkg	Room temperature
	88526		24 per pkg	
Pierce™ Protein Concentrator, 10K MWCO	88527		10 per pkg	
	88528		24 per pkg	
Pierce™ Protein Concentrator, 30K MWCO	88529		10 per pkg	
	88531		24 per pkg	
Pierce™ Protein Concentrator, 50K MWCO	88540		10 per pkg	
	88541		24 per pkg	
Pierce™ Protein Concentrator, 100K MWCO	88532		10 per pkg	
	88533		24 per pkg	

[1] Molecular weight cutoff

Appendix 1

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Swinging-bucket (2,000-5,000 × g) or fixed-angle (5,000-8,000 × g) centrifuge that accommodates 50-mL conical tubes	MLS
Pipet for retentate recovery	MLS
For desalting: Exchange buffer	MLS

Procedural guidelines

- For swinging-bucket centrifuges, models capable of generating 2,000–5,000 × *g* are recommended. Use a maximum centrifugal force of 4,000 × *g* for 5–20 mL, 3K, 10K, 30K, and 50K, and 3,000 × *g* for 100K Concentrators in a swinging-bucket rotor. For fixed-angle centrifuges, models capable of generating 5,000–8,000 × *g* are recommended. Use a maximum centrifugal force of 6,000 × *g* for 5–20 mL, 3K, 10K, 30K, 50K, and 100K Concentrators in a fixed-angle rotor.
- When using a fixed-angle rotor, position the Concentrator so that the printed sample window faces outward.
- Ensure the Concentrators are properly balanced in the rotor before centrifugation. The counterbalance must be another Concentrator to ensure proper balance due to changing sample distribution during processing. Do not use a filled conical tube as a counterbalance.
- For maximum protein recovery, samples should have a molecular weight two-fold greater than the MWCO of the device membrane. A slightly reduced recovery can occur with molecules that are < 2-fold the MWCO of the membrane. Recovery varies depending on the specific protein and starting concentration.
  Example: When using IgG (MW~150K) samples, use the 5K, 10K, 30K, or 50K MWCO Concentrators.
- The dead-stop volume for the 5–20 mL Concentrators is approximately 50 µL.
- Precipitation can occur at high concentration factors for some proteins. The maximum concentration factor is dependent on the specific protein, starting concentration, and buffer system. Unless the stability of the protein has been determined, avoid concentrating to dead-stop.
- Do not autoclave the Concentrators. High temperatures will significantly increase the membrane MWCO. To sterilize, use a 70% ethanol solution.
- The membrane is compatible with buffers at pH 1 to 9.
- The membrane is compatible with desalting and buffer exchange. The salt content can be reduced by ≥ 95% with one exchange as salts show minimal or no retention when they pass through the membrane. Buffer components larger than standard salt can require additional buffer exchange steps.
- For shortest concentration time we recommend using centrifuge setting near or at the maximum *g*-force recommended for a particular device and MWCO. For greater control of the concentration factor, reduce the centrifugation speed. For example concentration rates to achieve a desired volume for each MWCO, see Figure 1.
- Centrifugal force, temperature, sample volume, concentration, and viscosity affect filtration rate. Optimize centrifugal time for each application. Depending on conditions, centrifugation time to achieve desired result can differ significantly.

Concentrate the sample without desalting

1. Place sample into the Concentrator sample chamber.
2. Cap, then place the Concentrator assembly into the rotor with a proper counterbalance.

   IMPORTANT! The counterbalance must be another Concentrator (not a filled conical tube) to ensure proper balance because of changing sample distribution during centrifugation.
3. Centrifuge the sample until the desired concentration factor is achieved.
4. Use a pipet tip to gently aspirate the retentate from the bottom and center of the sample chamber.

Concentrate the sample with desalting

1. Place the sample into the Concentrator sample chamber.
2. Cap, then place the Concentrator assembly into the rotor with a proper counterbalance.

   IMPORTANT! The counterbalance must be another Concentrator (not a filled conical tube) to ensure proper balance because of changing sample distribution during centrifugation.
3. Centrifuge the samples until the volume is reduced by 90–95%.
4. Dilute the sample to the original volume with Exchange Buffer.
5. Repeat steps 3 and 4 until the desired solute removal has been achieved.

   Note: Precipitation can occur at high concentration factors for some proteins. If this occurs, reduce sample volume less in each step and increase the number of repeats.

Appendix 1

Troubleshooting

Observation	Possible cause	Recommended action
Protein precipitation.	Concentration was too high.	Reduce concentration factor.
		Try a different buffer system to increase protein solubility.
Low protein recovery.	The protein MW < two-fold higher than MWCO.	Select a new Concentrator with a MWCO at least 2-fold lower than the protein MW.
	The membrane was damaged and there is protein in the filtrate.	Use a new Concentrator and do not touch the membrane with the pipet tip.
		Do not exceed the recommended centrifugal force.

Flux curves and recovery data

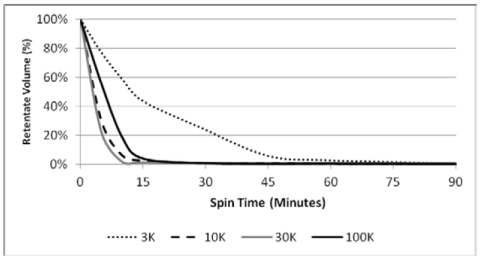

Figure 1 Protein concentration rates

Protein samples (20 mL of ~0.25 mg/mL starting concentrations) for each MWCO (3K, 10K, 30K, and 100K) were centrifuged in Pierce™ Protein Concentrators at 4,700 × *g* for 90 minutes to determine the rate at which the protein is concentrated. Within 15 minutes, the 10K, 30K, and 100K Concentrators had concentrated > 90% of the protein solution. Within 90 minutes, the 3K Concentrator had concentrated > 90% of the protein solution.

Table 2 Protein recoveries using Pierce™ Protein Concentrators

Starting volume of 20 mL in a swinging-bucket rotor at 4,700 × *g* and 25°C.

Protein Sample	Membrane MWCO (kDa)	Centrifuge Time (minutes)	Protein Retentate	
			Volume (µL)	Recovery (%)
Cytochrome C, 12 kDa (0.25 mg/mL)	3	90	121	100
Ovalbumin, 45 kDa (0.25 mg/mL)	10	15	528	94
BSA, 66 kDa (0.25 mg/mL)	30	15	216	100
Thyroglobulin, 660 kDa (0.25 mg/mL)	100	15	766	98

Appendix 1

Chemical compatibility

The PES membranes used in the Pierce™ Protein Concentrators are compatible with most standard aqueous biological samples, buffers, and salts, according to the following table. Samples containing high levels of cell membranes, fats, or lipids can reduce performance and result in membrane blockage.

Table 3 Concentrator chemical compatibility

Acids and Bases	Rating[1]	Organics	Rating[1]	Miscellaneous	Rating[1]
Acetic acid (25%)	A	Acetone	NR	Ammonium sulfate (saturated)	A
Formic acid (5%)	A	Acetonitrile	NR	Glycerine (70%)	A
Hydrochloric acid (1 M)	A	Benzene (100%)	NR	Guanidine HCl (5–5–20M)	A
Lactic acid (5%)	A	Chloroform (1%)	NR	Imidazole (300 mM)	A
Nitric acid (10%)	A	Dimethyl sulfoxide(5%)	A	Phosphate buffer (1.0 M)	A
Sodium hydroxide (2.5 M)	NR	Ethanol (70%)	A	Polyethylene glycol (10%)	A
Sulfamic acid (5%)	A	Ethyl Acetate (100%)	NR	Sodium carbonate (5–20%)	A
Trifluoroacetic acid (10%)	A	Formaldehyde (30%)	A	Sodium deoxycholate (5%)	A
		Hydrocarbons (aromatic)	NR	Sodium dodecylsulfate (0.1 M)	A
		Hydrocarbons (chlorinated)	NR	Sodium hypochlorite (5–200 ppm)	A
		Isopropanol (70%)	A	Sodium nitrate (1%)	A
		Mercaptoethanol (1.0 M)	NR	Tween™ 20 (0.1%)	A
		Pyridine (100%)	NR	Triton™ X-100 (0.1%)	A
		Tetrahydrofuran (5%)	NR	Urea (8 M)	A
		Toluene (1%)	NR		

[1] A = Acceptable, NR = Not Recommended

Note: Concentrations listed are provided as guidelines and do not necessarily represent maximum tolerances. Some compatible chemicals might modify the apparent molecular weight of molecules in the sample and/or the MWCO rating of the membrane.

Appendix 1

Related products

Product	Cat. No.	Capacity	Amount
Slide-A-Lyzer™ G2 Dialysis Cassettes, 2K MWCO	87717	0.5 mL	10 per pkg
Slide-A-Lyzer™ G2 Dialysis Cassettes, 3.5K MWCO	87722	0.5 mL	10 per pkg
Slide-A-Lyzer™ Dialysis Cassettes, 2K MWCO	66205	0.5 mL	10 per pkg
Slide-A-Lyzer™ Dialysis Cassettes, 3.5K MWCO	66333	0.5 mL	10 per pkg
Pierce™ Protein Concentrator, PES, 3K MWCO	88512	100–500 µL	25 per pkg
Pierce™ Protein Concentrator, PES, 10K MWCO	88513	100–500 µL	25 per pkg
Pierce™ Protein Concentrator, PES, 30K MWCO	88502	100–500 µL	25 per pkg
Pierce™ Protein Concentrator, PES, 50K MWCO	88504	100–500 µL	25 per pkg
Pierce™ Protein Concentrator, PES, 100K MWCO	88503	100–500 µL	25 per pkg
Pierce™ Protein Concentrator, PES, 3K MWCO	88514	2–6 mL	10 per pkg
	88515	2–6 mL	24 per pkg
Pierce™ Protein Concentrator, PES, 10K MWCO	88516	2–6 mL	10 per pkg
	88517	2–6 mL	24 per pkg
Pierce™ Protein Concentrator, PES, 30K MWCO	88521	2–6 mL	10 per pkg
	88522	2–6 mL	24 per pkg
Pierce™ Protein Concentrator, PES, 50K MWCO	88538	2–6 mL	10 per pkg
	88539	2–6 mL	24 per pkg
Pierce™ Protein Concentrator, PES, 100K MWCO	88523	2–6 mL	10 per pkg
	88524	2–6 mL	24 per pkg
Pierce™ Protein Concentrator, PES, 5K MWCO	88534	20–100 mL	4 per pkg
Pierce™ Protein Concentrator, PES, 10K MWCO	88535	20–100 mL	4 per pkg
Pierce™ Protein Concentrator, PES, 30K MWCO	88536	20–100 mL	4 per pkg
Pierce™ Protein Concentrator, PES, 50K MWCO	88542	20–100 mL	4 per pkg
Pierce™ Protein Concentrator, PES, 100K MWCO	88537	20–100 mL	4 per pkg
Pierce™ BCA Protein Assay Kit	23225	—	—

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

Revision history: Pub. No. MAN0015694

Revision	Date	Description
B.0	17 January 2019	Rebranding of document.

Appendix 1

INSTRUCTIONS

Pierce High-Capacity Endotoxin Removal Resin

MAN0016351
Rev A.0
Pub. Part No. 2162373.3

Number	Description
88270	**Pierce High-Capacity Endotoxin Removal Resin,** 10mL of settled resin supplied as 50% slurry in 20% ethanol
88271	**Pierce High-Capacity Endotoxin Removal Resin,** 100mL of settled resin supplied as 50% slurry in 20% ethanol
88272	**Pierce High-Capacity Endotoxin Removal Resin,** 250mL of settled resin supplied as 50% slurry in 20% ethanol
88273	**Pierce High-Capacity Endotoxin Removal Spin Column, 0.25mL,** 5 columns, each column contains 25% slurry in 20% ethanol
88274	**Pierce High-Capacity Endotoxin Removal Spin Column, 0.50mL,** 5 columns, each column contains 25% slurry in 20% ethanol
88275	**Pierce High-Capacity Endotoxin Removal Spin Column, 0.50mL,** 25 columns, each column contains 25% slurry in 20% ethanol
88276	**Pierce High-Capacity Endotoxin Removal Spin Column, 1.0mL,** 5 columns, each column contains 25% slurry in 20% ethanol
88277	**Pierce High-Capacity Endotoxin Removal Spin Column, 1.0mL,** 25 columns, each column contains 25% slurry in 20% ethanol

Storage: Upon receipt store at 4°C. Product shipped at ambient temperature.

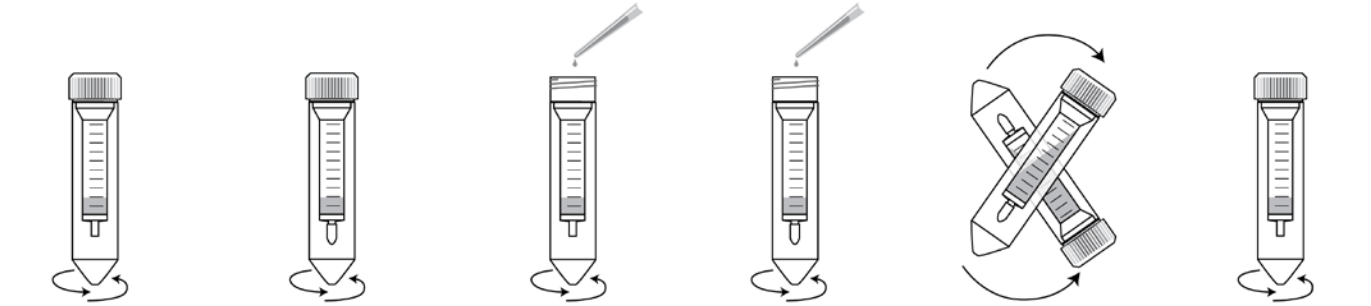
Introduction

The Thermo Scientific™ Pierce™ High Capacity Endotoxin Removal Resin contains porous cellulose beads that have been surface modified with covalently attached, modified ε-poly-L-lysine. Modified polylysine has a high affinity for endotoxins with the affinity ligand eliminating toxicity associated with alternative technologies using polymixin B ligands and sodium deoxycholate buffers. The binding capacity of 2,000,000 Endotoxin Units (EU)/mL allows endotoxin levels to be reduced by 99% in samples containing 10,000EU/mL; typical protein samples processed with the resin have a final endotoxin concentration below 5EU/mL. The resin is offered in a slurry format for custom packing of endotoxin removal columns for gravity flow or continuous flow (10-15 mL/hour) applications or a spin column format for the fast, single-use batch method. Removal of endotoxins from differing volumes of biological samples can be done in ≥ 1 hour (see Table 1).

Endotoxins consist of lipopolysaccharides (LPS), which are biologically active, structural components of the outer cell membrane of all gram-negative bacteria. Small amounts of endotoxin in recombinant protein preparations can cause side effects, including endotoxin shock, tissue injury and death in host organisms; therefore, it is essential to remove endotoxins from drugs, injectables and other biological products.

One milliliter of this resin can reduce the endotoxin concentration from a 1mL sample containing 10,000EU to ≤ 5EU.

Procedure Summary



1. Centrifuge at 500 x *g* for 1 minute to remove the storage buffer.
2. Regenerate with 0.2N NaOH (overnight) or 0.2N NaOH in 95% ethanol for 1-2 hours.
3. Wash with 2M NaCl followed by ET-free water.
4. Equilibrate with ET-free buffer, pH 6-8. Repeat three times.
5. Add sample and incubate at 4-22°C with gentle end-over-end mixing for 1 hour.
6. Centrifuge at 500 x *g* for 1 minute to collect the sample.

Important Product Information

- All materials (e.g., containers, buffers and pipette tips) must be endotoxin-free. Use endotoxin-free water to prepare buffers and other solutions.
- Endotoxin binding to the resin occurs at pH 6-8; Equilibrate the resin with an endotoxin-free buffer at neutral pH that includes 10-50mM sodium phosphate buffer or Tris-HCl buffer containing 0.1-0.2M NaCl. Check the sample pH and adjust to pH 6-8 with 0.1M NaOH or 0.1M HCl.
- The speed of sample application and wash depends on the sample endotoxin state. Free endotoxin can bind quickly to the resin, but protein-bound endotoxin may require longer incubation, slower flow rates and/or multiple recycling of the sample through the column. Tightly bound endotoxin may be incubated overnight at room temperature or 4°C in batch mode with gentle shaking.
- Resins can be used a minimum of five times without loss of endotoxin-removal efficiency.
- Sample volumes vary depending on the chosen pre-loaded column size (see Table 1).

Table 1. Sample volumes for the Thermo Scientific Pierce High-Capacity Endotoxin Removal Columns.

Column size (mL)	Sample volume (mL)
0.25	0.5-1.0
0.50	1.0-4.0
1.0	2.0-10.0

Additional Materials Required

- Endotoxin-free, ultrapure water
- Endotoxin-free 15mL conical collection tubes (for 0.25mL and 0.5mL spin columns)
- Endotoxin-free 50mL conical collection tubes (for 1.0mL spin columns)
- Regeneration buffer: 0.2N NaOH for overnight incubation at room temperature **OR** 0.2N NaOH and 95% ethanol for 1-2 hours of incubation at room temperature

  Note: Regenerate the column before the first use and after each subsequent use.
- 2M NaCl solution: If possible, use ready-made endotoxin-free NaCl solution; alternatively, dissolve pure sodium chloride crystals in endotoxin-free water
- Variable-speed centrifuge with rotor and carriers capable of handling 15mL and 50mL conical collection tubes

Endotoxin Removal Procedure using the Column Method

- Regenerate the resin before the first use and after each subsequent use.
- Equilibrate all solutions and the resin to room temperature before use.
- Degas the resin slurry before applying to the column to prevent air bubbles from clogging the column and reducing flow.

1. To degas the resin, place slurry in a suction filter flask and degas with gentle stirring.
2. Pour the degassed resin slurry into an appropriately sized column and allow the resin to settle.
3. Regenerate the resin by washing with five resin-bed volumes of 0.2N NaOH overnight at room temperature **OR** five resin-bed volumes of 0.2N NaOH in 95% ethanol for 1-2 hours at room temperature.
4. Wash with five resin-bed volumes of 2M NaCl.
5. Wash with five resin-bed volumes of endotoxin-free, ultrapure water.
6. Equilibrate with five resin-bed volumes of endotoxin-free buffer.
7. Apply sample to the column at a flow rate of 10-15mL/hr and collect the flow-through (see the Important Product Information Section).
8. Elute the protein by adding endotoxin-free buffer and collecting fractions; one or two resin-bed volume elutions are sufficient.
9. Determine the endotoxin concentration of the processed sample. **Use caution to prevent sample contamination from dust or contaminated tubes subsequent to endotoxin removal.** Store solutions frozen or assay them before use to ensure sterility.
10. Regenerate the resin as described in Steps 3-5 and store the column in 20% ethanol at 2-8°C.

Endotoxin Removal Procedure using the Batch Method with Spin Columns

- Regenerate the resin before the first use and after each subsequent use. For regeneration and equilibration (steps 1-10), use regeneration buffer in 2mL volumes for the 0.25mL column, 3.5mL volumes for the 0.5mL column and 8mL volumes for the 1mL column.
- Wear appropriate gloves while handling the spin columns. Use caution to prevent sample contamination during the endotoxin-removal procedure.

1. Equilibrate the spin column to room temperature.
2. Twist off the column's bottom closure and loosen the top cap. Place the spin column into a collection tube. Centrifuge the column at 500 × *g* for 1 minute to remove the storage solution. Discard the storage solution.
3. Remove the column cap and insert the bottom plug. To regenerate, add 0.2N NaOH, replace the cap, invert the column several times until the resin is suspended in the solution and incubate overnight at room temperature, **OR** add 0.2N NaOH in 95% ethanol, replace the cap, invert the column several times until the resin is suspended in the solution and incubate 1-2 hours at room temperature.
4. Loosen the cap and remove the bottom plug. Place the column in a collection tube and centrifuge at 500 × *g* for 1 minute to remove the solution. Discard the solution.
5. Remove the cap and insert the bottom plug. Add 2M NaCl, replace the cap and invert the column several times until the resin is suspended in the solution.
6. Loosen the cap and remove the bottom plug. Place the column in a collection tube and centrifuge at 500 × *g* for 1 minute to remove the solution. Discard the solution.
7. Remove the cap and insert the bottom plug. Add endotoxin-free, ultrapure water. Replace the cap and invert the column several times until the resin is suspended in the solution.
8. Loosen the cap and remove the bottom plug. Place the column in a collection tube and centrifuge at 500 × *g* for 1 minute to remove the solution. Discard the solution.
9. Remove the cap and insert the bottom plug. Add endotoxin-free buffer, replace the cap and invert the column several times until the resin is suspended in the solution.

10. Loosen the cap and remove the bottom plug. Place the column in a collection tube and centrifuge at 500 × *g* for 1 minute to remove the solution. Discard the solution. Repeat steps 9 and 10 two additional times.
11. Remove the cap and insert the bottom plug. Add the sample to the resin, replace the cap and invert the column several times until the resin is suspended in the solution.
12. Incubate the column with gentle end-over-end mixing at room temperature or 4°C for 1 hour. Incubation time can be extended depending on the sample type and requirements (see the Important Product Information Section).
13. Loosen the cap and remove the bottom plug. Place column in a collection tube and centrifuge at 500 × *g* for 1 minute to collect the sample.
14. Determine the endotoxin concentration of the processed sample. **Use caution to prevent sample contamination after endotoxin removal.** Store solutions at -20°C or assay before use to ensure sterility.
15. Regenerate the resin as described in step 3 and store the column in 20% ethanol at 2-8°C.

Troubleshooting

Problem	Possible Cause	Solution
Low endotoxin removal efficiency	Sample pH was not within a neutral range	Adjust sample to pH 6-8
	Incubation time was not sufficient	Increase the incubation time for the batch method
	Endotoxin was bound to the target protein	Recycle the sample through the column several times
	The removal or detection system was contaminated by extrinsic LPS	Use endotoxin-free labware and buffers
Low protein/sample recovery	Target protein aggregated with endotoxin and was removed	Increase NaCl concentration in the sample and equilibration buffer to 0.4M
	Nonspecific binding of sample to the resin	

Related Thermo Scientific Products

88282 **Pierce LAL Chromogenic Endotoxin Quantitation Kit**

89896, 7, 8 **Pierce Centrifuge Columns, (2mL, 5mL and 10mL, respectively),** 25/pkg

23225 **Pierce BCA Protein Assay Kit**

22660 **Pierce 660nm Protein Assay**

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thermo scientific USER GUIDE

Pierce™ Chromogenic Endotoxin Quant Kit

Catalog Numbers A39552S, A39552, and A39553

Doc. Part No. 2162713 **Pub. No.** MAN0017902 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Contents

Product	Cat. No.	Contents	Storage
Pierce™ Chromogenic Endotoxin Quant Kit	A39552S	Kit sufficient for 30 tests of standards and samples in a microplate Contents: Lyophilized *E. coli* (0111:B4) Endotoxin Standard, 1 vial, 10-50 endotoxin units (EU)/vial Lyophilized Amebocyte Lysate, 1 vial, 1.7 mL/vial upon reconstitution Lyophilized Chromogenic Substrate, 1 vial, 3.4 mL/vial upon reconstitution Endotoxin-Free Water, 1 vial, 50 mL	Store at 4°C.
	A39552	Kit sufficient for 60 tests of standards and samples in a microplate Contents: Lyophilized *E. coli* (0111:B4) Endotoxin Standard, 2 vials, 10-50 endotoxin units (EU)/vial Lyophilized Amebocyte Lysate, 2 vials, 1.7 mL/vial upon reconstitution Lyophilized Chromogenic Substrate, 2 vials, 3.4 mL/vial upon reconstitution Endotoxin-Free Water, 2 vials, 50 mL/vial	
	A39553	Kit sufficient for 240 tests of standards and samples in a microplate Contents: Lyophilized *E. coli* (0111:B4) Endotoxin Standard, 8 vials, 10-50 endotoxin units (EU)/vial Lyophilized Amebocyte Lysate, 8 vials, 1.7 mL/vial upon reconstitution Lyophilized Chromogenic Substrate, 8 vials, 3.4 mL/vial upon reconstitution Endotoxin-Free Water, 4 vials, 50 mL/vial	

Product description

The Thermo Scientific™ Pierce™ Chromogenic Endotoxin Quant Kit is an efficient, quantitative endpoint assay that uses amebocyte lysates derived from blood of the horseshoe crab to quantitate endotoxin in protein, peptides, antibodies or nucleic acid samples. Amebocyte lysates are widely used as a simple and sensitive assay for the detection of endotoxin lipopolysaccharide (LPS), the membrane component of gram-negative bacteria. When endotoxin encounters the amebocyte lysate, a series of enzymatic reactions results in the activation of Factor C, Factor B and pro-clotting enzyme. The activated enzyme catalyzes the release of p-nitroaniline (pNA) from the colorless chromogenic substrate, Ac-Ile-Glu-Ala-Arg-pNA, producing a yellow color. After stopping the reaction, the released pNA is photometrically measured at 405 nm. The correlation between absorbance and endotoxin concentration is linear in the 0.1-1.0 EU/mL and in 0.01-0.1 EU/mL range. The developed color intensity is proportional to the amount of endotoxin present in the sample and can be calculated using a standard curve.

Important product information

Note: Thorough cleanliness in labware, raw materials, and in lab technique is required to accurately detect levels of endotoxin in a given sample.

- Accurate pipetting is critical for maintaining consistent results. A repetitive pipettor can aid in normalizing volumes between samples. Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row.
- All materials (e.g., pipette tips, glass tubes, microcentrifuge tubes and disposable 96-well microplates) must be endotoxin-free.
- Maintaining the correct temperature is critical for reproducibility. Use a proper heating block at 37±1°C. Cabinet-style incubators are not recommended to perform the assay.
- Endotoxin adheres to glass and plastic surfaces; before pipetting, vortex solutions to ensure the correct endotoxin concentrations are measured.
- Glass tubes are recommended for making standard stock solutions.
- Each lysate lot is tested for functionality using the United States Reference Standard EC-6. The assay lot is then matched to a lot of our Endotoxin Standard (ES) by testing in parallel with the Reference Standard Endotoxin (RSE). The RSE/ES correlation assay determines the potency of each ES lot when used with each matching lysate lot.

Appendix 3

Materials required but not supplied

- Disposable endotoxin-free glass tubes or pyrogen-free 1.5 mL microcentrifuge tubes
- Disposable endotoxin-free pipette tips
- Disposable endotoxin-free 96-well microplates or plate strips
- Stable temperature plate heater (37±1°C)
- Pipettor
- Repetitive pipettor (optional) or multichannel pipettor
- Pyrogen-free reservoir
- Microplate reader
- 25% acetic acid (stop solution)

Prepare materials

Note: Equilibrate all reagents to room temperature before use.

Prepare Endotoxin Standard Stock Solutions

1. Each *E. coli* Endotoxin Standard vial contains 10-50 EU of lyophilized endotoxin; the actual potency is printed on the label. Reconstitute with room temperature Endotoxin-Free Water by adding 1/10 mL of the EU amount indicated on the vial to make Endotoxin Standard (ES) Solution at 10 EU/mL (e.g., a vial with potency of 15 EU, when reconstituted with 1.5 mL of Endotoxin-Free Water (EFW), will yield a concentration of 10 EU/mL).
2. Vortex the solution vigorously for 15 minutes (recommended <1500 rpm).

   Note: Reconstituted stock solution is stable for 4 weeks at 2-8°C. Prior to subsequent use, warm the solution to room temperature and vigorously mix for 15 minutes. This is important because the endotoxin adheres to the sides of the glass vial.
3. Prepare High Standards (0.1-1.0 EU/mL) (Table 1) or Low Standards (0.01-0.1 EU/mL) (Table 2) from the Endotoxin Standard Solution (10 EU/mL) using the dilutions and procedures in Tables 1 and 2.

Table 1 High Standards (0.1-1.0 EU/mL)

Vial	Volume of Endotoxin Standard Solution (mL)	Volume of Standard 1 (mL)	Endotoxin-Free Water (mL)	Final Endotoxin Concentration (EU/mL)	Vortex Time (min)
Standard 1	0.20	—	1.80	1.00	2
Standard 2	—	1.00	1.00	0.50	1
Standard 3	—	0.50	1.50	0.25	1
Standard 4	—	0.20	1.80	0.10	1
Blank	—	—	0.50	0	—

Table 2 Low Standards (0.01-0.1 EU/mL)

Vial	Volume of Endotoxin Standard Solution (mL)	Volume of Stock (mL)	Volume of Standard 1 (mL)	Endotoxin-Free Water (mL)	Final Endotoxin Concentration (EU/mL)	Vortex Time (min)
Stock	0.20	—	—	1.80	1.00	2
Standard 1	—	0.20	—	1.80	0.100	2
Standard 2	—	—	1.00	1.00	0.050	1
Standard 3	—	—	0.50	1.50	0.025	1
Standard 4	—	—	0.20	1.80	0.010	1
Blank	—	—	—	0.50	0	—

Reconstitute Lyophilized Amebocyte Lysate

1. **Reconstitute Lyophilized Amebocyte Lysate immediately before use** with 1.7 mL of Endotoxin-Free Water (EFW) and swirl gently to dissolve the powder. If more than 1 vial is required, pool 2 or more vials before use. **Avoid foaming; do not vortex the solution**.

   Note: Make sure to recover all of the powder from the sides and the cap of the vial by gently inverting end-over-end. Extreme care must be taken not to touch the inside part of the cap to avoid contamination.

   Note: Reconstituted amebocyte lysate solution is stable for 1 week at -20°C or colder if frozen **immediately** after reconstitution. Upon thawing, the reconstituted lysate solution may be used only 1 time. Once thawed, gently swirl the reagent to mix before use.

Appendix 3

Sample preparation

- Adjust the sample pH to 6-8 using endotoxin-free 0.1M NaOH or 0.1M HCl. Avoid pH-electrode contamination of the sample by testing the pH of a small sample taken from the bulk sample.
- Components of undiluted serum interfere in the assay. Serum samples must be diluted 50- to 100-fold to be compatible. The serum must be completely free of red blood cells, and the diluted sample may need to be heat-shocked (70°C for 15 minutes).
- To stop all bacteriological activity in test samples, store samples to be tested at 2-8°C for <24 hours or -20°C for >24 hours.

Chromogenic Substrate

Each vial contains 3.4 mg of lyophilized Chromogenic Substrate. Reconstitute the substrate by adding 3.4 mL of Endotoxin-Free Water.

Note: Reconstituted Chromogenic Substrate is stable for 4 weeks when stored at 2-8°C. **Pre-warm a sufficient substrate amount for the assay to 37°C for no more than 5-10 minutes prior to use.**

Assay procedure

Note: Equilibrate all reagents to room temperature before use. Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row throughout the procedure.

1. Prepare all reagents and standards as directed in previous section immediately before use.
2. Pre-equilibrate plate in a heating block at 37±1°C. **Throughout the assay procedure, maintain the plate at 37±1°C.**
3. Add 50 µL of Endotoxin Standard dilutions, blank, and samples per well.

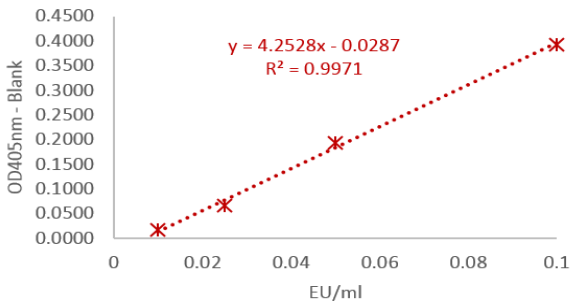
   Note: It is recommended to run each sample and standards in triplicate, including triplicate of a blank (50 µL of Endotoxin-Free Water).
4. Keeping the plate at 37±1°C, add 50 µL of the reconstituted Amebocyte Lysate Reagent per well. Begin timing as the lysate is added to the first well.
5. Once the Amebocyte Lysate Reagent has been added to the plate wells, briefly remove from the plate heater and mix by gently tapping 10 times on the side of the plate, avoiding spilling. Return the plate to the plate heater and incubate at 37±1°C for the time **T1 indicated on the lysate vial**.

   Note: T1 High = Time 1 for High Standards and T1 Low = Time 1 for Low Standards.
6. Reconstitute the Chromogenic Substrate as described in Material Preparation with 3.4 mL of Endotoxin-Free Water. Mix gently by tilting and swirling the vial. Pre-warm to 37±1°C for 5 minutes before use.
7. After exactly time T1, add 100 µL per well of pre-warmed reconstituted Chromogenic Substrate Solution.
8. Once the substrate solution has been added into all plate wells, briefly remove from the plate heater and mix gently by tapping 10 times to facilitate mixing. Return to the plate heater at 37±1°C for **T2 = 6 minutes**.
9. At exactly T2 = 6 minutes, add 50 µL per well of Stop Solution (25% acetic acid).
10. Once the stop solution has been added to the plate wells, remove the plate from the plate heater and mix by gently tapping 10 times on the side of the plate.
11. Read the optical density (OD) at 405 nm immediately after assay completion. If the plate is read at a later time, keep covered to avoid evaporation.
12. Subtract the average absorbance of the blank replicates from the average absorbance of all individual standards and sample replicates to calculate mean Δ absorbance.
13. Prepare a standard curve by plotting the average blank-corrected absorbance for each standard on the y-axis vs. the corresponding endotoxin concentration in EU/mL on the x-axis. The coefficient of determination, r^2, must be ≥0.98.

    Note: Do not include the blank OD in the calculation of the regression line.
14. Use the formulated standard curve (linear regression) to determine the endotoxin concentration of each sample.

Table 3 Example data and standard curve for Low Standard.

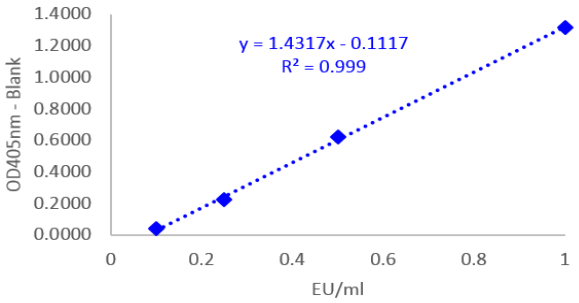
UE/mL	Avg. OD (405 nm)	Δ	Std. Dev.	%CV
0.1	0.5120	0.3937	0.0137	3
0.05	0.3129	0.1945	0.0015	1
0.025	0.1851	0.0668	0.0001	0
0.01	0.1353	0.0170	0.0003	2
0	0.1183	0	0.0024	—



Appendix 3

Table 4 Example data and standard curve for High Standard.

UE/mL	Avg. OD (405 nm)	Δ	Std. Dev.	%CV
1.0	1.4327	1.3162	0.0774	6
0.5	0.7382	0.6217	0.0158	3
0.25	0.3388	0.2223	0.0045	2
0.1	0.1581	0.0416	0.0018	4
0	0.1165	0	0.0033	—



Troubleshooting

Observation	Possible cause	Recommended action
Non-linear standard curve.	Endotoxin Standard Solution and dilutions were not mixed well.	Vortex the Endotoxin Standard Solution for 15 minutes before each use.
		Vortex all endotoxin standard dilutions for 1-2 minutes before each use.
		Vortex the endotoxin standard dilutions for 2 minutes if they were sitting for >10 minutes after preparation before adding into the plate wells.
	Pipetting order and rate of reagent addition were irregular.	Ensure pipetting order and rate of reagent addition remain consistent from well-to-well and row-to-row.
		Use a repetitive or multichannel pipettor.
	Incubation times were not followed.	Strictly adhere to the incubation times.
		Start the timer at the point of adding reagent into the first well.
Higher absorbance in blank than standard dilutions.	Materials (e.g., tips, vials, microplates) were contaminated.	Use endotoxin-free materials.
Higher absorbance in samples than standard curve.	Test sample endotoxin concentration is >1.0 EU/mL (for High Standard curve) or >0.1 EU/mL (for Low Standard curve).	Dilute the sample 5-fold in Endotoxin-Free Water. Re-test.
Samples turning yellow immediately after addition of the Stop Solution (25% acetic acid).	Samples contain substances that turns yellow in acidic environments (e.g., certain tissue culture media).	To determine if a sample's intrinsic color will alter the absorbance readings, construct a mock reaction tube by adding 50 µL of sample, 150 µL of Endotoxin-Free Water and 50 µL of Stop Solution with no incubation. Read the absorbance at 405 nm. If the absorbance is significantly greater than the absorbance of Endotoxin-Free water, then the intrinsic color will alter the correct sample absorbance readings. In such cases, include appropriate controls in the assay.

Interfering substances

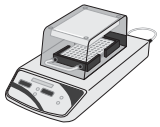
- The presence of interfering substances in test samples can cause product inhibition leading to false negatives. If unsure if your sample contains interfering substances, it is recommended to determine the potential product inhibition for each sample type undiluted or at an appropriate dilution (e.g., serum).
- To verify potential product inhibition, add a known amount of endotoxin to an aliquot or dilution of your test sample (e.g., 0.5 EU/mL). Assay the spiked sample and an unspiked sample to determine the respective endotoxin concentrations. The difference between the two calculated endotoxin values should equal the known concentration of the spike ±25%. See example below.
- Samples showing inhibition on the amebocyte lysate reaction may require further dilution to overcome the inhibitory effects. Once the non-inhibitory dilution is determined, the exact dilution can be found by testing two-fold dilutions near that dilution. The degree of inhibition or enhancement is dependent on the product concentration.

Table 5 Example with sample containing 20% glycerol.

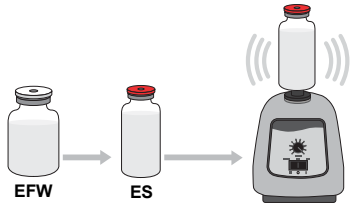
Sample Dilution	Observed Spiked[1] Sample Concentration (EU/mL)	Observed Unspiked Sample Concentration (EU/mL)	Δ	
Undiluted	0.103	0.099	0.004	Inhibitory
1:20	0.649	0.102	0.547	Non-inhibitory

[1] Spiked concentration should show a value of 0.50 EU/mL. The value of 0.103 is indicative of inhibition for sample containing 20% glycerol.

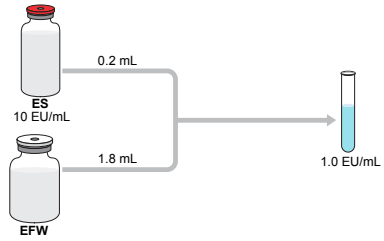
Appendix 3



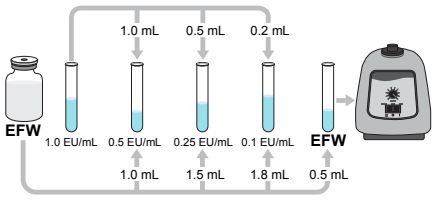
1. Pre-equilibrate the plate to 37±1°C for 10 minutes.



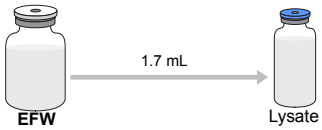
2. Reconstitute Endotoxin Standard (ES) to 10 EU/mL with Endotoxin-Free Water (EFW).
3. Mix vigorously for 15 minutes.



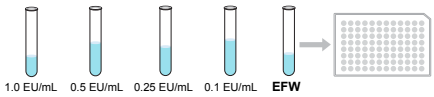
4. Prepare 1.0 EU/mL Stock from 10 EU/mL Endotoxin Standard Solution.
5. Vortex for 2 minutes.



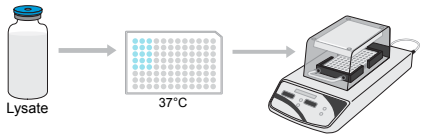
6. Using 1.0 EU/mL Stock, prepare a series of endotoxin dilutions.
7. Vortex for 1 minute.
8. Prepare samples according to information in the user guide.



9. Reconstitute the Amebocyte Lysate with 1.7 mL EFW.
10. Gently swirl and avoid foaming. Lysate reagent must be used within 5 minutes.

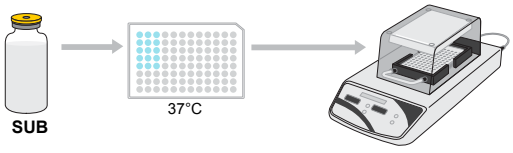


11. Keeping the plate at 37±1°C, add 50 µL of each endotoxin dilution, sample, and blank to the plate wells (triplicates).

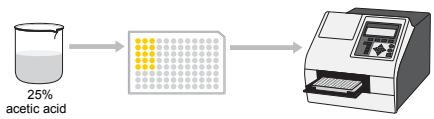


12. Keeping the plate at 37±1°C, add 50 µL of Amebocyte Lysate Reagent to the wells following the same order of addition as the endotoxin dilution and samples.
13. Mix well by gently tapping on the side of the plate.
14. Cover and incubate for time T1 (High or Low) as indicated on the Lysate label.

15. Reconstitute Chromogenic Substrate (SUB) with 3.4 mL of EFW.
16. Pre-warm the solution for 5 minutes at 37±1°C.



17. Keeping the plate at 37±1°C, add 100 µL of Chromogenic Substrate Solution to each well following the same order of addition as previous steps.
18. Mix well by gently tapping on the side of the plate.
19. Incubate for 6 minutes at 37±1°C.



20. Add 50 µL of 25% acetic acid to each well to stop the reaction.
21. Mix well by gently tapping on the side of the plate.
22. Read the absorbance at 405 nm.

Appendix 3

Related products

Product	Cat. no.
Pierce™ High Capacity Endotoxin Removal Resin, 10 mL	88270
Pierce™ High Capacity Endotoxin Removal Resin, 100 mL	88271
Pierce™ High Capacity Endotoxin Removal Resin, 250 mL	88272
Pierce™ High Capacity Endotoxin Removal Spin Columns, 0.25 mL	88273
Pierce™ High Capacity Endotoxin Removal Spin Columns, 0.50 mL	88274
Pierce™ High Capacity Endotoxin Removal Spin Columns, 1 mL	88276
Detoxi-Gel™ Endotoxin Removing Gel	20339
Detoxi-Gel™ Endotoxin Removing Columns	20344
Pierce™ Rapid Gold BCA Protein Assay Kit	A53225

[1]Roslansky, P.F. and Novitsky, T.J. (1991). Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. *J Clin Microbiol* **54** (5). Jcm.asm.org/content/29/11/2477.short

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Manufacturer: Thermo Fisher Scientific | 3747 N. Meridian Road | Rockford, Illinois 61101 USA

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Appendix 4

SDS-PAGE Protocol

SDS-PAGE Solutions

40% Acrylamide (37.5:1)

Acrylamide	116.8 g
N,N'-Methylene bisacrylamide	3.2 g
DDI H_2O	to 300 ml

Filter and store in a dark bottle at 4°C. (We buy this premade)

30% Ammonium Persulfate

Ammonium Persulfate	1.5 g
DDI H_2O	5 ml

Store at 4°C. Replace every month.

RG Buffer--1.5 M Tris•Cl, pH 8.8

DDI H_2O	300 ml
Tris–free base	90.75 g
Conc. HCl	8 ml

Adjust to pH 8.8 with conc. HCl, and bring final volume to 500 ml with DDI H_2O.

SG Buffer--1.0 M Tris•Cl, pH 6.8

DDI H_2O	300 ml
Tris–free base	60.54 g
Conc. HCl	36 ml

Adjust to pH 6.8 with conc. HCl, and bring final volume to 500 ml with DDI H_2O.

4x SDS-PAGE Sample Buffer

125 mM Tris•HCl, pH 6.8	1 M	5 ml
20% Glycerol		8 ml
4% SDS	20%	8 ml
10% ß-Mercaptoethanol		4 ml
0.5 mg/ml Bromophenol Blue		20 mg
DDI H_2O		15 ml
Total		40 ml

10x SDS-PAGE Running Buffer

30.3 g	Tris base
144.0 g	Glycine
10.0 g	SDS

Dissolve and bring total volume to 1,000 ml with deionized water. Do not adjust pH with acid or base (pH is normally 8.3 as prepared).

Coomassie Stain Solution

Ethanol	150 ml
Glacial Acetic Acid	50 ml
DDI H_2O	300 ml
Coomasie Brilliant Blue–R-250	1 g

Dissolve Coomasie Brilliant Blue–R-250 in EtOH first.

Destain Solution

Ethanol	1200 ml
Glacial Acetic Acid	400 ml
DDI H_2O	2.4 l

Appendix 4

SDS-PAGE Protocol

Casting the Gel

1] Assemble glass plates and spacers in gel casting apparatus–see BioRad instruction manual.

2] Mix the components for the resolving gel as described in the Mini-Protean II protocol.

3] Pour the resolving gel mixture into the gel plates to a level 2 cm below the top of the shorter plate.

4] Pace a layer of DDI H_2O over the top of the resolving gel to prevent meniscus formation in the resolving gel.

5] Allow resolving gel to stand 30 min at room temperature.

6] Drain the DDI H_2O from top of the resolving gel. Rinse with DDI H_2O, drain, and wick any remaining DDI H_2O away with a Kimwipe.

7] Mix components for stacking gel.

8] Pour stacking gel solution into gel plates (on top of running gel), so that gel plates are filled. Insert comb to the top of the spacers.

9] Allow gel to stand for at least 1 hr at room temperature, or overnigt at 4°C (wrapped in saran wrap).

Preparing Samples

Note: 10 well combs will hold up to 30 μl of prepared sample. 15 well combs will hold up to 20 μl of prepared sample.

Cell Samples

1] Harvest 100 μl of cells at O.D. > 0.6. Decant the supernatant media.

2] Resuspend cells in 20 μl of 2x sample buffer.

3] Incubate tubes in boiling water for 5 min.

4] Centrifuge at 12,000 x g for 30 s.

Solution Samples

1] Place a volume of protein solution (or 1 μl of standard) into a μfuge tube, such that there

is 5-10 μg of protein in the solution.

2] And an equal volume of 2x sample buffer (or 10 μl for standards).

3] Incubate tubes in boiling water for 5 min.

4] Centrifuge at 12,000 x g for 30 s.

Running the Gel

1] Remove comb and assemble cast gel into Mini-Protean II apparatus.

2] Add freshly prepared 1x running buffer (300 ml) to both chambers of the apparatus.

3] Load the prepared samples into the wells of the gel.

4] Run the gel at 100 V until the dye front migrates into the running gel (~15 min), and increase to 200 V until the dye front reaches the bottom of the gel (~45 min).

Staining & Destaining the Gel

1] Remove the run gel from the aparatus and remove the spacers and glass plates. Place the gel into a small tray. *Note:* Never us a metal spatula to separate the glass plates.

2] Add ~20 ml staining solution and stain for > 30 min with gentle shaking.

3] Pour off and save the stain.

4] Add ~5 ml destain solution and destain for ~1 min with gentle shaking.

5] Pour off and discard the destain solution. Add ~ 30 ml of destain solution.

6] Destain with gentle shaking until the gel is visibly destained (> 2 hr).

5] Pour off and discard the destain solution.

6] Rinse with DDI H_2O. Add ~30 ml DDI H_2O and rinse for 5 min with gentle shaking.

7] Dry the gel on the gel dryer at 60°C for 1 hr with a sheet of Whatman filter paper below the gel and a piece of Seran wrap over the gel.

Appendix 4

% Acrylamide in running gel	Separation size range (kDa)
Single percentage:	
5%	100–250
7.5%	40–200
10%	30–150
12%	20–120
15%	10–100
18%	6–50
Gradient:	
4–15%	20–250
4–20%	10–200
10–20%	10–100
8–16%	6–70

From the BioRad Readygel Manual.

Adapted from Laemmli, U.K. (1970) Nature 227, 680-685. (see page 681).

Appendix 4

SDS-PAGE Gels

Resolving Gel	**8 %**		**10%**	
	1 gel	2 gels	1 gel	2 gels
DDI H_2O	1.8 ml	3.6 ml	1.6 ml	3.2 ml
1.5 M Tris-HCl, pH 8.8 (RG Bfr.)	1.3 ml	2.6 ml	1.3 ml	2.6 ml
40% Acrylamide Stock	800 μl	1.6 ml	1.0 ml	2.0 ml
20 % SDS	100 μl	200 μl	100 μl	200 μl
30% Ammonium Persulfate	10 μl	20 μl	10 μl	20 μl
TEMED	4 μl	8 μl	4 μl	8 μl

	12 %		**15%**	
	1 gel	2 gels	1 gel	2 gels
DDI H_2O	1.4 ml	2.8 ml	1.1 μl	2.2 ml
1.5 M Tris-HCl, pH 8.8 (RG Bft.)	1.3 ml	2.6 ml	1.3 ml	2.6 ml
40% Acrylamide Stock	1.2 ml	2.4 ml	1.5 ml	3.0 ml
20 % SDS	100 μl	200 μl	100 μl	200 μl
10% Ammonium Persulfate	10 μl	20 μl	10 μl	20 μl
TEMED	4 μl	8 μl	4 μl	8 μl

Stacking Gel (4 %)

	2 gels
DDI H_2O	3.9 ml
1.0 M Tris-HCl, pH 6.8 (SG Bfr.)	500 μl
40% Acrylamide Stock	500 μl
20 % SDS	100 μl
30% Ammonium Persulfate	16 μl
TEMED	8 μl

Note: Always add Ammonium Persulfate and TEMED immediately before casting gel.

Number of gels refers to 1 mm thick gels. Use the 2 gel recipe for 1.5 mm thick gels.

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Version: 2 Revision Date: 07/26/2016

APC anti-rat CD3 Antibody

Catalog# / Size	201413 / 25 µg 201414 / 100 µg
Clone	1F4
Regulatory Status	RUO
Other Names	T cell antigen receptor complex, T3
Isotype	Mouse IgM, κ
Description	CD3 is a complex composed of δ, γ, ε, and ζ chains. They are 20-25 kD members of the immunoglobulin superfamily and associated with the T cell receptor (TCR). CD3 is expressed on thymocytes, peripheral T cells, some NK-T cells, and dendritic epidermal T cells. CD3 is involved in antigen recognition, signal transduction, and T cell activation.

Product Details

Reactivity	Rat
Antibody Type	Monoclonal
Host Species	Mouse
Immunogen	F344 rat spleen cells stimulated with PMA and calcium ionophore
Formulation	Phosphate-buffered solution, pH 7.2, containing 0.09% sodium azide.
Preparation	The antibody was purified by affinity chromatography and conjugated with APC under optimal conditions.
Storage & Handling	The antibody solution should be stored undiluted between 2°C and 8°C, and protected from prolonged exposure to light. **Do not freeze.**
Application	FC - Quality tested
Recommended Usage	Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. For flow cytometric staining, the suggested use of this reagent is ≤1.0 µg per million cells in 100 µl volume. It is recommended that the reagent be titrated for optimal performance for each application.
Excitation Laser	Red Laser (633 nm)
Application Notes	Immobilized 1F4 antibody can induce T cell proliferation *in vitro*. Additional reported applications (for relevant formats of this clone) include: immunohistochemistry of acetone-fixed frozen sections[1] and formaldehyde- fixed paraffin embedded sections[4,5] immunofluorescence microscopy[3], *in vivo* activation of T cell responses[1], and *in vivo* inhibition of T cell responses[2].

Application References

(PubMed link indicates BioLegend citation)

1. Tanaka T, et al. 1989. J. Immunol. 142:2791. (Activ, IHC, IP)
2. Nicholls MR, et al. 1993. Transplantation 55:459. (Block)
3. Elbe A, et al. 1993. J. Invest. Dermatol. 102:74. (IF)
4. Baba T, et al. 2006. Blood 107:2004. (IHC)
5. Fujishiro J, et al. 2010. Am. J. Transplant. 10:1545-55. (IHC-P)
6. Li X, et al. 2009. J. Immunol. 183:3955. (FC) PubMed

Product Citations

1. Zimmerman KA, et al. 2019. J Am Soc Nephrol. 30:767. PubMed

RRID

AB_2563365 (BioLegend Cat. No. 201413)
AB_2563366 (BioLegend Cat. No. 201414)

Appendix 5

Antigen Details

Structure	Ig superfamily, approximately 20-25 kD
Distribution	Thymocytes, peripheral T cells, dendritic epidermal T cells, NK-T cells
Function	Antigen recognition, TCR signal transduction, T cell activation
Ligand/Receptor	Peptide antigen/MHC complex
Cell Type	NKT cells, T cells, Thymocytes
Biology Area	Immunology
Molecular Family	CD Molecules
Antigen References	1. Tanaka T, et al. 1989 J. Immunol. 142:2791. 2. Elbe A, et al. 1993. J. Invest. Dermatol. 102:74.
Gene ID	25710 300678 315609 25300

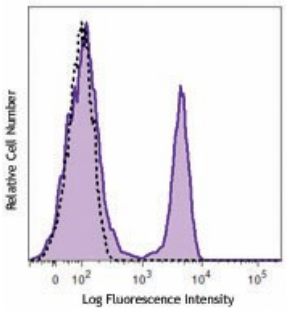
Related Protocols

Cell Surface Flow Cytometry Staining Protocol

Other Formats

Purified anti-rat CD3, FITC anti-rat CD3, PE anti-rat CD3, Alexa Fluor® 488 anti-rat CD3, Alexa Fluor® 647 anti-rat CD3, Ultra-LEAF™ Purified anti-rat CD3, PerCP/Cyanine5.5 anti-rat CD3

Product Data



Lewis rat splenocytes were stained with CD3 (clone 1F4) APC (filled histogram) or mouse IgM, κ APC isotype control (open histogram).

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Version: 1 Revision Date: 11/30/2012

APC/Cyanine7 anti-rat CD4 Antibody

Catalog# / Size	201517 / 25 µg 201518 / 100 µg
Clone	W3/25
Regulatory Status	RUO
Other Names	T4, L3T4, W3/25
Isotype	Mouse IgG1, κ
Description	CD4 is a 55 kD glycoprotein also known as T4. Rat CD4 is a member of the immunoglobulin superfamily and is expressed on majority of thymocytes, macrophages, and a peripheral T cell subset (T helper cells). CD4 is a T cell co-receptor that interacts with the MHC class II molecule and is involved in T cell activation.

Product Details

Reactivity	Rat
Antibody Type	Monoclonal
Host Species	Mouse
Immunogen	Rat thymocyte membrane glycoproteins
Formulation	Phosphate-buffered solution, pH 7.2, containing 0.09% sodium azide.
Preparation	The antibody was purified by affinity chromatography, and conjugated with APC/Cyanine7 under optimal conditions.
Concentration	0.2 mg/ml
Storage & Handling	The antibody solution should be stored undiluted between 2°C and 8°C, and protected from prolonged exposure to light. **Do not freeze.**
Application	FC - Quality tested
Recommended Usage	Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. For flow cytometric staining, the suggested use of this reagent is ≤0.5 µg per million cells in 100 µl volume. It is recommended that the reagent be titrated for optimal performance for each application.
Excitation Laser	Red Laser (633 nm)
Application Notes	The W3/25 antibody has been shown to inhibit IL-2 production by T helper cells and to prevent autoimmune T cell transfer in an MBP induced EAE model *in vivo*. Additional reported applications (for the relevant formats) include: immunohistochemistry of acetone-fixed frozen sections[1,2], inhibition of IL-2 production[3], inhibition of MBP-induced T cell activation in EAE transfer model[3].
Additional Product Notes	BioLegend is in the process of converting the name APC/Cy7 to APC/Cyanine7. The dye molecule remains the same, so you should expect the same quality and performance from our APC/Cyanine7 products. Please contact Technical Service if you have any questions.

Application References

(PubMed link indicates BioLegend citation)

1. Whiteland JL, et al. 1995. J. Histochem. Cytochem. 43:313. (IHC)
2. Shioji K, et al. 2001. Circulation Res. 89:540. (IHC)
3. Mannie MD, et al. 1993. J. Immunol. 151:7293.
4. Kurtz CC, et al. 2007. Dev. Comp. Immunol. 31:415. PubMed

Product Citations

1. Ruppert KA, et al. 2018. Sci Rep. 8:480. PubMed
2. Pai AV, et al. 2018. Physiology (Bethesda). 33:254. PubMed
3. Alsheikh AJ, et al. 2019. Am J Physiol Regul Integr Comp Physiol. 317:R182. PubMed

4. Li B, et al. 2020. Cell Mol Immunol. 1.386111111. PubMed
5. Almolda B, et al. 2011. PLoS One. 6:e27473. PubMed
6. Xie L, et al. 2014. J Immunol. 192:6009. PubMed
7. Fehrenbach DJ, et al. 2020. Exp Physiol. 105:864. PubMed

RRID AB_1186088 (BioLegend Cat. No. 201517)
AB_1186084 (BioLegend Cat. No. 201518)

Antigen Details

Structure	Ig superfamily, 55 kD
Distribution	Majority of thymocytes, macrophages, and a peripheral T cell subset (T helper cells)
Function	T cell co-receptor, T cell activation
Ligand/Receptor	MHC class II molecule
Cell Type	Macrophages, T cells, Thymocytes
Biology Area	Immunology
Molecular Family	CD Molecules
Antigen References	1. Brideau RJ, et al. 1980. Eur. J. Immunol. 10:609. 2. Clark SJ, et al. 187. P. Natl. Acad. Sci. USA 84:1649.
Gene ID	24938 24939

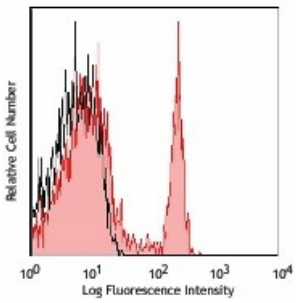
Related Protocols

Cell Surface Flow Cytometry Staining Protocol

Other Formats

Purified anti-rat CD4, FITC anti-rat CD4, PE anti-rat CD4, APC anti-rat CD4, Alexa Fluor® 488 anti-rat CD4, PE/Cyanine7 anti-rat CD4, PerCP/Cyanine5.5 anti-rat CD4

Product Data



LOU rat splenocytes stained with W3/25 APC/Cyanine7

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Version: 1 Revision Date: 05/19/2020

PE/Cyanine7 anti-rat CD161 Antibody

Catalog# / Size	205609 / 25 µg 205610 / 100 µg
Clone	3.2.3
Regulatory Status	RUO
Other Names	NKR-P1, CD161a/CD161b, NKR-P1a/KLRB1a
Isotype	Mouse IgG1, κ
Description	CD161 is a 30 kD type II transmembrane C-type lectin, expressed as a homodimer. Rat NKR-P1 receptors are primarily expressed on NK cells, a subset of T cells, dendritic cells, and activated monocytes. There are three different types of NKR-P in rat, namely NKR-P1a, NKR-P1b, and NKR-P1c. NKR-P1a does not contain an ITIM structure and is an activating receptor, while NKR-P1b contains an ITIM and displays inhibitory function. LLT-1 (ligand lectin like transcript 1) is the ligand, while KLR (killer cell lectin like) functions as a receptor.

Product Details

Reactivity	Rat
Antibody Type	Monoclonal
Host Species	Mouse
Immunogen	LEW rat splenic NK cells
Formulation	Phosphate-buffered solution, pH 7.2, containing 0.09% sodium azide.
Preparation	The antibody was purified by affinity chromatography and conjugated with PE/Cyanine7 under optimal conditions.
Concentration	0.2 mg/mL
Storage & Handling	The antibody solution should be stored undiluted between 2°C and 8°C, and protected from prolonged exposure to light. **Do not freeze**.
Application	FC - *Quality tested*
Recommended Usage	Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. For flow cytometric staining, the suggested use of this reagent is ≤ 0.06 µg per million cells in 100 µL volume. It is recommended that the reagent be titrated for optimal performance for each application.
Excitation Laser	Blue Laser (488 nm) Green Laser (532 nm)/Yellow-Green Laser (561 nm)
Application Notes	3.2.3 recognizes a common epitope of NKR-1P1a (CD161a) and NKR-P1b (CD161b).
Application References **(PubMed link indicates BioLegend citation)**	1. Chambers W, et al. 1989. J. Exp. Med. 169:1373. (FC)
RRID	AB_2860756 (BioLegend Cat. No. 205609) AB_2860757 (BioLegend Cat. No. 205610)

Antigen Details

Structure	Type II transmembrane C-type lectin, 30 kD.

Appendix 5

Distribution	NK cells, subset of T cells, dendritic cells, activated monocytes.
Function	NKR-P1a is a NK cell-activating receptor; NKR-P1b is a NK cell-inhibitory receptor.
Ligand/Receptor	LLT1-ligand lectin like transcript 1; KLR-killer cell lectin like receptor.
Cell Type	Dendritic cells, Monocytes, NK cells, T cells
Biology Area	Cell Biology, Immunology, Innate Immunity, Signal Transduction
Molecular Family	CD Molecules
Antigen References	1. Chambers WH, et al. 1989. J. Exp. Med. 169:1373. 2. Brink M, et al. 1990. J. Exp. Med. 171:197. 3. Li J, et al. 2003. Int. Immunol. 15:411.
Gene ID	362443

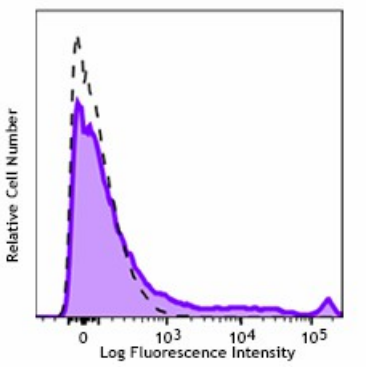
Related Protocols

Cell Surface Flow Cytometry Staining Protocol

Other Formats

PE anti-rat CD161, APC anti-rat CD161, FITC anti-rat CD161, PE/Cyanine5 anti-rat CD161

Product Data



Rat splenocytes were stained with CD161 (clone 3.2.3) PE/Cyanine7 (filled histogram) or mouse IgG1, κ PE/Cyanine7 isotype control (open histogram).

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Version: 1 Revision Date: 11/30/2012

FITC anti-rat IgG1 Antibody

Catalog# / Size	407405 / 50 µg 407406 / 500 µg
Clone	MRG1-58
Regulatory Status	RUO
Other Names	Imunoglobulin G1
Isotype	Mouse IgG
Description	The MRG1-58 monoclonal antibody reacts with rat immunoglobulin G1 (IgG1) in all tested rat strains (Lou, Lou/Ws1/M, Lewis, Wistar, DA, Sprague-Dawley). It does not react with other isotypes. The MRG1-58 monoclonal antibody may be used as primary or secondary reagent for ELISA or immunofluorescent analysis.

Product Details

Reactivity	Rat
Antibody Type	Monoclonal
Host Species	Mouse
Immunogen	Mixed rat Igs
Formulation	Phosphate-buffered solution, pH 7.2, containing 0.09% sodium azide.
Preparation	The antibody was purified by affinity chromatography, and conjugated with FITC under optimal conditions.
Concentration	0.5 mg/ml
Storage & Handling	The antibody solution should be stored undiluted between 2°C and 8°C, and protected from prolonged exposure to light. **Do not freeze.**
Application	FC - Quality tested
Recommended Usage	Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. For flow cytometric staining, the suggested use of this reagent is ≤0.25 µg per million cells in 100 µl volume. It is recommended that the reagent be titrated for optimal performance for each application.
Excitation Laser	Blue Laser (488 nm)
Product Citations	1. Li Q, et al. 2019. Neuron. 101:207. PubMed
RRID	AB_492921 (BioLegend Cat. No. 407405) AB_492922 (BioLegend Cat. No. 407406)

Related Protocols

Cell Surface Flow Cytometry Staining Protocol

Other Formats

Purified anti-rat IgG1, Biotin anti-rat IgG1, PE anti-rat IgG1, Alexa Fluor® 594 anti-rat IgG1

Appendix 5

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Version: 1 Revision Date: 11/30/2012

Purified anti-rat IgG2a Antibody

Catalog# / Size	407501 / 50 µg 407502 / 500 µg
Clone	MRG2a-83
Regulatory Status	RUO
Other Names	Immunoglobulin G2a
Isotype	Mouse IgG
Description	The MRG2a-83 monoclonal antibody reacts with rat immunoglobulin G2a (IgG2a) in all tested rat strains (Lou, Lou/Ws1/M, Lewis, Wistar, DA, Sprague-Dawley). It does not react with other isotypes. The MRG2a-83 monoclonal antibody may be used as primary or secondary reagent for ELISA or immunofluorescent analysis.

Product Details

Reactivity	Rat
Antibody Type	Monoclonal
Host Species	Mouse
Immunogen	Mixed rat Igs
Formulation	Phosphate-buffered solution, pH 7.2, containing 0.09% sodium azide.
Preparation	The antibody was purified by affinity chromatography.
Concentration	0.5 mg/ml
Storage & Handling	The antibody solution should be stored undiluted between 2°C and 8°C.
Application	ELISA, FC
Recommended Usage	Each lot of this antibody is quality control tested by ELISA. For ELISA capture, the suggested use of this reagent is ≤ 5.0 µg per ml. It's recommended that the reagent be titrated for optimal performance for each application.
Application References (PubMed link indicates BioLegend citation)	1. Inoue S, et al. 2006. Cancer Research 66:7741.
RRID	AB_345337 (BioLegend Cat. No. 407501) AB_345338 (BioLegend Cat. No. 407502)

Antigen Details

Gene ID	367586

Related Protocols

Sandwich ELISA Protocol

Appendix 5

Other Formats

Biotin anti-rat IgG2a, Alexa Fluor® 594 anti-rat IgG2a, PE anti-rat IgG2a, FITC anti-rat IgG2a, Alexa Fluor® 647 anti-rat IgG2a

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Version: 1 Revision Date: 06/29/2020

PerCP/Cyanine5.5 anti-rat IgM Antibody

Catalog# / Size	408915 / 25 µg 408916 / 100 µg
Clone	MRM-47
Regulatory Status	RUO
Other Names	Immunoglobulin M
Isotype	Mouse IgG
Description	The MRM-47 monoclonal antibody reacts with rat immunoglobulin M (IgM) in all tested rat strains (Lou, Lou/Ws1/M, Lewis, Wistar, DA, Sprague-Dawley). It does not react with other isotypes. The MRM-47 antibody can be used as a primary or secondary reagent for ELISA or immunofluorescent analysis.

Product Details

Reactivity	Rat
Antibody Type	Monoclonal
Host Species	Mouse
Immunogen	Mixed rat Igs
Formulation	Phosphate-buffered solution, pH 7.2, containing 0.09% sodium azide
Preparation	The antibody was purified by affinity chromatography and conjugated with PerCP/Cyanine5.5 under optimal conditions.
Concentration	0.2 mg/mL
Storage & Handling	The antibody solution should be stored undiluted between 2°C and 8°C, and protected from prolonged exposure to light. **Do not freeze**.
Application	FC - *Quality tested*
Recommended Usage	Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. For flow cytometric staining, the suggested use of this reagent is ≤ 0.5 µg per million cells in 100 µL volume. It is recommended that the reagent be titrated for optimal performance for each application. * PerCP/Cyanine5.5 has a maximum absorption of 482 nm and a maximum emission of 690 nm.
Excitation Laser	Blue Laser (488 nm)
RRID	AB_2876734 (BioLegend Cat. No. 408915) AB_2876734 (BioLegend Cat. No. 408916)

Antigen Details

Gene ID	299357

Related Protocols

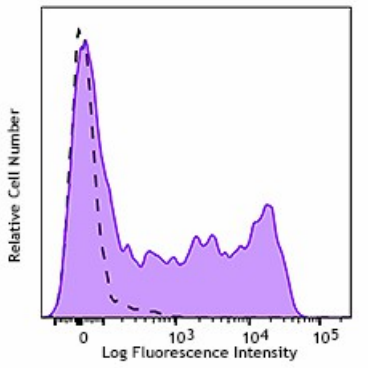
Appendix 5

Cell Surface Flow Cytometry Staining Protocol

Other Formats

Purified anti-rat IgM, Biotin anti-rat IgM, Alexa Fluor® 594 anti-rat IgM, FITC anti-rat IgM, Alexa Fluor® 647 anti-rat IgM, Alexa Fluor® 488 anti-rat IgM, PE/Cyanine7 anti-rat IgM

Product Data



Rat splenocytes were stained with rat IgM (clone MRM-47) PerCP/Cyanine5.5 (filled histogram), or mouse IgG isotype control (open histogram).

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Cell Surface Flow Cytometry Staining of Whole Blood

Protocol Steps

1. Add predetermined optimum concentrations of desired fluorochrome conjugated, biotinylated, or purified primary antibodies to 100µl of anti-coagulated whole blood.
2. Incubate at room temperature for 15-20 minutes in the dark.
3. Dilute 10X Red Blood Cell (RBC) Lysis Buffer (BioLegend Cat. No. 420301) to 1X working concentration with DI water. Warm to room temperature prior to use. Add 2ml of 1X RBC lysis solution to whole blood/antibody mixture. Incubate at room temperature for 10 minutes.
4. Centrifuge at 350xg for 5 minutes, discard the supernatant.
5. Wash 1X with at least 2ml of Cell Staining Buffer by centrifugation at 350xg for 5 minutes.

   If using a purified primary antibody, resuspend pellet in residual buffer and add a previously determined optimum concentration of anti-species immunoglobulin fluorochrome conjugated secondary antibody (e.g. FITC anti-mouse Ig) and incubate in the dark for 15-20 minutes.

   If using a biotinylated primary antibody, resuspend cell pellet in residual buffer and add a previously determined optimum concentration of fluorochrome conjugated Streptavidin (SAv) reagent (e.g. SAv-PE, BioLegend Cat. No. 405204) and incubate for 15-20 minutes in the dark.
6. Repeat step 5.
7. Resuspend cells in 0.5ml Cell Staining Buffer or 0.5ml 2% paraformaldehyde-PBS fixation buffer.

Tip: For gentler fixation (particularly with tandem fluors), FluoroFix™ Buffer (Cat. No. 422101) may be used.

8. Perform fluorescence activated cell sorting (FACS), or flow cytometric analysis.

Appendix 6

Note: If you are unable to immediately read your samples on a cytometer, keep them shielded from light and in a refrigerator set at 4-8°C. The samples should be resuspended in Cell Staining Buffer. Note that samples should not remain in a fixation buffer for extended periods of time as this can affect fluor conformation and fluorescence.

Appendix 7

Tissue Processing

Fixation was done as the first step to preserve the tissue substrate and render protein structure and other tissue components insoluble in all reagents exposed later in either the processing or staining process. This step must be done as soon as possible as most cells contain lysosomes which will carry out cell autolysis and release digestive enzymes to break down cell components after the cells have died (Bancroft & Gamble, 2002). Besides that, extracellular microorganisms, mostly bacteria, will take opportunities and break down the dead cell through putrefaction for nutrient absorption (Bancroft & Gamble, 2002). By fixation, autolysis and putrefaction can be halted and tissue substrates can be preserved. In fixation, ideal fixatives are listed below (Bancroft & Gamble 2002, pp 25-26)

- Do not shrink, swell or harden tissue substrates
- Do not dissolve tissue components
- Kill microorganisms
- Retain the original form of tissue constituents throughout subsequent processing of tissue
- Compatible with subsequent staining method
- Adequate penetration rate

In the experiment, 10% neutral buffered formalin, which is the chemical fixative, was used due to:

- High penetration rate
- Inexpensive
- Reaction slow and reversible for first 24 hour
- Preserve tissue for a quite long period if buffered
- Non coagulative gel: better preservation for cellular organelle

Formalin is inflammable and a strong mucous membrane irritant (Bancroft & Gamble 2002), thus it must be handled carefully.

After fixation, tissues were insufficiently firm and cohesive to allow thin sections to be cut (Gormley, 2012. This is why processing must be carried out. However,

before the processing, about 3-5mm thick of the tissue was obtained and put into tissue dek cassettes (Gormley, 2012). The cassettes were then labelled for their laboratory accession number with chemical resistant lead pencils (Gormley, 2012). Dehydration was then carried out to remove all water in the tissue substrate so that water droplets will not trap and affect the specimen from being processed (Gormley, 2012). Ethanol was used in the dehydration process as it has the least side effects on tissue substrates compared to other dehydration agents. Water molecules were removed in ascending grades of alcohols to absolute alcohol.

Wax is insoluble in ethanol. Clearing agent that was miscible in both ethanol and wax is required. As xylene has least effect on tissue substrate (Bancroft & Steven, 1990), it was used in the experiment. However, xylene can harden the tissue, thus the immersion time cannot be prolonged (Bancroft & Steven 1990).

After the clearing process, tissues were then infiltrated with wax. In this process, paraffin wax which had a melting point of about 540C to 580C was used (Gormley, 2012). Additives such as bee's wax or synthetic resin were added to increase the hardness and stickiness of wax in order to give better cutting properties on tissue by altering the size of wax crystals (Bancroft & Gamble, 2002). Temperature of the wax was kept 2 to 30C above the melting point of wax (~600C) so that the wax will remain as liquid form throughout tissue infiltration process (Bancroft & Gamble, 2002). However, it is important to make sure that the wax did not overheat as it will destroy plastic polymers (Bancroft & Steven, 1990) and cause the cutting process to become more difficult. In the overall view, the tissue substrates were immersed in the chemical as shown:

1) 70% ethanol for 1 hour
2) 95% ethanol for 1 hour
3) Absolute ethanol for 1 hour
4) Step 4 repeated for other 4 times
5) Xylene for 1 hour
6) Paraffin wax for 2 hour

7) The whole process (dehydration, clearing and infiltration of wax) was done in Shandon Duplex Automatic Tissue Processor.

Embedding

Before the cutting, processed tissue needs to be embedded in wax as a 'block' form to enable the section to be cut. Tissue cassettes moulds method was applied as it did not require further trimming of wax around the tissue. Different tissues have different embedding section. For instance, appendix was embedded with the cross section across lumen (Bancroft & Steven, 1990). Skin was embedded with the plane section across all tissue layers with the epithelial on top which was cut last to minimize pressure distortion of the epidermis (Bancroft & Steven, 1990). The embedding process (Gormley, 2012) was done as shown:

1) Little wax was poured into the tissue molds and allowed to form at the bottom layer of the molds.

2) Tissues were then picked with warm forceps and put onto the centre of the molds.

3) Plastic tissue cassettes were placed over the mold. More wax was added into the molds if necessary.

4) The molds were then moved to cold plate for rapid cooling and give fine crystalline structure to wax for better cutting properties.

Cutting

In this experiment, rotary microtome was used for cutting sections. This is because rotary microtome is good in cutting semi-thin section for light microscopy (Bancroft & Gamble, 2002). Besides that, it can be motorized to facilitate the cutting of plastic embedded tissue. The full cutting method is shown in appendix 1, while the faults and remedies are shown in Appendix 2. There are several precautions (Bancroft & Gamble, 2002) that needed to be considered during cutting section, such as:

 Microtome knife MUST be set at clearance angle about 50 to prevent compression and chatter in the section

 Water bath must be set at temperature about 450C to 500C. Small amount of alcohol or detergent should be added into water bath to reduce surface tension and allow the section to flatten out easily.

 Floating of tissue section should be done more carefully to prevent water bubbles from being trapped under section. Fold in section can be removed by simply teasing with forceps. Section
should be allowed to float for about 30s as prolonged floating will cause excessive expansion and distorting of tissue.

 Debris and tissue fragment MUST be cleaned after each block was cut to avoid any overlap of other debris and fragment on tissue section. This can be done by dragging tissue paper across water surface.

Staining

The constituent part of cell and intercellular material are usually transparent after fixation and processing (Gormley, 2012). Very little details can be viewed microscopically (Gormley, 2012). Thus it needs to be stained with coloured agents, such as dyes. Haemotoxylin and Eosin (H&E) stain, which is general stain, was used on ileum. Special stains were also used in the experiment to highlight either mature collagen or mucous substrates. For example, Periodic acid Schiff (PAS) was used in the ileum to highlight the goblet; Van Gieson stain was used to stain skin; Masson's Trichrome stain was used to stain the appendix. The staining processes for each stain were shown as below:

 Haemotoxylin and eosin (H&E) stain

Photometric Sample Table

Appendix 8

Print Date : 04/04/2021 04:30:48 PM

[Summary]

File Information
Filename: C:\UVVis-Data\Data\m.j protein.vphd
Parameter File Name: C:\UVVis-Data\Parameter \m.j.vphm
Analyst:
Date/Time: 04/04/2021 03:29:54 PM
Comments:
Report File Name:

Instrument Information
Instrument Name: UV-1900
Instrument Type: UV-1900
Model (S/N): 1900I (A12535800440)

Software Information
Software Name: LabSolutions UV-Vis
Version: 1.10

Instrument Information
Instrument Name: UV-1900
Instrument Type: UV-1900
Model (S/N): 1900I (A12535800440)

[Measurement Parameters]

[Wavelengths]
Type of Measuring Mode: Absorbance
rounded: OFF
Column Name: WL280.00
Measuring Method: Point (280.00nm)

[Formula]

[Unknown Sample]
Acquiring Method: Measurement
Repeat: OFF

[Instrument]
Slit Width: 1.0 nm
Light Source Switch Wavelength: 340.00 nm
S/R Switch: Standard

[Sample Table]

	Sample Name	Sample ID	Option	Type	Ex	WL280.00	Comments
1		23b		UNK		0.030	
2		23a		UNK		0.018	
3		3a		UNK		0.010	
4		3b		UNK		0.016	
5		23p		UNK		-0.001	
6		23p		UNK		-0.001	
7	P. aeroginosa flagellin	3 after ET removal		UNK		0.012	
8	P. aeroginosa flagellin	23 after ET removal		UNK		0.008	

الخلاصة

هدفت الدراسة الحالية إلى التحقيق من استراتيجيات التمنيع الفعالة باستخدام بروتين المستضد السوطي المنقى من بكتريا الزوائف الزنجارية من أجل توفير حماية كاملة ضد عدوى الجهاز التنفسي الحادة الناتجة عن الاصابة ببكتريا الزائفة الزنجارية شديدة المقاومة للمضادات الحياتية (XDR) في نموذج الجرذ.

بعد التأكد من تشخيص العزلات البكتيرية المشخصة باستخدام طرق التشخيص التقليدية فضلا عن استخدام جهاز VITEK 2 system، تم استخدام تقنية تفاعلات السلسلة المتبلمرة (PCR) للكشف جزيئيا عن نوع المستضد السوطي عن طريق تضخيم جين *fliC* .

أجريت عملية التنقية لبروتين المستضد السوطي نوع a ونوع b بشكل جزئي من عزلات بكتريا الزوائف الزنجارية رقم ٢٣ ورقم ٣ (على التوالي) باستخدام كبريتات الأمونيوم بتركيز ٧٠٪ بالاضافة الى أستخدام أنابيب خاصة فائقة الترشيح (بقطر ٣٠ كيلو دالتون) بينما تمت عملية ازالة السموم الداخلية (LPS) باستخدام اعمدة خاصة، و من ثم تم قياس تركيزها باستخدام تقنية LAL Chromogenic.

تم قياس تركيز المستضد السوطي نوع a ونوع b بواسطة مقياس الطيف الضوئي المرئي للأشعة فوق البنفسجية كما وتم التحري عن وجود المستضد السوطي المنقى باستخدام تقنية الترحيل الكهربائي العمودي (SDS-PAGE).

قسمت الحيوانات الى ستة مجاميع مختلفة وكالاتي: المجاميع الممنعة بالمستضد السوطي نوع a (مجموعة A1 بدون اصابة و مجموعة A2 المعرضة للإصابة بالعزلة MJ من بكتريا الزوائف الزنجارية)، و المجاميع الممنعة بالمستضد السوطي نوع b (مجموعة B1 بدون اصابة و مجموعة B2 المعرضة للإصابة بالعزلة MJ من بكتريا الزوائف الزنجارية)، و المجاميع غير الممنعة (مجموعة C1 بدون اصابة و مجموعة C2 المعرضة للإصابة بالعزلة MJ من بكتريا الزوائف الزنجارية).

وقد تم جمع عينات الدم من جميع الحيوانات عن طريق طعنة القلب بعد تخديرها لغرض إجراء فحوصات الدم والفحوصات المناعية الاخرى. حيث اجريت فحوصات الدم للكشف عن إجمالي الخلايا الليمفاوية بواسطة جهاز آلي لتعداد الدم الكامل (CBC)، كما وتم الكشف عن الخلايا المناعية البائية والخلايا التائية وكذلك تحديد أنواع الخلايا التائية المتمايزة الى الخلايا التائية المساعدة Th ومن ثم تمايزها الى Th1 و Th17 و Th2 وذلك باستخدام واسمات محددة بواسطة تقنية قياس التدفق الخلوي.

أظهرت النتائج ان تركيز المستضد السوطي نوع a و نوع b كانت ٣,٢ مجم/مل و ٤,٨ مجم/مل على التوالي، و ان مستوى السموم الداخلية قد انخفض بشكل ملحوظ الى أقل من ٢ EU/ml (ان مستوى السموم المسموح به عالميا للاستخدام في التجارب قبل السريرية اقل من ٢٠ EU/ml).

كما أظهرت النتائج ان الوزن الجزيئي للمستضد السوطي نوع a و نوع b بعد الترحيل الكهربائي العمودي كان ٣٩ كيلو دالتون و ٤٥ كيلو دالتون على التوالي.

أظهرت نتائج التعداد الالي للدم (CBC) وجود انخفاظ معنوي في الخلايا الليمفاوية من ٠,٦٥ في المجموعة C1 الى ٠,٥٩ في المجموعة C2 بينما لم يكن هذا الانخفاض معنويا عند المقارنة بين المجموعتين B1 و B2 (من ٠,٦٩ الى ٠,٦٧) ، وكذلك فأنه لم يلاحظ هناك فرق معنوي بين المجموعتين A1 و A2 (٠,٦٧ لكلى المجموعتين).

أظهرت الدراسات المناعية أن الكشف عن الخلايا الليمفاوية باستخدام واسمة CD3 كان متوافقًا مع نتائج تحليل تعداد كريات الدم الكامل (CBC) في تأثير المستضد السوطي نوع b و المستضد السوطي نوع a في منع البكتيريا من تقليل الخلايا الليمفاوية عند المقارنة بين المجاميع الممنعة بالمستضد السوطي نوع b أو المستضد السوطي نوع a والمجاميع غير الممنعة بعد الإصابة (قيمة الاحتمالية ٠,٠٣٨ و ٠,٠٣٩ على التوالي).

كما اظهر اختبار الكشف عن الخلايا التائية المساعدة (Th) ارتفاعا معنويا في الخلايا التائية المساعدة في المجاميع الممنعة بالمستضد السوطي نوع b بعد الإصابة أكثر مما هي عليه قبل الاصابة (القيمة الاحتمالية <٠,٠٥). في حين أظهرت المجاميع الممنعة بالمستضد السوطي نوع a و المجاميع غير الممنعة انخفاضًا في الخلايا التائية المساعدة (Th) بعد الإصابة.

ان قياس إجمالي الخلايا المناعية نوع Th17 و الخلايا Th17 المتفرعة من الخلايا المساعدة (Th) و الخلاياTh17 المتفرعة من CTL قد أظهر تأثيرًا جيدًا للمستضد السوطي نوع b في ارتفاع إجمالي الخلايا Th17 و الخلايا المساعدة نوع Th17 المتفرعة من الخلايا المساعدة (Th) في المجموعة الممنعة بعد الإصابة (B2) أكثر مما هي عليه قبل الإصابة (B1) (حيث كانت القيمة الاحصائية < ٠,٠٥). بينما أظهرت النتائج انخفاضًا في هذه الخلايا في المجموعة الممنعة بالمستضد السوطي نوع a و المجموعة غير الممنعة.

أظهر الكشف عن الخلايا المناعية المساعدة نوع Th1 ارتفاعًا في هذه الخلايا في المجاميع الممنعة بالمستضد السوطي نوع b (B1 وB2) أكثر من المجاميع الممنعة بالمستضد السوطي نوع a (A1 وA2) وأكثر من المجاميع غير الممنعة (C1 و C2). في حين اظهر الفحص عن الخلايا المناعية المساعدة نوع Th2 ارتفاعا في هذه الخلايا في المجاميع غير الممنعة (C1 و C2) في حين انخفاضها في المجاميع الممنعة (A1 و A2 و B1 و B2) قبل وبعد الإصابة.

كما أظهرت نتائج الدراسة تأثير المستضد السوطي نوع b في رفع مستوى الخلايا البائية (IgM+ B cells) في المجاميع الممنعة قبل وبعد الاصابة عند المقارنة مع المجاميع غير الممنعة في حين لم يكن هناك اي فرق معنوي عند المقارنة بين المجاميع الممنعة بالمستضد السوطي نوع a عند مقارنتها بالمجاميع غير الممنعة.

من جهة اخرى، أظهرت نتائج الفحص النسيجي لأعضاء الحيوانات المشرحة (القلب، الكلى، الكبد، الطحال، والرئة) أن جميع الأعضاء (باستثناء الرئة) كانت طبيعية في تركيبها النسيجي، بينما أظهر

الفحص النسيجي للرئة تجمعا خفيفًا للخلايا الالتهابية في داخل النسيج في المجاميع الممنعة على العكس من المجاميع غير الممنعة.

يمكننا أن نستنتج من الدراسة الحالية أن المستضد السوطي نوع b يعتبر لقاحًا مرشحًا جيدًا للقضاء على عدوى الجهاز التنفسي لبكتريا الزائفة الزنجارية شديدة المقاومة للمضادات الحياتية (XDR) عن طريق تحسين الاستجابة المناعية الخلوية وذلك من خلال رفع مستويات الخلايا المناعية المساعدة نوع Th17 والخلايا المناعية المساعدة نوع Th1 وتحويل الاستجابة المناعية ضد العدوى الرئوية نحو العلاج بدلاً من تطورها الى العدوى المزمنة. من جهة اخرى فأن المستضد السوطي نوع b يعمل ايضا على تنشيط الاستجابة المناعية الخلطية عن طريق زيادة الخلايا البائية (IgM+ B cells) المهمة في انتاج الاجسام المضادة، فضلا عن ان المستضد السوطي نوع b لم يظهر أي تغيرات نسيجية مرضية في الاعضاء الحيوية بل يعمل على زيادة مناعة الغشاء المخاطي عن طريق تجنيد الخلايا المناعية في أنسجة الرئة.

إن تحضير لقاح المستضد السوطي المنقى بواسطة طريقة تنقية محورة والذي يمكن استخدامه لحماية الجرذان من الالتهابات الرئوية التي تسببها بكتريا الزوائف الزنجارية يعد أول دراسة تجرى في العراق.

جمهورية العراق
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كليـــــــة الطـــــب

التأثير المناعي الدفاعي للمستضد السوطي نوع b كلقاح مرشح ضد الاصابات التنفسية المتسببة عن بكتريا الزوائف الزنجارية

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

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بإشراف
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