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Immunogenic activity of Liposome incorporated Lipopolysaccharide Antigen of *Salmonella enterica* serovar Typhimurium in Mice

A Thesis
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Acknowledgment

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الاهداء

إلى ملاكي الحارس ..... الى شمعتي التي أنارت دربي وأشرقت حياتي ....
الى الصدر الذي الجأ إليه من هموم الحياة
أمي الحبيبة

إلى من كان لي وطن ....إلى من حماني وآزرني ...إلى
من ضللني بحبه وحنانه
أبي الحبيب

إلى رفيق دربي وسندي في الحياة .....الذي أرى من خلال عينيه مستقبلي
المشرق ....وسعادة اسأل الله إن تدوم
زوجي الحبيب فراس

إلى إخوتي وعضدي ..... إلى رفقائي في مسيرتي ..... إلى من رافقوني في كل
خطواتي
خلدون - أسامه – شيماء

اهدي إليكم ثمرة جهدي

فرح

Summary

The study was conducted to assess the role of *Salmonella enterica* serovar Typhimurium as a causative pathogen in hospitalized diarrhoeal patients younger than five years old, extract and purify endotoxin (lipopolysaccharide; LPS) from isolated and identified *Salmonella enterica* serovar Typhimurium, determine the role of LPS-liposome conjugate as a potential vaccine against *Salmonella enterica* serovar Typhimurium in albino male mice, and finally evaluate humoral and cellular immune responses (total and absolute counts of leukocytes, phagocytosis, Arthus reaction; AR and delayed type hypersensitivity reaction; DTHR) of vaccinated mice, in addition to histopathological changes in liver and spleen.

Ninety five patient were admitted to the Central Pediatric Hospital and Al-Kadhiymiah Pediatric Hospital in Baghdad during the period 24/10/2010 - 30/11/2010, because of severe diarrhoea and fever. Bacterial evaluation of stool samples revealed the identification of two (2.1%) *Salmonella enterica* serovar Typhimurium isolates (S1 and S2). Antibiotic sensitivity test demonstrated that S1 isolate was more resistance than S2 isolate; therefore it was considered more virulent and subjected for further manipulations, which included extraction of LPS from the bacterial outer membrane. Chemical characterization of the extracted LPS revealed that the carbohydrate content was 2.34 mg/ml, while the protein concentration was very low (0.52 µg/ml). Partial purification extracted LPS by using gel-filtration chromatography (sephacryl 200 S) showed three peaks, and after determination of protein and carbohydrate concentrations for each peak, the second peak observed to have the highest carbohydrate content (25%) and the lowest contaminated protein (0.001%). The LPS of this peak was immunologically evaluated in mice at a concentration of 100 µg/ml, alone or in conjugation with a commercially available liposome (LIP).

The mice were distributed into eight groups (negative controls(NC), positive controls(PC), complete Freund's adjuvant (CFA), heat-killed bacteria;

(HKB), formalin-killed bacteria (FKB), (LPS), (LIP) and (LPS-LIP conjugate). Each mouse was injected intraperitoneally (IP) with 0.1 ml of the respective solution in day 1, and a further dose in day 8. These mice were considered as pre-challenged groups, and they were dissected for laboratory evaluations in day 15. Further similar groups were challenged with 0.1 ml live bacteria in day 15 (5 x 10^4 cell/ ml), and they were dissected for laboratory evaluations in day 29 (post-challenged groups). The following results were obtained:

1. The total leukocyte count in all groups (pre- and post-challenged) was almost approximated the normal leukocyte count range in mice, but mice vaccinated with HKB or FKB showed the highest count in pre-challenged animals, and such increase also contributed to increased counts of lymphocytes, neutrophils and monocytes. However, mice vaccinated with LPS showed a significant increased count of lymphocytes in post-challenged animals; an observation that suggests that LPS may be able to enhance the adaptive immunity against *Salmonella enterica* serovar Typhimurium.
2. The highest percentage of phagocytosis was observed in mice vaccinated with LPS+LIP in pre- and post-challenged groups. Such finding highlights the importance of conjugation between LPS and LIP in enhancing the function of phagocytic cells.
3. In most types of post-challenged vaccinated groups, AR and DTHR responses were increased, while in pre-challenged groups some variations were observed, but mice vaccinated with LPS+LIP conjugates were almost recorded the highest results.
4. Bacterial evaluation of liver and spleen in mice vaccinated with LPS-LIP conjugate in post-challenged mice showed no growth of bacteria (*Salmonella enterica* serovar Typhimurium for liver and a scantly growth for spleen. In addition, histological section of liver showed look-like normal hepatic tissue appearance especially near the portal area.

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List of Abbreviations

ALC	Absolute Lymphocyte Count
AMC	Absolute monocyte count
ANC	Absolute Neutrophil Count
AP-1	Activator protein 1
cya	Adenylate cyclase
AgH	Antigen H
AgO	Antigen O
APCs	Antigen presenting cells
aroA, aroC and aroD	Aromatic amino acids A, C and D
AR	Arthus reaction
BSA	Bovine serum albumin
CMI	Cell-mediated Immunity
C3	Complement component 3
CFA	Complete Freund's Adjuvant
COPS	Core polysaccharide-OPS
Crp	cyclic AMP receptor protein
DTHR	Delayed type hypersensitivity reaction
EDTA	Ethylene-diamine-tetra-acetic acid
FliC	Flagellin
FKB	Formalin-killed bacteria
HKB	Heat-killed bacteria
HIV	Human immunodeficiency virus
IFNγR	Interferon γ receptor
IFN-γ	Interferon-gamma

IL	Interleukin
LPS	Lipopolysaccharide
iNOS	Inducible nitric oxide synthase
LPS-LIP Conjugate	Lipopolysaccharide –Liposome Conjugate
LIP	Liposome
LB	Luria-Bertani
MAP	Mitogen activated protein
MYD88	Myeloid differentiation primary response gene 88
NC	Negative control
NCCLS	National committee for clinical laboratory standards
NTS	Non-typhoid *Salmonella*
NF-κB	Nuclear factor kappa-light-chain B cell
PAMPs	Pathogen associated molecular patterns
PI	Phagocytic index
PBS	Phosphate buffered saline
PC	Positive control
purA and purE	Purines A and E
Vi-rEPA	Recombinant *Pseudomonas aeruginosa* exoprotein A
S-S agar	*Salmonella-Shigella* agar
T_H	T helper
TLRs	Toll-like receptors
TLC	Total leukocyte count
TNF-α	Tumor necrosis factor-alpha
T3SS-1	Type III secretion system-1

Chapter One

Introduction
and
Literature Review

Chapter One
Introduction and Literature Review

1.1 Introduction

Enteric infections take a heavy toll on the world's population, particularly children living in the developing world. Estimates of World Health Organisation (WHO) in 2004 placed the death toll from diarrhoeal diseases at nearly 1.9 million deaths per year; ranking third among all causes of disease burden worldwide, and in developing countries, diarrhoeal diseases account for 15-34% of all deaths. Most of these deaths occurred in infants and young children under 5 years of age, (WHO, 2004). In addition, evidence has been emerging for long-term consequences of early childhood diarrhoeal disease on growth and physical and cognitive development that may translate into costly impairment of human potential and productivity (Guerrant *et al*., 2002).

Different pathogens can cause diarrhoeal diseases, and these include enteric adenoviruses, astroviruses, human caliciviruses, rotaviruses, *Campylobacter jejuni*, toxigenic *Escherichia coli*, Shigellas, Salmonellas and *Vibrio cholerae* (Girard *et al*., 2006). Therefore, *Salmonella* are also involved as an important cause of a diahoreal disease, and in the United States of America (USA), there were an estimated 1.5 million new cases of non-typhoidal *Salmonella* infections annually (Mead *et al*., 1999). The most common clinical presentation is gastroenteritis with nausea, vomiting, and diarrhoea with or without fever (Miller and Pegues, 2000). A small percentage (5%) of patients develops invasive *Salmonella* infections and some of these patients may progress to extragastrointestinal infections including bacteremia, and 5-10% of these bacteremic persons develop localized infections (Lesser and Miller, 2003). Invasive *Salmonella* infections can be caused by many serovars (serotypes) and begin with a series of events characterized by adhesion and epithelial entry of the *Salmonella* organism. Invasion of intestinal cells is a hallmark of *Salmonella*

pathogenesis and a trait shared by many serovars, and it is also capable of invading non-phagocytic cells of the intestinal epithelium and causing gastroenteritis by multiplying within the gut associated lymphoid tissues (Bopp *et al*., 2003). In this regard, *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) is considered as an important pathogen that can causes diarrhoea, especially in infants and young children (Litrup *et al*., 2010).

Salmonella Typhimurium is one of the non-typhoidal *Salmonella* serovars and being the most common serovar in the USA. Infection always occurs via ingestion of water or food contaminated with animal waste rather than human waste (Gray and Fedorka-Cray, 2002). The emergence of multidrug-resistant *S*. Typhimurium DT104 has been associated with outbreaks related to food contamination and resulted in increased rates of hospitalization (Yousef and Carlstrom, 2003). Therefore, *S*. Typhimurium continues to represent a major public health problem worldwide, and vaccine development has been an important target for researches in salmonellosis. The prevention of salmonellosis by vaccination has been the subject of many investigations, and despite this, the mechanism of protective immunity against *Salmonella* infections remains a controversial subject (Singh, 2009).

Salmonella Typhimurium is classified as a facultative, intracellular bacterium by virtue of its ability to survive and multiply within the specialized phagocytic cells of the host reticuloendothelial system (Groisman and Mouslim, 2000). In their sheltered intracellular environment, the salmonellae are provided with a considerable amount of protection from several host defence mechanisms, and although both cell-mediated (CMI) and humoral immune responses are evoked in naturally occurring and in experimentally induced salmonellosis, the relative degree of protection afforded by each of the two arms of the specific immune response is still unclear (Sa´nchez-Vargas *et al*., 2011). Thus, it has been observed that mice vaccinated with killed *Salmonella* bacteria or with soluble *Salmonella* antigens were not sufficiently protected from a lethal

challenge inoculum despite the presence of a substantial amount of specific antibody. However, adoptive transfer experiments suggest that cellular immunity is the overriding protective factor against *Salmonella* infections (Girard *et al*., 2006).

Several studies have demonstrated that the lipopolysaccharide (LPS) of the *S*. Typhimurium is a key component associated with bacterial virulence, but in terms of vaccine potential, the LPS itself is not very immunogenic, and as a result, attempts have been made at synthesizing a vaccine which incorporates *Salmonella* LPS antigenic determinants, but is devoid of the toxic properties inherent in the lipid A moiety of the LPS, through the covalent attachment to carrier molecules, such as phospholipid bilayered vesicles (liposomes) (Masoud, 2007). Liposomes have received a considerable amount of attention as carriers for the delivery of a wide variety of biologically active substances to cells and tissues *in vitro* and *in vivo*, and they have been employed as immunological adjuvants for the enhancement or modulation of immune responses, especially CMI, to various antigens (Christensen *et al*., 2011; Henriksen-Lacey *et al*., 2011).

Aims of study:

- Assessing the role of *S*. Typhimurium as a causative potential in hospitalized diarrhoeal patients younger than five years old.
- Extraction and purification of LPS from isolated and identified *S*. Typhimurium.
- Determining the role of LPS-liposome conjugate as a potential vaccine against *S*. Typhimurium in albino male mice.
- Evaluating the humoral and cellular immune responses (total and absolute counts of leukocytes, phagocytosis, Arthus reaction and delayed type hypersensitivity reaction) of vaccinated mice, in addition to histopathological changes in liver and spleen.

1.2 Literature Review

1.2.1 *Salmonella*

Salmonella species are Gram-negative bacilli associated with animal and human infections, which lead to high morbidity rates, not only in the developing world but also in industrialized countries, and the high mortality is mainly observed in the poorest nations of the developing world. It is believed that epidemics caused by *Salmonella* spp. may have significantly affected the history of humankind, even at present, the effect of *Salmonella* infections on entire communities result in economic burden to developing and also industrialized nations (Cunha, 2004). The interest generated with *Salmonella* spp. as pathogens over the last decades and their implications on history, economics and biomedical science, is represented by close to 70,000 articles posted in Medline, and close to 15 million internet entries (Sa´nchez-Vargas *et al*., 2011).

1.2.2 Historical Profile

The genus *Salmonella* was ultimately named after Dr. Salmon, an American veterinary pathologist, while Smith was the actual discoverer of the type bacterium (*Salmonella enterica* serovar choleraesuis) in 1885. Dr. Salmon was the administrator of the USDA (United State Department of Agriculture) research programme, and thus the organism was named after him by Smith (Cunha, 2004). Smith and Salmon had been searching for the cause of common hog cholera and proposed this organism as the causal agent. Later research, however, showed that this organism (now known as *Salmonella enterica*) rarely causes enteric symptoms in pigs, and was thus not the agent they were seeking (which was eventually shown to be a virus). However, related bacteria in the genus *Salmonella* were eventually shown to cause other important infectious diseases. The genus *Salmonella* was finally formally adopted in 1900 by J. Lignières for the many species of *Salmonella*, after Smith's first type-strain *Salmonella choleraesuis* (Ryan and Ray, 2004).

1.2.3 Classification and Nomenclature

The genus *Salmonella* incorporates Gram-negative, facultative anaerobic rod-shaped bacilli that are classified as members of the family Enterobacteriaceae. This genus, which is estimated to have diverged from *Escherichia coli* approximately 100–150 million years ago, is genetically diverse and has adapted to colonize many different hosts. For example, *Salmonella* bacteria can be found both as commensal and pathogen in a range of warm and cold-blooded animals and they are capable of surviving free in the environment for extended periods of time (Ryan and Ray, 2004).

Historically, *Salmonella* naming was based on the original places of isolation such as *Salmonella* London and *Salmonella* Indiana. However, Kauffmann-White scheme classifies *Salmonella* according to three major antigenic determinants composed of flagellar H antigens, somatic O antigens and virulence (V_i) capsular K antigens. This was adopted by the International Association of Microbiologists in 1934 (Scherer and Miller, 2001). Agglutination by antibodies specific for the various O antigens is employed to group Salmonellae into six serogroups: A, B, C1, C2, D and E. For instance, *S*. Paratyphi A, B, C and *S*. Typhi express O antigens of serogroups A, B, C1 and D, respectively. More than 99% of *Salmonella* strains causing human infections belong to *Salmonella enterica* subspecies *enterica* (Andrews and Baumler, 2005).

Bacteria can also be classified on the basis of phylogeny, and a phylogenetic tree can be derived from the comparison with 16S rRNA or other gene sequences. There are 2463 *Salmonella* serotypes which are now placed under two species due to the difference in 16S rRNA sequence analysis: *Salmonella enterica* (2443 serotypes) and *Salmonella bongori* (20 serotypes). This system is currently used by the WHO Collaborating Centre, Centers for Disease Control and Prevention (CDC) and some other organizations (Pui *et al*., 2011). *Salmonella enterica* is further divided into six subspecies, which are

designated by roman numerals: enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI). *Salmonella enterica* subspecies I is mainly isolated from warm-blooded animals and accounts for more than 99% of clinical isolates whereas remaining subspecies and *S. bongori* are mainly isolated from cold-blooded animals and account for less than 1% of clinical isolates. As an example, the Kauffmann species *Salmonella typhimurium* is now designated as *Salmonella enterica* subspecies I serovar Typhimurium. Under the modern nomenclature system, the subspecies information is often omitted and culture is called *S. enterica* serovar Typhimurium and in subsequent appearance, it is written as *S.* Typhimurium. This system of nomenclature is used to bring uniformity in reporting them (Center for Disease Control, 2006). Cardona-Castro *et al*. (2009) reported that *S.* Typhimurium is one of the most prevalent serovars among *Salmonella* spp. causing gastroenteritis in 49 countries and accounting for an estimated 15% of all food-borne infections in the USA.

1.2.4 Epidemiology

The epidemiology of *Salmonella*-associated infections varies widely depending on the type of *Salmonella* spp. involved, and despite the improvement in sanitation and hygiene, non-typhoid *Salmonella* (NTS) illnesses continue to impose a significant burden on the population's health in industrialized and underdeveloped countries (Westrell *et al*., 2009). It is estimated that 93.8 million cases of gastroenteritis due *Salmonella* spp. occur worldwide leading to 155000 deaths each year, and according to a WHO data from 2001 to 2005, *S.* Enteritidis was the most common serovar worldwide (65% of the isolates), followed by *S.* Typhimurium (12%) and *S.* Newport (4%) (Majowicz *et al*., 2010).

In Africa, *S.* Enteritidis and *S.* Typhimurium represented 26 and 25% of the isolates, respectively, and in Asia, Europe and Latin America, *S.* Entiritidis was the most frequent isolate (38, 87 and 31%, respectively). In North America, *S.* Typhimurium was the most frequently reported (29%) followed by *S.* Enteritidis

(21%) and other *Salmonella* spp. (21%) (Galanis *et al*., 2006). Sub-Saharan Africa hospital-based studies reported blood stream *Salmonella* spp. infections more frequently associated to NTS, particularly *S*. Enteritidis and *S*. Typhimurium, than *S*. Typhi or *S*. Paratyphi. In this region, invasive NTS is endemic and has elevated morbidity and mortality in children less than 3 years old and adults with human immunodeficiency virus (HIV) infection (Mandomando *et al*., 2009).

In industrialized countries the increasing incidence of NTS has become a public health concern. The estimated NTS-associated illnesses incidence in Europe is 690 per 100000 inhabitants per year. This incidence varies between regions from 240 per 100000 in Western Europe to 2390 per 100000 person-years in Central Europe. The incidence of NTS bacteremia in Finland, Australia, Denmark and Canada during 2000-2007 was estimated at 0.81 per 100000 per year (Laupland *et al*., 2010). In the USA, the Food-borne Diseases Active Surveillance Network (FoodNet) found that NTS infections were the most commonly reported (17.6 cases per 100000 inhabitants) and the incidence has not declined since 1996, when the surveillance was initiated, and FoodNet data from 1996 to 2005 reported that NTS infections have been the leading cause of death (39%) among food-borne bacterial pathogens with highest mortality among adults more 65 years and highest incidence among children less than 5 years of age (69.5 infections per 100000 children) (Center for Disease Control, 2011).

The number of multidrug-resistant NTS has increased in many countries since the 1990 report of the multidrug-resistant *S*. Typhimurium DT104 strain that spread around the globe (Helms *et al*., 2005). According to the National Antimicrobial Resistance Monitoring System (NARMS), 4.1% of the USA isolates from 2005 to 2006 had decreased susceptibility to cephalosporins and 84% had multidrug-resistance phenotypes, and more comprehensive NARMS data from 1996 to 2007 showed also that invasive NTS were more likely to be

multidrug-resistant, but more importantly, it reported that isolates began to show resistance to nalidixic acid (2.7%) and ceftriaxone (2.5%), rising concern about clinical management and public health surveillance and prevention and augmenting the importance of vaccine development (Crump *et al*., 2011).

1.2.5 Characteristic Features and Transmission

Salmonellae are non-fastidious as they can multiply under various environmental conditions outside the living hosts. They do not require sodium chloride for growth, but can grow in the presence of 0.4 to 4%. Most *Salmonella* serovars grow at a temperature range of 5 to 47°C with optimum temperature of 35 to 37°C but some can grow at temperature as low as 2 to 4°C or as high as 54°C (Gray and Fedorka-Cray, 2002). They are sensitive to heat and often killed at temperature of 70°C or above. Salmonellae grow in a pH range of 4.0 to 9.0 with the optimum between 6.5 and 7.5. They require high water activity (a_w) between 0.99 and 0.94 (pure water $a_w = 1.0$) yet can survive at $a_w < 0.2$ such as in dried foods. Complete inhibition of growth occurs at temperatures < 7°C, pH < 3.8 or water activity < 0.94 (Hanes, 2003).

Salmonella infections are transmitted by ingestion of contaminated materials. The most common contaminated foods are ice cream, eggs, shellfish, undercooked meat, raw fruits and vegetables, and contaminated water (Lesser and Miller, 2003). Non-typhoid *Salmonella* transmission to humans can occur by consumption of food animal products, non-animal food products, contaminated water, or by contact with animals. Food products mass production and distribution disseminates pathogens rapidly to communities. Furthermore, antibiotic resistance among NTS organisms makes more difficult the control and prevention of these infections (Majowicz *et al*., 2010). Farm animals are the major reservoir for NTS in industrialized countries with transmission by their contaminated products, and NTS are naturally found in chickens, ducklings, sheep, goats, pigs, reptiles, amphibians, birds, pet rodents, dogs, cats, and in a

variety of wild animals making infection control a challenge to public health authorities (Wacheck *et al*., 2010).

1.2.6 Clinical Presentation, Virulence and Pathogenesis

The most common clinical presentation of NTS is gastroenteritis that is associated with nausea, vomiting, and diarrhea with or without fever. A small percentage (< 5%) of patients develops invasive *Salmonella* infections and some of these patients may progress to extragastrointestinal infections including bacteremia, and 5 to 10% of these bacteremic persons develop localized infections (Gray and Fedorka-Cray, 2002). Invasive *Salmonella* infections can be caused by many serovars and begin with a series of events characterized by adhesion and epithelial entry of the *Salmonella* organism, which are facilitated by several types of bacterial fimbrae or pili. Invasion of the intestinal mucosa results in an extrusion of the infected epithelial cells into the intestinal lumen and a destruction of microvilli, which leads to a loss of absorptive surface. The invasion of epithelial cells elicits the production of the proinflammatory cytokines (Lesser and Miller, 2003), which stimulate the influx of polymorphonuclear leukocytes into the infected mucosa. Experiments in mice suggest that bacterial entry and destruction of enterocytes (macrophages and dendritic cells) play a major role in the invasion process. Via this route, the bacteria can reach the Peyer's patches, and here, the bacteria can be taken up by macrophages that are believed to carry these engulfed bacteria to systemic sites through the lymphatic system (Hardy, 2004).

Upon ingestion, *S*. Typhimurium colonizes the terminal ileum and colon, commonly eliciting symptoms of gastroenteritis within less than 24 hours. The signs of disease and the pathological changes in the human terminal ileum and colon have been experimentally examined in a calf model. Studies in this animal model identified that motility and two type III secretion systems as the main *S*. Typhimurium virulence factors important for triggering intestinal inflammation (Schmitt *et al*., 2001). Motility and the invasion-associated type III secretion

system (T3SS-1) work in concert to enable a fraction of the *S*. Typhimurium population to invade intestinal epithelial cells. Acting as a molecular syringe, the T3SS-1 injects proteins, termed effectors, into host cells. Five T3SS-1 effectors, named SipA, SopA, SopB (SigD), SopD and SopE2, act in concert to trigger alterations in the actin cytoskeleton of host cells, thereby promoting epithelial invasion and intestinal inflammation (Raffatellu *et al*., 2005). Once *S*. Typhimurium has crossed the epithelial lining, a second type III secretion (T3SS-2) system enables the pathogen to survive within tissue mononuclear cells. Finally, *S*. Typhimurium invasion of host tissue is detected by the innate immune surveillance, resulting in a rapid induction of proinflammatory cytokines-mediated intestinal inflammation, which is largely responsible for the signs of disease (Santos *et al*., 2009). The disease is usually self-limiting and recovery follows within a few days to a week but, occasionally, systemic infection may occur in vulnerable human patients such as infants and elderly peoples, leading to serious syndromes (Sa´nchez-Vargas *et al*., 2011).

1.2.7 Role of Immunity

The innate immune system detects the presence of *S*. Typhimurium in tissue by two distinct mechanisms, each involving a multitude of receptors. The first mechanism, termed pattern recognition, enables the host to distinguish self from bacteria by detecting conserved microbial structures, known as pathogen associated molecular patterns (PAMPs) (Janeway, 1989), by humoral proteins, such as complement, or by host cell receptors, such as Toll-like receptors (TLRs). For example, the O-antigen of the *S*. Typhimurium lipopolysaccharide (LPS) is a PAMP detected by complement component 3 (C3), thereby initiating the alternative pathway of complement activation. The complement fragments C3a and C5a generated during this process are also known as the anaphylatoxins, owing to their potency in inducing inflammatory responses (Haas and van Strijp, 2007). Besides the O-antigen, *S*. Typhimurium LPS contains a lipid A moiety, which is a powerful agonist of TLR4. Curli, an

amyloid fibril present in the extracellular matrix of *S*. Typhimurium biofilms, is the main TLR1/TLR2-ligand detected on intact bacterial cells (Vazquez-Torres *et al*., 2004). Finally, motility of *S*. Typhimurium is mediated by flagella, whose major protein subunit, flagellin (FliC), is a PAMP stimulating TLR5 (Tukel *et al*., 2009). TLR4, TLR1/TLR2 and TLR5 engage a common adaptor protein, myeloid differentiation primary response gene 88 (MYD88), to initiate mitogen activated protein (MAP) kinase signal transduction pathways that induce expression of proinflammatory genes by activating two transcription factors; activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Tukel *et al*., 2010).

Components of the bacterial cell wall such as LPS, flagella, and certain lipoproteins activate TLRs on host cells, which in turn induces a robust inflammatory response within tissues, characterized by the production of T_H1-like cytokines such as IFN-γ, TNF-α, and IL-1, IL-6, IL-12, and IL-18, as well as macrophage migration inhibitory factor and iNOS (Young *et al*., 2002). IFN-γ has been shown to be vital for resistance against infections involving intracellular pathogens, including *S*. typhimurium, in part because of stimulation of the antibacterial activity of macrophages. Mice treated with anti-IFN-γ antibodies or gene-targeted mice lacking the IFNγR are impaired in their ability to clear a sub-lethal dose of virulent *S*. typhimurium, and they eventually succumb to infection (Price *et al*., 2007). IL-12 and IL-18, secreted by activated macrophages, act both independently and synergistically on natural killer cells and helper T cells to induce the production of further IFN-γ, which activates the macrophages through a positive feedback loop. Additionally, IL-12 is important for the polarization of T-helper cells toward the T_H1 response (Gaspal *et al*., 2008).

T cells do not play a key role during the early phases of infection control in naive animals, as nude, αβT-cell knockout, as well as CD4+ or CD8+T-cell-depleted mice are all capable of suppressing the early growth of *Salmonella* (van

der Velden *et al*., 2005). Despite the lack of an apparent role early in infection, studies in the mouse have suggested that T cells may be activated soon after oral and parenteral *Salmonella* infection. Dendritic cells in the Peyer's patches may be involved in local T-cell activation after oral challenge and B-cells are also implicated in T-cell activation (Bueno *et al*., 2008). However, while the innate immune response is highly successful in controlling the initial growth of *S*. Typhimurium, it is insufficient for achieving full protective immunity. Effective control and eventual eradication of bacteria during the late phases of primary infection and the generation of protective immunity against subsequent infections requires the development of a *Salmonella*-specific T-lymphocyte response and the active recruitment of such cells to the site of infection (Dougan *et al*., 2011).

1.2.8 Prevention and Vaccines

The most important measures for enteric infection prevention are provision of safe water access, safe food handling practices, sanitation measures, public education and vaccination. For NST, measures to limit the number of infections from animals may include proper hand washing after being in contact with farm animals (WHO, 2003). Another essential measure is antibiotic resistance, and to prevent the increased number of antibiotic resistant NTS strains is the restriction to the indiscriminate use of antibiotics in livestock animal food, along with improved farm-based infection control measures (Talbot *et al*., 2006). The high morbidity and mortality associated with *Salmonella* spp. infections and the emergence of multidrug resistance strains highlights the importance of vaccination. While no vaccines are available for human NTS infections, two types of vaccines are approved for typhoid fever prevention; the oral live attenuated vaccines and the inactivated or subunit parenteral vaccines, however, the main limitations of currently licensed typhoid vaccines are that they do not protect infants and they do not protect against *S*. Paratyphi or NTS, for which there are no licensed vaccines (Whitaker *et al*., 2009).

1.2.8.1 Parenteral Vaccines

Parenteral vaccination against typhoid fever has been used since the 19th century. The whole-killed cell vaccine has high reactogenicity and efficacy between 50 and 94%. The high rate of side effects with this vaccine has limited its use. Side effects include fever, severe headache, and pain at the site of the injection (Paterson and Maskell, 2010).

The parenteral Vi vaccine, a significantly less reactogenic vaccine, and it is based on a capsular polysaccharide antigen of *S*. Typhi. This vaccine, widely used in developing and industrialized countries, is licensed for adults and children more than two years. Vi vaccine is safe in immunocompromised host and administrated in a single parenteral dose with a booster recommended every two years. The protection starts seven days after vaccination with maximal protection after 28 days. The parenteral Vi vaccine has an efficacy between 61 and 80% with protection that last only two years and it is well tolerated (Yang *et al*., 2001). In a cluster-randomized study in India with 37673 subjects, who were two years of age or older, one single dose of Vi vaccine conferred 61% total protection. Children between two and five years of age had a higher level of protection (80%) and side effects were minimal. However, the Vi capsular polysaccharide vaccine is a T cell-independent vaccine, does not generate immunological memory and is not immunogenic in children below two years of age (Sur *et al*., 2009).

To improve capsular polysaccharide immunogenicity, it was bound to the recombinant *Pseudomonas aeruginosa* exoprotein A (Vi-rEPA). This conjugation switches the response from T cell-independent response to T cell-dependent response and as a consequence it induces immunological memory that may result in a prolonged protection (Mai *et al*., 2003). In a double blind, randomized trial, Thiem *et al*. (2011) evaluated in VietNam the Vi-rEPA in 11091 children 2-5 years old who were followed 27 months. The efficacy with two vaccine doses was 91.5%. The Vi-rEPA conjugate vaccine was safe and

immunogenic with significant persistence of antibodies at two years of vaccination. At over 46 months of follow up, the efficacy in the vaccinated group was 89%, and in healthy test infants aged two months and above, it showed safety, protective immunity, and compatibility with the expanded programme on immunization vaccines from the WHO. Vi capsular polysaccharide based vaccines, however, are unlikely to provide effective protection against *S*. Paratyphi, since they lack of Vi antigen, similarly this vaccine may be ineffective against *S*. Typhi strains not expressing the Vi polysaccharide (Vi negative strains) (Baker *et al*., 2010).

1.2.8.2 Attenuated Life Oral *Salmonella* Vaccine Ty21a

The currently approved oral live attenuated *S*. Typhi Ty21a vaccine was derived from the wild-type Ty2 *S*. Typhi strain after chemical mutagenesis. Oral administration mimics the infection route that the wild-type strain would take and it induces not only humoral and cellular immune responses at the mucosal level but also systemically. Oral live attenuated *S*. Typhi vaccine is proven to induce sufficient immune response to protect individuals against infections with wild-type *S*. Typhi infections, with an efficacy of 50 - 80% (Sa´nchez-Vargas *et al*., 2011). The live attenuated *S*. Typhi Ty21a vaccine is given orally in three to four doses for optimal immunogenicity. The vaccine elicits protection from 10 to 14 days after the third dose. This vaccine is well tolerated, and field studies in school age children in Egypt and Chile have demonstrated significant protection. The duration of protection is at least five years at which time boost is recommended. It is licensed in the United States for use in adults and children more than 6 years of age. Side effects are mild, infrequent and limited to gastrointestinal discomfort or fever (Paterson and Maskell, 2010). Therefore, new vaccine development research concepts are necessary in response to the need for enteric fever prevention against *S*. Typhi, *S*. Paratyphi and S. Typhimurium. New vaccine candidate proposals should also consider cost,

single rather than multiple doses and optimal immunogenicity in all age groups especially in children younger than two years.

1.2.8.3 Novel Attenuated *Salmonella* Vaccine Strains

Over the past 35 years, bacterial genetics combined with emerging molecular biology tools have allowed the dissection of the mechanisms of bacterial virulence and have highlighted the complexity of host pathogen interactions. Especially for *S*. Typhimurium, the availability of an appropriate animal model deepened the understanding of the intracellular life style and associated virulence factors. This knowledge has been instrumental in the development of new attenuation strategies, resulting in genetically defined attenuated strains of bacteria (Spreng *et al*., 2006). The introduction of defined non-reverting mutations affecting metabolic functions or critical virulence factors from *Salmonella* has been used to generate an array of *S*. Typhimurium-based live vaccine carriers. The most widely studied metabolically attenuated strains include mutants deficient in the biosynthesis of aromatic amino acids (i.e., *aroA*, or *aroC* and *aroD*), purines (*purA*, *purE*), in the production of adenylate cyclase (*cya*) or the cyclic AMP receptor protein (*crp*). For instance, deletions in the *aro* genes render these bacteria auxotrophic for aromatic amino acids, as well as for p-aminobenzoic acid and 2,3-dihydroxybenzoate. The mutant bacteria become attenuated under *in vivo* conditions because they are unable to scavenge these compounds that are unavailable in the human gut (Burns-Guydish *et al*., 2007). Such attenuation methods may hold a good promise for the development of vaccines against salmonellosis, and the investigations are still engaged in their evaluation because of some limitations; for instance, an *aroC aroD* derivative of the serovar Typhi isolate ISP1820 (strain CVD906), and *aroA aroD* (strain PBCC211) or *aroA aroD htrA* (strain PBCC222) derivatives of the CDC10-80 strain were found to cause fever and other adverse reactions, including vaccinemia (Karasova *et al*., 2009).

1.2.8.4 *Salmonella* Lipopolysaccharides and their Vaccine Potential

As mentioned earlier, *Salmonella* species are classified based on Kauffman-White method in which more that 2300 serovars is derived according to the antigenic structure of LPS of the cell surface (AgO) and flagellate proteins (AgH) (Scherer and Miller, 2001). Lipopolysaccharides have a complex structure in Gram-negative bacteria and is composed of three parts including: complex part named lipid A (the source of LPS is on the membrane and has immunologic property), polysaccharide (is composed of 10 to 15 sugar and has a special role in penetration membrane) and specific O side chain (O side chain composed of repetitive units of 7 sugar and has a special role in bacteria antigenicity) as shown in figure 1-1 (Andrews and Baumler, 2005). The LPS combination is the most surface part of the cell wall of Gram-negative bacteria and is concerned as their endotoxin, and causes the severe reactions in immune system and highly poisonous for animals (Pakzad *et al*., 2003). Endotoxins of Gram-negative bacteria are attached to cell wall of bacteria and cannot separate from it unless the bacteriolysis is done and the bacterium is decomposed. When LPS is degraded, the toxic part will be attached to lipid A. Polysaccharides are the formers of surface antigens of bacteria and are called Antigen O, and LPS molecule is attached to outer membrane of cell wall by hydrophobic bands. The place of LPS synthesis is in the cytoplasm membrane and after synthesis is transferred to its final location whiles it is ready (Rastegaret *et al*., 2008).

Lipopolysaccharides are one of the strong stimuli for immune responses. The B cells are activated and stimulate other immune cells for releasing IL-1 and IL-6 and TNF-α and other factors. The pure LPS can induce strong reaction by itself, and phagocyte cells from the macrophage-monocyte group are important intermediates to these responses. Monocytes are very sensitive to LPS and can produce inflammatory protein products such as TNF-α and IL-1β at the very low level of LPS. These proteins have very important roles in defence of host against Gram negative infections and also are concerned as key

intermediate of septic shock (Kim *et al*., 2007). However, it has been suggested that *Salmonella* LPS does not confer immunological memory; a situation that limit its potential as a vaccine, and to overcome such limitation, core polysaccharide-OPS (COPS) of *Salmonella* Enteritidis LPS was conjugated to flagellin protein from a homologous strain, and the results revealed that COPS-flagellin conjugates were significantly protective from lethal challenge with wild-type *S*. Enteritidis (80 to 100% vaccine efficacy) (Simon *et al*., 2011). In the present study, the concept of conjugation was further explored, but with artificial vesicles made of phospholipid, which were liposomes.

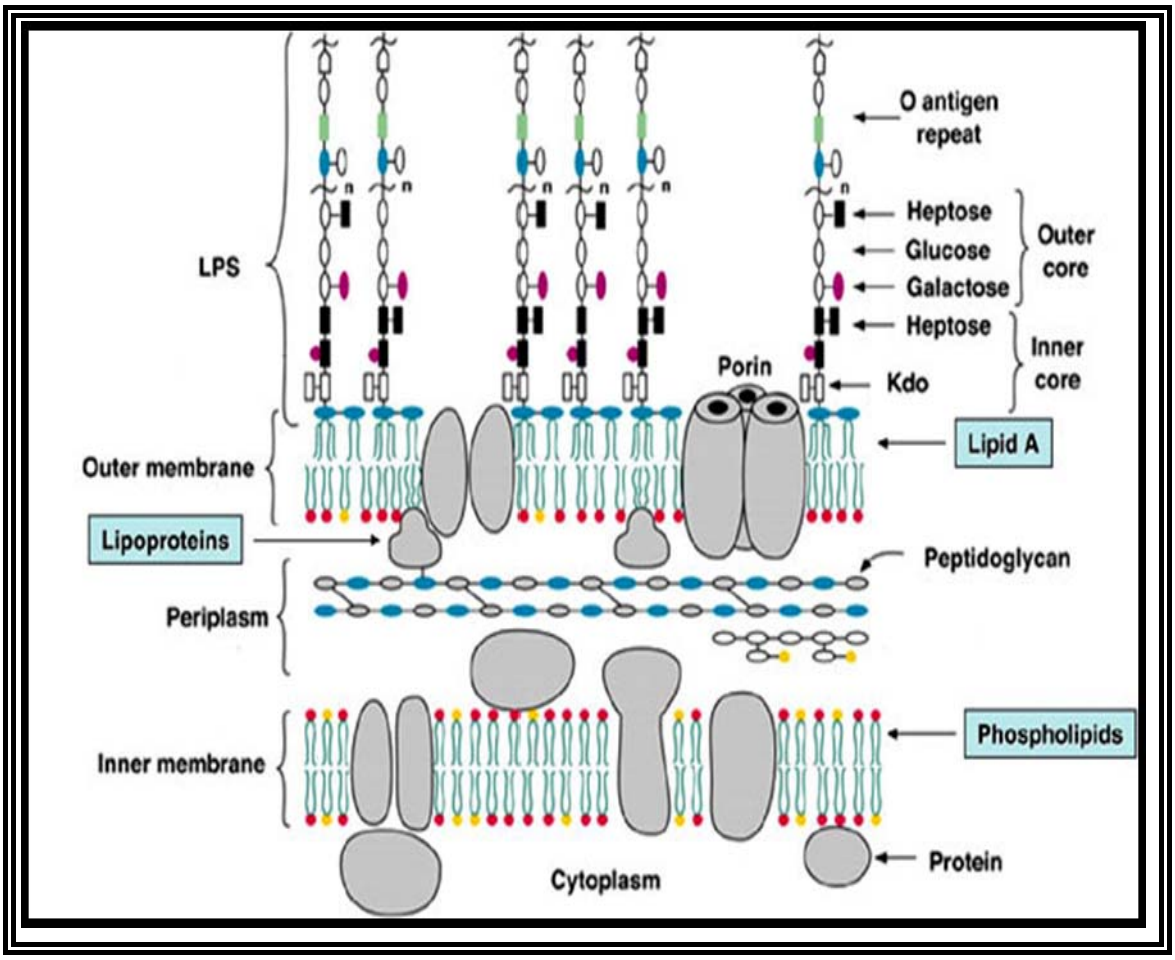


Figure 1-1: The structure of lipopolysaccharides in the cell wall of Gram negative bacteria (Andrews and Baumler, 2005).

1.2.9 Liposomes

Liposome science and technology is one of the fastest growing scientific fields contributing to areas such as drug delivery, cosmetics, structure and

function of biological membranes. This is due to several advantageous characteristics of liposomes such as ability to incorporate not only water soluble but also lipid soluble agents, specific targeting to the required site in the body and versatility in terms of fluidity, size, charge and number of lamellae (Mozafari, 2005). Liposomes are vesicles consisting of one or a number of concentrically oriented phospholipid bilayers. They are used as model systems for biological membranes, e.g., to study functions of membrane proteins and membrane fusion phenomena, to study the immune system and as delivery systems for bioactive compounds. In addition, they are relatively safe; i.e., they have a low toxicity and a low intrinsic immunogenicity (Kersten and Crommelin, 1995; 2003). The idea to use liposomes as vehicles for the presentation of antigens was tested when it was shown that diphtheria toxoid incorporated in liposomes is more immunogenic than free diphtheria toxoid. Since then, the influence of several variables on the immune response has been studied: charge of liposomes, epitope density, rigidity of the bilayer and the association of the antigen with the liposome (in the bilayer or in the aqueous phase) (Wilson-Welder *et al*., 2009).

A lot of work has been done to study the interaction of liposomes with cells of the immune system and to elucidate the mechanism of induction of immune reactions, and this stimulated possibilities to manipulate liposomes *in vivo*. Through studying the uptake of immunoglobulin-coated liposomes by rat liver macrophages, it was clear that macrophages function as the main antigen presenting cells (APCs) for liposomes, and for liposome-associated antigens, it was shown that the humoral, as well as, cellular immune response in macrophage depleted animals was suppressed. Inhibition of the humoral response was also observed after macrophage suppression *in vivo*. Upon reappearance of macrophages the response recovered (Ahsan *et al*., 2002). B-cells also have antigen processing and presentation capacity, but for the presentation of liposomal antigens this is not a major route because of their low

tendency to phagocytose liposomes. B-cells nevertheless play a role in the activation (i.e., increased phagocytic activity) of macrophages by liposomes. In general, liposomes not only augment the humoral response, but also the cellular response, and *in vitro* T-cell activation can also occur in a non-restricted manner, i.e. directly, in the absence of APCs and the presence of anti-MHC monoclonal antibodies (Vangasseri *et al*., 2006).

In bacterial vaccines, Allison and Gregoriadis (1974) were the first to demonstrate that liposomes may be useful antigen carriers in vaccines. They used diphtheria toxoid as antigen. A toxoid that since then has been investigated more extensively in liposomes is TT. The effect of epitope density and membrane fluidity was determined. It was shown that high phospholipid:TT ratios induced higher humoral responses than liposomes with low ratios (Gregoriadis *et al*., 1999). The humoral reaction against liposome-associated antigens was extended to involve *Neisseria* species, and experiments with pore protein I from *N. gonorrhoeae* in liposomes demonstrated that the humoral response against protein I was lower compared with protein-detergent-AIPO 4 complexes and outer membrane complexes. An increase in immunogenicity and a reduction in toxicity were observed when LPS from *N. meningitidis* was used in liposome-associated form compared to free LPS. In addition, liposomal-LPS conjugate was less pyrogenic in rabbits than free LPS (Zollinger *et al*., 2011). The immune stimulating effect of liposomal-LPS conjugate was also observed with LPS from *Brucella*, and a positive effect of liposomal-LPS conjugate on the delayed type hypersensitivity response and survival after challenge in mice was reported, especially when the animals were co-immunized with IL-18 (Singha *et al*., 2011).

Chapter Two

Patients, Materials and Methods

Chapter two
Patients, Materials and Methods

2.1 patients

Subjects of present study were hospitalized children and infants (95 cases: 60 males and 35 females) under the age of five years (40 days – 2.5 years old). They were admitted to the Central Pediatric Hospital and Al-Kadhiymiah Pediatric Hospital in Baghdad during the period 24/10/2010 - 30/11/2010, because of severe diarrhoea and fever.

2.2 Materials

2.2.1 Equipments

Equipment	Company	Origin
Autoclave	Koksun	Japan
Candal jar	BDH	U.K.
Centrifuge	GallenKamp	U.K.
Cooled centrifuge	Beckman	U.S.A
Disposable micropipette tips	Walter	Germany
Disposable Petri-dishes	Grenier	Germany
Disposable swab collectors	BDH	U.K.
Disposable syringes	CMP	Turkey
Glass slides and cover slips	Sail Brand	China
Incubator	Fisher	U.K.
Light microscope	Olympus	Japan
Micropipette	Brand	Germany
Pasteur pipette	Biomerux	France
pH-meter	Beckman	U.S.A.
Precision pipettes 50µl, 100 µl, 200µl, 1000 µl	Eppendorf	Germany
Oven	Fisher	U.K.
Sensitive balance	Matller	U.K.
Spectrophotometer	Shimadzu	Japan
Test tubes	Grenier	Germany
Lyophilizer	Fisher	U.K.

2.2.2 Chemicals and Biological Materials

Material	Company	Origin
Aceton	BDH	U.K.
Bovine serum albumin	Himedia	India
Canada balsam	BDH	U.K.
Chloroform	Applichem	Germany
Complete Freund's adjuvant	Behring	Germany
Coomassie brilliant blue G- 250	GT Beaker	Holland
EDTA	BDH	U.K.
Eosin stain	BDH	U.K.
Ethanol	BDH	U.K.
Formalin	Scharlau	Spain
Glucose	Fluka	Holland
Glycerol	BDH	U.K.
Glycial acetic acid	Bisolve	France
H_2SO_4	Fluka	Holland
H_2O_2	Himedia	India
H_3PO_4	GT Beaker	Holland
Haematoxylin stain	BDH	U.K.
HCl	BDH	U.K.
Human plasma	Biotest	Germany
K_2HPO_4	BDH	U.K.
KCl	Himedia	India
KH_2PO_4	BDH	U.K.
KOH	BDH	U.K.
Kovacs reagent	Himedia	India
Methanol	BDH	U.K.
Methyl red	Himedia	India
N,N,N,N-tetramethyl-P-phenylene diamine dihydrochloride	BDH	U.K.
Na_2HPO_4	Himedia	India
NaCl	BDH	U.K.
NaH_2PO_4	Himedia	India
α-Nephthol	BDH	U.K.
Peptone	Himedia	India
Phenol	BDH	U.K.

Phenol red	Merck	Germany
Saccharomyces cervisiae	Pakmaya	Turkey
Sephacryl 200 S	Sigma	U.S.A
Sodium Chloride	ADWIC	Egypt
Tris-base	Himedia	India
Tris-HCl	Himedia	India
Tryptone	Biolife	Italy
Trypan blue	BDH	U.K.
Urea base	Himedia	India
Yeast extract	Oxiod	England

2.2.3 Enzymes

Enzyme	Company	Origin
DNase	Cinnagen	Iran
RNase		
Proteinase K		

2.2.4 Kits

Kit	Company	Origin
Antisera for identification of *S. Typhimurium*	BioRad	U.S.A.
Giemsa stain	Himedia	India
Leishman stain	Himedia	India
Liposome	Promega	U.S.A.

2.2.5 Culture Media

Media	Company	Origin
Agar-agar	Himedia	India
Brain heart infusion agar		
Brain heart infusion broth		
MacConkey agar		
Nutrient agar		
Nutrient broth		
S-S agar		
Simmon citrate agar		

Tetrathionate broth		
Tripl sugar iron		
Urea agar base	Biolife	Italy

2.2.6 Antibiotic Discs

Antibiotic Disc	Symbol	Company	Origin
Ampicilin (10 mcg)	AM	Bioanalysis	Turkey
Augmantin (5 mcg)	AMC		
Amoxicillin (25 mcg)	AX		
Bactracin (10 mcg)	B		
Chloramphenicol(30 mcg)	C		
Ciprofloxacin (10 mcg)	CIP		
Gentamycin (10 mcg)	GN		
Cefotaxime (10 mcg)	CTX		
Imipeneme (10 mcg)	IPM		
Neomycin (10 mcg)	N		
Nalidixic acid (30 mcg)	NAL		
Optichin (5 mcg)	O		
Piperacillin (10 mcg)	PRL		
Streptomycine (10 mcg)	S		
Tetracyclin (10 mcg)	TE		
Trimethoprim (10 mcg)	TMP		

Augmentin=Amoxicillin+Clavulanic acid

2.3 Preparation of Media

The media used for isolation and identification bacteria were prepared according to the instructions of manufacturer, unless otherwise are cited. They were sterilized by autoclaving at 121°C (15 Pound/In^2) for 15 minutes.

2.3.1 Isolation and Identification Media

- **MacConkey agar**: It is a selective and differential medium used for identifying Gram-negative bacteria and detecting their ability to ferment lactose.
- **Motility test medium**: The medium was prepared by adding of agar-agar to nutrient broth at a final concentration of 0.3%, boiled for 1 minute, sterilized,

distributed into sterile tubes then inoculated by stabbing. It was used for the detection of bacterial motility (Collee *et al.*, 1996).

- **Pepton water**: The medium was prepared by dissolving 10 g of pepton and 5 g of NaCl in 800 ml of distilled water and the volume was made up to 1000 ml. The pH was adjusted to 7.4, sterilized and then it was distributed into sterile tubes. It was used for detecting bacterial ability to produce indole from tryptophan (Atlas and Snyder, 2006).
- **MR-VP medium**: The medium was used for detecting bacterial ability to ferment glucose, and differentiation of bacteria was based on acid production (methyl red test) and acetoine production (vogas-proskauer reaction) (Tang and Stratton, 2006).
- **Simmon citrate medium**: The medium was used for detecting bacterial ability to utilized citrate as a sole source for carbon and energy (Atlas *et al.,* 1995).
- **Kligler iron medium**: The medium was used for detecting bacterial ability to ferment sugars and produce H_2S and CO_2 (Atlas *et al.,* 1995**)**
- **S-S agar medium (*Salmonella-Shigella* agar)**: The medium was not autoclaved, but it was boiled to 100°C.
- **Glucose fermentation medium**: The medium was prepared by dissolving 1 g of glucose in 100 ml of brain heart infusion broth, and then few drops of phenol red indicator was added, pH was adjusted to 7.2 and the medium was sterilized by autoclaving (Atlas *et al.,* 1995).
- **Urea agar medium**: It was prepared by adjusting the pH of 95 ml urea agar base (Christensen's media) to 7.2 and then autoclaved. After cooling to 50°C, 5 ml of 40% urea solution (sterilized by filtration using Millipore filter; 0.22 µm) was added, mixed and distributed into sterilized test tubes in a slant position (Atlas *et al.,* 1995).
- **Luria-Bertani (LB) Broth**: Tryptone (10 g), yeast extract (5 g) and NaCl (5 g) were supplemented with 10mM glucose. After dissolving the components

in 1000 ml distilled water, the pH was adjusted to 7.2 and sterilized by autoclaving. For LB agar preparation, agar was added at a final concentration of 1.5% (Maniatis *et al*., 1982).

2.3.2 Cultivation, Maintaining and Preservation Media

- **Brain heart infusion broth**: It was used for cultivation of bacteria.
- **Nutrient broth**: It was used for cultivation and maintenance of bacteria.
- **Nutrient agar**: It was used for cultivation and maintenance of bacteria.
- **Nutrient broth + glycerol**: The medium was prepared by adding glycerol to nutrient broth 15% and then it was dispensed into sterile and well-capped screwed test tube, autoclaved, and then used for preserving bacterial isolates for several months at -20°C (Benson, 2001).
- **Brain heart infusion agar**: The medium was used for preserving bacterial strains for several days or weeks at 4°C.

2.4 Preparation of Solutions

2.4.1 Reagents

The following reagents were prepared according to methods presented by Colle *et al*. (1996), with the exception of API 20 E reagents:

- **Catalase Reagent**: It was hydrogen peroxide (H_2O_2) at a concentration of 3%.
- **Methyl Red Reagent**: It was prepared by dissolving 0.1 g of methyl red pigment in 300 ml of ethanol (99%), and then 200 ml of distilled water were added.
- **Voges-Proskaur Reagent:** Two solutions were first prepared. The first (A) was prepared by dissolving 40 g of KOH in 100 ml of distilled water (40% KOH), while the second (B) was prepared by dissolving 5 g of α-naphthol in 100 ml of absolute ethanol.

- **API 20 E Kit (BioMerieux, France)**: The kit consisted of galleries (the gallery is plastic strip with 20 microtubes containing dehydratea reactive ingredients) and API 20 E reagents (Oxidase, Voges-Proskaure reagents [VPI: 40% potassium hydroxide and VPI: 6% alpha-naphthol], 10% Ferric chloride and Kovac's reagents).

2.4.2 Lipopolysaccharide (Endotoxin) ExtractionSolutions

- **Phosphate Buffer (0.025M)**: It was prepared by dissolving 1.21 g K_2HPO_4 and 0.34 g KH_2PO_4 in 1000 ml distilled water**.** The pH was adjusted to 7.2 and sterilized by autoclaving and stored at 4°C (Silipo *et al*., 2002).
- **Ethylene-diamine-tetra-acetic acid (EDTA) solution (0.5M)**: It was prepared by dissolving 8.1 g EDTA in 50 ml phosphate buffer (0.025M) (Chandan and Fraser, 1994).
- **DNase Solution**: A quantity of 1 mg of DNase was dissolved in 10 ml of 10mM Tris-base (pH 7.5) and stored at -20ºC (Maniatis *et al*., 1982).
- **RNase Solution**: RNase powder was dissolved at concentration of 1 mg/ml in 10mM Tris-base (pH 7.5) and 15mM NaCl. It was heated to 100ºC for 15 minutes and then allowed to cooling slowly to room temperature and storeed at -20ºC (Maniatis *et al*., 1982).
- **Proteinase K solution:** A quantity of 1 mg proteinase K was dissolved in 10 ml sterilized distilled water and stored at -20ºC (Maniatis *et al*., 1982).

2.4.3 Solutions of Protein Concentration Determination

They were prepared according to Bradford (1976)

- **Coomassie brilliant blue G-250 stain**: It was prepared by dissolving 0.1 g of coomassie brilliant blue-G-250 in 50 ml of 95% ethanol, and then 100 ml of 85% phosphoric acid was added with agitation and the volume was made-up to 1000 ml with distilled water. The stain solution was filtered Whattman filter paper (No.1) and kept in a dark bottle.

- **Tris-HCl buffer**: It was prepared by dissolving 0.3 g of Tris-HCl in 100 ml distilled water, and the pH was adjusted to 7.5.
- **Bovine Serum Albumin (BSA) Stock Solution**: It was prepared by dissolving 10 mg of BSA in 10 ml of Tris-HCl buffer(1mg/ml).

2.4.4 Solutions of Carbohydrate Concentration Determination

They were prepared according to Dubois *et al*. (1956).

- **Glucose stock solution**: It was prepared by dissolving 1mg of glucose in 10 ml distilled water.
- **Phenol solution (5%)**: Five grams of phenol powder was dissolved in 100 ml distilled water.
- **Concentrated H_2SO_4 solution**: Ready used solution.

2.4.5 Immunological and Histopathological Solutions and Stains

- **Phosphate Buffered Saline (PBS)**: The following chemicals were dissolved in 500 ml of distilled water, and then the volume was made up to 1000 ml. The pH was adjusted to 7.2, and the solution was autoclaved and stored at 4°C (Hudson and Hay, 1989).
    - Sodium chloride (NaCl): 8.00 g
    - Potassium chloride (KCl): 0.20 g
    - Di-sodium hydrogen phosphate (Na_2HPO_4): 1.15 g
    - Potassium di-hydrogen phosphate (KH_2PO_4): 0.20 g
- **Haematoxylin and Eosin stains:** The stain solutions were ready supplied by the Histopathology Unit at Teaching Laboratories of Baghdad Medical City.
- **Trypan blue**: One gram of trypan blue powder was dissolved in 100 ml of normal saline. The stain solution was filtered (Whattman filter paper No.3) before use (Ad'hiah, 1990).
- **Normal Saline**: A ready solution (0.85% NaCl) was used. It was the product of ADWIC Company (Egypt).

- **Heat-killed Yeast** Suspension: Ten grams of the yeast *Saccharomyces cervisiae* were suspended in warm (37ºC) physiological saline (150 ml). The cell suspension was heated in boiling water both for 60 minutes. After heating, the cell suspension was cooled to 37ºC, and filtered using sterile double layers of gauze. The filtered cell suspension was assessed for yeast cell viability by a dye exclusion test (trypan blue) to assure that all cells were dead. Then, the cell suspension was divided into aliquots (5 ml) after adjusting the cell count to 10^7 cell / ml, and stored at -20ºC until use (Metcalf *et al*., 1986).

2.5 Laboratory Methods

2.5.1 Bacterial collection from Stool Specimens

A stool sample was taken randomly from each pediatric patient upon admission to the hospital and before taking any medication using a disposable swab applicator, which was transferred to a test tube containing 10 ml sterilized peptone water. After 2-3 hours, the sample was incubated at 37°C with a total time of approximately 24 hours.

2.5.1.1 Bacterial Isolation

After incubation, 5 ml of cultured stool sample were transferred to 250ml flask containing 50 ml tetra-thionate broth (an enrichment medium for the selection of *Salmonella*), and incubated at 37°C for 24 hours. After incubation a loopful from each flask was streaked on SS agar (selective medium) plates, and incubated at 37°C for 24 hours. The suspected colonies were subjected to further identifications.

2.5.1.2 Identification of Suspected Colonies (Bacterial Isolates)

A. Gram Stain: A smear of the bacterial isolates was made on slide and subjected to the conventional Gram staining. Stained slides were examined for Gram stain pattern under light microscope using oil immersion lens (100X).

B. Catalase test: The test colony was transferred with a sterile loop onto a clean glass slide, and then few drops of 3% hydrogen peroxide (H_2O_2) were added. The release of oxygen bubbles indicated the presence of catalase (Collee *et al.*, 1996).

C. Indole test: Pepton water (3-4 ml) was inoculated with overnight tested bacterial culture and incubated at 37°C for 24-48 hour, and after that, 0.5 ml of Kovac's reagent was added directly to the culture vial. The immediate formation of a red ring at the top of the broth indicated a positive result (Collee *et al.*, 1996).

D. Methyl red test: MR-VP medium (3-4 ml) was inoculated with the tested bacterial culture and incubated at 37°C for 24-48 hour, and then few drops of methyl red solution were added to the broth culture. An immediate red-coloured reaction indicated positive result, while yellow-coloured reaction was a negative result (Collee *et al.*, 1996).

E. Vogas-Proskauer test: MR-VP broth medium (3-4 ml) was inoculated with the tested bacterial culture and incubated at 37°C for 24-48 hour, and then 3 ml of Vogas-Proskauer solution A and 1 ml of Vogas-Proskauer solution B were added. The appearance of red colour after 5 minutes indicated a positive result (Collee *et al.*, 1996).

F. Citrate utilization test: Simmon's citrate agar slant was inoculated with tested bacterial culture by streaking with sterile loop and incubated at 37°C for 24-48 hour. A positive result was indicated by changing the colour of medium from green to blue (Collee *et al.*, 1996).

G. Kligler iron test: Kligler iron slant was inoculated with tested bacterial culture by streaking on the surface and stabbing in button of the medium, and incubated at 37°C for 24-48 hour. The positive result was noticed by changing the colour of medium red slant and yellow bottom and associated with production of H_2S (Collee *et al.*, 1996).

H. Glucose Fermentation Test: The glucose fermentation medium was inoculated with a fresh culture of each suspected isolate and incubated at 37°C for 24 hours. Changing the color from red to yellow indicated a positive result (Atlas *et al*., 1995).

2.5.1.3 Using API 20 E system and serotyping

The *Salmonella* was identified by APi 20E system, while *S.* Typhimurium was identified with serotyping.

2.6.1.3.1 API 20E System

Identification of *Salmonella* isolates was carried out by sub-culturing representative colonies from MacConkey Agar plates on APi 20E microtubes system. This system is designed for the performance of 20 standard biochemical tests from a single colony on plate medium. Each test in this system is preformed within a sterile plastic microtube that contains the appropriate substrates and affixed to an impermeable plastic strip (gallery). Each gallery contains 21 microtubes. The biochemical tests included in APi 20E system were:

1. β-Galactosidase test (ONPG).
2. Arginine dihydrolase test (ADH).
3. Lysine decarboxylase test (LDC).
4. Ornithine decarboxylase test (ODC).
5. Citrate utilization test (CIT).
6. Hydrogen sulphide test (PLS).
7. Urease test (URE).
8. Tryptophane deaminase test (TDA).
9. Indole test (IND).
10. Voges-Proskauer test (VP).
11. Gelatin liquefaction test (GEL).
12. Glucose fermentation test (GLU).

13. Manitol fermentation test (MAN).
14. Inositol fermentation test (INO).
15. Sorbitol fermentation test (SOR).
16. Rhamnose fermentation test (RHA).
17. Sucrose fermentation test (SAL).
18. Melibiose fermentation test (MEL).
19. Amygdalin fermentation test (AMY).
20. Arabinose fermentation test (ARA).
21. Oxidase test (OXI).

A. Preparation of galleries: Five milliliters of tap water were dispensed into the incubation tray to provide a humid atmosphere during incubation.

B. Preparation of bacterial suspension: Single colony from plating medium was picked and suspended in 5 ml sterile distilled water by rubbing against the side of the tube and mixed thoroughly.

C. Inoculation of galleries: With a sterile Pasteur pipette, the 21 microtubes were inoculated with the bacteria suspension. According to the manufacture instructions, both the tubes and couple section of CIT, VP and GEH microtubes were filled. After inoculation, couple section of the ADH, LDC, ODC, H_2S and URE microtubes were completely filled with mineral oil.

D. Incubation of the Galleries: After inoculation, the plastic lid was placed on the tray and the galleries were incubated for 18-24 hours at 37°C.

E. Reading the Galleries: All reactions that not requiring reagents were recorded first, and then the following reagents were added to the corresponding microtubes:

- One drop of 3.4% ferric chloride was added to the TDA microtube.
- One drop of Kavoc's reagent was added to the IND microtube.
- One drop of Voges-Proskauer reagent was added to VP microtube.
- One drop of oxidase reagent was added to either H_2S or ONPG

microtube.

The biochemical reaction performed by the APi 20E and their interpretations are listed in table 2-1.

Table 2-1: Interpretation of reactions performed by APi 20E system.

Negative	Positive	Microtube
Colorless	Yellow	ONPG
Yellow	Red/Orange	ADH
Yellow	Orange	LDC
Yellow	Red/Orange	ODC
Pale green/Yellow	Blue-Green	CIT
Colorless/Grayish	Black deposit	H2S
Yellow	Red/Orange	URE
Yellow	Dark brown	TDA
Yellow ring	Red ring	IND
Colorless	Pink/Red	VP
No diffusion	Diffusion of black pigment	GEL
Blue/Blue green	Yellow	GLU
Blue/Blue green	Yellow	MAN
Blue/Blue green	Yellow	INO
Blue/Blue green	Yellow	SOR
Blue/Blue green	Yellow	RHA
Blue/Blue green	Yellow	SAC
Blue/Blue green	Yellow	MEL
Blue/Blue green	Yellow	AMY
Blue/Blue green	Yellow	ARA
Colorless/Light purple	Violet/Dark purple	OXI

F. Identification of isolates: Identification of the isolates using the analytical profile index (Numerical Coding) for rapid identification at species and biotype level were done as supplied by the manufacturer. To use the index, the biochemical profile obtained was transformed into a numerical profile and to compare it with those listed in the index by transforming all 21 biochemical results into a seven-figure numerical profile (seven-digit number), by placing them into groups of three consigning a specific value for each of the positive as follows:

Group 4			**Group 3**			**Group 2**			**Group 1**		
GLU	GEL	VP	IND	TDA	URE	H_2S	CIT	ODC	LDC	ADH	ONPG
4	2	1	4	2	1	4	2	1	4	2	1
Group 7			**Group 6**			**Group 5**		
OXI	ARA	AMY	MEL	SAC	RHA	SOR	INO	MAN
4	2	1	4	2	1	4	2	1

Each positive result is given a value equal to 1, 2 or 4 according to the position of the test in its group. The sum of these three values was given the corresponding figure. Thus, the figure can have a value from 0 to 7 (zero for negative reaction), and the seven numerical digit profile is then looked up in the index and the identification is determined.

2.5.1.3.2 Serotyping of Salmonella

An Antisera kit was used for the identification of *S.* Typhimurium that was manufactured by BioRad Company and contained the following antisera:

- Anti-Omni serum.
- Anti-OMA serum.
- Anti-O serum.
- Anti-HMA serum.
- Anti-H serum.

2.5.1.4 Antibiotic Susceptibility Test of Isolates

Susceptibility of *S.* Typhimurium isolate to different antibiotics was studied by the standard disc diffusion test of National Committee for Clinical Laboratory Standards (NCCLS) of 2010. Five milliliters of sterile brain heart infusion broth were inoculated with 0.1 ml of the fresh culture of *Salmonella* Typhimurium isolate and incubated at 37°C for 4 hours in shaking incubator (100 rpm). Then, ten serial dilutions were prepared, and 0.1 ml of the fourth dilution (10^{-5}) was spread on brain heart infusion agar plate. The inoculated plates were placed at room temperature for 30 minutes (to allow absorption of

excess moisture), before antibiotic disks were placed on the inoculated plates (5 discs / plate), and incubated at 37°C fo18 hours. After incubation, diameters of the inhibition zones were measured and compared with that of NCCLS.

2.5.2 Preservation of Bacterial Isolates

For short-time preservation, brain heart infusion agar was inoculated with bacterial strain by streaking on the agar plates and agar slant, incubated at 37°C for 24 hours, sealed well and preserved at 4°C for a maximum of two weeks. In the case of long-time preservation, nutrient broth with glycerol 15% medium was inoculated with single colony of bacterial strain then preserved at -20°C (Manintis *et al.*, 1982).

2.5.3 Extraction of Lipopolysaccharide (Endotoxin)

2.6.3.1 Cell Preparation

Bacterial cells (*S* Typhimurium isolate) for lipopolysaccharide (LPS) extraction were obtained by growing each isolate in flask containing 25 ml of LB broth (for bacterial activation) at 37ºC for 18 hours. The fresh cultures were used to inoculated 3.5 L of LB broth suspended in 500ml conical flasks containing 200 ml broth. The inoculated flasks were incubated at 37ºC for 24 hours with shaking at 150 rpm. After incubation, cultures were centrifuged (3000 rpm for 15 minutes), and the pellet was washed twice with phosphate buffer. Cells were suspended in phosphate buffer containing 0.5% formalin (pH= 7.2) and were kept at 4ºC for 18 hours. After that, the cells were centrifuged (3000 rpm for 15 minutes) and washed with phosphate buffer. Finally, cells were dried using cold acetone by ten times the sample's volume **(**Silipo *et al*., 2002).

2.5.3.2 Lipopolysaccharide Extraction

The *Salmonella* LPS was extracted by a method given by Chandan and Fraser (1994), which is summarized in the following steps:

- The dried cells (20 g) were suspended in 50 ml phosphate buffer, and then 0.5 ml of 0.5M EDTA solution was added. The suspension was then homogenized with magnetic stirrer for 2 minutes.
- The homogenized suspension was autoclaved and then left to cool. After cooling, DNase and RNase solutions were added to at a final concentration of 1μg/ml for each solution and incubated at 37ºC for 10 minutes.
- Proteinase K solution at a final concentration of 0.1mg/ml was added and incubated at 56ºC for 10 minutes and then the temperature was increased to 60ºC for further 10 minutes. Finally the mixture was left to cool.
- The extraction mixture was centrifuged at 10000 rpm for 15 minutes. Two phases were formed; the aqueous phase (upper phase) was aspirated off with sterilized Pasteur's pipette and dialyzed for 4-6 days against distilled water at 4ºC with changing the water every day. The dialyzed sample that contained endotoxin was lyophilized to obtain crude endotoxin.

2.5.3.3 Partial Purification of LPS by Gel Filtration

- **Preparation and packing of the gel (Sephacryl 200 S)**: Sephacryl 200 S gel was prepared according to the instructions of the manufacturer company. It was washed and suspended in 0.025 M of PBS (pH 7.2), degassed by using vacuum pump and then poured with care to avoid bubbles into a column with dimension of 75×2 cm. The final volume of the column was 235.5 cm^3. The column was equilibrated with 0.025 M of PBS (pH 7.2), and the flow rate was 75 ml/hour.
- **Recovery of LPS**: According to Morrison and Leive (1975), 5 ml of crude LPS was applied gently to the column, and flow rate was approximately 75 ml/hour. Five milliliters fractions were collected, and absorption was read at 280 nm for protein determination (Bruck *et al.*, 1982). Determination of

carbohydrates content in the collected fractions was made by phenol-sulphuric acid method, by transferring 0.5 ml from each fraction in a sterile tube, then 0.5 ml of 5% phenol and 2.5 ml of sulphuric acid were added to each tube, and cooled in ice bath. Then, the absorbance was measured at 490 nm (Dubois *et al.*, 1956).

2.5.4 Chemical Analysis of Lipopolysacharride

2.5.4.1 Determination of Protein Concentration

Protein concentration was determined according to Bradford, (1976) and as the following:

- Standard solutions (20, 40, 60, 80 and 100 µg/ml) of BSA were prepared from the BSA stock solution (1 mg/ml), in order to plot the standard curve. Therefore, five tubes were set-up as the following:

Final Volume (ml)	Protein Concentration (µg)	Tris-HCl Buffer (µl)	BSA (µl)
0.1	20	80	20
0.1	40	60	40
0.1	60	40	60
0.1	80	20	80
0.1	100	0	100

- Then, 2.5 ml of Coomassie brilliant blue G-250 dye was added, mixed and left to stand for 2 minutes at room temperature.
- The absorbance at 595 nm was measured. The blank was prepared from 0.1 ml of Tris-HCl buffer and 2.5 ml of the dye reagent.
- A standard curve was plotted between the BSA concentrations against the corresponding absorbance of the bovine serum albumin.
- The protein concentration of LPS in the sample was estimated by taking 0.1 ml of 1mg/ml of LPS solution (dissolved in Tris-HCl buffer), and subjected

to similar steps of standard curve estimation. The protein concentration was estimated from the standard curve using curve fitting equation (Figure 2-1).

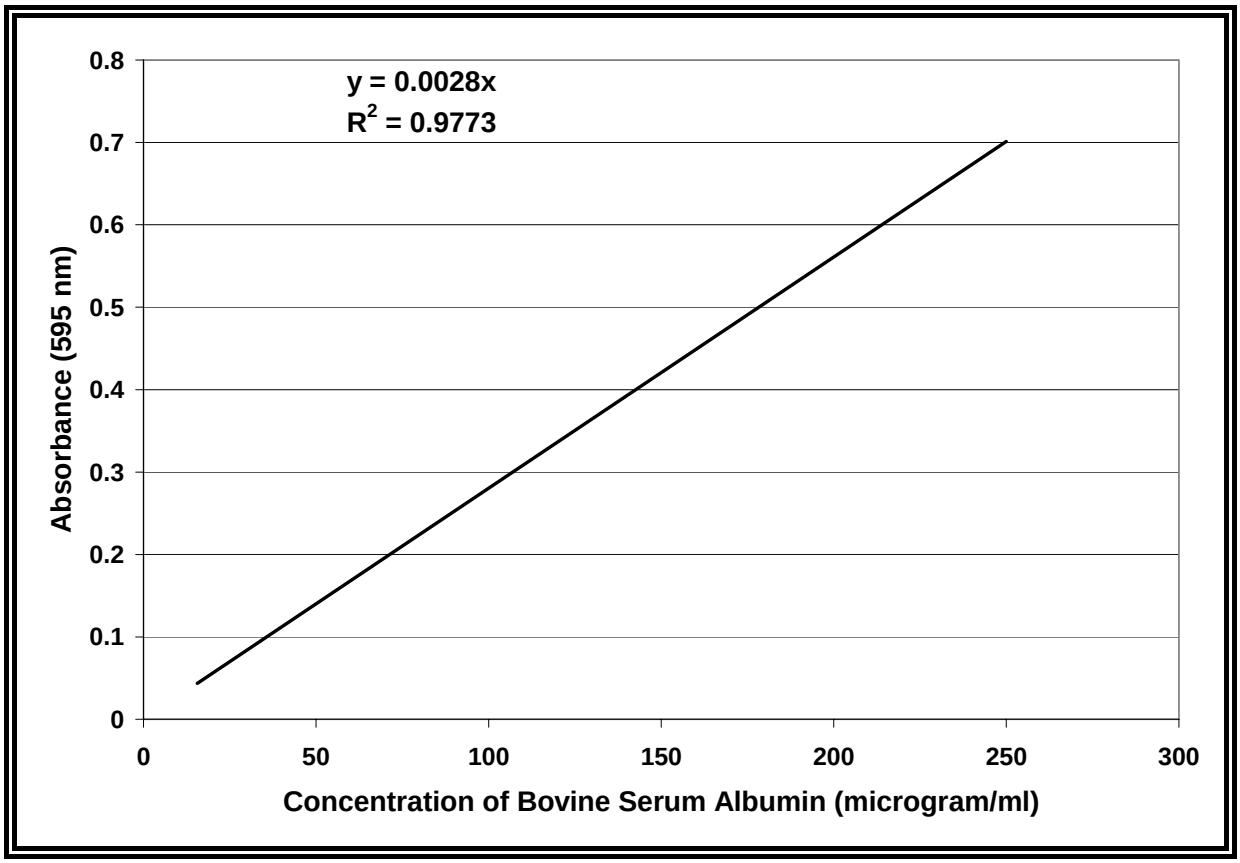


Figure 2-1: Standard curve of bovine serum albumin.

2.5.4.2 Carbohydrate Determination

According to Dubois *et al.* (1956), the phenol-sulphuric acid method was used to determine carbohydrate concentration in LPS, in which the following steps were adopted:

- Standard solutions (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml) of glucose were prepared from the glucose stock solution (100 µg/ml), in order to plot the standard curve. Therefore, 10 tubes were set-up, in addition to a blank tube, as the following:

Final concentration (µg/ml)	Final volume (ml)	Distilled water (ml)	Glucose stock solution (ml)	Tube No.
0	1	1.0	0	1
10	1	0.9	0.1	2
20	1	0.8	0.2	3
30	1	0.7	0.3	4
40	1	0.6	0.4	5
50	1	0.5	0.5	6
60	1	0.4	0.6	7
70	1	0.3	0.7	8
80	1	0.2	0.8	9
90	1	0.1	0.9	10
100	1	0	1.0	11

- One ml of 5% phenol was added to each tube and shaken well, followed by addition of 5 ml of H_2SO_4 was added to each tube. After well-shaking, the tubes were cooled in ice bath.
- Absorbance was read at 490 nm for each tube, and then the standard curve was plotted.
- The total carbohydrates concentration in LPS sample was determined by transferring 0.5 ml of each gel-filtration fraction to a test tube, and then 0.5 ml of 5% phenol and 2.5 ml of H_2SO_4were added. The tubes were shaken well, and then cooled in ice bath. The absorbance was read at 490 nm, and the total carbohydrate concentration was estimated from the standard curve using curve fitting equation (Figure 2-2).

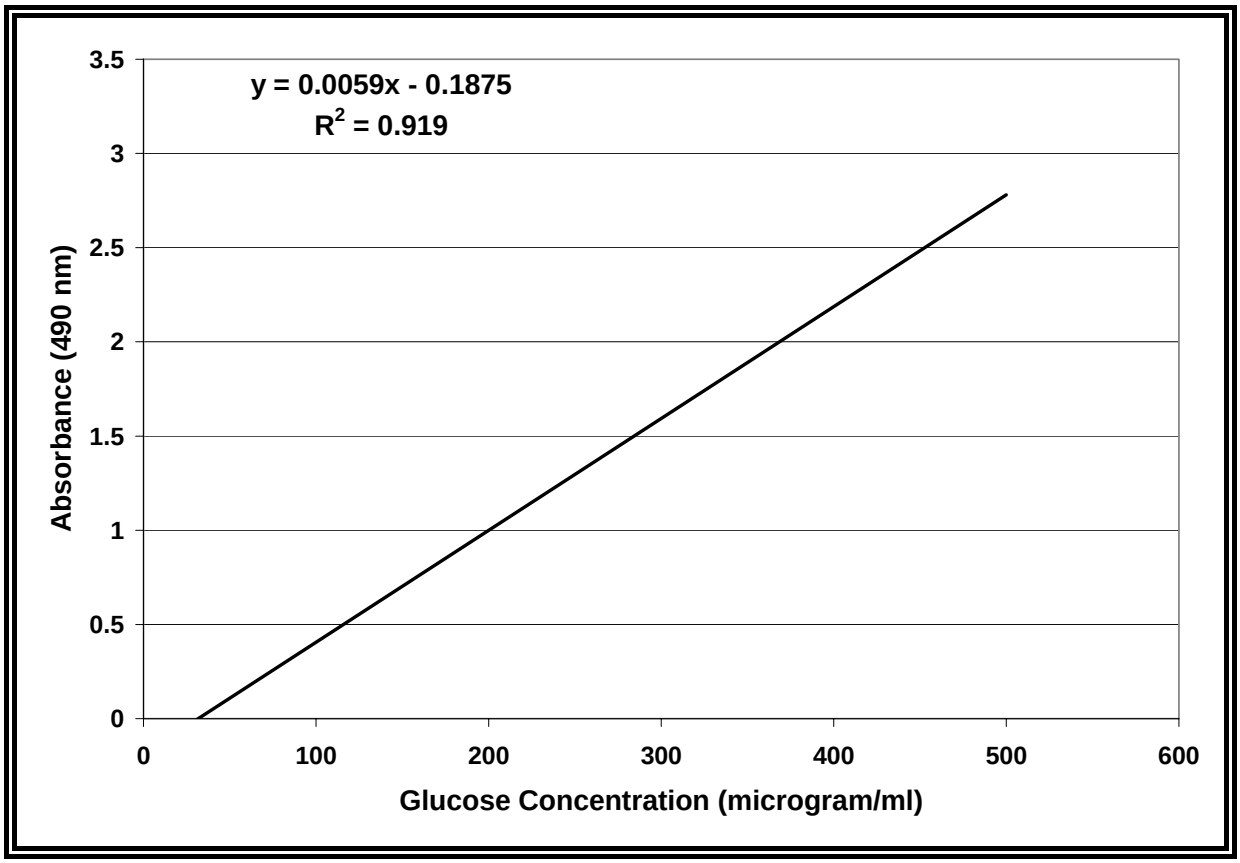


Figure 2-2: Standard curve of glucose.

2.6 Experimental Design

2.6.1 Laboratory Animals

Albino male mice (*Mus musculus*) were the laboratory animals, which were used to carry out the investigations of the present study. They were obtained from Biotechnology Research Centre (Al-Nahrain University). Their age range was 8-9 weeks, and their weight was 23-27 grams at the beginning of experiments. They were caged in the animal house of the supplier, in which the temperature was 23-26°C, and a light:dark periods of 10:14 hours/day. The animals had free excess to food (standard pellets) and drinking water (*ad libitium*) during all experiments.

2.6.2 Preparation of Doses (Desiderio and Campbell, 1983)

A. Live Bacteria: The bacteria (*Salmonella* serovar Typhimurium) were adjusted at a concentration of 5 x 10^4 cell/ ml PBS using McFarland method.

B. Complete Freund's Adjuvant (CFA): It was ready solution (1 mg/ml).

C. **Heat-Killed Bacteria (HKB)**: Brain-heart infusion broth (10 ml) containing over-night bacterial growth of *Salmonella* serovar Typhimurium was placed in a water bath (80ºC) for 60 minutes, and after cooling, it was centrifuged (3000 rpm) for 10 minutes. The cell pellet was washed twice with PBS, and then the cell concentration was adjusted to 5 x 10^9 cell/ ml PBS by using McFarland method

D. **Formalin-Killed Bacteria (FKB)**: Brain-heart infusion broth (10 ml) containing over-night bacterial growth of *Salmonella s*erovar Typhimurium was centrifuged (3000 rpm) for 10 minutes, and the cell pellet was suspended in 10 ml of 0.5% formalin solution, and incubated over-night at 4 ºC. After that, the bacterial suspension was centrifuged (3000 rpm) for 10 minutes, and the cell pellet was washed twice with PBS, and then the cell concentration was adjusted to 5 x 10^9 cell/ ml PBS by using McFarland method

E. **Lipopolysaccharide (LPS) Solution**: Partially-purified LPS (section 2.5.3.3) solution was adjusted at a concentration of 100 µg/ml PBS.

F. **Liposome (LIP) Solution**: It was ready prepared solution (TransFastTM Transfection Reagent, Promega Corporation, U.S.A.), and supplied in glass vials (3 x 0.4 mg) provided with nuclease free water (2 ml). One vial (0.4 mg) was reconstituted with 200 µl (stock solution: 2 µg/µl**)**. The stock solution was diluted with injection distilled water in proportion of 100 µl: 900 µl (0.001 µg/ml).

G. **LPS-LIP Conjugate**: Partially purified LPS at a concentration of 2.5 mg/ml PBS (900 µl) was mixed with 100 µl (stock solution: 2 µg/µl**)** of LIP solution.

2.6.3 Vaccination Protocol and Laboratory Evaluation

Each mouse of the groups presented in next section (2.6.4) was injected intraperitoneally (IP) with 0.1 ml of the respective solution in day 1, and a further dose in day 8. These mice were considered as pre-challenged groups (8 mice per group), and they were dissected for laboratory evaluations in day 15. Further similar groups were challenged with 0.1 ml live bacteria in day 15 (5 x 10^4 cell/

ml PBS by using McFarland method) and they were dissected for laboratory evaluations in day 29 (post-challenged groups).

2.6.4 Pre- and Post-Challenged Groups

Eight groups of albino male mice (16 mice per group) were vaccinated according to the protocol given in section 2.6.3, and they are as the following:

- **Negative Control (NC)**: The injected solution was PBS.
- **Positive Control (PC)**: The injected solution was a suspension of live *S*. Typhimurium.
- **Complete Freund's Adjuvant (CFA)**: The injected solution was CFA.
- **Heat-Killed bacteria (HKB)**: The injected solution was a suspension of heat-killed *S*. Typhimurium.
- **Formalin-Killed Bacteria (FKB)**: The injected solution was a suspension of formalin-killed *S*. Typhimurium.
- **Lipopolysaccharide (LPS)**: The injected solution was partially purified LPS.
- **Liposome (LIP)**: The injected solution was LIP.
- **LPS-LIP Conjugate**: The injected solution was LPS-LIP conjugate.

The total number of mice in these groups was 128.

2.6.5 Laboratory Investigations

2.6.5.1 Total and Absolute Counts of Leukocytes

Both counts were carried out on blood obtained by heart puncture using insulin disposable syringe (1 ml) pre-coated with heparin.

- **Total Count of Leucocytes (TLC)**: The conventional method of blood cell counting was employed, following the procedure of Sood (1986). A volume of 0.02 ml blood was dispensed in a test tube containing 0.38 ml of leukocyte diluent solution, and then the contents were mixed and the tube was left for three minutes. One drop of the diluted blood was applied to the surface of a counting chamber (Neubauer hemocytometer) under the cover slip. After that,

the chamber was left for two minutes to settle the cells, and by then, the leukocytes were counted using the following equation:

$$\text{Total Count (cell/cu.mm.blood)} = \left(\frac{\text{Number of Cells Counted}}{4}\right) \text{x } 20 \text{ x } 10$$

- **Absolute Count of Leukocytes**: A blood smear was made on a clean slide and left for air drying. Then, the slide was stained with Leishman's stain for two minutes and buffered for 10 minutes with Leishman's buffer. After that, the slide was rinsed with tap water and left for air-drying (Sood, 1986). The stained smear was examined under oil immersion power (100X), and at least 200 leukocytes were randomly counted. Then, the percentage of each cell type was obtained. The absolute count of leukocytes (lymphocytes, neutrophils and monocytes) was calculated according to the following equation:

$$\text{Absolute Count (cell/cu. mm. blood)} = \left(\frac{\text{Percentageof Cells x Total Count}}{100}\right)$$

2.6.5.2 Phagocytic Index (PI)

The mouse was anesthetized with chloroform, and 3 ml of warm (37°C) sterile saline was injected in the peritoneum. Then, the region was carefully massaged for 3 minutes. After that, the injected saline was drawn back, and transferred to a test tube. The test tube was centrifuged (2000 rpm) for 5 minutes, and the cell deposit was gently suspended in 1 ml of PBS. The cell suspension was subjected to cell counting, and the cell number was adjusted to 1 x 10^6 cell/ml.

To carry out the assay of phagocytosis, a mixture of cell suspension (150 µl), heat-killed yeast suspension (50 µl) and human AB plasma (20 µl) was incubated for 30 minutes at 37ºC. After incubation, a drop of the mixture was taken and a thin film was prepared on a clean slide. The slide was air-dried and stained with Giemsa stain for 15 minutes. After staining, the slide was rinsed with distilled

water, and left at room temperature to dry (Metcalf *et al.*, 1986). The slide was examined under oil immersion objective lens (100X), and at least 100 phagocytic and non-phagocytic cells were counted. The PI was calculated according to the following equation:

$$\text{Phagocytic Index (\%)} = \left(\frac{\text{Number of Phagocytic Cells}}{\text{Total Count}} \right) \text{x } 100$$

2.6.5.3 Hypersensitivity Reactions

After the immunization protocol that were presented in section 2.6.3, the left foot pad of mouse was injected with 0.05 ml of LPS-LIP conjugate, while the right foot pad was injected with 0.05 ml of PBS. Four hours later, the thickness of both pads was measured using a vernier, and the difference represented Arthus reaction (AR) index, which was given in units of millimeter (mm). The thickness measurement was repeated 24 hours later, and the difference represented the delayed type hypersensitivity reaction (DTHR) index (Triolo *et al*., 1989).

2.6.5.4 Bacterial Isolation from Liver and Spleen

The mice were dissected by using sterile instruments after cleaning of abdominal area with alcohol, and then a longitudinal incision was made and the two organs were removed, and then the bacteriological evaluation was made through the inoculation of one loopfull from liver and spleen after cutting the organ into two pieces on SS agar plates. After that, the organs were transferred immediately to 10% formalin for a histopathological manipulation (section 2.6.5.5). The inoculated media were incubated at 37°C for 24 hours, and after incubation, the plate was inspected for the formation of bacterial colonies, which were scored as 1 (scanty), 2 (moderate), 3 (heavy) or (0) no growth as suggested by Buchanan (1974).

2.6.5.5 Histopathological Examination of Liver and Spleen

The liver and spleen were fixed in 10% formalin, and the procedure of Bancroft and Stevens (1982) was followed to prepare sections for histopathological examinations. The procedure is outlined as the following:

- **Washing:** The sample was placed in 70% ethanol overnight.
- **Dehydration:** The sample was dehydrated with ascending concentrations (70, 80, 90 and 99%) of ethanol. There were two hours for each concentration.
- **Clearing:** The sample was placed in xylene for two hours.
- **Infiltration**: The sample was first placed in paraffin-xylene (1:1) for 30 minutes at 57-58ºC, and then in paraffin alone for 2 hours at 60-70ºC.
- **Embedding**: The sample was embedded in pure paraffin wax (melting temperature: 60-70ºC) and left to solidified at room temperature.
- **Sectioning**: The paraffin block was sectioned (rotary microtome) at a thickness of 5 microns, and then the sections were transferred to a slide covered with Mayer's albumin. The section of tissue was placed in a water bath (35-40ºC) for few seconds.
- **Staining**: The slide was first placed in xylene for 15-20 minutes, descending concentrations (100, 90, 80 and 70%) of ethanol (two minutes for each concentration) and finally distilled water. After that, the slide was stained with haematoxylin for 10-20 minutes and then washed with distilled water for 5 minutes. Then, the slide was placed in acidic alcohol for one minutes and washed with distilled water. After washing, the slide was placed in eosin stain for 10-15 seconds, and then in ascending concentrations (70, 80, 90 and 99%) of ethanol (two minutes for each concentration). Finally, the slide was cleared with xylene for 10 minute.
- **Mounting**: The slide was mounted with a Canada balsam and covered with a cover slip. Then, the slide was examined microscopically to inspect the histopathological changes.

2.7 Statistical Analysis

The data were tabulated in a data sheet, and they were analyzed using the computer programme SPSS (Statistical Package for Social Sciences) version 13.0. The investigated parameters were presented in terms of means ± standard errors (S.E.), and differences between means were assessed by ANOVA (analysis of variance), followed by LSD (least significant difference) or Duncan test. The difference was considered significant when the probability (P) value was ≤ 0.05, 0.01 or 0.001.

Chapter Three

Results and discussion

Chapter Three
Results and Discussion

3.1 Isolation and Identification of *Salmonella* Typhimurium

Out of 95 stool samples, *Salmonella* Typhimurium was isolated and identified from two samples only (2.1%), which were belong to two children; the first was at age 25 months, while the second was 4 months older. Both children had severe diarrhea that was associated with fever. Enteric infection with *Salmonella* spp. is an important cause of children diarrheal disease worldwide, but the frequency of children with the infection shows variations between studies. A study from Bolivia of 133 consecutive children less than 5 years old presenting with bloody diarrhea revealed bacterial etiology in 41%, and out of this percentage, *Salmonella* spp. accounted for 4% (Townes *et al*., 1997). However, a more recent study from Vietnam, no *Salmonella* infection was reported in 587 children with diarrhea under the age 24 months, although other potential pathogens were identified in 67.3% of children with diarrhea, including Gram negative bacteria (Vu Nguyen *et al*., 2006). Recently, Fischer Walker and colleagues (2010) searched PubMed/Medline, System for Information on Grey Literature in Europe (SIGLE), and all World Health Organization (WHO) Regional Databases for studies published from January 1, 1980 through December 31, 2008 using all combinations of the following search and MeSH terms: ‘‘diarrhea’’, ‘‘etiology’’, ‘‘pathogen’’, ‘‘incidence’’, ‘‘mortality’’, ‘‘cause of death’’, and ‘‘gastroenteritis’’, with the objectives to determine the causative agents of diarrhea worldwide. They identified 25,701 papers with possible etiology data, in which *Salmonella* spp. was considered most frequently encountered, in addition to *Shigella* spp., and *Entamoeba histolytica*, and Because little is known about the care-seeking behavior for community acquired diarrhea among children 5 years of age, they suggested that additional data are needed in this age group to determine the distribution of

pathogens in the community. Accordingly, understanding the burden of pathogen specific diarrheal disease and the variation by region is important for planning effective control programs for the overall reduction of diarrhea disease among persons of all ages, especially in children under the age of 5 years.

The isolation of *S.* Typhimurium was performed by cultivating the stool samples in flasks containing 50 ml tetra-thionate broth medium, which was used as a selective enrichment for the cultivation of *Salmonella* spp. that may be present in small numbers and compete with intestinal flora. Selectivity is accomplished by the combination of sodium thiosulfate and tetra-thionate, which suppresses common intestinal organisms (Eckner *et al*., 1994)

For pure cultures, 100µl aliquots from tetra-thionate broth cultures were transferred and spread onto the surface of *Salmonella-Shigella* agar plates (SS agar), which is highly selective and differential medium formulated to inhibit the growth of most coliform organisms and permit the growth of *Salmonella* and *Shigella* species from environmental and clinical specimens(Murray *et al*., 2007) The high bile salt concentration and sodium citrate inhibit all Gram-positive bacteria and many Gram-negative organisms including coliforms Lactose is the sole carbohydrate and neutral red is the indicator for acid detection. Sodium thiosulfate is a source of sulfur and the production of H_2S is indicated by black precipitate formed with ferric citrate (Atlas *et al*., 1995). The growth of *Salmonella* spp. in SS agar shows colorless colonies with black centers owing to H_2S production, while *Shigella* spp. does not blacken (Wells and Butterfield, 1997) As a result, 18 isolates, which showed black colonies in SS agar, were suspected as *Salmonella* spp. and subjected for further identifications.

3.1.1 Microscopical and Morphological Characterization

The suspected isolates were first identified depending on their Gram stain and microscopical characteristics. *Salmonella* isolates were found to be Gram-negative, rod or bacilli and non-spore forming. Morphological characteristics

of the suspected isolates were done depending on the colonial shape and form when re-cultured on the surface of MacConkey agar and SS agar. The colonies were pale yellow or nearly colorless, 1-3 mm in diameter and non-lactose fermented. In addition, the colonies in SS agar appeared to be circular, colorless, 1-2 mm in diameter with gas bubbles and black spots due to the production of H_2S. Such characteristics were applied to only 18 isolates that were suspected to be candidate for the biochemical tests.

3.1.2 Biochemical Tests

Biochemical tests were achieved on the suspected 18 isolates that showed growth and morphological characteristics, which were closely related to *S.* Typhimurium. For this purpose eight biochemical tests were carried out. Results showed that only two isolates gave negative reactions for indole, urease and Voges-Proskaur tests, but they gave positive reactions for citrate utilization, catalase, methyl red and glucose fermentation. In triple sugar iron test, both isolates were H_2S and gas producers. Table 3-1 demonstrates that the suspected two isolates were identified as *S.* Typhimurium by the criteria of Bergey's Manual of Systematic Bacteriology ((Holt *et al*., 1994).

Table 3-1: Morphological, microscopical, and biochemical characteristics of locally isolated *Salmonella* Typhimurium.

Characteristics	Isolate No. 1 (S1)	Isolate No. 2 (S2)
Shape	Rod	Rod
Gram stain	Negative	Negative
Motility test	Motile	Motile
Indole test	Negative	Negative
Methyl Red test	Positive	Positive
Voges-Proskaur test	Negative	Negative
Citrate Utilization test	Positive	Positive
Urease test	Negative	Negative

Catalase test		Positive	Positive
Glucose Fermentation test		Positive	Positive
Triple Sugar Iron Test	H_2S	Positive	Positive
	Gas	Positive	Positive
	Alkaline/Acid	+/+	+/+

Enterobacteriaceae members are Gram-negative bacteria and can grow in the intestinal tract of human and animals (Prescott *et al*., 1996). Many convention biochemical tests have been used to distinguish between Enterobacteriaceae family. *Salmonella* is a pathogenic enterobacter, which can be identified from coliforms in many tests; for instance, H_2S production, blackening and non- lactose fermentation (Woo *et al*., 2001). Within the genus *Salmonella*, species can be characterized by carrying out more biochemical tests (i.e. fermentation of different sugars), serological and genetic examinations; however species of *Salmonella* also differ from each other in the conventional biochemical tests; for instance, *S*. Typhimurium can utilize citrate but not *S*. Typhi or *S*. Paratyphi A (Tindall *et al*., 2005).

A further identification of the two *S*. Typhimurium isolates was achieved by using Api system (Api 20E). Results in figure 3-1 shows that these isolates were able to give positive results for ornithine decarboxylase, citrate utilization, H_2S production, gelatin liquefaction, and they were glucose, arabinose, sorbitol, rhamnose, melibiose, inositol and manitol fermentors. While, they gave negative reactions for β-galactosidase, arginine dihydrolase, lysine decarboxylase, urease, tryptophane deaminase, indole, Vogas-proskauer, amygdaline fermentation and sucrose fermentation. The results mentioned above were in agreement with those described by Holt *et al*. (1994); indicating that the two isolates were belong to the genus *Salmonella*., and by applying serotyping, they were both *S*. Typhimurium.

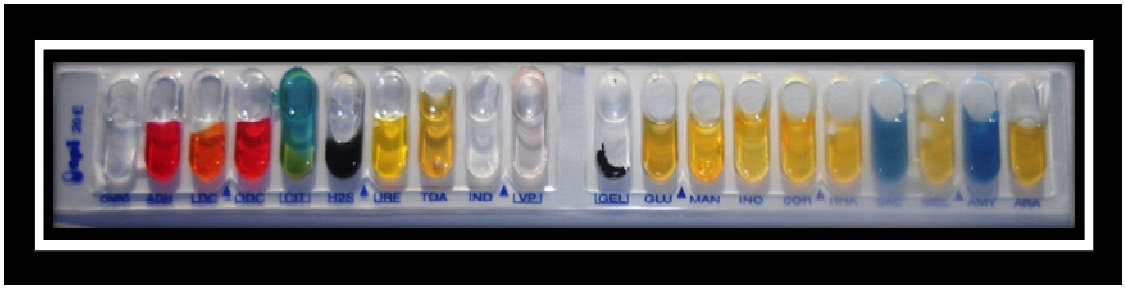


Figure 3-1: Api 20E system for identification of *Salmonella* spp.

3.2 Antibiotic Sensitivity Test

The two isolated *S*. Typhimurium were tested for their sensitivity to16 antibiotics, and the results showed that S1 isolate was resistant to 12 antibiotics, while S2 isolate was resistant to 10 antibiotics (Table 3-2), according to the table of NCCL (2002). Therefore S1 isolate was considered more virulent than S2 that agreement with (Giridhara Upadhyaya *et al*., 2009) bacteria was more virulens when resistant to antibiotic, for that S1 was selected for the extraction of lipopolysaccharide (LPS).

Table 3-2: Antibiotic sensitivity test for the two *S*. Typhimurium isolates.

Antibiotics	Symbol	Antibiotic Sensitivity	
		S1	S2
Ciprofloxacin	CIP	Sensitive	Sensitive
Cefotaxime	CTX	Resistant	Resistant
Gentamycin	GN	Resistant	Intermediate
Bactracin	B	Resistant	Resistant
Optochin	O	Resistant	Resistant
Trimethoprime	TMP	Resistant	Resistant
Tetracycline	TE	Resistant	Sensitive
Imipeneme	IPM	Sensitive	Sensitive
Chloramphenicol	C	Resistant	Resistant
Augmentin*	AMC	Resistant	Resistant
Ampicillin	AM	Resistant	Resistant
Naldixic acid	NAL	Sensitive	Sensitive
Amoxicillin	AX	Resistant	Resistant
Streptomycin	S	Resistant	Resistant
Neomycin	N	Intermediate	Intermediate
Piperacillin	PRL	Resistant	Resistant

*Augmentin: amoxicillin + clavulanic acid.

3.3 Isolation of Lipopolysaccharide from S1 Isolate

Lipopolysaccharide (endotoxin) is the major constituent of the outer membrane of Gram-negative bacteria (Martin *et al*, 2007). Bacterial LPSs generally consist of three regions; lipid A, core saccharide and O-antigenic side chain with covalent attachment between each other (Raetz and Whitfield, 2007). Due to the importance of bacterial endotoxin as a membrane barrier, bacterial recognizing site, induction of immune system and therapeutic activity (Tanamoto *et al*., 2001), several procedures have been developed for bacterial endotoxin extraction depending on the chemical nature, bacterial type and purpose of extraction (Apicella, 2008).

The endotoxin of *S*. Typhimurium isolates was extracted using a procedure developed by Chandan and Fraser (1994). This method is a combination between the application of pressure and hot water for bacterial cell destruction and hydrolytic enzyme treatment for protein and nucleic acid removal. The S1 isolate was grown in LB broth at 37ºC with shaking, because LB broth is effectively employed for cultivation of *S*. Typhimurium isolate, and beside that the supplementation of 10mM glucose results in a higher biomass yield and recovery of LPS (Guard-Petter *et al*., 1999), and in agreement with this, the present employed method yielded a bacterial mass of 20 grams dry weight bacteria.

Extraction of LPS by the method of Chandan and Fraser (1994) can be considered as simple and easy to run procedure for the extraction of LPS, and in the present study, 20 grams of dry weight bacteria gave LPS yield of 3.65 mg/ml. However, by using different methods for LPS extraction, the overall yield of LPS was reported to be higher with a range between 100-500 mg of LPS from 20 grams dry weight cells (Gerhardt *et al*., 1981), as well as Kato *et al*. (1998) gave a yield of 7.1 mg LPS /gram dry weight of cells.

It was noticed in the present method of extraction that the suspension of dried bacterial cells in EDTA solution and the application of autoclaving

resulted in destruction of bacteria cells and the denaturation of bacterial proteins. Following centrifugation at 10000 rpm, two phases were separated, the upper phase (aqueous phase) contains the LPS and nucleic acids and the lower phase contains denatured proteins and cell debris, as suggested by Carlson *et al*. (1987). The aqueous phase was aspirated off and dialyzed against distilled water in order to remove salts and other impurities (Weber *et al*., 1997). Due to the amphopathic nature of the LPS, this procedure takes the advantage that the majority of bacterial LPS show hydrophilic ability and hence become soluble within the aqueous phase (Pier *et al*., 1981; Godhaux *et al*., 1990; Apicella, 2008). In addition this procedure is suitable for extraction of both smooth and rough bacteria with low amount of contaminated proteins (due to autoclaving and proteinase K application) and nucleic acids (due to nucleases treatment).

3.3.1 Chemical Characterization

Chemical characterizations of the crude endotoxin extracted from *S*. Typhimurium (S1 isolate) were performed by estimating the carbohydrate contents according to Dubbois *et al*. (1956) depending on the standard curve of glucose, and estimating the protein contents according to Bradford, (1976) depending on the standard curve of bovine serum albumin. Accordingly, the carbohydrate content was 2.34 mg/ml, while the protein concentration was very low (0.52 µg/ml). In agreement with such findings, extraction of Gram negative endotoxin revealed that the aqueous phase contains low proportions of protein associated endotoxin (Kirikae *et al*., 1998). In addition, Fischer, (1990) and Helander *et al*. (1992) studied the chemical characterization of endotoxin separated from different Gram negative bacteria (*Pseudomonas* spp., *Escherichia coli* and *Salmonella* spp.), and their results demonstrated that the main constituents of endotoxin are phosphate group, fatty acids and different forms of sugars (glucose, manose, galactose and glucose-amine).

3.3.2 Partial Purification

The 50 collected fractions were first assessed for the determination of protein by reading the absorbance of each fraction at 280 nm as suggested by Bruck *et al.* (1982). After that, each fraction was processed by a method of phenol-sulphuric acid (Dubois *et al.*, 1956) to determine carbohydrate content, and then the absorbance was read at a wave length of 490 nm. The relationship between absorbency and fraction number of each constituent (protein and carbohydrate) was drawn (Figure 3-2).

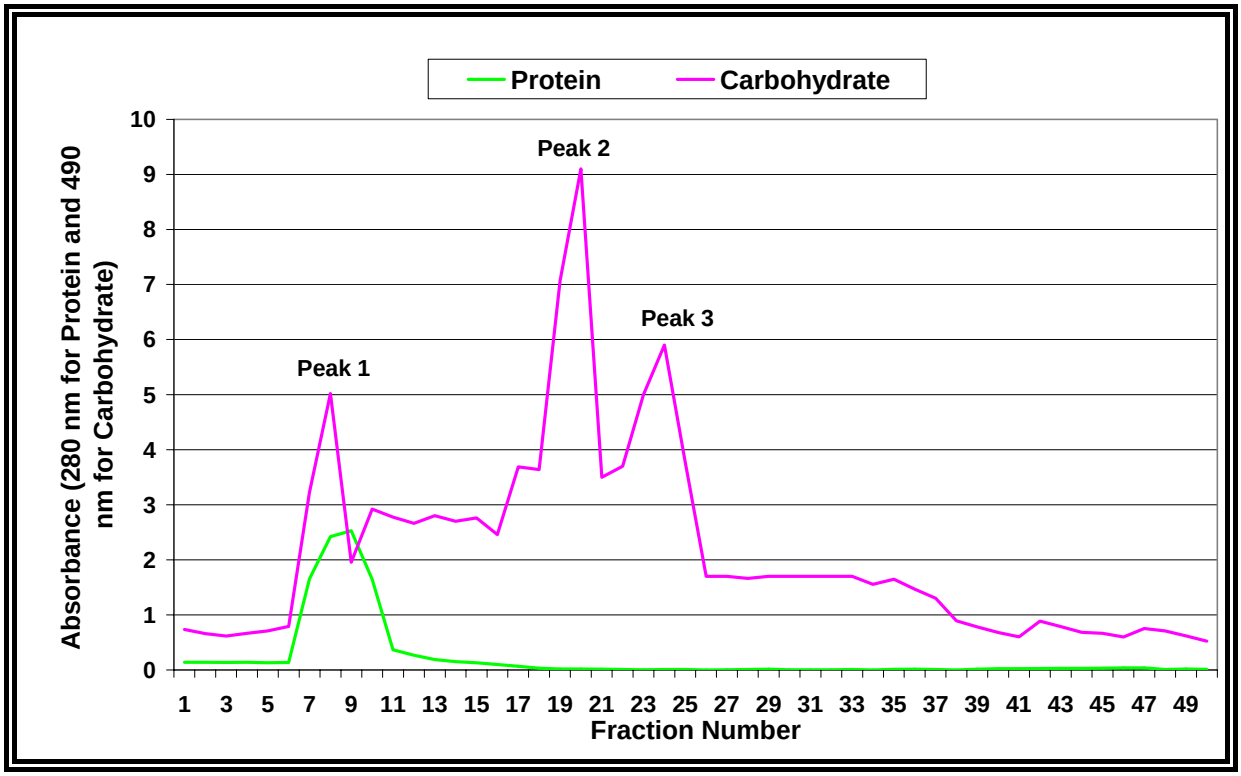


Figure 3-2: Gel-filtration chromatography for LPS partial purification from *S.* Typhimurium (S1 isolate) by using Sephacryl 200 S, 75×2 cm column equilibrated and eluted with 0.025 M PBS pH 7.2 with a flow rate of 75 ml/hour.

The figure demonstrates that at 490 nm three peaks were observed for carbohydrate. The first and third peaks were minor while the second peak was major. At 280 nm (protein), there was one peak separated at the position of carbohydrate peak one. Such finding suggests that there was a small amount of

protein bound to the LPS and it was difficult to separate it from the LPS. Similar findings have been reported by Al-Azzawi (1998), as well as Johnson and Perry (1975), Pier *et al.* (1978) and Darveau and Hancok (1983).

Chemical analysis of the partially purified endotoxin in the three observed peaks was carried out, and involved determination of carbohydrate, protein and nucleic acid contents. The results indicated that the percentage of carbohydrate was 11.0, 25.0 and 12.0% for peaks 1, 2 and 3, respectively. The percentage of carbohydrate in peak 2 was even higher than that of the crude LPS (25.0 vs. 17.2%) as shown in table 3-3.

Table 3-3: Carbohydrate and protein contents of crude and partially purified lipopolysaccharide.

Lipopolysaccharide		**Carbohydrate (%)**	**Protein (%)**
Crude		17.2	6.3
Partially Purified	Peak 1	11.0	3.0
	Peak 2	25.0	0.001
	Peak 3	12.0	0.001

Most studies are in favour of that the percentage yield of carbohydrates following endotoxin purification may vary widely. Horton *et al*. (1977) obtained 12-18% carbohydrate from partially purified endotoxin, while Wilkinson and Galibraith, (1975) reported a less percentage range, which was16.2-24.8%. In addition, 33.3% was obtained by Vinh *et al*. (1986). These differences can be attributed to the types of bacterial species from which LPS was extracted, method of extraction and purification process.

The present finding (carbohydrate yield of 25%) differs from that recorded by Al-Azzawi (1998), who demonstrated that the carbohydrate percentage in the partially purified LPS of local *P. aeruginosa* isolate was 15%, and further contradicting findings was also recorded by Horton *et al*. (1977), in which 12-18% range was observed. However, an agreement was also reached with further

studies. Chester and Meadow (1975) demonstrated a yield of 16-24%, and the same finding was reported by Wilkinson and Galibraith (1975), in which the carbohydrate percentage in purified LPS was 16.2-24.8%.

In table 3-3, we can notice that the protein percentage in partially purified LPS was 3% in peak 1 and 0.001% in peak 2 and 3. This percentage (peak 1) differs from the percentage recorded by Al-Azzawi (1998), who recorded that the protein percentage in partially purified LPS from *P. aeruginosa* was 2%. It also differs from the percentages recorded by Pier *et al*. (1978), who stated that the percentage of protein bound to the purified LPS was 4.3%. However, an agreement with Darveau and Hancock (1983) is reached, because they found that the percentage was less than 0.1%. Furthermore, Wilkinson and Galibraith (1975) recorded that the percentage of contaminated protein was very little, so they neglected such finding.

The differences in the protein and carbohydrate percentages in the purified LPS may be related to the differences in the bacterial strains and their content of LPS, the differences in the methods used in extraction and purification of LPS and the experiments circumstances. The results also showed that there were no nucleic acids in the partially purified LPS.

It was also observed that the carbohydrate percentage in the partial purified LPS (25% in peak 2) was higher than that of the crude LPS (17.2%), and a similar observation was made for the protein (3.0 and 0.001 *vs*. 6.3%). Both observations suggest the efficiency of the applied method of purification by gel filtration.

3.4 Immunological Evaluation

3.4.1 Leukocyte Counts

- **Total Leukocyte Count (TLC)**

The TLC showed different distributions in the investigated groups of mice (Table 3-4). Comparing pre- and post-challenged groups revealed significant

difference in the groups of negative control (NC), heat-killed bacteria (HKB) and formalin-killed bacteria (FKB). In NC group, the TLC was significantly ($P \leq 0.01$) increased in post-challenged mice as compared with pre-challenged mice (10400 *vs*. 7966 cells/cu. mm blood), while an opposite outcome was obtained in HKB (4700 *vs*. 11250 cells/cu. mm blood) and FKB (4466 *vs*. 12083 cells/cu. mm blood) mice, and at a higher level of significance ($P \leq 0.001$). In pre-challenged mice, the highest TLC was observed in HKB and FKB groups (11250 and 12083 cells/cu. mm blood, respectively), followed by lipopolysaccharides+liposomes (LPS+LIP: 9366 cells/cu. mm blood), while NC group were observed with the highest TLC (10400 cells/cu. mm blood) in post-challenged mice, and again followed by LPS+LIP mice (8533 cells/cu. mm blood).

Table 3-4: Total leukocyte count in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Total Leukocyte Count: cells/cu. mm blood)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	7966 ± 380^{C}	10400 ± 400^{A}	0.01
Positive Control	5200 ± 171^{D}	4800 ± 115^{D}	N.S.
Complete Freund's Adjuvant	5466 ± 133^{D}	6633 ± 270^{C}	N.S.
Heat-Killed Bacteria	11250 ± 1295^{A}	4700 ± 480^{D}	0.001
Formalin-Killed Bacteria	12083 ± 1719^{A}	4466 ± 480^{D}	0.001
Lipopolysaccharides	6500 ± 152^{D}	7733 ± 197^{C}	N.S.
Liposomes	6333 ± 168^{D}	6766 ± 238^{C}	N.S.
Lipopolysaccharides+Liposomes	9366 ± 95^{B}	8533 ± 240^{B}	N.S.

Different letters: Significant difference ($P \leq 0.05$) between means of columns.
P: Probability of difference between means of pre- and post-challenge.

- **Absolute Lymphocyte Count (ALC)**

A significantly ($P \leq 0.05$) increased ALC was observed in NC post-challenged mice as compared with pre-challenged mice (6198 *vs*. 5020 cells/cu.

mm blood). In contrast, a significantly decreased ALC was observed in HKB (2761 *vs*. 5833 cells/cu. mm blood; $P \leq 0.001$), FKB (2469 *vs*. 5075 cells/cu. mm blood; $P \leq 0.001$) and LPS+LIP (4389 *vs*. 5731 cells/cu. mm blood, $P \leq 0.05$) post-challenged mice as compared with pre-challenged mice. In pre-challenged mice, NC, HKB, FKB and LPS+LIP groups shared an approximated mean of ALC (5020, 5833, 5075 and 5731 cells/cu. mm blood, respectively), but it was significantly higher than the corresponding mean in positive control (PC), complete Freund's adjuvant (CFA), LPS and LIP groups (2711, 2688, 3527 and 3343 cells/cu. mm blood, respectively). However, in post-challenged mice, the most significant increase of ALC was observed in NC, followed by LPS and LPS+LIP groups (6198, 4970 and 4389 cells/cu. mm blood, respectively) (Table 3-5).

Table 3-5: Absolute lymphocyte count in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	**Mean ± S.E. (Absolute Lymphocyte Count: cells/cu. mm blood)**		**P ≤**
	Pre-Challenge	**Post-Challenge**	
Negative Control	5020 ± 149^{A}	6198 ± 355^{A}	0.05
Positive Control	2711 ± 97^{B}	2993 ± 30^{C}	N.S.
Complete Freund's Adjuvant	2688 ± 95^{B}	3447 ± 190^{C}	N.S.
Heat-Killed Bacteria	5833 ± 763^{A}	2761 ± 313^{C}	0.001
Formalin-Killed Bacteria	5075 ± 1222^{A}	2469 ± 297^{C}	0.001
Lipopolysaccharides	3527 ± 149^{B}	4970 ± 118^{AB}	0.01
Liposomes	3343 ± 108^{B}	3483 ± 175^{C}	N.S.
Lipopolysaccharides+Liposomes	5731 ± 116^{A}	4389 ± 108^{B}	0.05

Different letters: Significant difference ($P \leq 0.05$) between means of columns.
P: Probability of difference between means of pre- and post-challenge.

- **Absolute Neutrophil Count (ANC)**

In post challenged mice, a significantly ($P \leq 0.01$) decreased ANC was observed in NC group as compared with the corresponding ore-challenged group (3016 *vs*. 2098 cells/cu. mm blood). In contrast, a significantly ($P \leq 0.001$) decreased ANC was observed in post-challenged HKB (1318 *vs*. 4355 cells/cu. mm blood) and FKB (1471 *vs*. 4623 cells/cu. mm blood) groups in comparison with the corresponding pre-challenged groups. The latter two groups demonstrated the highest ANC in pre-challenged mice (4355 and 4623 cells/cu. mm blood, respectively), while in post-challenged mice; NC and LPS+LIP groups (3930 and 3016 cells/cu. mm blood, respectively) reported the highest ANC (Table 3-6).

Table 3-6: Absolute neurophil count in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Absolute Neutrophil Count: cells/cu. mm blood)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	2098 ± 63^{C}	3016 ± 134^{B}	0.01
Positive Control	1872 ± 106^{C}	1538 ± 66^{D}	N.S.
Complete Freund's Adjuvant	2136 ± 27^{C}	2596 ± 103^{B}	N.S.
Heat-Killed Bacteria	4355 ± 441^{A}	1318 ± 109^{D}	0.001
Formalin-Killed Bacteria	4623 ± 618^{A}	1471 ± 159^{D}	0.001
Lipopolysaccharides	2240 ± 67^{C}	2168 ± 99^{C}	N.S.
Liposomes	2483 ± 60^{C}	2656 ± 91^{B}	N.S.
Lipopolysaccharides+Liposomes	3637 ± 154^{B}	3930 ± 163^{A}	N.S.

Different letters: Significant difference ($P \leq 0.05$) between means of columns.
P: Probability of difference between means of pre- and post-challenge.

- **Absolute Monocyte Count (AMC)**

The observations that were made in ANC were almost noticed in AMC, which was significantly ($P \leq 0.01$) increased in post-challenged NC group (946

vs. 566 cells/cu. mm blood), and it was significantly ($P \leq 0.001$) decreased in HKB (549 *vs*. 1010 cells/cu. mm blood) and FKB (401 *vs*. 1205 cells/cu. mm blood) groups, as compared with corresponding groups in pre-challenged mice. However, it was interesting to note that post-challenged LPS+LIP mice demonstrated four-times increase in AMC as compared with the corresponding pre-challenged mice (213 *vs*. 49 cells/cu. mm blood), but the difference did not attend a significant ($P > 0.05$) level (Table 3-7).

Table 3-7: Absolute monocyte count in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Absolute Monocyte Count: cells/cu. mm blood)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	566 ± 194^{B}	946 ± 51^{A}	0.01
Positive Control	535 ± 44^{B}	329 ± 31^{BC}	N.S.
Complete Freund's Adjuvant	583 ± 49^{B}	628 ± 39^{B}	N.S.
Heat-Killed Bacteria	1010 ± 157^{A}	549 ± 61^{B}	0.001
Formalin-Killed Bacteria	1205 ± 242^{A}	401 ± 50^{BC}	0.001
Lipopolysaccharides	691 ± 45^{B}	595 ± 39^{B}	N.S.
Liposomes	585 ± 59^{B}	627 ± 62^{B}	N.S.
Lipopolysaccharides+Liposomes	49 ± 31^{C}	213 ± 76^{C}	N.S.

Different letters: Significant difference ($P \leq 0.05$) between means of columns.
P: Probability of difference between means of pre- and post-challenge.

Leukocytes are considered as the active cells in carrying out the functions of the immune system, both non-specifically and specifically, and their count may give a general picture about the function of the immune system (Lydyard and Grossi, 1998).These cells are originated in the bone marrow through two cell lineages; myeloid progenitor and lymphoid progenitor, which give rise to the five types of leukocytes (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Each type of these cells is commenced to carry out an immune function. The neutrophils are mainly involved in the innate immune system to

carry out phagocytosis (it will be discussed later), while lymphocytes represent the humoral and cellular arms of specific immunity. Monocytes are involved in carrying out phagocytosis, but they are also professional antigen presenting cells. Eosinophils are involved in allergic and inflammatory reactions, as well as, parasitic infections. Basophils release histamine, heparin and some pharmacological mediators of immunological reactions (Kramer, 2003). Due to these diverse immunological functions, the normal counts of leucocytes (total and absolute) can be deviated by infections (Ad'hiah *et al*., 2002). In agreement with such theme, both counts of leukocytes were deviated in the present study, but the deviation was subjected to the group investigated and whether pre- or post-challenged. In general, mice vaccinated with HKB or FKB showed the highest count of leukocytes in pre-challenged animals, and such increase also contributed to increased counts of lymphocytes, neutrophils and monocytes. These counts were almost approximated the counts of the other investigated post-challenged groups. However, it is worth to mention that TLC in all groups (pre- and post-challenged) approximated the normal leukocyte count range in mice, which was depicted by Everds (2007) as 2000 – 10000 cells/cu.mm.blood, and in a more recent estimation, the range was 5000 – 12000 cells/cu.mm.blood (McGarry *et al*., 2010). Therefore, it is not possible to draw a clear effect of the present vaccination protocol on total and absolute counts of leukocytes. An exception was observed in mice vaccinated with LPS, in which the ALC was significantly increased in post-challenged animals as compared with pre-challenged mice; an observation that may suggest that LPS may able to enhance the adaptive immunity against *S*. Typhimurium. Such suggestion has some support from a study carried out by Mahieu *et al*., (2006), in which they demonstrated that the wild-derived inbred mouse strain SPRET/Ei is resistant to LPS, although an enhanced immunity was observed. In addition, Dejager *et al*. (2010) demonstrated that such resistance in SPRET/Ei mice to *S*. Typhimurium infection is associated with increased leukocyte counts reaching the upper limit

of the range in the circulation and enhanced neutrophil influx into the peritoneum during the course of infection. However, when these results were compared with results in a further inbred mouse (C3H/HeN), some variations were observed, and they suggested that genetic differences account for the variation in leukocytes of mice.

3.4.2 Phagocytic Index (PI)

The PI was significantly ($P \leq 0.001$) increased in post-challenged NC (64.83 *vs*. 39.00%) and CFA (62.33 *vs*. 55.16%) mice as compared with the corresponding mice in pre-challenged groups, while an opposite observation was made in post-challenged HKB (61.16 *vs*. 66.50%) and FKB (62.13 *vs*. 66.66 %) groups. However, pre- and post-challenged LPS+LIP mice demonstrated the highest PI (73.66 and 72.50%, respectively), and without significant difference between their means (Table 3-8).

Table 3-8: Phagocytic index in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	**Mean ± S.E. (Phagocytic Index: %)**		**P ≤**
	Pre-Challenge	**Post-Challenge**	
Negative Control	39.00 ± 1.31^{D}	64.83 ± 1.30^{B}	0.001
Positive Control	67.50 ± 1.40^{B}	63.50 ± 1.72^{B}	N.S.
Complete Freund's Adjuvant	55.16 ± 1.07^{C}	62.33 ± 0.88^{B}	0.001
Heat-Killed Bacteria	66.50 ± 1.94^{B}	61.16 ± 1.57^{B}	0.01
Formalin-Killed Bacteria	66.66 ± 2.15^{B}	62.13 ± 1.04^{B}	0.01
Lipopolysaccharides	69.50 ± 1.17^{AB}	68.50 ± 0.76^{A}	N.S.
Liposomes	63.50 ± 0.76^{B}	60.83 ± 1.35^{B}	N.S.
Lipopolysaccharides+Liposomes	73.66 ± 1.14^{A}	72.50 ± 1.40^{A}	N.S.

Different letters: Significant difference ($P \leq 0.05$) between means of columns.
P: Probability of difference between means of pre- and post-challenge.

As presented, the highest percentage of phagocytosis was observed in mice vaccinated with LPS+LIP in pre- and post-challenged groups. Such finding highlights the importance of conjugation between LPS and LIP in enhancing the function of phagocytic cells. The adjuvant action of LIP in the stimulation of phagocytosis may reasoned by the fact that LIP possess many of the characteristics associated with inducers of cell-mediated responses. It is well established that the majority of LIP are taken up by phagocytic cells of the lymphoreticular system. Thus, the association of antigens with LIP provides a means of targeted delivery of the associated antigens directly to antigen-processing cells of the reticuloendothelial system (Cluff *et al*., 1987). Further, the incorporation of LPS into LIP renders the soluble LPS molecule particulate and much more hydrophobic. Furthermore, it has been shown that incorporation of LPS into phospholipids bilayers markedly reduced the adverse biological effects of lipid A. Additionally, since LIP are rapidly removed from the circulation by the reticuloendothelial system, the half-life in the blood of liposome-associated LPS is very short. Therefore, it is likely that the LPS-LIP complex is considerably less toxic than the equivalent amount of free LPS (Chhibber *et al*., 2004). After endocytosis of the LPS-LIP complex, it is assumed that disruption of the LIP bilayer must occur within the phagolysosomes to expose free LPS molecules for processing. This, along with the fact that nearly the entire antigenic mass is concentrated in the macrophage population of the reticuloendothelial system, may result in a longer retention time of the LPS and therefore prolonged antigenic stimulation (Hashioka *et al*., 2007).

3.4.3 Hypersensitivity Reactions

- **Arthus Reaction (AR) Index**

The most significant increase in Arthus reaction (AR) index was observed in post-challenged mice of LPS (1.350 *vs*. 0.466 mm; $P \leq 0.001$) groups, followed by LIP (1.150 *vs*. 0.566 mm; $P \leq 0.01$) and NC (1.050 *vs*. 0.450; $P \leq$

0.01) group, as compared with the corresponding pre-challenged groups, while an opposite observation was made in FKB group (1.019 *vs*. 2.216 mm; P ≤ 0.001). However, pre- and post-challenged LPS+LIP mice were among the highest AR values (1.683 and 1.533 mm, respectively), and without significant difference between their means (Table 3-9).

Table 3-9: Arthus reaction index in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Arthus Reaction: mm)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	0.450 ± 0.092^{C}	1.050 ± 0.180^{B}	0.01
Positive Control	1.680 ± 0.337^{B}	1.300 ± 0.096^{B}	N.S.
Complete Freund's Adjuvant	0.433 ± 0.098^{C}	1.000 ± 0.112^{B}	0.01
Heat-Killed Bacteria	1.516 ± 0.231^{B}	1.116 ± 0.142^{B}	N.S.
Formalin-Killed Bacteria	2.216 ± 0.157^{A}	1.019 ± 0.134^{B}	0.001
Lipopolysaccharides	0.466 ± 0.055^{C}	1.350 ± 0.095^{B}	0.001
Liposomes	0.566 ± 0.084^{C}	1.150 ± 0.085^{B}	0.01
Lipopolysaccharides+Liposomes	1.683 ± 0.166^{B}	1.533 ± 0.098^{A}	N.S.

Different letters: Significant difference (P ≤ 0.05) between means of columns.
P: Probability of difference between means of pre- and post-challenge.

- **Delayed Type Hypersensitivity Reaction (DTHR) Index**

The DTHR index was significantly (P ≤ 0.001) increased in post-challenged NC (1.933 *vs*. 0.400 mm) and CFA (1.450 *vs*. 0.500 mm) groups as compared with the corresponding pre-challenged groups, while an opposite observation (P ≤ 0.05) was made in FKB (1.883 *vs*. 2.350 mm) and LPS+LSO (1.932 *vs*. 2.466 mm) mice. However, the post-challenged groups showed no significant variation between their means, while in pre-challenged groups, PC, HKB, FKB, LPS and LPS+LIP mice (2.166, 1.700, 2.350, 2.050 and 2.466 mm, respectively) demonstrated the highest significant means (Table 3-10).

Table 3-10: Delayed type hypersensitivity reaction index in groups of mice pre- and post-challenged with *S.* Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Delayed Type Hypersensitivity Reaction: mm)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	0.400 ± 0.063^{C}	1.933 ± 0.066^{A}	0.001
Positive Control	2.166 ± 0.168^{A}	1.966 ± 0.142^{A}	N.S.
Complete Freund's Adjuvant	0.500 ± 0.036^{C}	1.450 ± 0.156^{A}	0.001
Heat-Killed Bacteria	1.700 ± 0.222^{A}	1.783 ± 0.218^{A}	N.S.
Formalin-Killed Bacteria	2.350 ± 0.330^{A}	1.883 ± 0.288^{A}	0.05
Lipopolysaccharides	2.050 ± 0.088^{A}	1.666 ± 0.135^{A}	N.S.
Liposomes	1.100 ± 0.139^{B}	1.500 ± 0.112^{A}	N.S.
Lipopolysaccharides+Liposomes	2.466 ± 0.142^{A}	1.945 ± 0.241^{A}	0.05

Different letters: Significant difference ($P \leq 0.05$) between means of columns.
P: Probability of difference between means of pre- and post-challenge.

In most types of post-challenged vaccinated groups, AR and DTHR responses were increased, while in pre-challenged groups showed some variations, but mice vaccinated with LPS+LIP conjugates were almost recorded the highest results. Arthus reaction is a type III hypersensitivity reaction, which is mediated by immune complex formation in a second challenge with the same antigen. The immune complexes are formed between antibodies and the challenged antigen in the area of injection. Such formation leads to the activation of the classical pathway of complement system, which in turn leads to the generation of chemo-attractants factors (C3a and C5a) that enhance the migration of neutrophils to the area of injection. As a consequence, a local inflammatory response is generated with the manifestation of a local erythema and oedema after 3-4 hours of the injection (Szalai *et al*., 2000; Cruse and Lewis, 2000).

Delayed type hypersensitivity reaction represents the fourth type of hypersensitivity reaction (Type IV), which differs from Arthus reaction in the

immunological constituents that participate in its generation. It is cell-mediated reaction, in which a specific T-helper lymphocyte, called T_{DTH}, plays a major role in its initiation together with macrophages. It occurs locally after 24-48 hours of a second challenge with the same antigen. Such time is required to activate the T_{DTH} by the antigen that is presented by macrophages, a process that requires the production of cytokines (IL-2, IFN-γ and tumour necrosis factor-β). These cytokines stimulate the migration of more macrophages to the area of injection, which in turn produce extracellular lysozymes that are responsible for the inflammatory reaction in the area of injection (Moore *et al*., 1999; Jacyan *et al*., 2001).

Accordingly, it is possible to suggest that LPS+LIP conjugate enhanced both immune responses; humoral and cellular. These findings can be justified by increased internalization of hydrophobic lipid antigens by macrophages that ultimately improved antigen presentation to cells. These macrophages function as antigen-presenting cells which take up antigen, catabolize them, and express the antigenic determinants in an energy requiring process before being presented to antigen specific T-cells (Chhibber *et al*., 2004. It is also possible that these LPS+LIP activated macrophages contribute towards positive regulating effects on the induction of specific immune response. Thus, the results of the present study elucidate that incorporation of *S*. Typhimurium LPS into LIP not only makes it possible to achieve a considerable immune response to lipid A-based immunogens by circumventing toxicity associated with lipid A, but also to convert polysaccharide antigens into thymus-dependent antigens (Hashioka *et al*., 2007).

3.5 Bacterial Isolation from Liver and Spleen

The liver and spleen of pre- (day 15) and post-challenged (day 29) groups of mice were the bacteriologically evaluated. For pre-challenged groups, only PC group demonstrated a heavy growth (score 3), while the other seven showed

no growth (score 0). However, for post-challenged groups some variations were observed and they are given in table 3-11. For liver; NC, PC, HKB and FKB groups shared a similar score (3: heavy growth), and CFA and LIP groups also shared a similar score, but at a lower level (2: moderate growth). However, in LPS and LPS+LIP groups, the scores were 1 (scanty growth) and 0 (no growth), respectively. The observations in spleen were almost similar, with the exception that mice vaccinated with LPS+LIP showed scant growth (score 1).

Table 3-11: Scores of bacterial Isolation from Liver and Spleen of groups of mice post-challenged with *S.* Typhimurium after different vaccination protocols.

Groups	Bacterial Isolation Score (Four Mice Each)	
	Liver	Spleen
Negative Control	3	3
Positive Control	3	3
Complete Freund's Adjuvant	2	2
Heat-Killed Bacteria	3	2
Formalin-Killed Bacteria	3	2
Lipopolysaccharides	1	1
Liposomes	2	2
Lipopolysaccharides+Liposomes	0	1

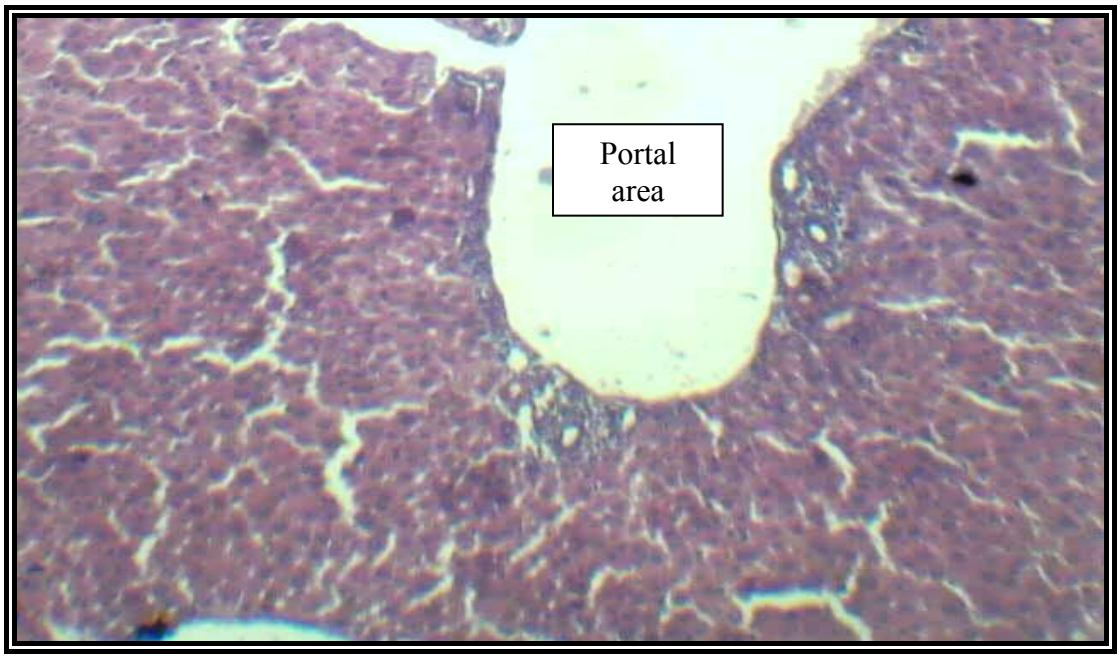
0: No growth; 1: Scanty growth; 2: Moderate growth; 3: Heavy growth.

The immune enhancement potential of LPS+LIP observed in phagocytosis and delayed type hypersensitivity reactions is sustained by the results of bacterial isolation from liver and spleen, in which the bacterial recovery after challenge with live *S.* Typhimurium in post-challenge mice vaccinated with LPS+LIP conjugate, was almost not detected and clearance of the bacteria by the immune system could have been occurred. Therefore, it is possible to hypothesized that the improved host defense of LPS+LIP vaccinated mice is mediated by immune mechanisms, in which macrophages and neutrophils influenced a critical role in controlling invading pathogens by phagocytosis and

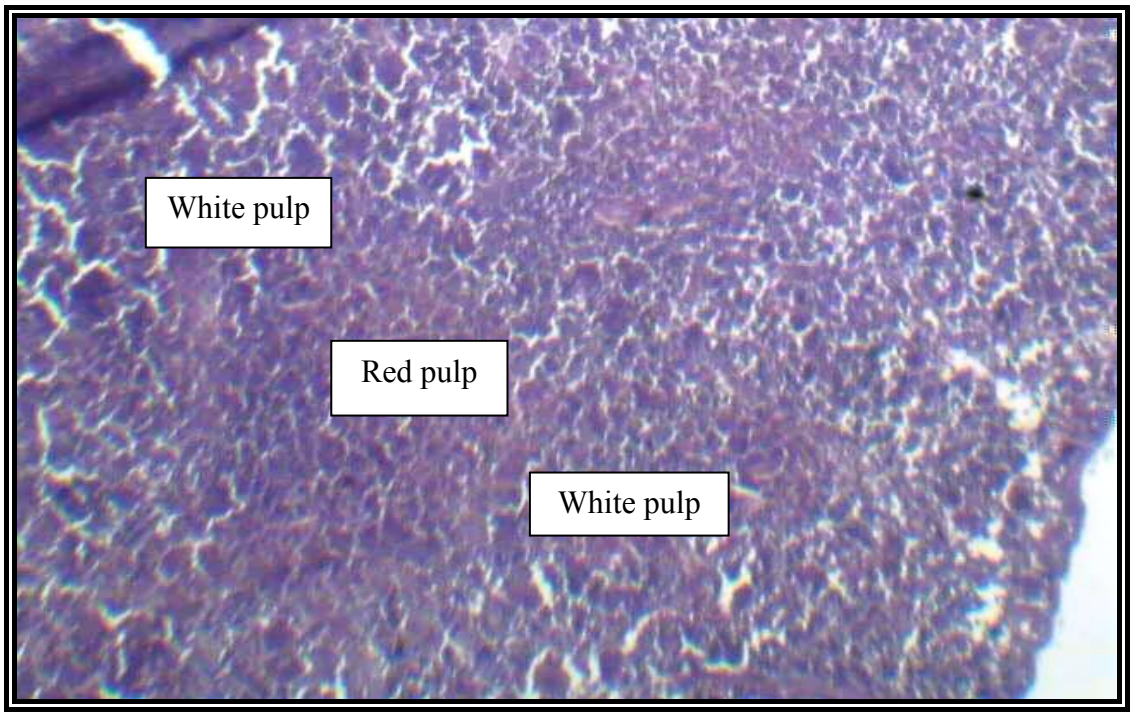
production of antimicrobial proteins. This has been demonstrated by Lehner *et al*. (2001), who showed that endotoxin-tolerant mice showed increased resistance to *S*. Typhimurium and that this resistance was caused by increased peritoneal accumulation of neutrophils. Furthermore, it has been demonstrated an elevated influx of polymorphonuclear cells in the peritoneal cavity, and this has been suggested to contribute to enhanced control of bacterial replication and, thus, increased resistance (Dejager *et al*., 2010). Furthermore, LPS-LIP conjugates are potent B-cell mitogens, and also activate T cells to produce IFN- and TNF and thereby enhance cellular immune responses. The major structural element responsible for their toxicity and adjuvant effect is Lipid A. In low acid conditions, lipid A can be hydrolysed to obtain monophosphoryl lipid A, a compound which retains the adjuvant activity of Lipid A with reduced toxicity, and the role of LIP in this regard is extend the half-life of antigens in blood ensuring a higher antigen exposure to antigen presenting cells after vaccination (Petrovsky and Aguilar, 2004). It has also been aimed to assess the contribution of neutrophils in the clearance of bacterial infection in a mouse model of tolerance to LPS. After tolerance was developed, Verónica *et al*. (2012) investigated *in vivo* different mechanisms of bacterial clearance. The elimination of a locally induced polymicrobial challenge was more efficient in tolerant mice. This was related to a higher number of neutrophils migrating to the infectious site. They finally suggested that the higher chemotactic response from an increased neutrophils marginal pool enhanced forming capacity is the main mechanisms mediating bacterial clearance in tolerant mice.

3.6 Histopathological Evaluation of Liver and Spleen

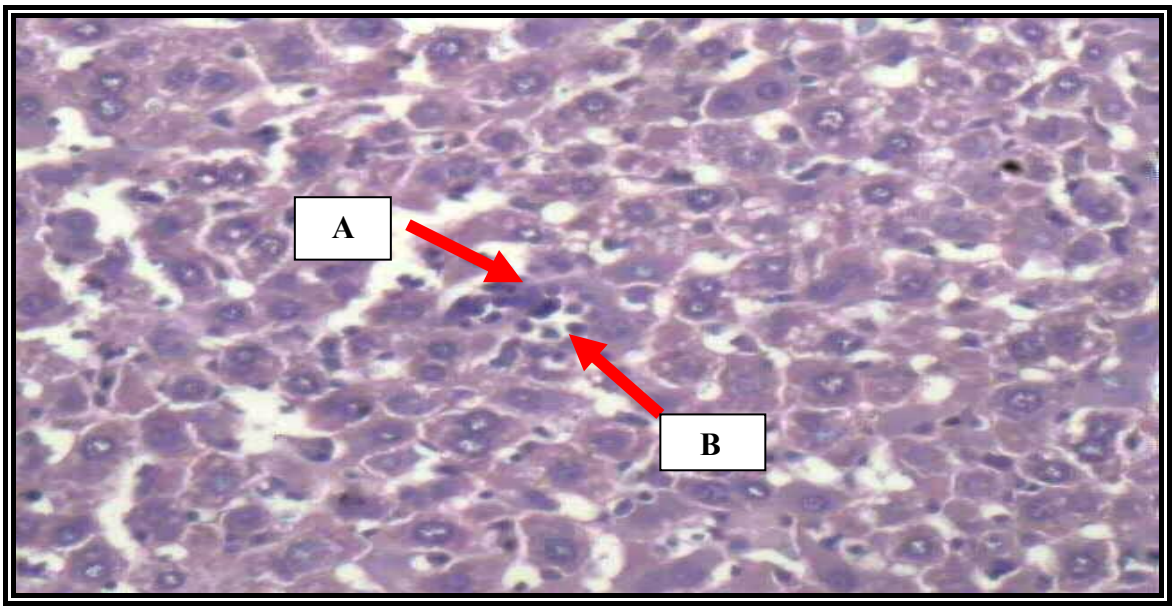
Different histopathological changes were observed in liver and spleen of pre- (day 15) and post-challenged (day 29) groups of mice, but such changes were subjected to the type of group and if it was pre- or post-challenged. For the ease of presentation, under each picture, the histopathological profile is given.



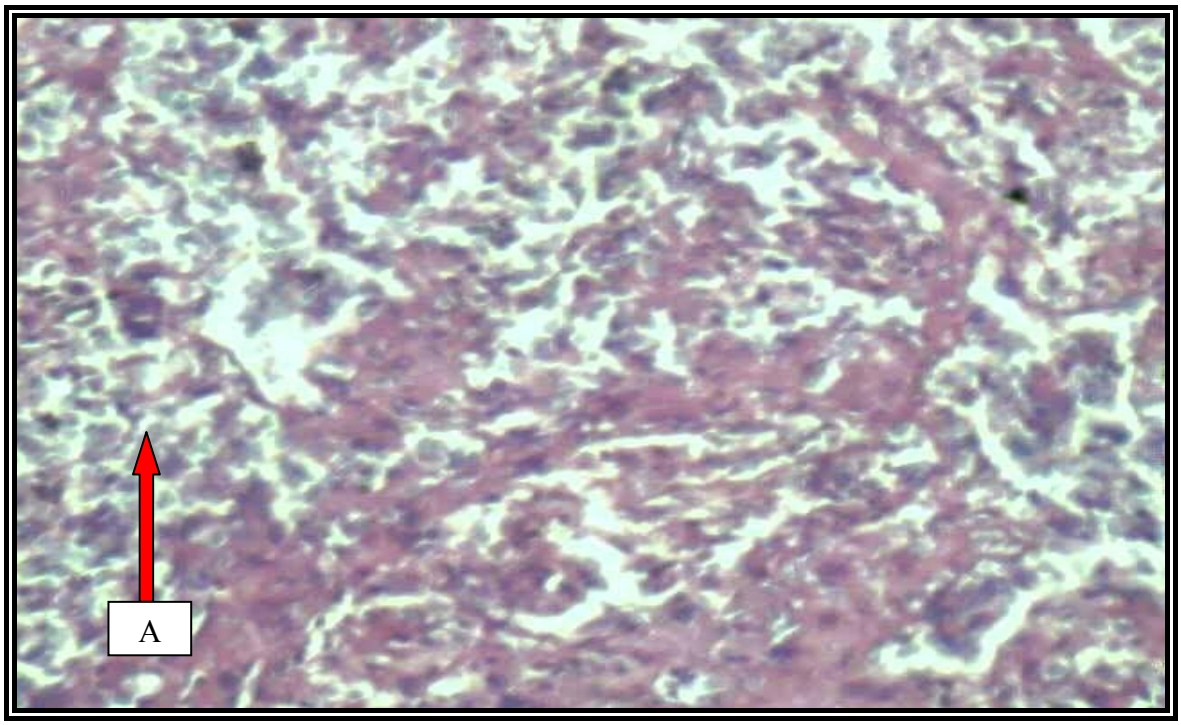
Picture 3-1: Histological section of mouse liver (pre-challenged negative control) showing normal looking hepatocytes (H and E; 200X).



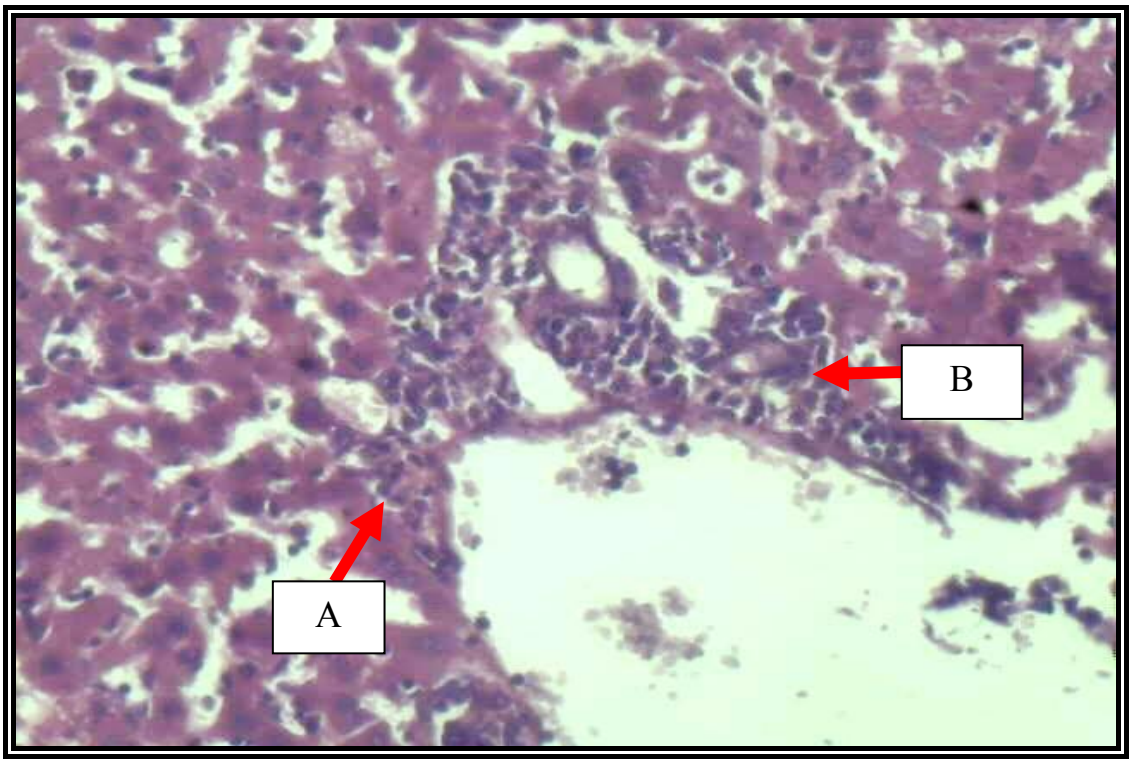
Picture 3-2: Histological section of mouse spleen (pre-challenged negative control) showing normal structure appearance of white and red pulp of the lymphoid parenchymal tissue (H and E; 200X).



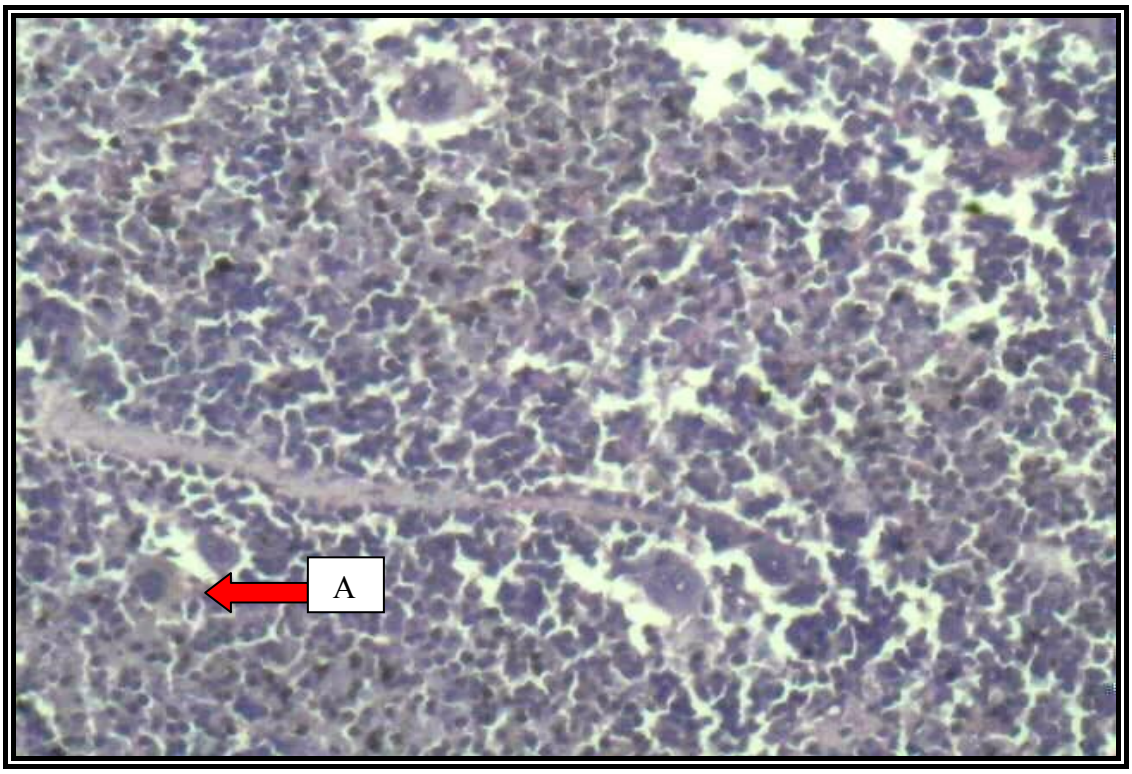
Picture 3-3: Histological section of mouse liver (pre-challenged positive control) showing degenerative change and necrosis (A) with inflammatory cells infiltration (B) (H and E; 250X).



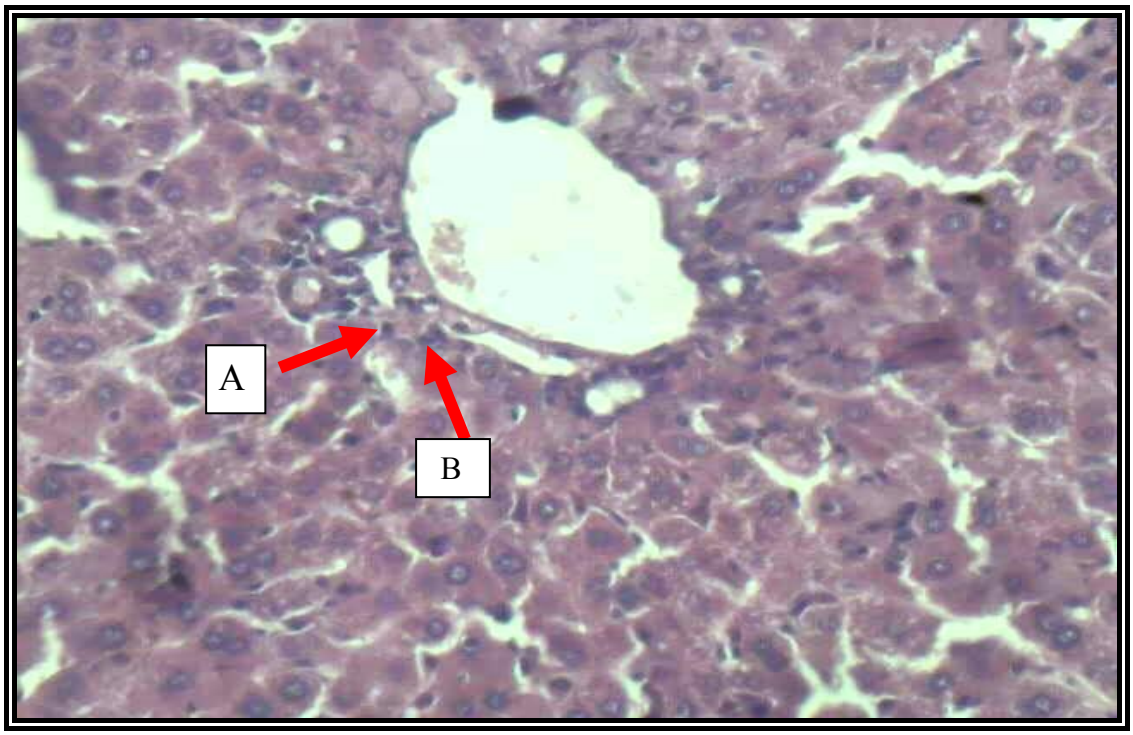
Picture 3-4: Histological section of mouse spleen (pre-challenged positive control) showing diffuse hyperplasia of the lymphoid parenchymal tissue(A) (H and E; 200X).



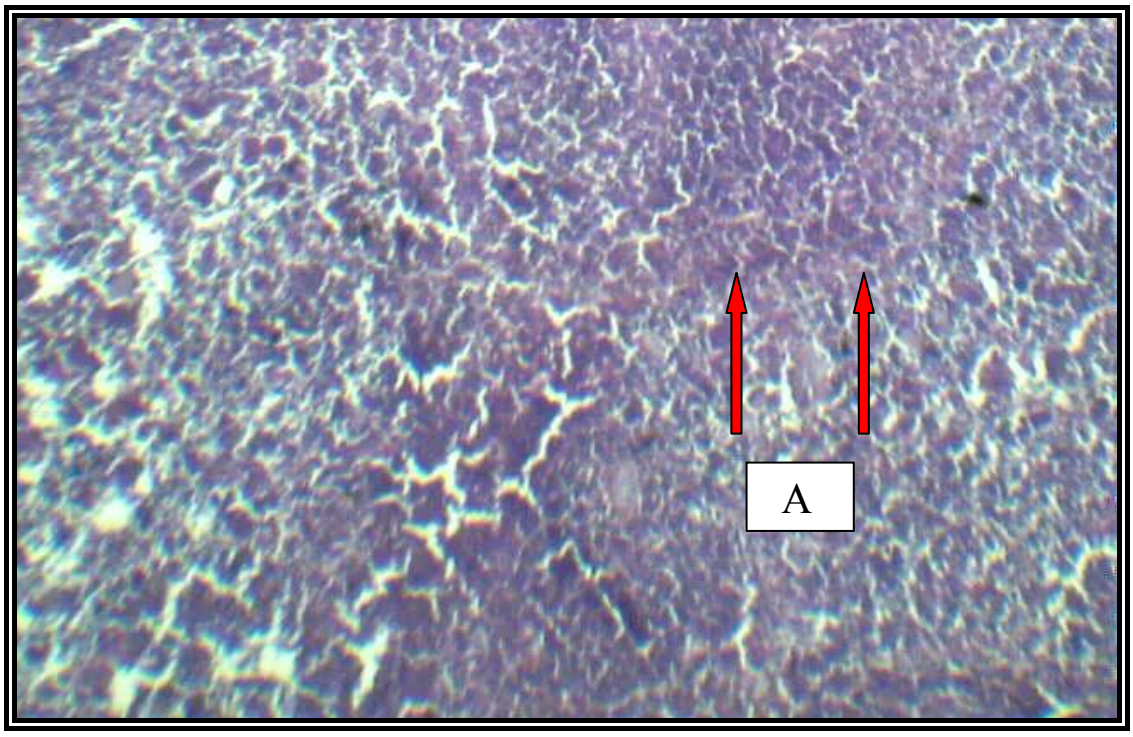
Picture 3-5: Histological section of mouse liver (pre-challenged heat-killed bacteria group) showing area of necrosis (A) with mild inflammatory cell infiltration (B) (H and E; 200X).



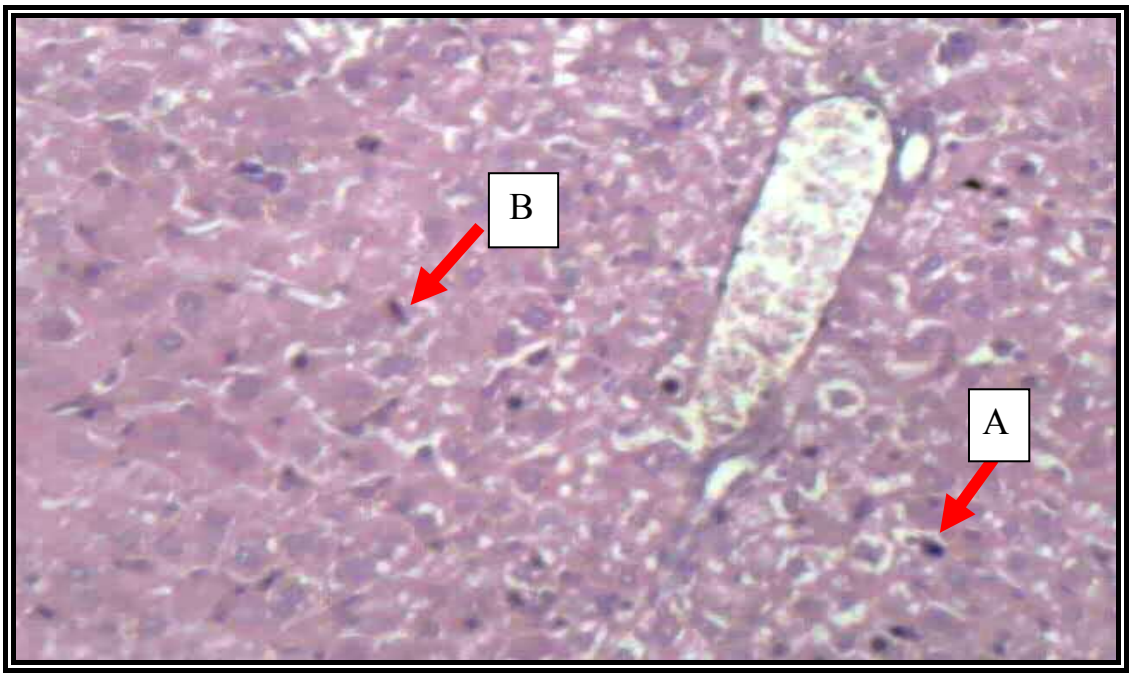
Picture 3-6: Histological section of mouse spleen (pre-challenged heat-killed bacteria group) showing diffuse hyperplasia of the lymphoid parenchymal tissue and presence of megakaryocytes(A) (H and E; 200X).



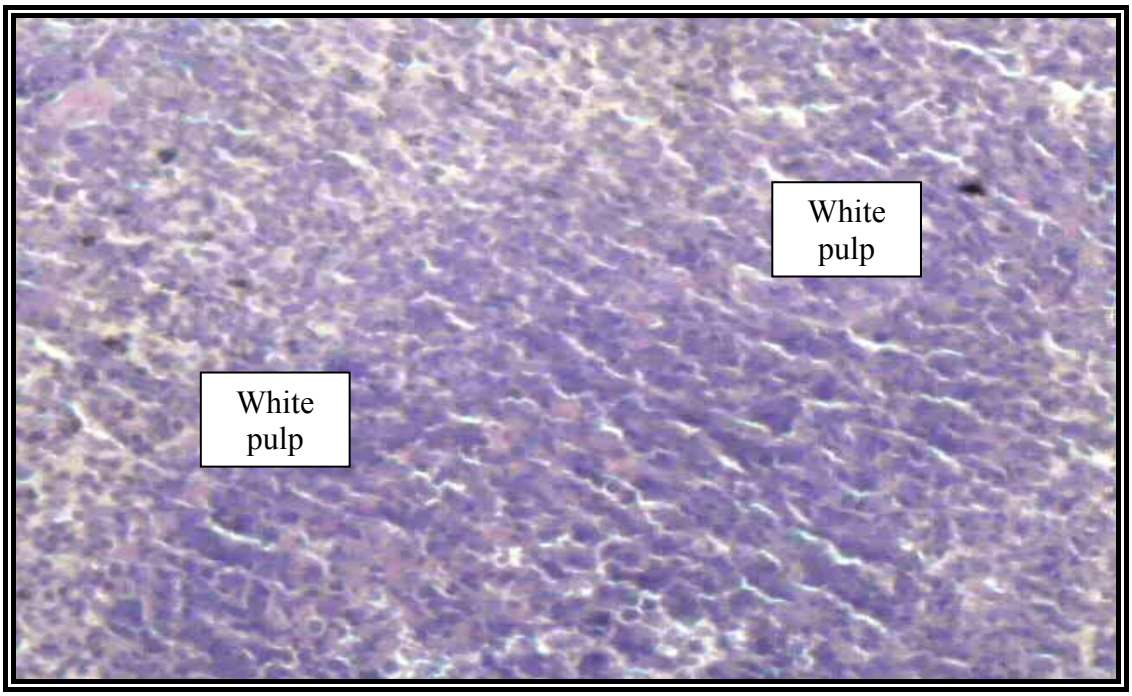
Picture 3-7: Histological section of mouse liver (pre-challenged formalin-killed bacteria group) showing mild degenerative changes and necrosis (A) with mononuclear cells infiltration (B) especially near the portal area (H and E; 200X).



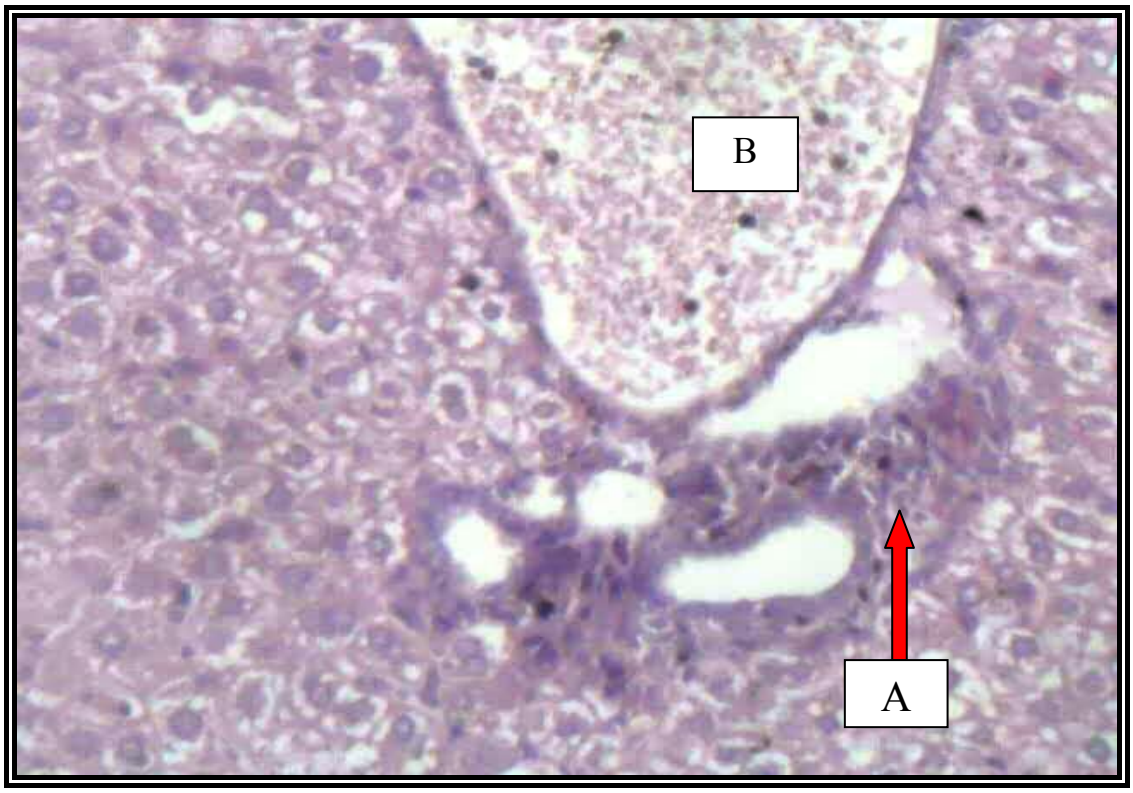
Picture 3-8: Histological section of mouse spleen (pre-challenged formalin-killed bacteria group) showing diffuse hyperplasia of the lymphoid parenchymal tissue(A) (H and E; 200X).



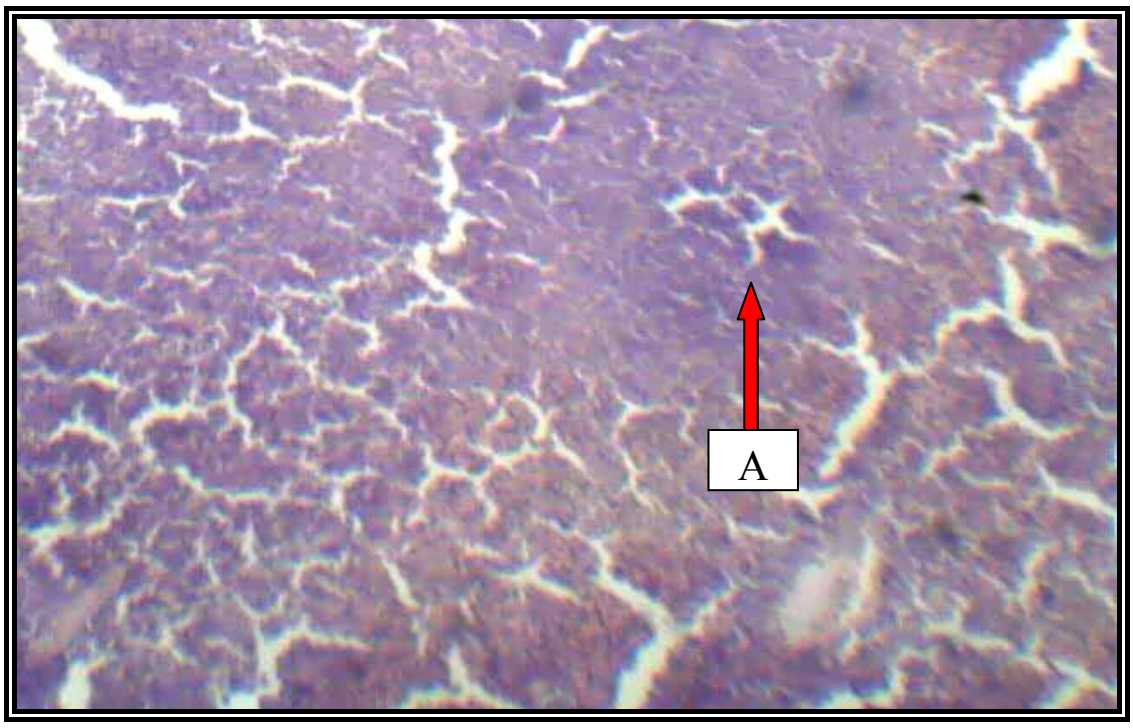
Picture 3-9: Histological section of mouse liver (pre-challenged complete Freund's adjuvant group) showing mild degenerative changes (A) with kupffer cells (B) hyperplasia (H and E; 200X).



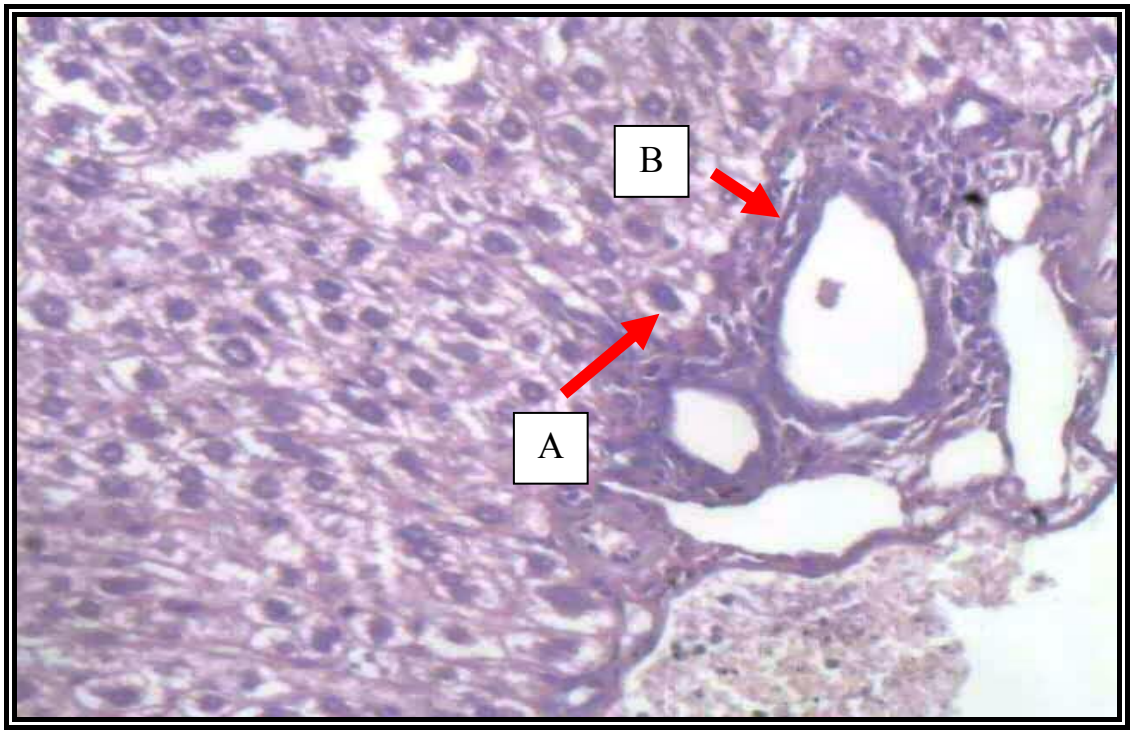
Picture 3-10: Histological section of mouse spleen (pre-challenged complete Freund's adjuvant group) showing follicular hyperplasia with widening of white pulp (H and E; 200X).



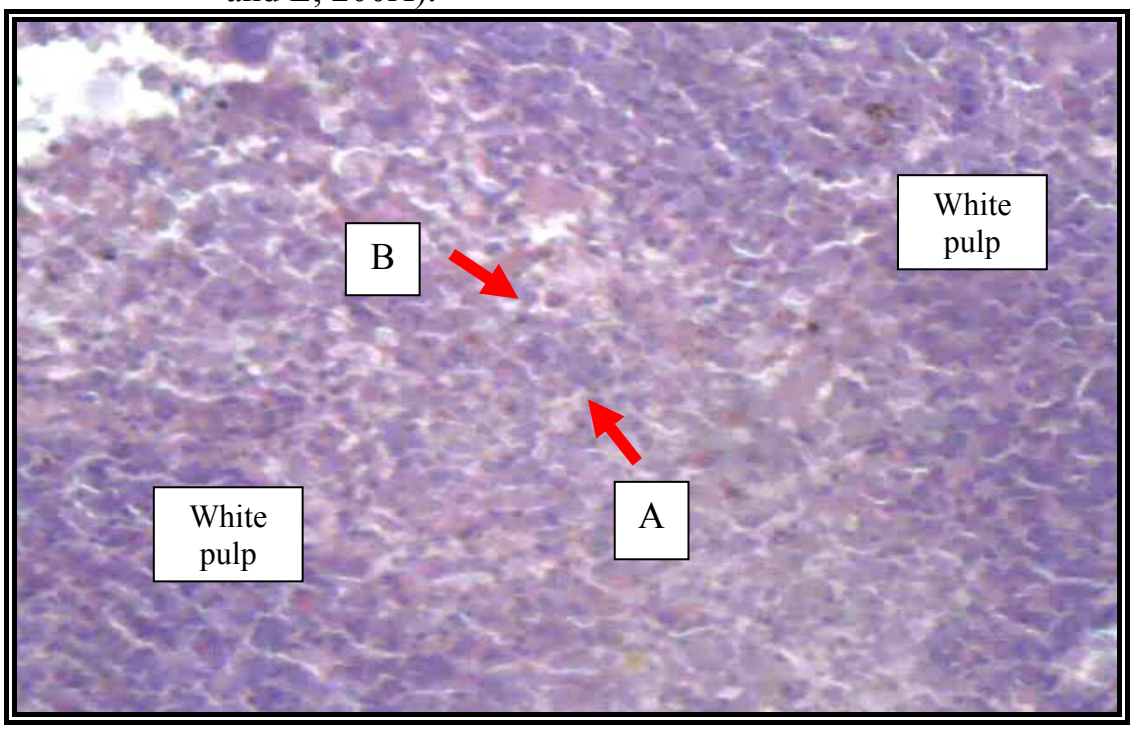
Picture 3-11: Histological section of mouse liver (pre-challenged liposome group) showing mild inflammatory cells infiltration(A) especially in portal area and congestion of blood vessel (A) (H and E; 200X).



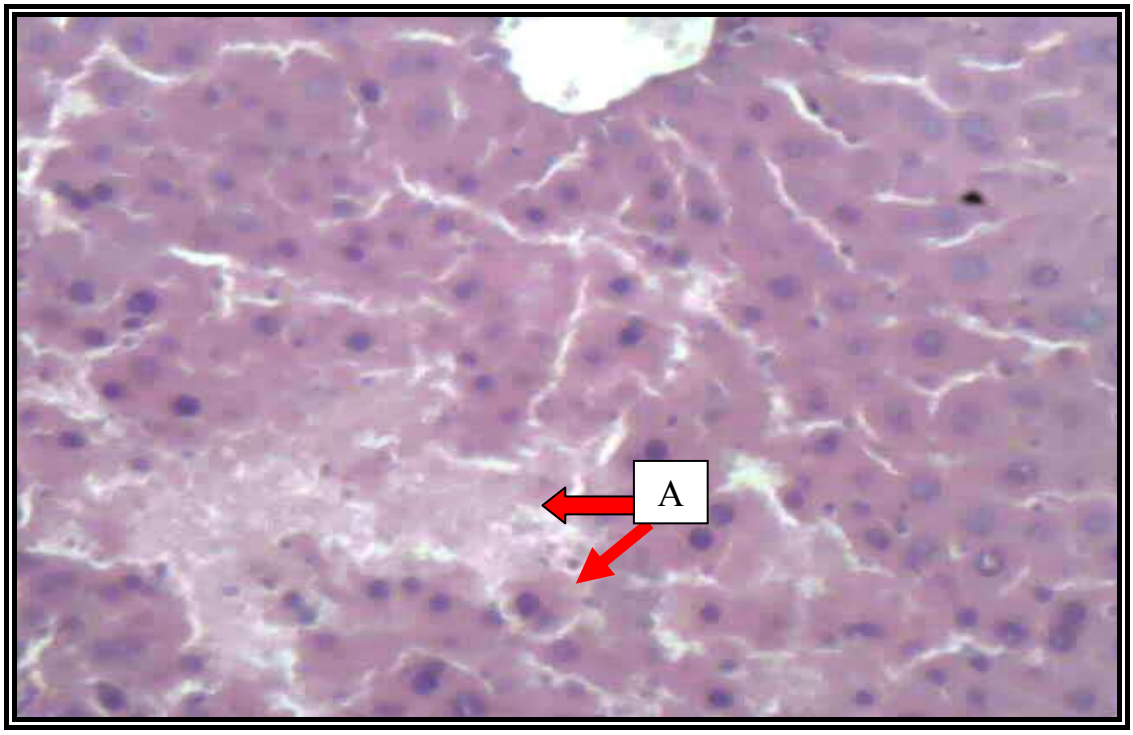
Picture 3-12: Histological section of mouse spleen (pre-challenged liposome group) showing area of necrosis with mild follicular hyperplasia (A) (H and E; 200X).



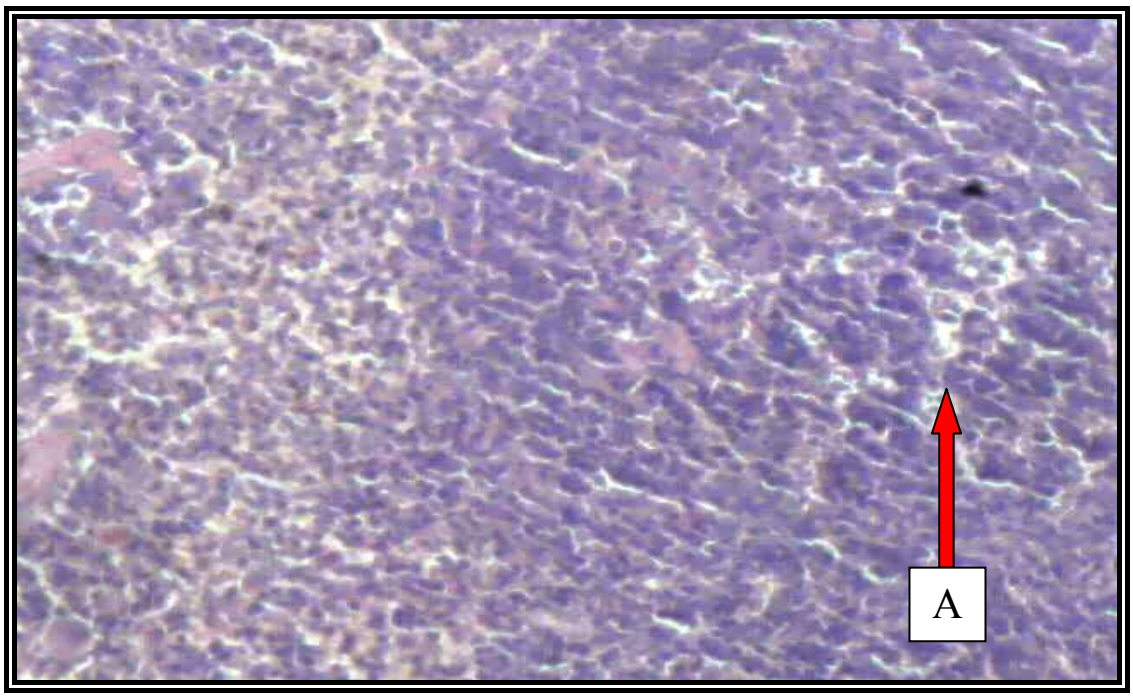
Picture 3-13: Histological section of mouse liver (pre-challenged lipopolysaccharide group) showing disperse necrotic cell (A) with mononuclear cells infiltration near the portal area (B) (H and E; 200X).



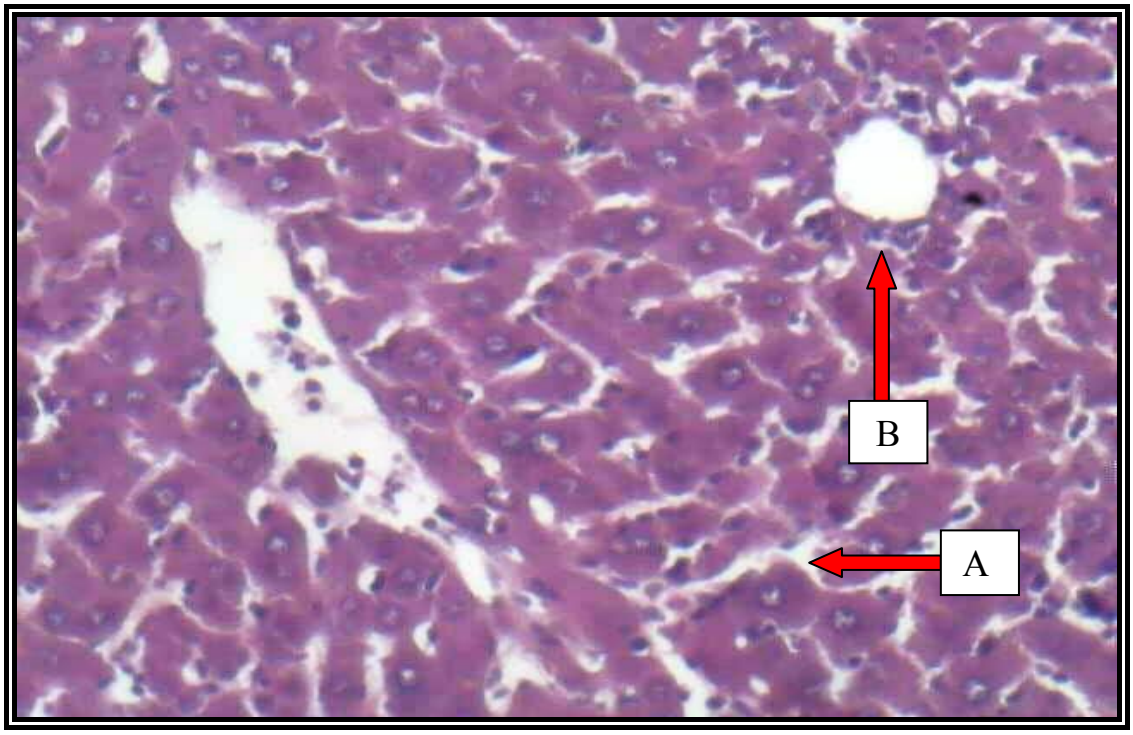
Picture 3-14: Histological section of mouse spleen (pre-challenged lipopolysaccharide group) showing certain area of splenic paranchymal tissue necrosis (A) with inflammatory cells infiltration (B) especially in region area in red pulp(B) (H and E; 200X).



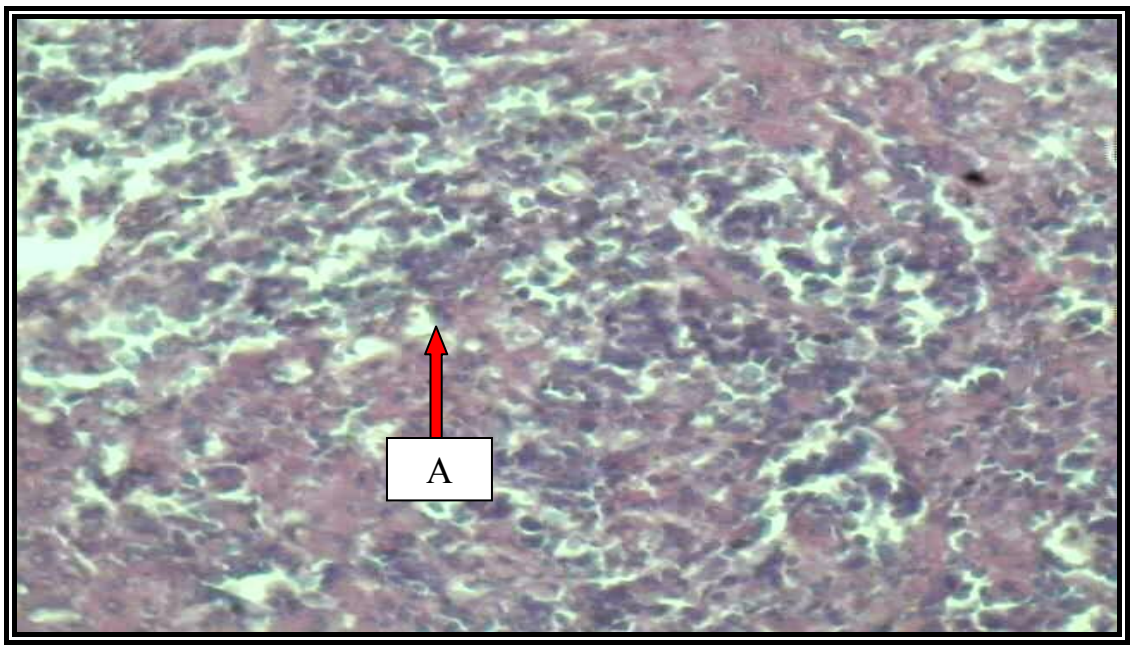
Picture 3-15: Histological section of mouse liver (pre-challenged lipopolysaccharide+lipsome conjugate group) showing certain necrosis area (A) of hepatic paranchymal tissue (H and E; 200X).



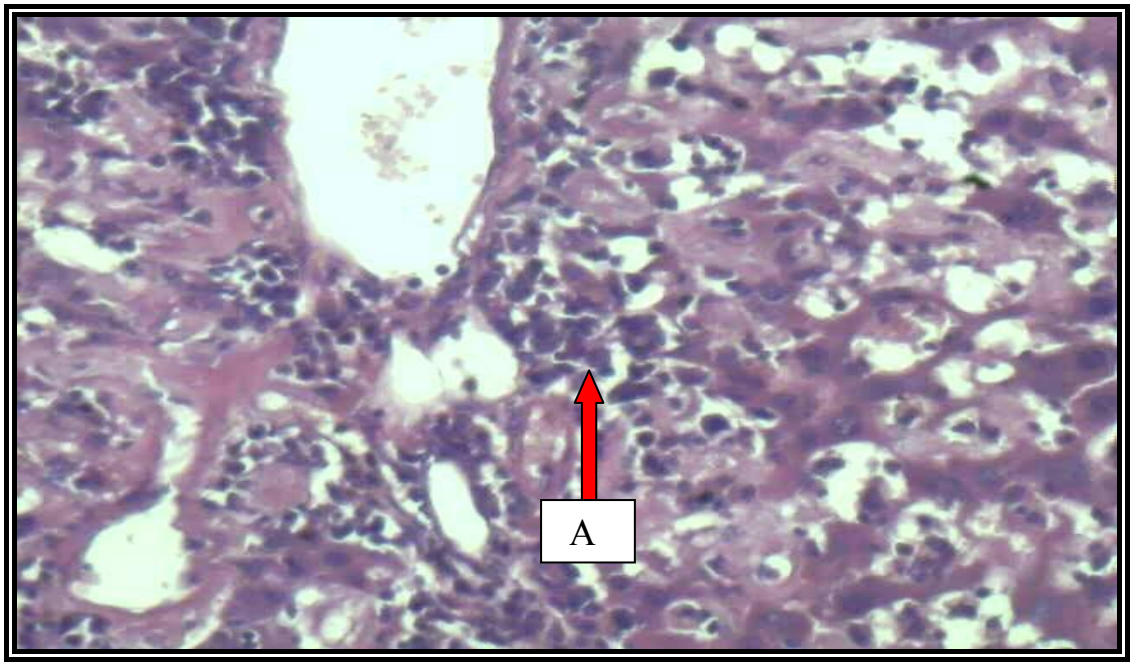
Picture 3-16: Histological section of mouse spleen (pre-challenged lipopolysaccharide+lipsome conjugate group) showing widening of white pulp (A)and follicular lymphoid hyperplasia (H and E; 200X).



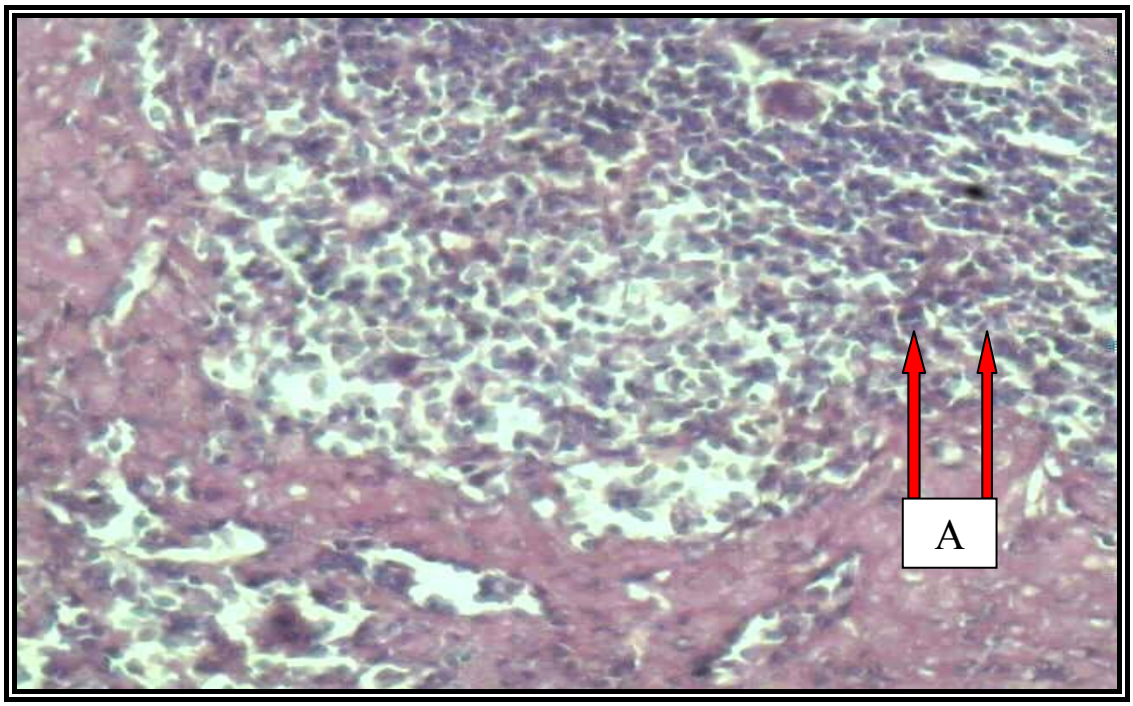
Picture 3-17: Histological section of mouse liver (post-challenged negative control group) showing mild degenerative changes, dilatation of sinusoid(A) and mild inflammatory cells infiltration(B) (H and E; 200X).



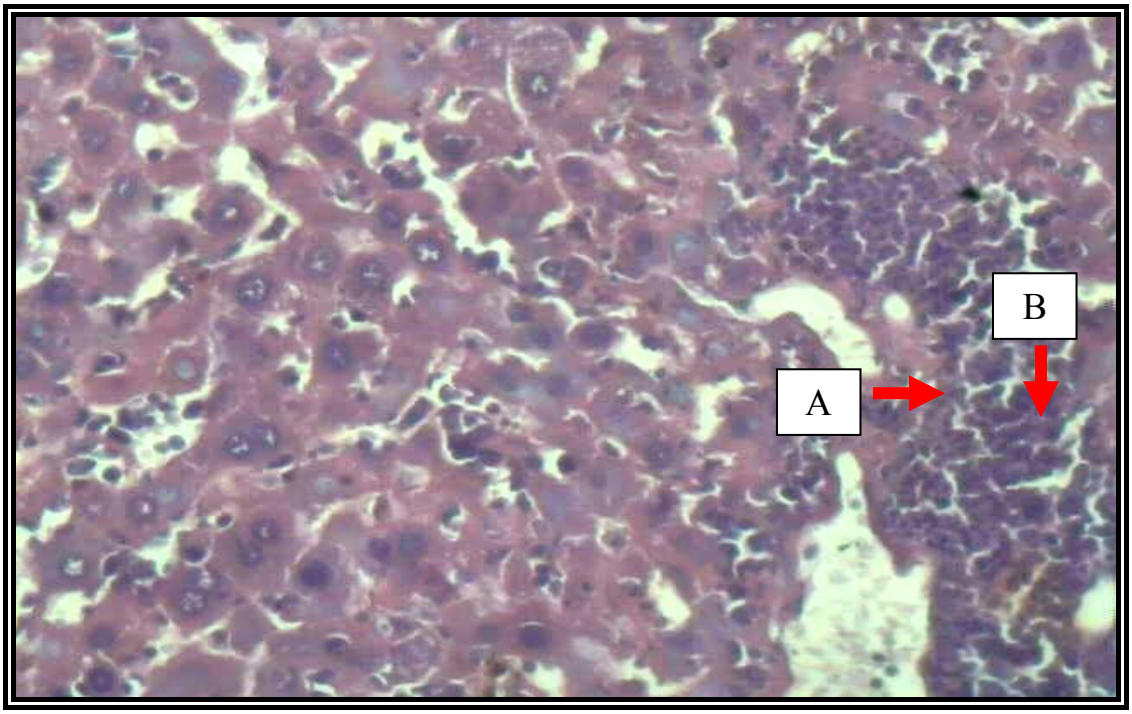
Picture 3-18: Histological section of mouse spleen (post-challenged negative control group) showing follicular lymphoid hyperplasia with mild infiltration of inflammatory cells in red pulp(A) (H and E; 200X).



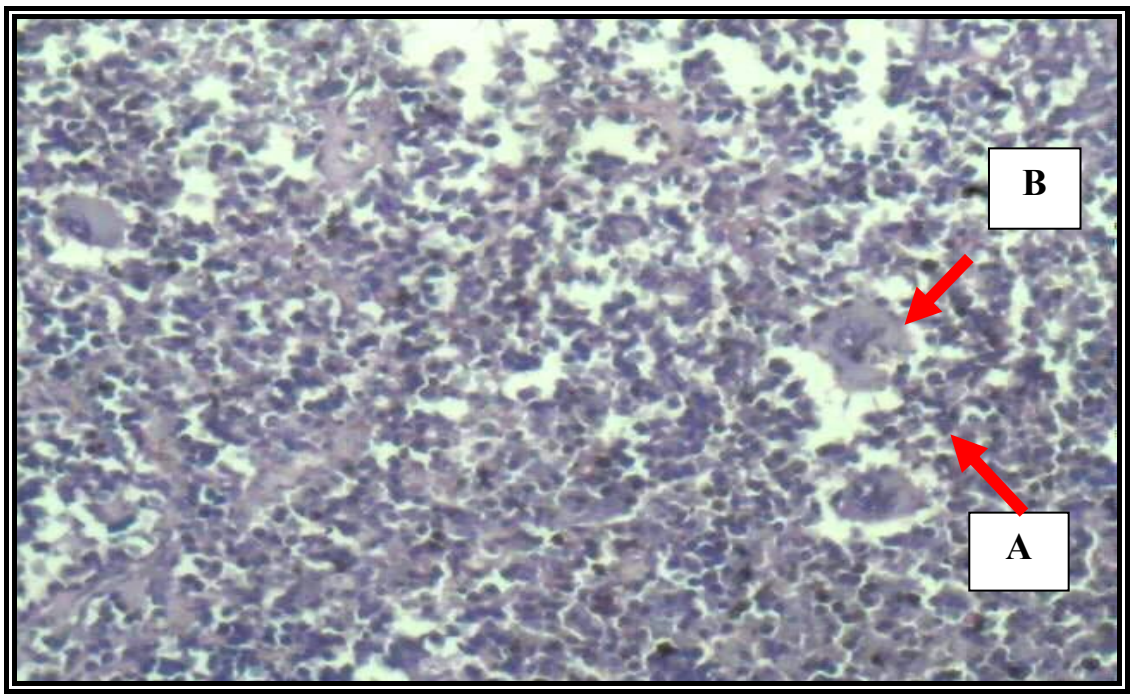
Picture 3-19: Histological section of mouse liver (post-challenged positive control group) showing necrosis of hepatocytes with heavy inflammatory cells infiltration(A) (H and E; 200X).



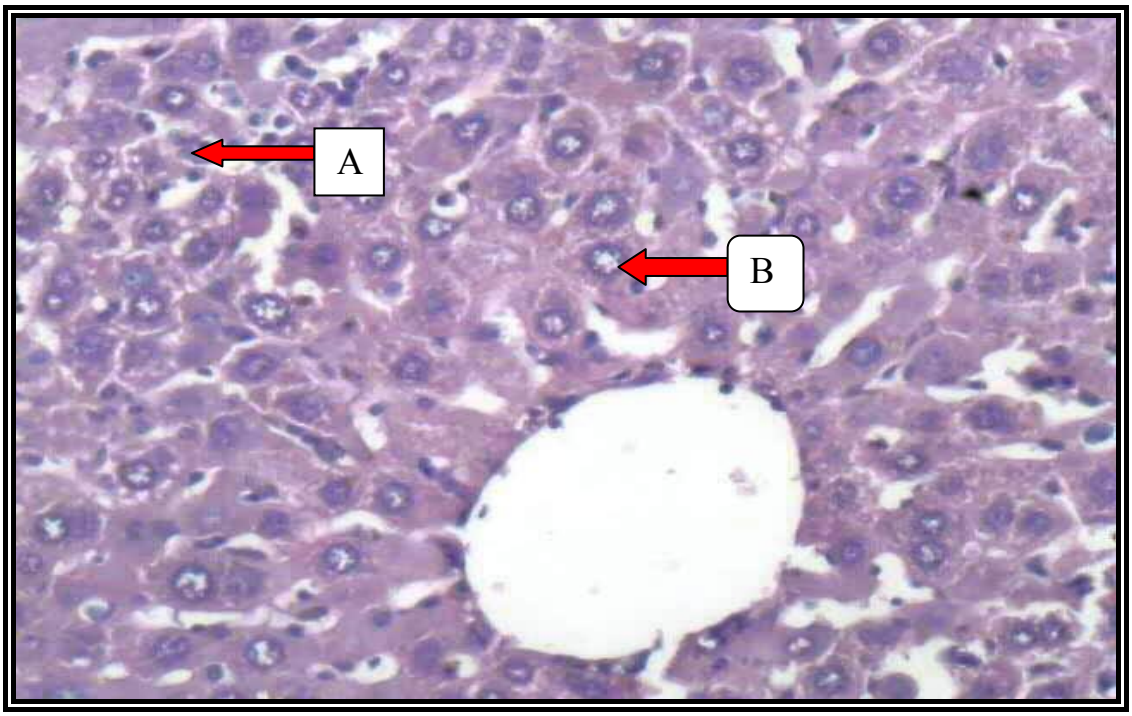
Picture 3-20: Histological section of mouse spleen (post-challenged positive control group) showing degenerative changes and necrosis of the lymphoid paranchymal tissue (H and E; 200X).



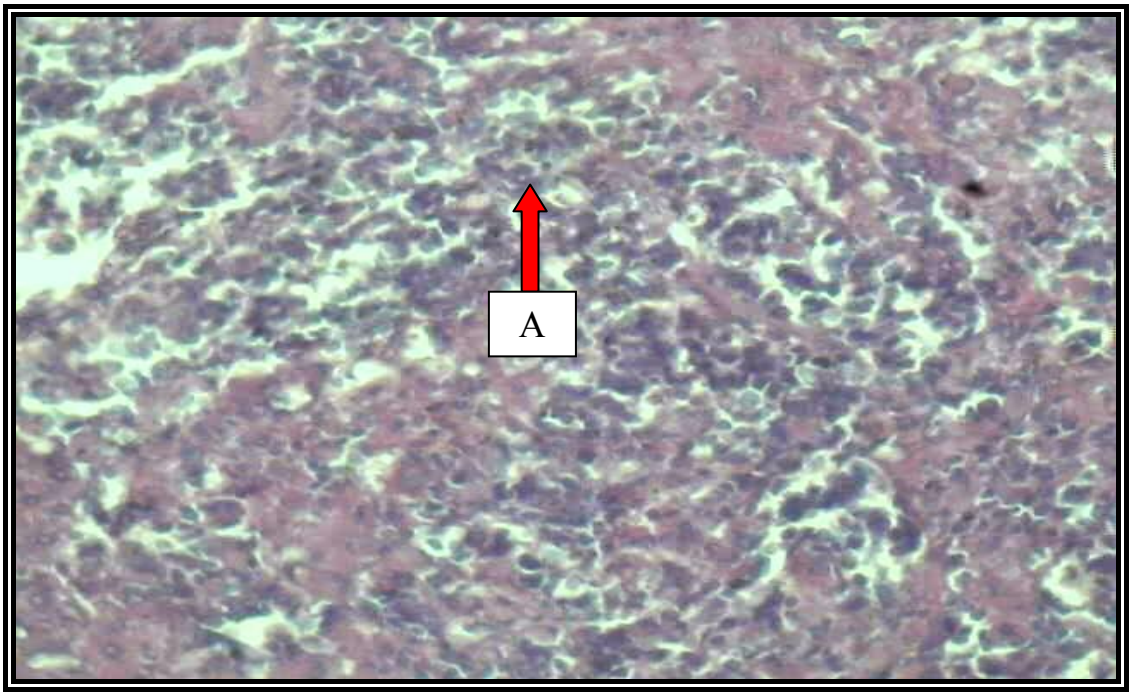
Picture 3-21: Histological section of mouse liver (post-challenged heat-killed bacteria group) showing certain degenerative changes and necrosis (A) with heavy mononuclear cells infiltration (B) (H and E; 200X).



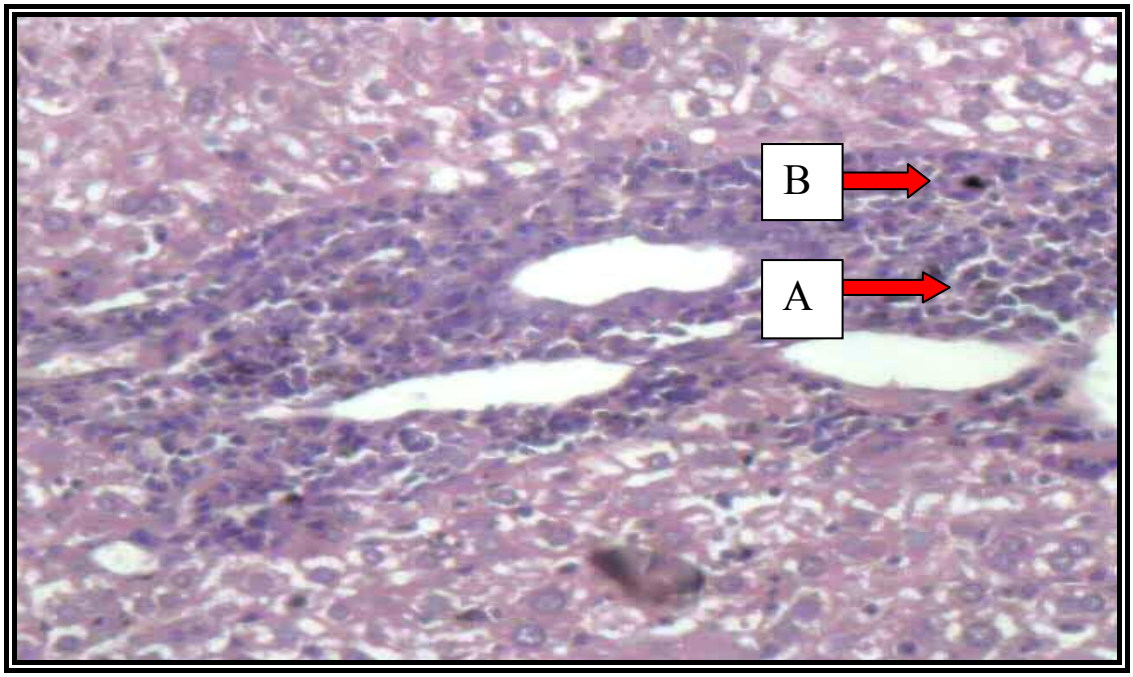
Picture 3-22: Histological section of mouse spleen (post-challenged heat-killed bacteria group) showing necrosis(A)with infiltration of inflammatory cells and presence of megakaryocytes (B) (H and E; 200X).



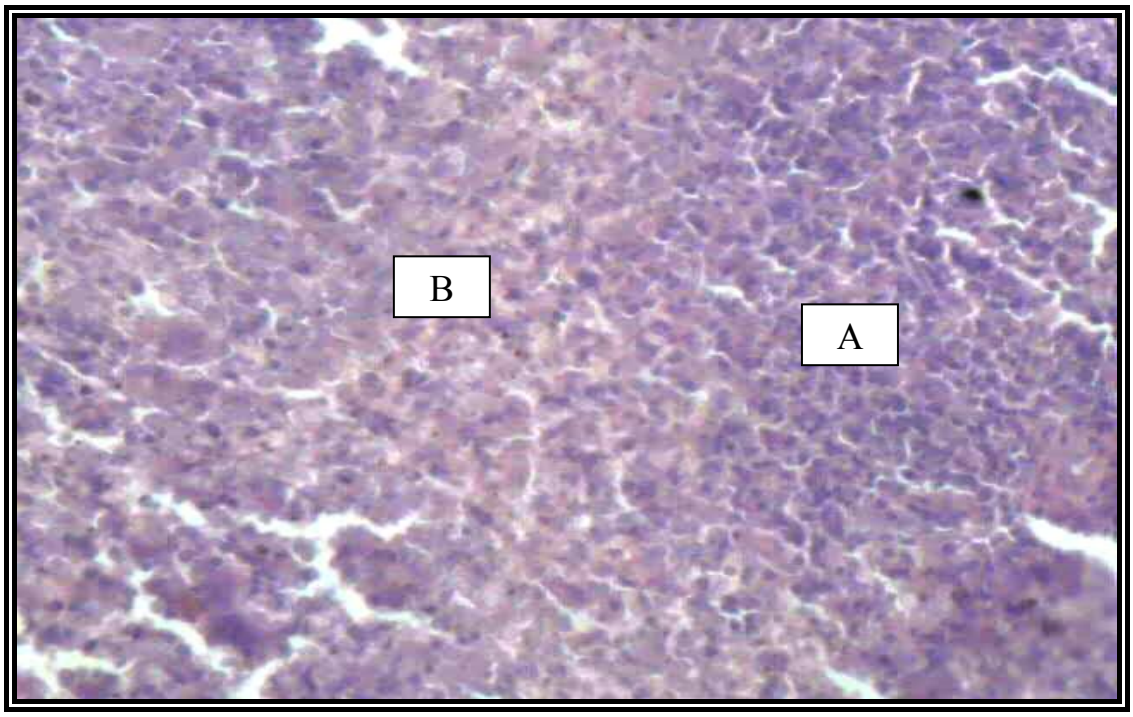
Picture 3-23: Histological section of mouse liver (post-challenged formalin-killed bacteria group) showing degenerative and necrosis changes with mononuclear cells infiltration(B) (H and E; 200X).



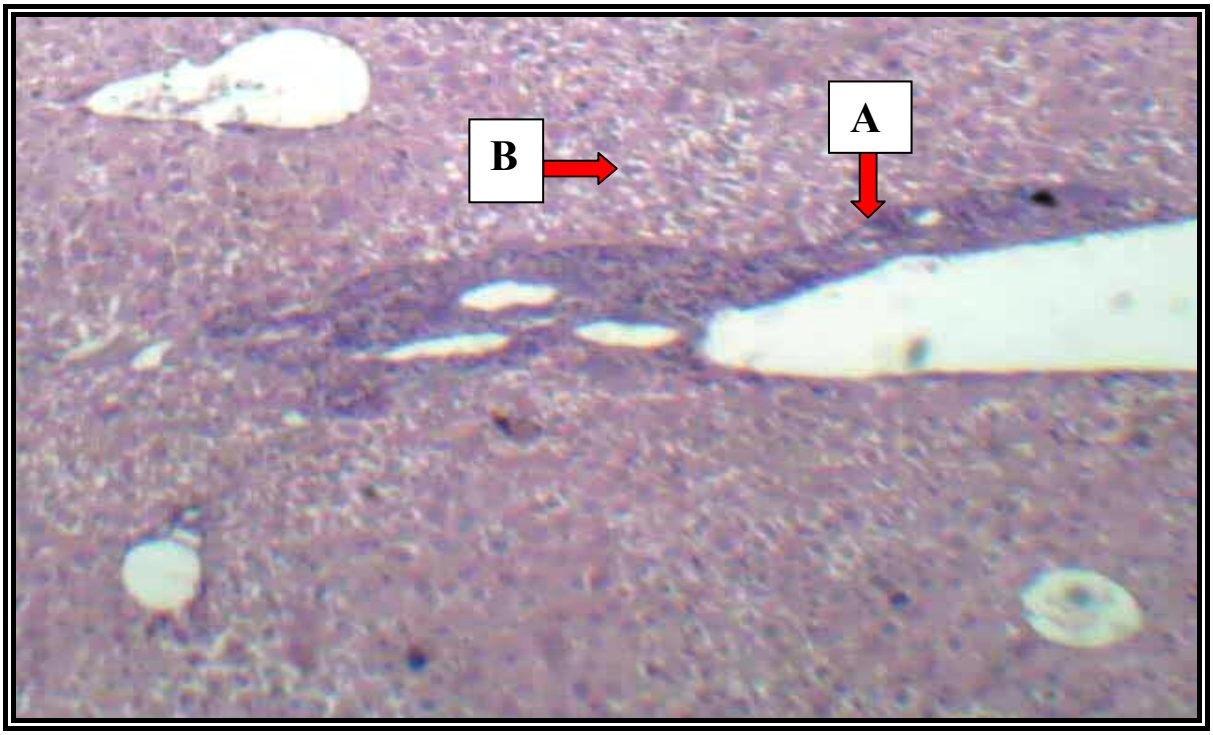
Picture 3-24: Histological section of mouse spleen (post-challenged formalin-killed bacteria group) showing certain necrosis areas of the paranchymal tissue with evidence of fibrosis(A) (H and E; 200X).



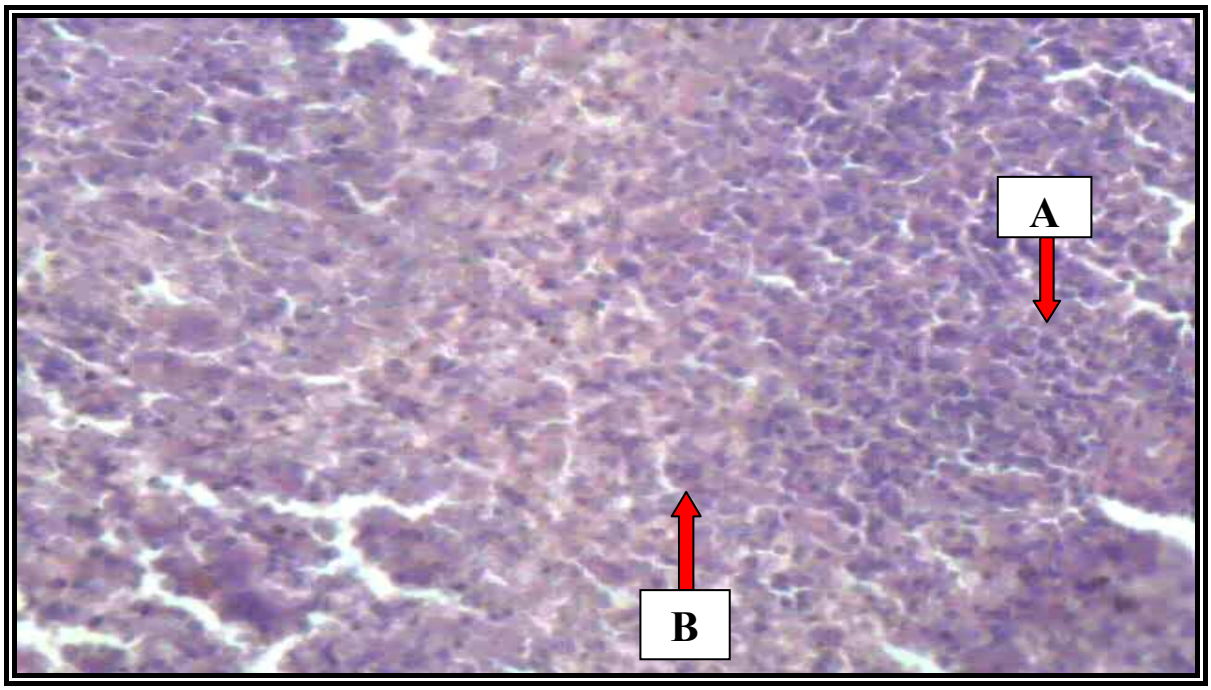
Picture 3-25: Histological section of mouse liver (post-challenged complete Freund's adjuvant group) showing certain cell necrosis of hepatocytes(A) with mononuclear inflammatory cells infiltration especially around portal area(B) (H and E; 200X).



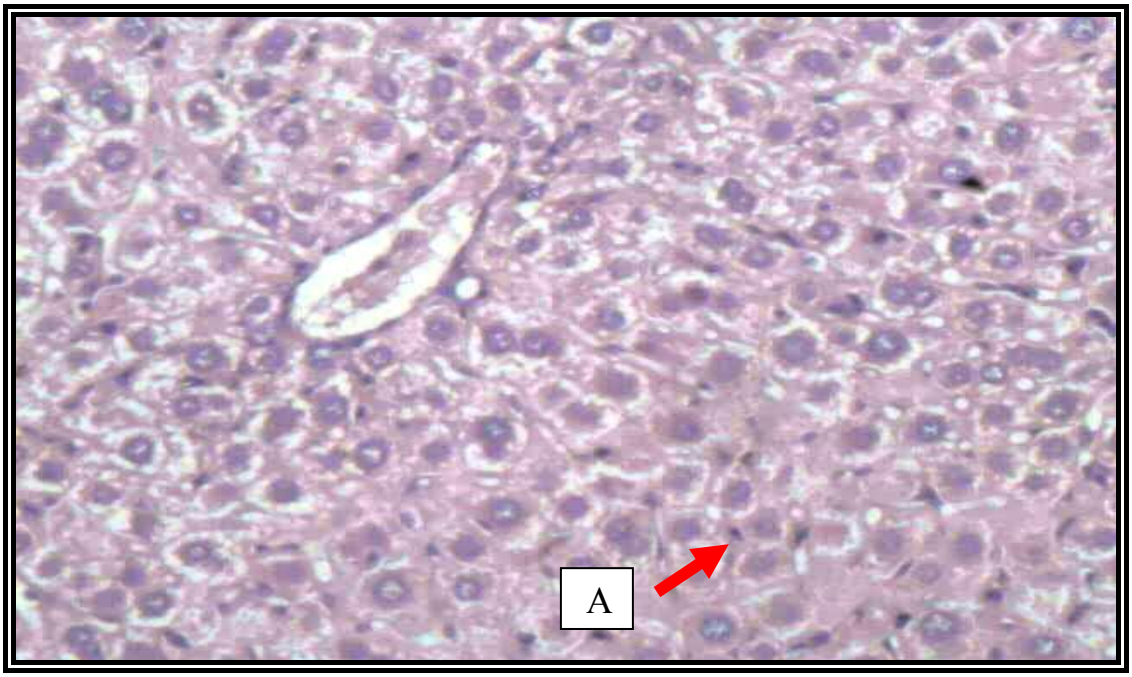
Picture 3-26: Histological section of mouse spleen (post-challenged complete Freund's adjuvant group) showing follicular lymphoid hyperplasia (A) with mild infiltration of inflammatory cells in red pulp (B) (H and E; 200X).



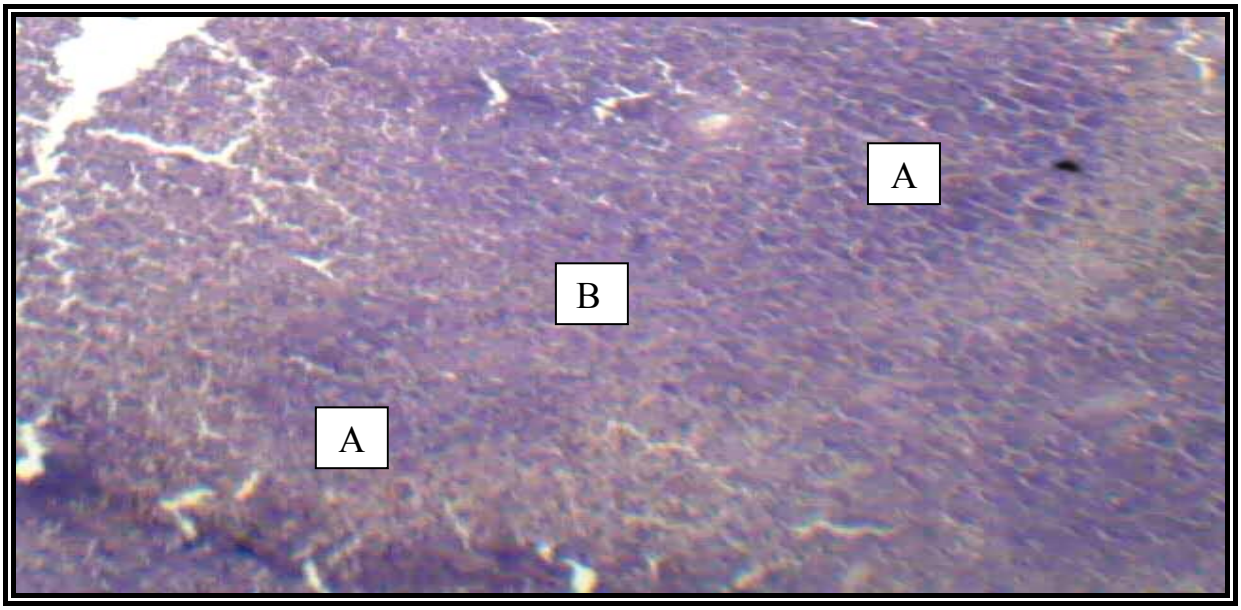
Picture 3-27: Histological section of mouse liver (post-challenged liposome group) showing certain cell necrosis of hepatocytes(A) and degenerative change with mononuclear cell infiltration(B) (H and E;100X).



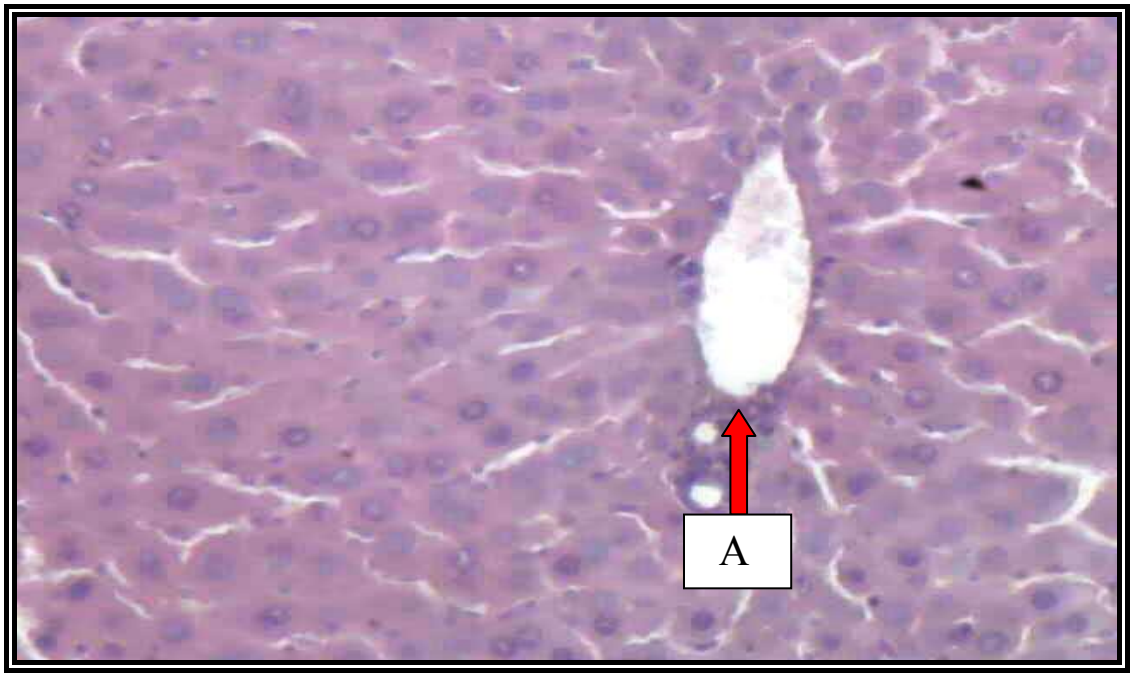
Picture 3-28: Histological section of mouse spleen (post-challenged liposome group) showing follicular lymphoid hyperplasia(A) with mild inflammatory cells infiltration(B) (H and E; 200X).



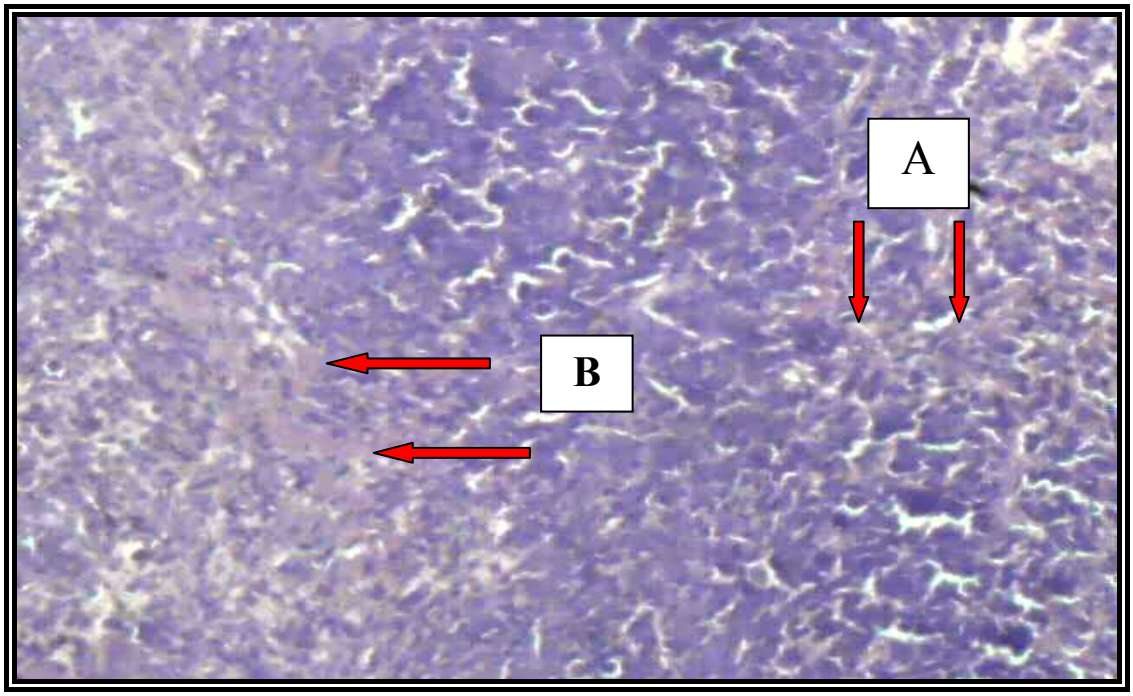
Picture 3-29: Histological section of mouse liver (post-challenged lipopolysaccharide group) showing look-like normal structure with kupffer cells (A) hyperplasia (H and E; 200X).



Picture 3-30: Histological section of mouse spleen (post-challenged lipopolysaccharide group) showing follicular hyperplasia and widening in white pulp (A) with reduction of red pulp (B) (H and E; 200X).



Picture 3-31: Histological section of mouse liver (post-challenged lipopolysaccharide+liposome conjugate group) showing look-like normal hepatic tissue appearance especially near portal area((A) (H and E; 200X).



Picture 3-32: Histological section of mouse spleen (post-challenged lipopolysaccharide+liposome conjugate group) showing follicular hyperplasia, in which widening of white pulp(A) and reduction in red pulp are observed(B) (H and E; 200X).

The most important finding of evaluating histopatholigical changes in mice of different groups in the present study, is post-challenged mice vaccinated with LPS+LIP, in which the liver histological section showed look-like normal hepatic tissue appearance especially near the portal area; a finding that confirms the other laboratory evaluations (phagocytosis, delayed hypersensitivity reactions and bacterial isolation). The reduced load of liver bacteria and the enhanced humoral and cellular immunity could have contributed to the observed re-normalling the histopathological profile of liver tissues. In agreement with such finding, Dejager *et al*. (2010) demonstrated that bacterial dissemination to the blood and, subsequently, to the reticuloendothelial system and other organs was diminished in vaccinated mice, and as a consequence, fewer histopathological lesions were evident in the liver of mice. This led the authors to hypothesize that more effective early inactivation of *S*. Typhimurium might be responsible for the increase in mean survival time of mice. This is also raised the question of which immune mechanisms contribute to the enhanced host defence, and their answer was in favour of enhanced phagocytic and killing activity of neutrophils.

Conclusions and Recommendation

Conclusions and Recommendations

I. Conclusions

Based on the findings of the present study, it is possible to reach the following conclusions:

1. Although the observed frequency of *Salmonella* infection was 2.1%, it still represents an important pathogen that causes diarrheal disease in children younger than five years, especially if we consider the wide range of antibiotic resistance exhibited by the two identified *S.* Typhimurium isolates.
2. The partial purification of lipopolysaccharide (LPS) be by gel chromatography (sephacryle 200 S) method was successful in yielding LPS rich in carbohydrate with a minimum contamination with protein.
3. It was also possible to conjugate the partially purified LPS with liposome (LIP), and such conjugate was able to modulate the humoral and cellular immune responses against live *S.* Typhimurium, as well as, reduced the bacterial load in liver, which almost showed normal histological appearance in mice vaccinated with LPS-LIP conjugate.

II. Recommendations

Based on the presented conclusions, it is possible to make the following recommendations:

1. It is of importance to screen the same group of children for other pathogens that are involved in diarrheal disease of children younger than five years, because we still have 97.9% of them with unidentified causative pathogens.
2. Further purification of LPS is required, and its conjugation with LIP has to be further evaluated from the point view of vaccination, especially if we consider advanced immunological parameters in the evaluation; for instance, profile of T_H(helper) cytokines (T_H1, T_H2, T_H17 and Treg) and characterization of these cells in terms of their expression for CD (cluster designation) markers.

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الخلاصة

اجريت الدراسة لتقييم دور بكتريا *Salmonella enterica* نوع Typhimurium كجرثومة مسببة للإسهال للمرضي في المستشفى الذين اعمارهم اقل من خمس سنوات . متعدد السكريد الشحمي (السم الداخلي) المعزول والمنقى من بكتريا *Salmonella enterica* serovar Typhimurium المعزولة والمشخصة لتحديد دور(متعدد السكريد الدهني- الجسم الدهني)المرتبط كلقاح محتمل ضد *Salmonella enterica* serovar Typhimurium في ذكور الفئران البيض .واخيرا تقييم الاستجابة المناعية الخلطية والخلوية (العدد الكلي والمطلق لكريات الدم البيض وخلايا الالتهام وتفاعل الحساسية وتفاعل فرط الحساسية المتأخرة) للفئران الممنعة بالإضافة للتغيرات النسيجية المرضية للكبد والطحال .

(٩٥مريض) الذين ادخلوا لمستشفى الطفل المركزي ومستشفى اطفال الكاظمية في بغداد خلال الفترة من ٢٠١٠/١٠/٢٤-٢٠١٠/١١/٣٠ بسبب الاسهال والحمى الحادة .التقييم البكتيري لعينات البراز اظهرت الكشف عن عزلتين (٢,١)٪ من بكتريا *Salmonella enterica* serovar Typhimurium المعزولة (S1 وS2).اظهر تفاعل الحساسية للمضادات الحيوية ان العزلة S1 هي اكثر مقاومة من العزلة S2 لذلك اعتبرت اكثر ضراوة واخضعت بالتالي لمزيد من الاختبارات التي تتضمن عزل متعدد السكريد الدهني من الغشاء الخارجي للبكتريا .الصفات الكيميائية لمتعدد السكريد الدهني المعزول اظهرت ان كمية الكربوهيدرات كانت ٢,٣٤ مليغرام/ملليتر بينما تركيز البروتين كان قليل ٥٢ ,٠ مايكروغرام /ملليتر. التنقية الجزيئية لمتعدد السكريد الدهني المعزول عن طريق استخدام كروموتوغرافيا الترشيح الهلامي باستخدام الهلام (سفأكريل٢٠٠ اس) اظهرت ثلاث قمم وبعد تحديد تركيز الكربوهيدرات لكل قمة اظهرت القمة الثانية كمية كربوهيدرات عالية ٢٥٪ واقل تركيز للبروتين الملوث ٠,٠٠١٪ متعدد السكريد الدهني لهذه القمة قيم مناعيا للفئران بتركيز ١٠٠مايكروغرام/ملليتر لوحده او مرتبط مع الجسم الدهني المتوفر تجاريا.

الفئران وزعت في ثمان مجاميع (سيطرة سالبة ، سيطرة موجبة ،المساعد المكمل فروند، البكتريا المقتولة بالحرارة ، البكتريا المقتولة بالفورمالين ، متعدد السكريد الدهني ، الجسم الدهني ، متعدد السكريد الدهني- الجسم الدهني المرتبط) حقنت كل فارة داخل البريتون ٠,١ ملليتر من المحلول الخاص في اليوم الاول وجرعة اضافية في اليوم الثامن هذه الفئران اعتبرت مجاميع قبل جرعة التحدي وشرحت للتقييم المختبري في اليوم الخامس عشر .المجاميع الاضافية المشابهة حقنت بجرعة التحدي ٠,١ ملليتر من البكتريا الحية في اليوم الخامس عشر بتركيز(٥×١٠^{٤}) خلية/ملليتروشرحت للتقييم المختبري في اليوم التاسع والعشرين (مجموعة بعد جرعة التحدي) واظهرت النتائج الاتي:

١- العدد الكلي لخلايا الدم البيض لكل المجاميع (قبل وبعد جرعة التحدي) كانت قريبة للعدد الطبيعي لكريات الدم البيض في الفئران ما عدا الفئران المقتولة بالحرارة والمقتولة بالفورمالين اظهرت زيادة في عدد الخلايا اللمفية والخلايا العدلة والخلايا الوحيدة ،بينما الفئران الممنعة بمتعدد السكريد الدهني اظهرت زيادة معنوية عددية في الخلايا اللمفية في ما بعد جرعة التحدي

للحيوانات مع ملاحظة اقتراح ان متعدد السكريد الدهني قد تكون له القابلية على تحفيز المناعة التطبعية ضد *Salmonella enterica* serovar Typhimurium.

٢-النسبة العالية للخلايا الملتهمة لوحظت في الفئران الممنعة (بمتعدد السكريد الدهني- الجسم الدهني) المرتبط في الجرعة قبل وبعد جرعة التحدي وهذا الاكتشاف يبين اهمية الارتباط بين متعدد السكريد الدهني والجسم الدهني في تحفيز وظيفة الخلايا البلعمية.

٣- في معظم المجاميع الممنعة بعد جرعة التحدي اظهر كل من تفاعل الحساسية وتفاعل فرط الحساسية المتاخرة اظهر زيادة في الاستجابة بينما في مجاميع قبل جرعة التحدي اظهرت تغاير ،لكن الفئران الممنعة (بمتعدد السكريد الدهني – الجسم الدهني) المرتبط اظهرت نسبة عالية من الاستجابة.

٤ - التقييم البكتيري للكبد والطحال في الفئران الممنعة (بمتعدد السكريد الدهني – الجسم الدهني)المرتبط في جرعة بعد التحدي اظهرت عدم نمو البكتريا *Salmonell enterica* serovar Typhimurium في الكبد ونادر في الطحال هذا بالاضافة للمقطع النسيجي للكبد اظهرت نسيج كبد مشابه للطبيعي وبالاخص قرب الجزء البوابي .


سُورَةُ الفَاتِحَةِ
بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ ١
الْحَمْدُ لِلَّهِ رَبِّ الْعَالَمِينَ ٢ الرَّحْمَٰنِ
الرَّحِيمِ ٣ مَالِكِ يَوْمِ الدِّينِ ٤
إِيَّاكَ نَعْبُدُ وَإِيَّاكَ نَسْتَعِينُ ٥
اهْدِنَا الصِّرَاطَ الْمُسْتَقِيمَ ٦ صِرَاطَ
الَّذِينَ أَنْعَمْتَ عَلَيْهِمْ غَيْرِ الْمَغْضُوبِ
عَلَيْهِمْ وَلَا الضَّالِّينَ ٧

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

الدور التمنيعي لمستضد متعدد السكريد الدهني لبكتريا *Salmonella enterica* serovar Typhimurium المقترن في الجسم الدهني في الفئران

رسالة

مقدمة إلى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الإحيائية

من قبل

فرح تركي عريبي الجميلي

بكالوريوس علوم الحياة، كلية العلوم، جامعة بغداد (٢٠٠٥)

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ربيع الثاني ١٤٣٣

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