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Effect of Liposome Conjugated Lipopolysacchariede of *Shigella flexneri* Against Shigellosis in Mice

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الإهداء

- 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 ظللني بحبه وحنانه ... **أبي الحبيب**
- إلى إخوتي وعضدي ..... إلى رفقائي في مسيرتي ... إلى من رافقوني في كل خطواتي ... **أخوتي وأصدقائي**

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جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

التاثير المناعي لمستضد متعدد السكريد الدهني لبكتريا *Shigella Flexneri* المغروز في الجسم الدهني ضد امراض الشكيلا في الفئران

رسالة

مقدمة الى كلية العلوم / جامعة النهرين

وهي جزء من متطلبات نيل درجة الماجستير في علوم التقانه الاحيائيه

مقدمة من قبل الطالب

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ايلول ٢٠١٢ شوال ١٤٣٣

Conclusions and Recommendations

I. Conclusions

- Lipopolysaccharide-liposome conjugate conferred immunoprotective effect against shigellosis and led to ameliorate the histopathological effect of *S. flexneri.*

II. Recommendations

- Studying the effect of Lipopolysaccharide-liposome conjugate of *Shigella* on CD4-Tcell, CD8-Tcell and Th1, Th2 cytokine production.
- Studying the protective effects of conjugative vaccine made from outer membrane proteins and liposome.

- Studying the effect of Lipopolysaccharide for other bacteria and liposome as conjugative vaccine against disease.

1.1 Introduction

Shigellosis is an acute invasive enteric infection caused by bacteria belonging to the genus *Shigella*; it is clinically manifested by diarrhea that is frequently bloody. Shigellosis is endemic in many developing countries and also occurs in epidemics causing considerable morbidity and mortality. Among the four species of *Shigella*, *S. dysenteriae* type 1 is especially important due to its toxin. It causes the most severe disease and may occur in large regional epidemics (WHO, 2005).

Few studies provide data on the global morbidity and mortality caused by infection with *Shigella* spp.; such estimates are needed, however, to plan strategies of prevention and treatment (Kotloff *et al*., 1999). It is estimated to cause at least 80 million cases of bloody diarrhea and 700,000 deaths each year. Ninety-nine percent of infections caused by *Shigella* occur in developing countries, and the majority of cases (70%), and of deaths (60%), occurs among children less than five years of age. Probably less than one percent of cases are treated in hospital (WHO, 2005).

Several studies have demonstrated that the lipopolysaccharide (LPS) of the *S. flexneri* is a key component associated with bacterial virulence, since the prevention of Shigellosis by vaccination has been the subject of many investigations, and despite this, the mechanism of protective immunity against *shigella* infections remains a controversial subject (Amy and Naresh, 2004).

Many attempts have been made at synthesizing a vaccine which incorporates *Shigella* LPS antigenic determinants, via 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 cell mediated immunity, to various antigens (Christensen *et al*., 2011; Henriksen-Lacey *et al*., 2011).

Aims of study:.

• Determining the role of LPS-liposome conjugate as a potential vaccine against *S. flexneri* in mice infected with *S. flexneri*.

• Evaluating the humoral and cellular immune responses (total and absolute counts of leukocytes, IgG titer) of vaccinated mice, in addition to histopathological changes in intestine, liver and spleen.

1.2 Literature Review

1.2.1 *Shigella*

In spite of some reports mentioned the discovery of *Shigella* in France by Widal and Chantemesse 1888 and in Japan by Ogata 1892, the description of such organism causing human bacterial dysentery was made by Japanese Shiga in 1898 (Percival *et al*., 2004), Flexner, two years later in 1900, identified a serologically related organism from patients in the Philippine Islands with dysnteriae. In the same year, Kruse isolated a nearly identical etiological agent from many cases of dysentery in Germany. Another serotype was isolated and identified in India in 1929 by Boyd (Lampel, 2005).

Shigella organisms are Gram-negative rods that belong to the family Enterobacteriace. This genus consists of four species, *S. dysenteriae*, *S. flexneri*, *S. boydii and S. sonnei.* and they often referred to as subgroups A, B, C and D respectively (Rowe and Gross, 1994). Germani and Sansonetti (2006) mentioned that the genus is divided into four groups (designated species), on the basis of their capacity to ferment sugars and on their O-antigen serotypes. These groups are:

- Group A: *S. dysenteriae* comprised of 15 serotypes.
- Group B: *S. flexneri* comprised of 6 serotypes.
- Group C: *S. boydii* comprised of 18 serotypes.
- Group D: *S. sonnei* comprised of a single serotype.

The four species can be differentiated from each other by the fermentation of sugars, production of indole, and the synthesis of ornithine decarboxylase or arginine dehydrolase. (Lampel *et al.*, 2000) .

1.2.2 General characteristics

Shigella spp. are Gram negative, short (1-3µm) non-motile, non-pigmented, non-encapsulated, non-spore forming, facultatively anaerobic rods. An important biochemical characteristic that distinguishes these bacteria from other enterics is the ability to ferment lactose, unlike other members in the enterobacteriaceae group, *Shigella* are non-lactose fermentor on MacConkey agar or deoxycholate citrate agar after 24 hrs of incubation (Percival *et al.*, 2004). However, some strains of *S. sonnei* may ferment lactose slowly or utilize citric acid as a sole carbon source. Only *S. flexneri* serotype 6 and *S. boydii* serotypes 13 and 14 are able to produce H_2S and unable to produce gas from glucose *Shigella* spp. are inhibited by potassium cyanide and do not synthesize lysine decarboxylase or hydrolyse arginine, they are oxidase-negative, and ornithine decarboxylase negative (*S. sonnei* is positive).

Actually, there is a confused between biotypes and bioserotypes of typical *Shigella* and *Escherichia* spp., and the so-called enteroinvasive *E. coli* (EIEC) strains are responsible for a disease similar to shigellosis. *E. coli* and the four groups of *Shigella* are so closely related that they constitute a single spp., and the decision to maintain *Shigella* and *E.coli* as separate entities was made only in the interest of epidemiology and clinical medicine (Parsot and Sansonetti, 1999). Montville and Matthews (2008) mentioned that enteroinvasive *E. coli* (EIEC) has pathogenic and biochemical properties similar to those of *Shigella* spp. and this similarity possess

problem in distinguishing these pathogens. For example, EIEC is non-motile and is unable to ferment lactose. Some serotypes of EIEC also have O antigens identical to those of *Shigella*.

Shigella grows less profusely on artificial media than coliform bacteria and other members of the family Enterobacteriaceae. They are less active in their utilization of carbohydrates than *E. coli* and do not form visible gas from carbohydrates (except for certain biotypes of *S. flexneri* 6). Urease, phenylalanine deaminase and hydrogen sulfide are not produced. The Voges-proskauer test is negative, and methyl red reaction is positive. Sodium malonate is not utilized, gelatin is not liquefied and growth does not occur in Simmon's citrate agar or on potassium cyanide medium (ICMSF, 1996).

Shigella spp. have a minimum temperature for growth is 6.1°C, and a maximum is 47°C. *Shigella* does not need to grow in food to cause illness and low infective dose means that the presence of the organism in food is sufficient to cause infection. *Shigella* spp. are able to survive at frozen and chill temperature, although the time of survival depends on the type of food environment as well as temperature. At room temperature *S. sonnei* rapidly increased in numbers when stored in vacuum/modified-atmosphere (30% N2, 70% CO2) packagies, and *Shigella* numbers remained static when stored under similar conditions at chilled temperature (Lawley *et al*., 2008).

The reported pH range allowing growth of *Shigella* spp. is (4.8-9.3), although actual values will depend on acid type. *Shigella*.spp. are gradually inactivated at pH values lower than 4.0, but the organism can survive for some time in acid conditions (Lawley *et al*., 2008).

Shigella spp. can grow at water activities down to 0.96 (maximum salt conc. 5.2% NaCl). They die out slowly at low water activities. Even at high NaCl concentration (10%) some strains can survive for 4 days (Lawley *et al*., 2008).

1.2.3 *Shigella flexneri*

Shigella flexneri causes the most infectious form of bacterial dysentery, shigellosis. Shigellosis causes acute mucosal inflammation in the colonic and rectal epithelium of humans and higher primates. Destruction of the epithelial layer causes watery diarrhoea and severe abdominal pain, which progresses into the characteristic bloody mucoid stool (Jennison & Verma, 2004).

The high incidence of shigellosis in developing countries, approximatelly 99% of all cases, can be attributed to the insufficient sanitation, deficiency of clean water, malnutrition and limited health services. While antibiotics can be used to reduce the period of bacterial excretion, the increasingly common resistance to cheaper and more readily available antibiotics makes this treatment option prohibitive for many developing countries (Ashkenazi *et al*., 2003). Consequently, the construction of a safe and effective vaccine against *S. flexneri* is of major concern to the World Health Organization (Kotloff *et al*., 1999).

Shigella flexneri is the predominant species in developing countries. Identification of *S. flexneri* is based on biochemical and serological properties. As *Shigella* strains are all negative for H antigen, *Shigella* serotyping is based on O antigen only. *S. flexneri* is divided into 15 serotypes. Some *S. flexneri* serotypes are more prevalent than others, with the three most prevalent being serotypes 2a, 3a, and 1a in Asian countries

including China (Wang *et al*., 2006). All *S. flexneri* serotypes except serotype 6 share the backbone of the basic O-antigen repeat unit, which is a tetrasaccharide consisting of a single N-acetylglucosamine and three rhamnose residues. Glucosylation of any of the four sugars and/or O acetylation of the last rhamnose gives rise to more than 13 known serotypes,

both processes are performed by genes carried by bacteriophages, glucosylation involves 3 gtr genes with one being type specific while O acetylation involves only one gene, oac (Allison *et al*., 2000) This O-antigenic variation is a major strategy used by the organism to evade host immunity, these bacteriophage-encoded modifications allow *S. flexneri* to change its O antigenicity rather simply(West *et al*., 2005).

1.2.4 Pathogenicity

S. flexneri is highly infectious, requiring as little as 100 cells to cause disease in adults volunteers (Jennison and Verma, 2004). This low infective dose is in part attributed to *S. flexneri's* ability to survive the low acidity of the host's stomach, via an up-regulation in acid resistance genes (Small *et al*., 1994).

Shigellosis is characterized by a severe inflammatory response at the colonic mucosa and destruction of colonic epithelial cells (Bennish and Albert, 2011). Pathogenesis can be divided into five stages (Bennish and Albert, 2011):

- Entry of the bacterium into the host colonic cell.
- Lysis of phagosomes (the vacuole that forms from the fusion of the cell membrane around the phagocytized *Shigella*) and bacterial multiplication.

- Intra- and intercellular spread of the bacterium.
- The death of the host cell and ulceration of the mucosa.
- Resultant of inflammatory response.

1.2.4.1 Mechanism of pathogenicity

Shigella spp. possess several key properties that are responsible for their virulence. The ingested microbes must survive the acidic environment of the stomach, which is the first significant host defensive barrier encountered by the bacteria (Jennison and Verma, 2004). To produce clinical symptoms, *Shigella* must attach to and invade the epithelial cells of the colon, multiply and disseminate intracelluarly through adjacent colonic epithelial cells, and cause abscesses and ulcerations of the intestinal lining cells leading to the bloody mucoid stools characteristic of dysentery. Bacterial invasion and replication also lead to an intense inflammatory response that serves both the host and the pathogen (Figure1-1) (Jennison and Verma, 2004; Day and Maurelli, 2006).

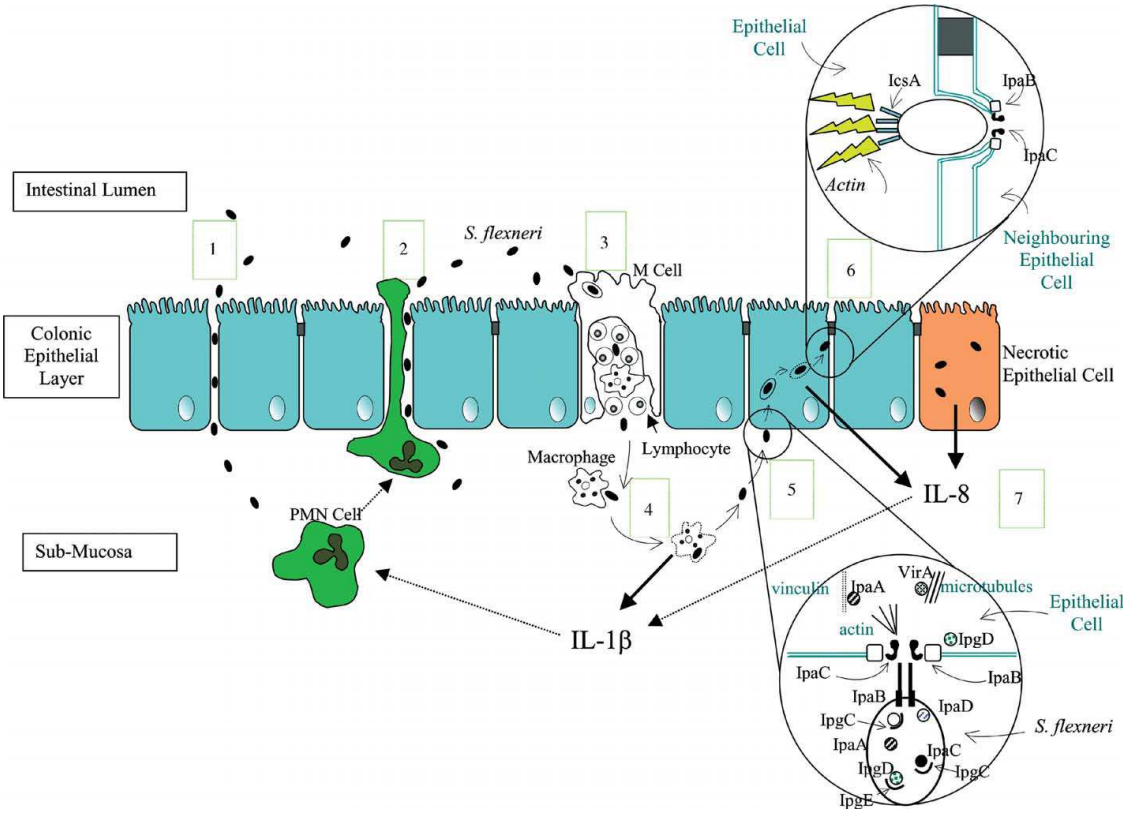


Figure (1- 1): Pathogenesis of *S. flexneri*.(Philpott *et al*., 2000)

Shigella spp. have a preference for M cells of the colon; these are specialized epithelial cells associated with mucosal lymphoid tissue. After adherence to and uptake into colonic M cells, *Shigella* are engulfed by phagosomes and approximately 1.5 hrs later lyse the M cells vacuoles. The pathogen multiplies and spreads intracellularly from the basolateral side into the submucosa of the colon. Further events are: (1) the interaction with host immune effector cells, (2) apoptotic lysis of macrophages, and (3) cytokine release and infiltration of polymorphonuclear leukocytes (Lampel *et al.*, 2000).

Essential virulence attributes of *Shigella* are the abilities to enter into and disseminate within epithelial cells, as well as the ability to induce apoptosis in macrophages (Parsot and Sansonetti, 1999).

It is currently unclear whether *Shigella* kills its phagocytic host cells by apoptosis or necrosis. A study by Nonaka *et al*., (2003) shows that rapid necrosis ensues in macrophage-like cell lines infected with the *S. flexneri*, since the infected cells rapidly lose membrane integrity, a typical feature of necrosis, as indicated by the release of the cytoplasmic lactate dehydrogenase and the exposure of phosphatidylserine (PS) associated with the rapid uptake of propidium iodide (PI). The infected cells exhibit DNA fragmentation without nuclear condensation, and substantial involvement of either caspase-3/-7 or caspase-1 was not detected, which is also contrary to what is normally observed in apoptosis. Other studies suggested that Cytochalasin D potently inhibited *Shigella*-induced cell death, indicating that only internalized *Shigella* can cause necrosis and osmoprotectants such as polyethylene glycols could suppress cell death, suggesting that insertion of a pore by *Shigella* into the host cell membrane induces the necrosis. *Shigella* can induce rapid necrosis of macrophage-like cells in a virulence-related manner by forming pores in the host cell membrane while some cells can be killed through apoptosis in a virulence-independent fashion (Nonaka *et al*., 2003). It was found that virulent strains of *S. dysenteriae* 1 produced disease in volunteers in doses as low as ten orgainsms, but large numbers of a noninvasive toxigenic strain (10^6-10^{11} organisms) were well tolerated by 85 of 86 men (Levine *et al*., 1973).

1.2.4.2 Shiga toxin

The name Shiga toxin is derived from a toxic activity originally discovered in Shiga's bacillus, *S. dysenteriae*. Credit for the discovery of Shiga toxin is generally accorded to Conardi, who described many of its properties in 1903, This activity was known as Shiga neurotoxin because

when injected parenterally into mice or rabbits it resulted in limb paralysis followed by death of the animal. It has been realized that the Shiga family of toxins are in fact a major cause of disease in many developed countries (Acheson *et al*., 2000).

The nomenclature for the Shiga toxin family has become confusing. In 1972, a toxin causing fluid secretions by rabbit small bowel was identified in *S. dysenteriae* 1 and named *Shigella* enterotoxin. This toxin was subsequently proved to be identical to the originally described Shiga neurotoxin. Acheson *et al*., (2000) proved that of that *E. coli* cytotoxins were active on Vero cells and named Verotoxins. This name is still used by many workers in the field who identify Verotoxin-producing *E. coli* as VTEC. However, when it became apparent in the early 1980s that these newly described *E. coli* toxins were very similar to Shiga toxin and were neutralized by antisera to Shiga toxin, other workers referred to them as Shiga-like toxins in 1996, when the common mechanism of action and cellular binding site was proven. An international group of investigators decided to designate this group of biologically homogenous toxins simply as Shiga toxin (Stx), irrespective of their bacterial origin. The gene designation (Stx) for Shiga toxin from *S. dysenteriae* 1 was already well established and the new nomenclature therefore maintained the *stx* gene designation for the *E. coli* derived toxins (Acheson *et al*., 2000).

Shiga toxin is a heat-labile exotoxin. The site of action of this toxin is on the vascular system of the central nervous system but it does not directly affect neurons. The toxin does inhibit protein synthesis in the host target cell. Shiga and Shiga-like toxin of *E. coli* have common properties (Lampel *et al.* 2000):

- They share sequence homology (the Shiga toxin and Stx1 from *E. coli* O157:H7 are nearly identical.)
- They have similar modes of action.
- They bind to eukaryotic cell membrane receptors with a specific galactose moiety.
- They possess the same stoichiometry of one A to five B subunits.

The toxin has a molecular weight of 70 KDa and consists of two polypeptide subunits A and B, which are combined in ratio 1:5.7. The B subunit mediates biding of the toxin to surface receptors on target cells. The A subunit enters cells by endocytic transport, binding to 60S ribosomes and inhibiting protein and DNA synthesis, leading to cell death (Sutherland and Varnam, 2002).

Shiga toxin clearly causes fluid secretion when placed in the small bowel lumen of rabbits and results in inflammatory enteritis in this model. Although it is cytotoxic to human colonic epithelial cells and thus can mimic colonic manifestations of clinical shigellosis, the interpretation is complicated because *Shigella* are invasive and multiply within epithelial cells (Acheson *et al*., 2000).

Bacterial Endotoxin (as a Biological Toxin) in general terms, endotoxin refers to any cell-bound bacterial toxin – a characteristic that distinguishe it from exotoxins which are secreted by bacterial cells (Grandel and Grimminger, 2003). The term endotoxin was first coined in 1893 by Pfeiffer to distinguish the class of toxic substances released after lysis of bacteria from the toxic substances (exotoxins) secreted by bacteria (Peterson, 1996).

1.2.4.3 Structure of Endotoxin

Lipopolysaccharide (LPS) forms the major constituent of the outer membrane of Gram – negative bacteria. They are believed to play a key role in the processes that govern microbial metal binding, surface adhesion and microb-mediated oxidation – reduction reaction (Soares *et al*., 2008). It protects the bacterial pathogen from host defenses and mediates the entry of the bacteria into eukaryotic cell (Brandenburg and Wiese, 2004). It is resistant to phagocytosis and serum; it also acts as a receptor for adsorption of some bacteriophages. It has also a role in the outer membrane permeability barrier (Darveao and Hancock, 1983). LPS possesses biologically important properties such as adjuvant activities that stimulate the immune systems of animal hosts and endotoxic effects that are deleterious to the health of the host (Lam *et al*., 1992).

LPS is synthesized on the cytoplasmic membrane and transported to its final exterior position (Brooks *et al*., 2007).

LPS molecule is composed of three regions, lipid A, rough core oligosaccharide and O antigenic side chain (Figure1-2), which are covalently

attached to one another (Darveao and Hancock,1983; Pitt and Simpson, 2006):

I. Lipid A is the lipid component of LPS. It contains the hydrophobic membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine dimer with 6 or 7 fatty acids attached. All fatty acids in Lipid A are saturated (Brandenburg *et al*.,2000). The structure of Lipid A is highly conserved among Gram negative bacteria (Perez-Perez *et al*., 1986).

The primary structure of Lipid A has been elucidated and Lipid A has been chemically synthesized (Galanos *et al*., 1985). Lipid A confers toxicity which appears to depend on a peculiar conformation that is determined by the glucosamine disaccharide, the PO_4 groups, the acyl chains, and also the KDO-containing inner core (Silipo *et al*., 2002a).

II. Core (R) antigen or R polysaccharide The core region consists of a hetero-oligosaccharide that can formally be subdivided into an outer and an inner portion. The outer core consist of short chains of hexose sugars. The inner core consists of two unusual sugars, heptose and 2-keto-3-deoxyoctonoic acid (KDO) (Caroff and Karibian, 2003). KDO is unique and invariably present in LPS and so it has been used as an indicator in assays for LPS (endotoxin) (Holst, 1999). With minor variations (mainly outer core), the core polysaccharide is common to all members of a bacterial genus (e.g. *Shigella*), but it is structurally distinct in other genera of Gram-negative bacteria. *Salmonella*, *Shigella* and *Escherichia* have similar but not identical cores (Rietschel *et al*.,1994).

III. Somatic (O) antigen or O polysaccharide: The O-specific chain constitutes a polymer of oligosaccharides subunits made up of (3 – 8) glycosyl residues. The O polysaccharide is much longer than the core

polysaccharide, and it maintains the hydrophilic domain of the LPS molecule (Raetz and Whitfield, 2002). The structure of repeating units differs from strain to strain within a serotype and thus exhibits an enormous structural variability, determines the serological specificity of the LPS and of bacteria containing it, and therefore function as an important surface antigen (Jansson, 1999). O-specific chain process intrinsic biological activity involving both immunogenicity an pathogenicity. In addition, O-specific chain plays an important role in bacterial protection, e.g. smooth pathogenic *Shigella* in tissue and body fluids persist and survive only if they express an O-specific chain,which in this case protects bacteria from phagocytosis and serum mediated lysis (Watson *et al*., 1992; Vinogradov *et al*., 2004). Loss of the O specific region by mutation results in the strain becoming a "rough" or R strain (Bayston and Cohen, 1990).

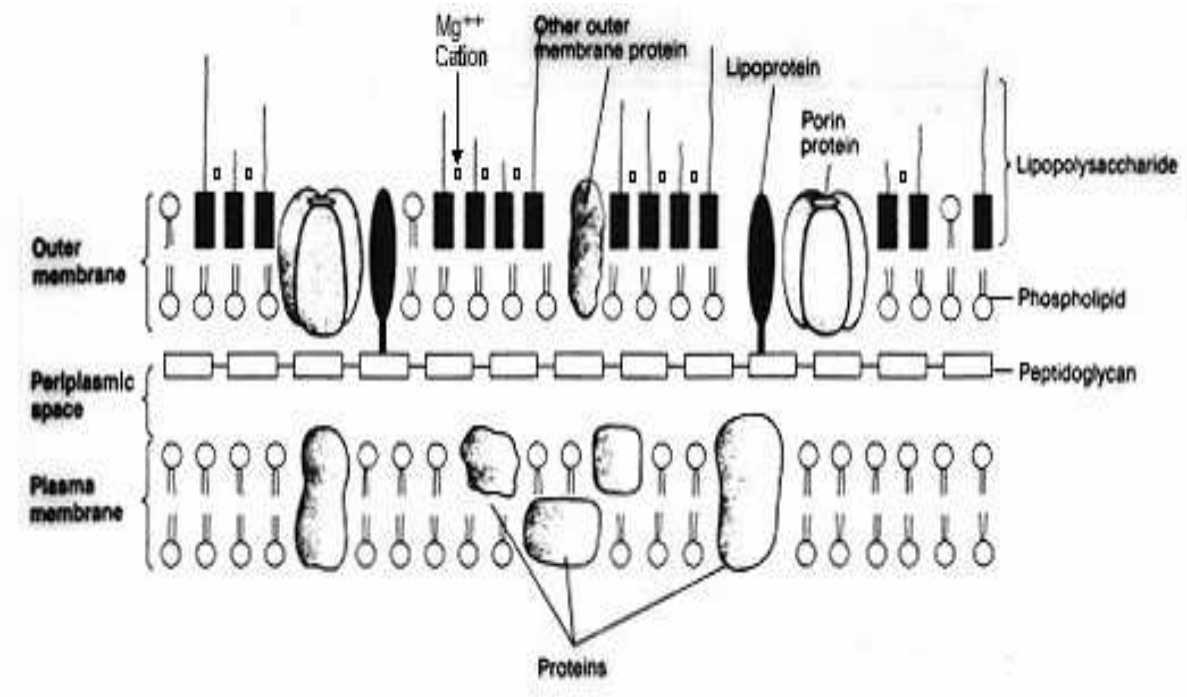


Fig (1-2): Structure of the cell envelope of a Gram-negative bacterium (Darveau and Hancock, 1983).

1.2.5 Symptoms and Characteristics of the shigellosis

Shigellosis is a self-limiting disease, lasting 5 to 6 days if untreated; however, in young malnourished children, the elderly, and the immunocompromised, the disease may be fatal. It is estimated that shigellosis is responsible for the death of 500,000 children worldwide each year (Lampel *et al*., 2000).

Shigella dysentery, or bacillary dysentery, is characterized by the sudden onset of abdominal cramps, diarrhea, and fever after 1-4 days incubation. Mucus and sometimes blood appear in the feces. Bacteraemia occurs, occasionally in the compromised host, with a high fatality rate. The triad of symptoms – fever, abdominal pain and watery diarrhea – are not enough to implicate *Shigella* as the etiological agent, because other organisms, e.g. pathogenic *E. coli*, *Salmonella*, and *Campylobacter*, cause similar symptoms. If the illness progress to the colonic phase (usually within 1-3 days), in which the scanty stool becomes bloody and mucoid, specific diagnosis can be suspected. The severe (colonic) phase is characterized by waves of intense cramps, frequent bowel movements producing only scanty quantities of blood and mucus and acute pain with each motion (ICMSF, 1996).

Shigellosis is differentiated from diseases caused by most other foodborne pathogens by at least two important characteristics:

- The production of bloody diarrhea or dysentery.
- The low infectious dose.

Dysentery involves bloody diarrhea, but the passage of bloody mucoid stools is accompanied by severe abdominal and rectal pain, cramps and fever. While abdominal pain and diarrhea are experienced by nearly all patients with shigellosis, fever occurs in about one-third and gross blood occurs in about 40% of cases (Montville and Matthews, 2008). The clinical features of shigellosis range from a mild watery diarrhea to severe dysentery. The dysentery stage caused by *Shigella* spp. may or may not be preceded by watery diarrhea. Montville and matthews (2008) reported that during the dysentery stage, there's extensive bacterial colonization of the colon and invasion of the cells of the colon. As the infection progresses, dead cells of the mucosal surface slough off. This leads to the presence of blood, pus and mucus in the stool .

The incubation period is usually (12-50 hrs); onset is rapid and accompanied by fever and severe abdominal pain. Symptoms usually last 3 to 4 days, but can persist for 14 days or longer. In healthy adults, death is rare, but *Shigella* dysentery is a major cause of death among infants in countries where hygiene is poor. Infections with *S. dysenteriae* almost always develop full and severe symptoms of dysentery and may be associated with many complications like leukaemoid reaction and haemolytic-ureamic syndrome. It is known to produce protracted epidemics and pandemics and is usually multi-drug resistant (Sutherland and Varnam, 2002).

Similar symptoms, although often less severe, can also be associated with *S. boydii* and *S. flexneri*. Most adult infections by these spp., however, and virtually all by *S. sonnei* do not progress beyond relatively mild, non-bloody diarrhea. Symptoms may differ in young children and be of greater

severity, possibly involving extra intestinal symptoms, including convulsions, headaches and delirium (Bhattacharya and Sur, 2003).

The only natural hosts of *Shigella* are humans and monkeys; infections are localized in the colon and restricted to the outermost layer of the intestinal wall, where they elicit a strong inflammatory reaction (Parsot and Sansonetti, 1999).

Other clinical manifestations can occur from infections due to certain *Shigella* spp. Reactive arthritis or Reiter's syndrome may follow infections with *S. flexneri*. Reiter's syndrome can include three clinical symptoms: urethritis, conjunctivitis, and arthritis. One possible explanation for these sequelae of shigellosis is that antibodies produced in response to antigens of *S. flexneri* cross-react with the human leukocyte antigen HLA-B27. Hemolytic uremic syndrome (HUS) is caused by *S. dysenteriae* type 1 and other pathogens, notably enterohemorrhagic *E. coli* O157:H7. HUS is a common cause of pain in the joints, irritation of the eyes, painful urination and renal failure in children, although *Shigella* may be responsible for 5% to 15% of all cases of diarrhea worldwide, *Shigellae* are associated with 30 to 50% of cases of dysentery. Other symptoms include thrombocytopenia and microangiopathic hemolytic anemia (Lampel *et al.*, 2000).

1.2.6 Immune response to *S. flexneri*

1.2.6.1 Innate immunity

The severe inflammation generated by shigellosis can be regulated up of a variety of cytokines (IL-1, TNF, IL-6, IFN-Y, TNF-B, IL-4, IL-10, TGF-B and IL-8) (Raqib *et al.*, 1995). Although some of the clinical symptoms of shigellosis may actually be a direct consequence of the

cytokines, they also assist in controlling and containing the infection .Resident macrophages and infiltrating monocytes are unable to efficiently kill *S. flexneri* in their phagosomes and instead succumb to apoptosis (Hathaway *et al*., 2002, Zychlinsky *et al*., 1992), and Biet *et al*., (2002) stated that the IL-18 released by apoptotic macrophages can target NK cells and T lymphocytes, inducing production of IFN-Y.

IFN-Y deficient mice are five times more susceptible to a *Shigella* infection, as IFN-Y activates macrophages and fibroblast cells, which promote bacterial clearance and possibly inhibit bacterial replication within epithelial cells (Way *et al*., 1998).

The most important consequence of the hosts innate immune response appears to be the cytokine induced migration of PMN cells. The transcription factor NF-kB is activated in *Shigella*-infected epithelial cells in an LPS-dependant mechanism, leading to the production and secretion of IL-8 by the infected cells (Kohler *et al*., 2002). IL-8 is a potent chemo attractant for PMN cells, as well as IL-1released from apoptotic macrophages .*Shigella* is unable to escape the phagocytic vacuole of PMN cells and are killed inside the phagosome (Mandic-Mulec *et al*., 1997).

Recent research has implicated the human neutronphile elastase (NE) as a key host defence protein of the neutrophil, capable of degrading *Shigella* virulence proteins within 10 min of *Shigella* infecting the neutrophil (Weinrauch *et al*., 2002). PMN cells ultimately play a crucial role in controlling the *Shigella* infection, confining extracellular bacteria to the mucosa, preventing deeper tissue invasion and systemic spread (Sansonetti *et al*., 1999., Zhang *et al*., 2001).

Another host defence mechanism directed against *Shigella* has recently been discovered. The glycoprotein, lactoferrin, present in mucosal secretions, breast milk and phagocytic cells can impair the ability of *S. flexneri* to invade HeLa cells, exposing IpaB–IpaC complexes to protease degradation by disrupting the bacterial surface(Gomez *et al*., 2003) Additionally, a study in transgenic mice expressing a human intestinal defensin has demonstrated an important role as intestinally-secreted antibiotic peptidesin controlling a *Salmonella typhimurium* enteric infection, therefor it is highly likely that intestinal defensins would display similar antibiotic properties against enteric *S. flexneri*. Persist in the gut for over a month, with a general up regulation (Salzman *et al*., 2003).

1.2.6.2 Cellular immunity

Very little data is available on the hosts cellular immune response to *S. flexneri*, especially in comparison to other intracellular bacteria. Studies have shown increased T cell activation in shigellosis patients and T cell clones have been isolated which proliferate in response to *S. flexneri* antigen (Amy and Naresh, 2004). The cytokines induced by *Shigella* antigens in vaccine studies are suggestive of Th1and Th2 lymphocyte responses (van de Verg *et al*., 1995; Kotloff, *et al*., 2000). Additionally, the increased susceptibility of AIDS patients, deficient in CD4 T cells, to shigellosis could suggest that cell mediated immunity can play a protective role in shigellosis (Nelson *et al*., 1992).

However, the contribution of T lymphocytes to the hosts protective immunity against *Shigella* was studied in the mouse pulmonary model where mice deficient in T cells were vaccinated with attenuated *S. flexneri*. These mice were suitably protected from challenge with wild type bacteria despite

their deficiency in T lymphocytes, suggesting that even if T cell responses develop to *Shigella* they are not essential for protection (Way *et al*., 1999).

1.2.6.3 Humoral immunity

Information about the hosts humoral response to *Shigella* infection has been collected from numerous serological studies of infected humans and experiments performed in animal models. The data suggests that the humeral immune response is a major component of protective immunity to shigellosis with both systemic and mucosal responses activated against the LPS and some virulence plasmid encoded proteins, including the Ipa proteins. The serotype-specific structure of the LPS is assumed to be the major target of the hosts immune responseas. Natural and experimental infections with *Shigella* confer serotype-specific immunity, where previous infection or vaccination provides little to no protection against heterologous serotypes (Formal *et al*., 1991). However, antibodies directed against epitopes shared between certain O-antigen structures do appear to show some cross-reactivity (Van De Verg *et al*., 1996). The overall importance of an antibody response to *Shigella* infection has been confirmed in a study which showed that a reduced and delaye humoral immune response in comparison to adult patients is the likely cause of the increased susceptibility of children to shigellosis (Raqib *et al*., 2002) .

It appears that both the systemic and mucosal arms of the humoral response are activated as serum IgG , IgM and secretory IgA have all been implicated in the generation of serotype-specific immunity against *S. flexneri*. Secretory IgA (sIgA) is made up of 2 IgA units and two polypeptides, the J chain and the secretory component(SC). sIgA transcytoses into the lumenal cavity of the intestine where the secretory

component binds the mucosal coating of the epithelial cells, forming an antibody shield over the cells (Phalipon *et al*., 2002) sIgA can also coat the outer membrane of lumenal bacteria, impeding invasion by preventing their attachment to the mucosal surfaces, mediate antibody-dependant cell-mediated cytotoxicity and interfere with bacterial utilization of growth factors. IgA especially anti-LPS IgA have been detected in humans suffering natural shigellosis in a number of studies and is thought to play an important role in immunity to re-infection (Rasolofo-Razanamparany *et al*., 2001). Anti-LPS secretoryIgA antibodies in the breast milk of mothers exposed to shigellosis appear to be responsible for the decreased severity of shigellosis in *Shigella*-infected infants (Clemens *et al*., 1986). Additionally, the implantation of a serotype-specific sIgA hybridoma on the back of mice protected them against intranasal challenge with a lethal dose of *S. flexneri* organisms (Phalipon *et al*., 1995). This experiment suggests that a mucosal antibody directed against a single LPS epitope of *Shigella* could be sufficient for protective immunity against re-infection by the homologous serotype. Despite shigellosis generally being a localized mucosal infection, serum antibodies IgG and IgM are detected in natural human infections directed against the LPS and virulence plasmid antigens (Amy and Naresh, 2004). IgG and possibly IgM directed against the LPS have been shown to play a protective role in immunity to *Shigella* in mice studies. Way *et al* (1999) reported that IgA deficient vaccinated mice are fully protected against pulmonary *Shigella* challenge, suggesting that IgG or IgM are able to provide immunity. Immunised mice deficient in all T lymphocytes were protected from wild-type *Shigella* challenge by a predominantly anti-LPS IgM response .

1.2.7 *Shigella flexneri* vaccine development

The cost of treating shigellosis with antibiotics, especially in the developing world, is unrealistic and the serotype-specific immunity generated by *S. flexneri* provides protection against reinfection by the homologous serotype, making vaccination a viable option for controlling shigellosis (Amy and Naresh, 2004). A suitable vaccine for shigellosis must fulfil certain requirements: the mucosal immune system must be activated and this immunity should be long-lasting, the vaccine must be cheap to manufacture, induce minimal side effects and be simple to administer, as children in developing countries will be the main recipients .Since the 1940s a number of candidate vaccines for *S.flexneri* have been developed but as yet none have been successful enough for field release, and early attempts to develop *S. flexneri* vaccines consisted of inactivated bacteria delivered parenterally, which failed to induce aprotective immune response, despite inducing a high titre of serum anti-LPS antibody (Amy and Naresh, 2004).

The lack of protection was most likely due to the failure of the parenteral vaccine in inducing a mucosal immune response. Consequently, many recent vaccine strategies have concentrated on developing live vaccine strains which can be administered orally and will activate the effectors of mucosal immunity.

1.2.7.1 Subunit vaccines

Subunit *Shigella* vaccines may avoid the safety issues associated with live vaccines, that LPS can be complexed to proteosomes and delivered intranasally to humans. Clinical trials have revealed that a *S. flexneri* LPS–proteosome vaccine is capable of generating a serotype-specific immune

response in humans (Fries *et al*., 2001) *S. flexneri* LPS has also been attached to proteins and delivered parenterally to volunteers as potential vaccines. These vaccines were safe in humans and induced strong serum antibody responses (Passwell *et al*., 2001). Other subunit vaccines are yet to be evaluated in humans.

1.2.7.2 Killed oral vaccines

Early challenge experiments in monkeys revealed that orally administered acetone-killed and dried *Shigella* was unable to protect monkeys from infection (Amy and Naresh, 2004). More recently however, an oral heat-killed *S. flexneri* vaccine evaluated in a rabbit model was shown to be 100% protective (Chakrabarti *et al*., 1999). Thus, further studies are required to determine the protective capabilities of killed oral vaccines for *S. flexneri* in humans.

1.2.7.3 Non-invasive live vaccines

Mutations in either the *S. flexneri* chromosome or the plasmid have been used to generate non-invasive live vaccine strains. Most of these strains were safe in humans and were able to induce some degree of protective immunity in volunteers. Probably the most successful of these vaccines is the invasion plasmid mutant, *S. flexneri* 2a Istrati T32 which is 100% safe in humans and provides up to an 85% protective efficacy. However, it must be administered in large ($1x10^{11}$ CFU) multiple doses every six months which is expensive and difficult to implement in developing countries (Meitert *et al*., 1984).

1.2.7.4 Invasive live vaccines

Invasive oral *Shigella* vaccine strain strategies are increasingly being explored as invasive strains deliver antigen to the mucosal immune system, provoking a strong immune response. As the genetic understanding of *S. flexneri* virulence has improved to construct safe invasive vaccines (Kotloff *et al*., 2000). Invasive vaccine strains are generally attenuated by mutations in either virulence genes necessary for pathogenesis after cell entry or in metabolic genes which prevent the bacteria from replicating and spreading in the host after invasion (Karnell *et al.*, 1995). Mutations in either icsA and/or in a variety of metabolic genes have produced attenuated invasive vaccine strains which are safe and capable of up to 100% protection with multiple doses in monkeys (Kotloff *et al*., 1996).

The *S. flexneri* vaccine strain SC602 has proceeded to phase 2 clinical trials in humans. This strain carries deletions in icsA as well as the aerobactin iuc locus, which is involved in iron transport. SC602 is safe in humans at low doses ($1x10^4$ CFU) and capable of providing protection to immunised humans challenged with wild type 2a *S. flexneri*. However, the vaccine is only weakly attenuated causing symptoms such as diarrhea and fever when administered in doses higher that $1x10^4$ CFU (Coster *et al*., 1999). Thus, despite promising results with *S. flexneri* invasive vaccine candidates, further work is required to achieve a balance between immunogenicity and safety in humans.

1.2.7.5 Hybrid vaccines

E. coli vaccine candidates have also been used to develop hybrid vaccines expressing *Shigella* antigens. Early attempts using S*higella–E. coli*

hybrid vaccines developed invasive vaccines which caused symptoms in human volunteers or which were not protective (Amy and Naresh, 2004). Strains based on *E. coli* K12 carrying the group- and type-specific antigen of *S. flexneri* 2a and the virulence plasmid from *S. flexneri* were unable to induce significant protection in immunised volunteers (Kotloff *et al*., 1995).

Additionally, *S. flexneri* candidate vaccine strains are being engineered to express the O-antigen's of other *Shigella* species. The *S. flexneri* 2a vaccine strain T32 carrying a plasmid containing the gene cluster coding for *S. sonnei* O-antigen, was capable of providing 100% protection to mice against challenge with both virulent *S. flexneri* and *S. sonnei* (Rui *et al*., 1996). The *S. dysenteriae* O antigen biosynthesis genes were integrated into the SFL124 (serotype Y) vaccine strain, generating strains able to induce antibodies specific to both homologous and heterologous O-antigen structures in mice (Klee *et al*., 1997).

1.2.8 Liposomes

Liposomes 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 (figure 1-3). 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 Crommlin, 1995). 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).

Ahsan *et al*., (2002) studied 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 liposome-associated antigens. It was shown that the humoral, as well as, cellular immune response in macrophage deplated animals was suppressed. Inhibition of the humoral response was also observed after macrophage suppression *in vivo*. Upon reappearance of macrophages the response recovered. Vangasseri *et al* (2006) reported that B-cells also have antigen processing and presentation capacity, but for the presentation of liposomal of 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, 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.

An increase in immunogenicity and a reduction in toxicity were observed when LPS from *N. meningitides* was used in liposome-associated from 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).

1.2.8.1 Type of Liposomes

Depending upon the structure there are two type of liposomes (Thomas and Joseph, 2001).

a) Unilamellar liposomes: Unilamellar vesicles has a single phospholipids bilayer sphere enclosing aqueous solution (figure 1-3).

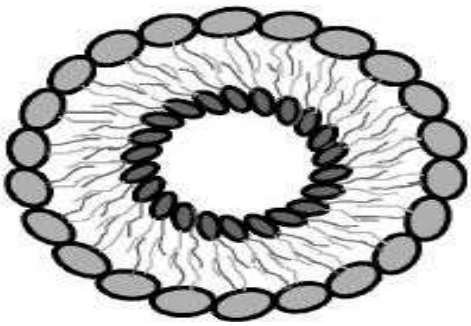
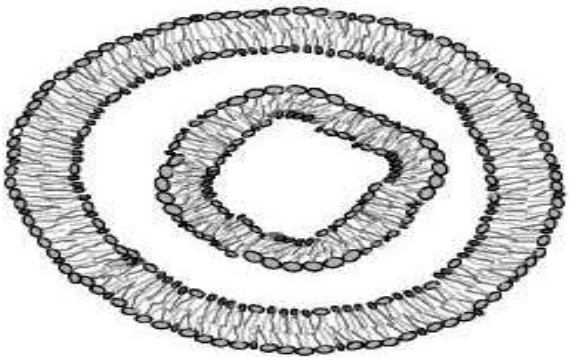


Fig (1- 3):Very Small, Single Layer liposome (Thomas and Joseph, 2001).

b) Multilamellar Liposomes: Multilamellar vesicles have onion structure. Typically, several Unilamellar vesicles will form one inside the other in diminishing size, creating a multilamellar structure of concentric phospholipid spheres separated by layers of water (figure 1-4).



Fig(1-4): Large Vesicle, Multilayer Liposme (Thomas and Joseph, 2001).

1.2.8.2 Composition and Characteristics of liposome

Usually liposomes composed of cholysterol and phospholipids. The structure, composition and proportion being practically the same as in the host cell membranes (Bangham, 1983). The phospholipids possess a hydrophobic tail structure and a hydrophilic head componrnt and organize in the following when dissolved in water, the hydrophobic tails mutually attract while the hydrophilic heads contact with the aqueous medium external and internal to the liposome surface (Figure 1-5). In this way, double lipid layers are formed which seal off to form small vescicles similar to the body cells and oraganelles. These sphere or liposomes constitute small deposits that can be made to contain an antigen, an antibiotic, an allergen, a drug substance or a gene. The liposomes can in turn be introduced in the body without trigrring immune rejection reaction. Phospholipid bilayers are the core structure of liposomes and cell membrane formation (Sharma *et al.*, 2009) .

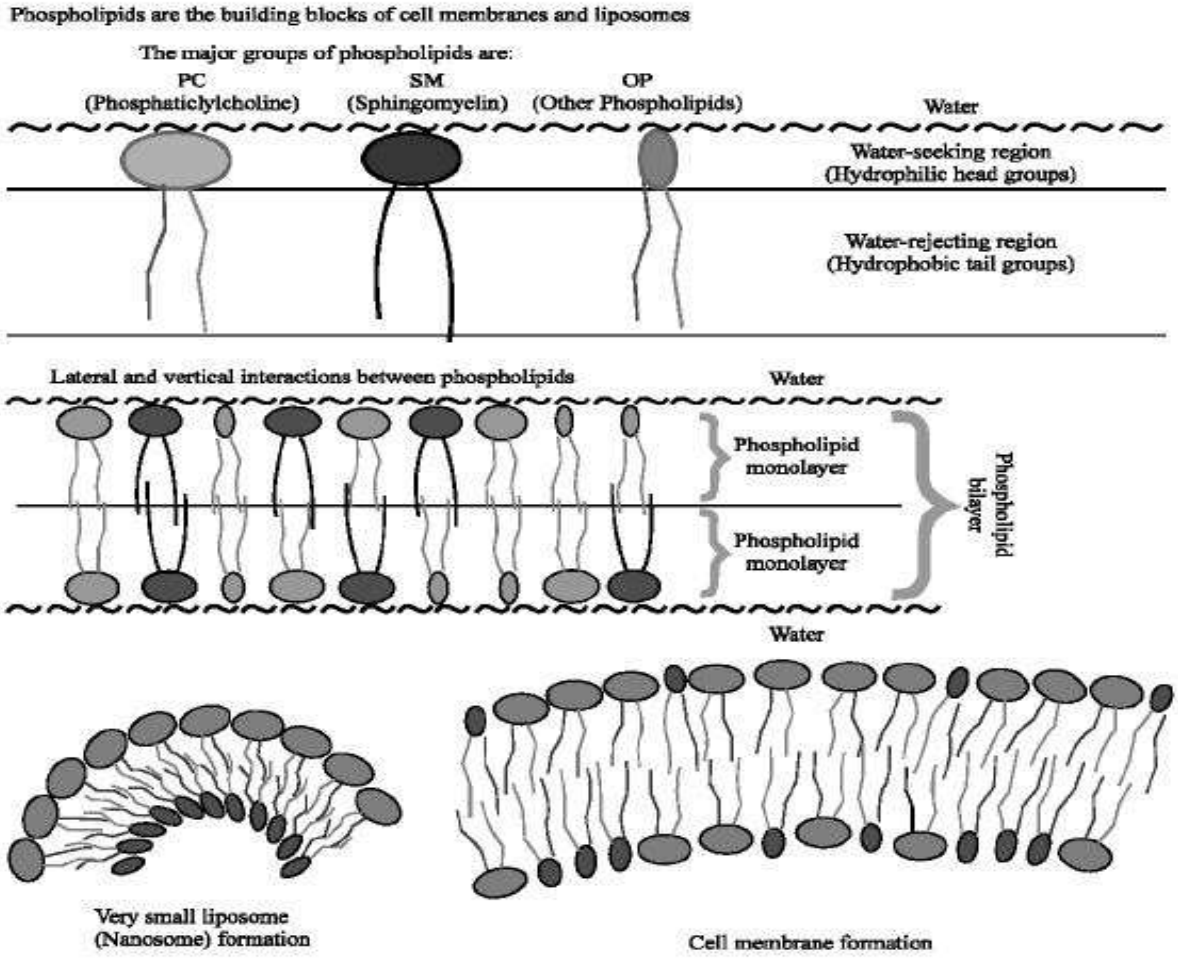


Fig (1- 5): Compositional Structure of Liposome (Sharma *et al*., 2009)

1.2.8.3 Mechanism of transportation through liposome

Liposome can interact with cells by four different mechanism (Sharma *et al*., 2009):

- Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils.
- Adsorption to the cell surface either by nonspecific weak hydrophobic or electrostatic forces or by specific interactions with cell-surface components.

- Fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal content into the cytoplasm .
- Transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. It often is difficult to determine what mechanism is operative and more than one may operate at the same time.

Materials and Methods

2.1 Materials

2.1.1 Equipments & Apparatus

The equipments and apparatus used throughout this study are mentioned in Table (2-1).

Table 2-1: The equipments and apparatus used throughout the study.

Equipment and Apparatus	Company	Origin
Autoclave	Fanem	Brazil
Candal jar	BDH	England
Centrifuge	GallenKamp	England
Cooled centrifuge	Beckman	U.S.A
Distillator	Kottermann	Germany
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	Nuve	Turkey
Micropipettes	Dragonmed	China
Microscope	Zeiss	Germany

Oven	Memmert	Germany
Pasteur pipette	Biomerux	France
pH meter	Milwaukee	USA
Precision pipettes 50µl, 100 µl, 200µl, 1000 µl	Eppendorf	Germany
Refrigerator	Ishtar	Iraq
Sensitive balance	Kern	Germany
Spectrophotometer	Shimadzu	Japan
Test tubes	Grenier	Germany
Lyophilizer	Fisher	England
Vortex mixer	Fanem	Brazil
Sonicator	Fanem	Brazil
Rotary evaporator	Memmert	Germany
Water bath	Memmert	Germany

2.1.2 Biological and chemical materials

The biological and chemical materials that were used are listed in Table (2-2).

Table 2-2: The biological and chemical materials used in the study.

Chemicals	Company	Origin
Aceton	BDH	England
Bovine serum albumin	Himedia	India
Chloroform	Applichem	Germany
Complete Freund's adjuvant	Behring	Germany

Coomassie brilliant blue G- 250	GT Beaker	Holland
EDTA	BDH	England
Eosin stain	BDH	England
Ethanol	BDH	England
Formalin	Scharlau	Spain
Glucose	Fluka	Holland
Glycerol	BDH	England
Glycial acetic acid	Bisolve	France
H_2SO_4	Fluka	Holland
H_2O_2	Himedia	India
H_3PO_4	GT Beaker	Holland
Haematoxylin stain	BDH	England
HCl	BDH	England
Human plasma	Biotest	Germany
K_2HPO_4	BDH	England
KCL	Himedia	India
KH_2PO_4	BDH	England
KOH	BDH	England
Methanol	BDH	England
Methyl red	Himedia	India
N,N,N,N-tetramethyl-P-phenylene diamine dihydrochloride	BDH	England
Na2HPO4	Himedia	India
NaCl	BDH	England
NaH2PO4	Himedia	India
α-Nephthol	BDH	England
Peptone	Himedia	India
Phenol	BDH	England
Phenol red	Merck	Germany
Sephacryl 200 S	Sigma	U.S.A
Sodium Chloride	ADWIC	Egypt

Tris-base	Himedia	India
Tris-HCl	Himedia	India
Tryptone	Biolife	Italy
Urea base	Himedia	India
Yeast extract	Oxiod	England
Giemsa stain	Himedia	India
Leishman stain	Himedia	India

2.1.3 Antibiotic discs

All antibiotics that were used are mentioned in Table (2-3).

Table 2-3: The Antibiotics used in the study.

Antibiotics	Code	Antibiotic conc. µg/disc	Manufacturer (origin)
Amikacin	AK	30	Bioanalyse(turkey)
Ampicillin	Amp	10	Bioanalyse
Cefotaxime	CTX	30	Bioanalyse
Ceftriaxone	Cro	30	Bioanalyse
Chloramphenicol	C	10	Bioanalyse
Ciprofloxacin	Cip	5	Bioanalyse
Nalidixic acid	NA	30	Bioanalyse
Streptomycin	S	10	Bioanalyse
Tetracycline	TE	30	Bioanalyse
Trimethoprime	TMP	5	Bioanalyse

These discs were used to determine the susceptibility of the isolates using disc diffusion method.

2.1.4 Culture Media

2.1.4.1 Diagnostic media (manufactured).

Table 2-4: Diagnostic media used throughout the study.

Medium	Company	Origin
Brain heart infusion broth	Himedia	India
MacConkey agar	Himedia	India
Muller-Hinton agar	Oxoid	England
Nutrient agar	Himedia	India
Nutrient broth	Himedia	India
Peptone water	Himedia	India
Simmon Citrate agar	Himedia	India
Triple sugar Iron agar	Himedia	India
Trypton soya broth	Himedia	India
Urea agar base	Himedia	India
Xylose Lysine Deoxycholate (XLD) agar	Oxoid	England

2.1.5 Kits

Kit	Company	Origin
ELISA KIT (IgG)	Sigma	U.S.A
Liposome	Sigma	U.S.A

2.2 Methods

2.2.1 Diagnostic media (manufactured).

All ready culture media listed in table (2-4) were prepared according to manufacturer company instructions. The pH was adjusted as mentioned and sterilized by autoclaving at 121ºC/15 pounds for 15 mins (except for XLD agar which sterilized only by heating) then dispensed into sterile Petri dishes or tubes as required and stored in refrigerator at 4ºC until use.

2.2.2 Laboratory prepared culture Media

2.2.2.1 Semi solid media (Kumar, 2007).

Composition per liter:

Nutrient broth	13g
Agar agar	4g

This medium was prepared by dissolving the components in 1 liter of distilled water, distributed into tubes and sterilized by autoclave at 121ºC/15 pounds for 15 mins. This was used for motility test.

2.2.2.2 *Shigella* broth (Van der zee, 2003; ISO 21567, 2004).

Composition per liter:

Casein	20g
Dextrose	1g
Potassium hydrogen phosphate	2g
Potassium dihydrogen phosphate	2g
Sodium chloride	5g
Polisorbate 80 (Tween 80)	1.5g

Final pH 7.0

It was prepared by dissolving 31.5 g of the powder in 1 litre of distilled water. Distributed in vials and sterilized in the autoclave at 121ºC for 15 mins. Cooled to 45ºC and aseptically novobiocin (sterilized by filteration using 0.45µm milipore filter) was added to reach a final concentration of 0.5 µg/mL. The complete medium must be used of preparation. The basal broth without antibiotic can be stored in refrigerator for 4 weeks.

2.2.2.3 Urea agar (Kumar, 2007).

It was prepared by dissolving 24 grams of urea agar base in 950 ml of distilled water and sterilized by autoclave, after cooling to 45°C, 50 ml of

40% urea solution which is sterilized by filtration using 0.45µm milipore filter was added then distributed into tubes and let them to solidify as a slant. This was used to indicate the ability of bacteria to produce urease.

2.2.3 Solutions, Reagents and Stains

2.2.3.1 Solutions

2.2.3.1.1 Physiological saline (Prescott, 2002).

It was prepared by adding 8.5 gm of NaCl to 900 ml of distilled water, then completing the volume to 1 liter and sterilized by autoclaveing at 121ºC/15 pounds for 15 minuets.

2.2.3.1.2 Standard turbidity solution (McFarland tube 0.5) (Kumar, 2007).

It was prepared as the following:

- Solution A: 1.175 gm of barium chloride was dissolved in 100 ml of distilled water.
- Solution B: 1 ml of concentrated Sulfuric acid was added to 100 ml of distilled water.

A volume of 0.5 ml from solution A was added to 99.5 ml from solution B and mixed well in glass tubes with tighten covers to avoid steaming, stored in dark at room temperature and mixed well before every time used. This solution was used to approximate the turbidity of bacterial suspension (It's density is equal to 1×10^9 cells per 1 ml).

2.2.3.1.3 Lipopolysaccharide (Endotoxin) Extraction Solutions

• **Phenol solution (90%)**: Ninty grams of phenol powder were dissolved in 100 ml distilled water.

• **Phosphate Buffer (0.025M) (Silipo *et al.*, 2002):** It was prepared by dissolving 1.21 g K_2HPO_4 and 0.34 g KH_2PO_4 in 950 ml distilled water**.** The pH was adjusted to 7.2 and the volume was completed to 1L and sterilized by autoclaving and stored at 4°C.

2.2.3.1.4 Solutions for Protein estimation

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 via 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).

2.2.3.1.5 Solutions for Carbohydrate 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.

2.2.7 Solutions used in Sodium Dodecyl Sulphate-PolyAcryl amid Gel Electrophoresis of proteins (Lammili, 1970)

2.2.7.1 Resolving gel buffer : (0.375M Tris-HCl at pH 8.8)

It was prepared by dissolving 18.17 g of Tris base in 2.2 ml of 37% HCl, then the volume was completed to 100 ml with D.W, and pH was adjusted to 8.8.

2.2.7.2 Stacking gel buffer: (0.125M Tris-Hcl at pH 6.8)

It was prepared by dissolving 6.06 g of Tris base in 3ml of 37% HCl then the volume was complete to 100 ml with D.W, and pH was adjusted to 6.8.

2.2.7.3 Reservoir buffer: (Tris-glycine pH 8.3)

It was prepared by dissolving 3 g of Tris base; 14.4 g glycine and 1 g of SDS in 1000 ml of D.W, and then pH adjusted to 8.3.

2.2.7.4 Acryl amide solution:

Quantities of 24 g of acrylamide and 1 g of N, N, methylen-bisacrylamide were dissolved in 100 ml distilled water, then solution was filtered through whatman filter paper No.1, then stored in dark bottle at 40˚C.

2.2.7.5 Ammonium persulfate solution:

It was freshly prepared by dissolving 250 mg of ammonium persulfate in a quantity of distilled water, then the volume was completed to 10 ml with distilled water and stored in a dark bottle at 4˚C.

2.2.7.6 SDS – solution (10 %)

It was prepared by dissolving 10 g of SDS in 90 ml distilled water, and then the volume was completed to 100 ml with distilled water.

2.2.7.7 Bromophenol blue solution (0.25 %)

It was prepared by dissolving 250 mg of Bromophenol blue in 100 ml distilled water.

2.2.7.8 Lammili sample buffer

It was prepared by mixing the following components:

Component	Quantity (ml)
Staking buffer (0.125M Tris-Hcl PH6.8)	1
Glycerol	0.8
10% SDS	1.6
2-Mercaptoethanol	0.02
Bromophenol blue	0.05

2.2.7.9 Staining solution

It was prepared by dissolving 0.25 g of coomassie brilliant blue R-250 in 48 ml of methanol, then 4 ml of glacial acetic acid was added, and the volume was completed to 100 ml with D.W.

2.2.7.10 Destaining solution

It was prepared by adding 7 ml of glacial acetic acid gradually to a mixture of 5ml methanol and 88 ml distilled water.

2.2.7.11 Fixing solution

It was prepared to be consisting of the following components:

Component	Quantity
Trichloroacetic acid	57 g
Sulphosalicylic acid	17 g
Methanol	159 ml
Distilled Water	350 ml

2.2.7.12 EDTA solution (0.02 M)

It was prepared by dissolving 0.744 g of EDTA in 100 ml D.W. pH was adjusted to 7.5.

2.2.7.13 Tris-hydrochloride Solution (0.5 M)

A quantity of 6 g Tris-hydrochloride was dissolved in 90 ml distilled water then the volume was completed to 100 ml with D.W.

2.2.7.14 Guanidine hydrochloride (6 M):

It was prepared by dissolving 4.2 g of guanidine hydrochloride in 10 ml D.W.

2.2.7.15 Gel preparation

Components of resolving and stacking gels were prepared from the Following solutions:

Solution	Resolving gel (10%)	Stacking gel (3%)
Distilled Water	9.32 ml	8.8 ml
Stacking gel Buffer	-	3.68 ml
Resolving gel Buffer	8 ml	-
SDS 10%	0.64 ml	0.32 ml
TEMED	0.04 ml	0.02 ml
Ammonium per sulfate	0.36ml	0.15 ml

2.2.3.1.6 Immunological and Histopathological Solutions and Stains

- **Haematoxylin and Eosin stains:** The stain solutions were ready and 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).

- **Reagents**

  - **Kovac's reagent** (Prescott, 2002).

  It consists of the followings:

α- dimethyl amino benzaldehyde	5g
Amyl alcohol	75ml
HCl	25ml

It was prepared by dissolving α- dimethyl amino benzaldehyde in alcohol then HCl was added.

- **Methyl red reagent** (Kumar, 2007).

It consists of the followings:

Methyl red	0.1 g
Ethanol 95%	300ml
Distilled water	200ml

This was prepared by dissolving methyl red in alcohol then diluted by distilled water.

- **Voges- Proskauer reagents** (Kumar, 2007).

  This was composed of two reagents:

- VP reagent 1: prepared by dissolving 5 grams of α- naphthol in 100 ml ethyl alcohol.
- VP reagent 2: prepared by dissolving 40 grams of potassium hydroxide in 100 ml distilled water.
- **Catalse reagent** (Kumar, 2007).

  This was composed of 3% Hydrogen peroxide (H_2O_2).

- **Oxidase reagent** (Prsecott, 2002).

This reagent was prepared by dissolving 1 gm of N,N,N,N, tetra methyl para phenyl diamine dihydrochloride in 100 ml of distilled water, stored in dark bottle. Used immediately after preparation.

- **Gram stain kit** (Kumar, 2007).

  Consists of: Crystal violet, Ethanol(95%), Iodine and Safranin.

- **API 20E Kit.** (Analytical profile index for Enterobacteriaceae) **–** Bio-Merieux, France.
- **Antisera kit:** used for stereotyping of *Shigella*. Difco-England.

Consists of: Shigella antiserum poly group A, A_1, B, C, C_1, C_2, D and Alkalescence-Dispar antiserum poly.

٢.4 Isolation and Identification

2.2.4.1 Clinical samples:

One hundred stool samples were collected from children and infants (100cases: 63 males and 37 females) under the age of five years (40 days – 2.5 years old) with diarrhea from different hospitals in Baghdad. Those specimens comprised bloody and watery diarrhea where *Shigella* spp. are

expected to be found. Different biochemical tests applied after streaking stool samples on selective media as in the figure (2-1):

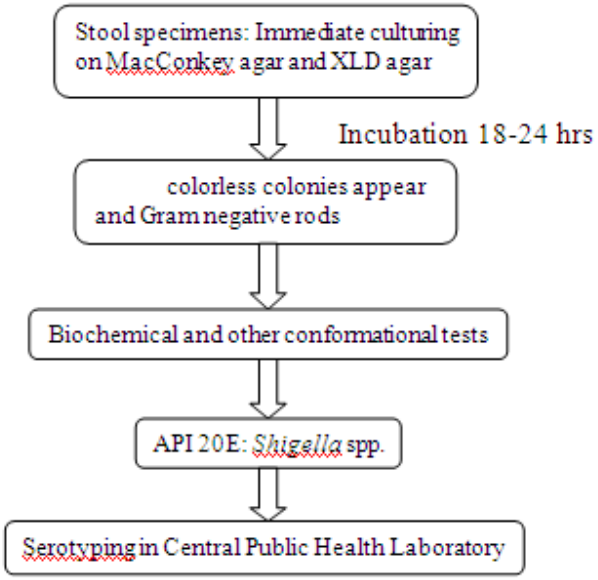


Figure 2-1: Isolation & Identification steps of *Shigella* from clinical samples (Kumar, 2007).

2.2.4.2 Microscopic examinations:

The bacterial isolates also identified through Gram stain to indicate their microscopic characteristics.

2.2.4.3 Biochemical tests

- **Triple sugar iron test (TSI)** (Morello *et al*., 2002).

The TSI test includes fermentation of 3 kinds of sugars, CO_2 and H_2S production. After dissolving, sterilization and setting as slant with 1 inch bottom, fresh pure isolated colony was cultivated by using needle through stabbing the bottom and streaking the slant, incubated for 24-48 hrs.

- **Urease test** (Morello *et al*., 2002; Kumar, 2007).

This test indicates the ability of bacteria to produce urease enzyme. By streaking with loop on the slant surface after touching pure fresh colonies then incubate for 24-48 hrs. No change in the color of the medium indicates negative result.

- **Lactose fermentation test** (Kumar, 2007).

This test can be done by culturing the bacteria on MacConkey agar, if the color of medium change to yellow, the organism is able to ferment lactose. Since *Shigella* is lactose non-fermenter; therefore, color of the medium will remain unchanged.

- **Oxidase test** (Kumar, 2007).

One drop of oxidase reagent was placed on filter paper, with the use of wood stick after touching pure colonies with one side and drawn on the filter paper with reagent. No color formation indicates negative test.

- **Catalse test** (Kumar, 2007).

One drop of 3% Hydrogen peroxide (H_2O_2) was placed on a glass slide and a loopful of pure colonies were placed over the drop. The appearance of gas bubbles indicates a +ve result.

- **Motility test** (Morello *et al*., 2002; Kumar, 2007).

By stabbing the semisolid medium with loop after touching pure fresh colonies and incubated overnight. If hazy growth appeared from the stab location directed to the tube wall indicates that the organism is motile.

- **Indole test** (Morello *et al*., 2002).

A volume of 2 ml of peptone water in a test tube was inoculated by a single colony of test organism and incubated over night then 5 drops of

Kovac's reagent were added to the peptone water culture. The appearance of red color on the surface indicates production of indole.

- **Methyl red-Voges Proskaure test** (Morello *et al*., 2002).

Test tubes containing 2.5 ml of peptone water were inoculated with suspected bacteria and incubated at 37°C for 24 hrs. 5 drops of methyl red reagent were added to one of the tubes and the second for VP test (as prepared in section 2-2-3-2), 6 drops of reagent 1 and 2 drops of reagent 2 were added. The positive results for both appears by changing the color of medium to red.

- **Simmons citrate test** (Morello *et al*., 2002).

A slant of Simmons citrate agar was inoculated with suspected organism and incubated at 37°C overnight. By changing the color of medium from green to blue indicated the positive result.

2.2.4.4 Biochemical Identification of *Shigella* spp. with API 20E (Analytical profile Index): (Castillo and Bruckner, 1984; Neubauer *et al.*, 1998).

The API 20E is a standardized identification system for Enterobacteriaceae and other non-fastidious, Gram negative rods. The API 20E strip consists of 20 microtubes containing dehydrated substrates. These tests were inoculated with a bacterial suspension that reconstitutes the media. The reactions are read according to the reading table and the identification is obtained by referring to the analytical profile index. Oxidase test was performed separately and the result recorded on a sheet as an integral part of the final profile (21 identification test).

Preparation of the strip:

The incubation box was prepared by distributing 5ml of water into the honey combed wells of the tray to create a humid atmosphere, then the strip placed in the incubation box.

Preparation of the inoculum:

A single well-isolated bacterial colony was removed from XLD plate and emulsified in the ampule of API NaCl 0.85% to achieve a homogeneous bacterial suspension.

Inoculation of the strip:

With a sterile pipette, the bacterial suspension was distributed into the tubes of the strip:

- For the CIT, VP and GEL tests, both tubes and cupules were filled.
- For the other tests, only the tubes were filled.
- For the tests ADH, LDC, ODC, H_2S and URE, anaerobiosis was created by overlying with mineral oil.
- The box was closed and incubated at 37°C for 18-24 hrs.
- For purity confirmation of suspension, it was streaked on MacConkey agar and incubated overnight.

Reading the strip:

- After the incubation period, the strip was read by referring to the reading table.
- For TDA test: 1 drop of TDA reagent was added.
- For IND test: 1 drop of JAMES reagent was added.
- For VP test: 1 drop each of VP 1 and VP 2 reagents were added.

The recorded results were compared and wrote on the report sheet.

2.2.4.5 Serololgical identification of *Shigella*: (Lefebvre *et al*., 1995).

Shigella Antisera are used for the identification of *Shigella* sp. By the slide agglutination test.

Principle:

Serological confirmation involves the reaction in which the microorganism (antigen) reacts with its corresponding antibody. This *in vitro* reaction produces macroscopic clumping called agglutination. The desired homologous reaction is rapid, does not dissociate and binds strongly (high affinity).

Procedure:

- One drop (35µl) of the antiserum to be tested was dispensed on an agglutination slide.
- A portion of a loopful of an isolated colonies from a MacConkey's agar plate was transferred to the reaction area above and mixed thoroughly.
- Negative control: 1 drop of sterile 0.85% NaCl solution was dispensed on an agglutination slide. One drop of the Difco Shigella antiserum was added to be tested on the agglutination slide and mixed thoroughly.
- Positive control: 1 drop of the Difco *Shigella* antiserum was dispensed on an agglutination slide. One drop of an appropriate Difco QC antigen *Shigella* or stock cultures of known serological identification was added on the agglutination slide.

- Each reaction area was mixed with a separate stick. After 1 minute the agglutination was read.

If the background of the agglutination was clear to slightly hazy or slightly cloudy the result is positive. If the background was moderately cloudy or cloudy the result is negative. Table (2-6) shows the type of antiserum that reacts with *Shigella* groups.

Table 2-5 Antisera types that agglutinates with different *Shigella* groups (Lefebvre *et al*., 1995).

***Shigella* Antiserum**	**Reacts with**
Poly group A	*S. dysenteriae* types 1-7
Poly group A_1	*S. dysenteriae* type 8ab, 8ac, 9,10
Poly group B	*S. flexneri* 1-6
Poly group C	*S. boydii* 1-7
Poly group C_1	*S. boydii* 8-13
Poly group C_2	*S. boydii* 14-18
Poly group D	*S. sonnei* I and II

2.2.5 Preservation of isolates (Prsecott, 2002).

2.2.5.1 Short-Term Preservation: Pure isolated colonies are inoculated on nutrient agar slants and incubated overnight at 37 °C, stored in refrigerator at 4°C.

2.2.5.2 medium -Term Preservation: Heavy bacterial growth on nutrient agar was inoculated by stabbing with the use of needle in test tubes containing nutrient agar and incubated at 37 °C for 24 h, stored in refrigerator.

2.2.6 Extraction of Lipopolysaccharide (Endotoxin)

2.2.6.1 Cell Preparation (Silipo *et al*., 2002).

- Bacterial cells were grown in flask containing 25 ml of brain heart infusion broth at 37ºC for 18 hours.
- The fresh cultures were used to inoculated 3.5 L of brain heart infusion broth in 500 ml conical flasks (each of them containing 200 ml broth).
- The inoculated flasks were incubated at 37ºC for 24 hours with shaking at 150 rpm.
- After incubation, cultures were centrifuged at (3000 rpm for 15 minutes) and the pellet was washed twice with phosphate buffer.

2.2.6.2 LPS extraction.

LPS was extracted according to method demonstated by (Alexander *et al*., 1992), which is summarized as in the following :

Ten grams of bacterial cells were suspended in 250 ml of water, the suspension was heated to 70°C, and an equal volume of a 90% aqueous phenol solution at the same temperature was added. This mixture was stirred for 30 min at 70°C, left in refrigerator over night, and centrifuged at 3000 rpm for 10 min to obtain 3 layers. Aqueous layer was collected and dialysed (8000-14000 kDa) for three days against D.W. then the dialysate was concentrated in a rotary evaporator at 70°C.

2.2.6.3 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). The carbohydrate concentration was estimated according to the method described by Dubois *et al* (1956).

2.2.7 Chemical Analysis of Lipopolysacharride

2.2.7.1 Estimation 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 0.1ml 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 2-2)

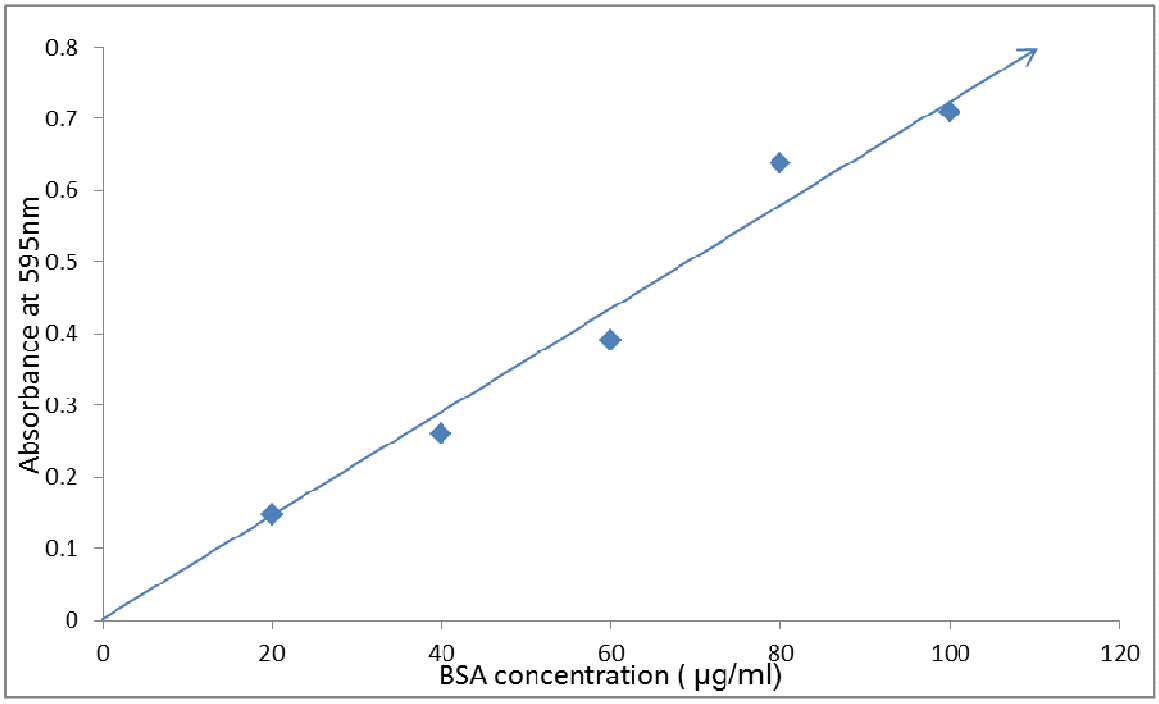


Figure 2-2: Standard curve of bovine serum albumin

2.2.7.2 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_4 were 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 2-3).

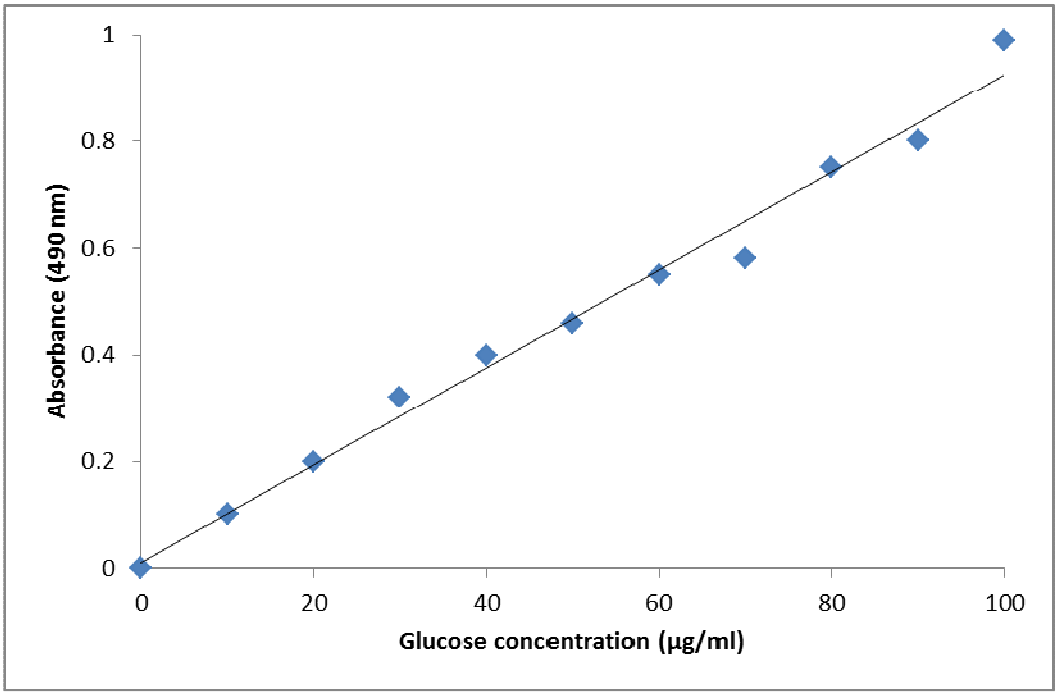


Figure 2-3: Standard curve of glucose

2.2.7.3 Protein detection by Sodium Dodecyl Sulphate-PolyAcryl amid Gel Electrophoresis (Lammili, 1970)

- Resolving gel (10%) was poured in electrophoresis tubes, and these tubes were then left for 30 minutes to ensure complete solidification.
- Staking gel (3%) was added to resolving gel in tubes, the tubes were then left for 15 min for complete polymerization. After 24 hr. they were placed in the electrophoresis unit.
- The electrophoresis system was connected to the power supply with current density 2mA/tube for 30 min to remove positive ions for free protein movement.

2.2.8 Preparation of liposome incorporated LPS (LPS – LIP conjugate) (Dijkstra *et al.*, 1988)

- Five ml of chloroform was added to phospholipids vesicles which were commercially supplied from sigma and evaporated by rotary evaporation at 45° for 1hr.
- The dried lipid mixture was resuspended by vigorously vortexing in 4 ml Tris-buffer (6mM. pH 8), and this treatment was repeated five times over the next hour to obtain multilamellar vesicles .
- One ml of LPS solution at a concentration 100 µg/ml was mixed with 2 ml of lipid suspension at (100 µmol/package).
- The mixture was heated to 45°C for 5 min, sonicated (50 Hz) for 1 min , and vortexed for 1 min . This treatment was repeated 3 times.

2.2.9 Experimental Design

2.2.9.1 Laboratory Animals

Twenty five Albino male mice (*Mus musculus*) 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.

2.2.9.2 Preparation of Doses (Desiderio and Campbell, 1983)

A. Live Bacteria: The bacteria (*S. flexneri*) were adjusted at a concentration of 1 x 10^9 cell/ ml PBS using McFarland method.

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

C. Lipopolysaccharide (LPS) Solution: Partially-purified LPS (section 2.5.3.3) solution was adjusted at a concentration of 100 µg/ml Tris- Hcl (buffer 6mM pH 8).

D. Liposome (LIP) Solution: prepared as mentioned in section (2-2-8) before incorporation to LPS .

E. LPS-LIP Conjugate: prepared as mentioned in section (2-2-8)

2.2.9.3 Protection schedule of liposome incorporation LPS in mice infected with *shigella flexneri*

Group number	First dose	Second dose after 14 days	Third dose after 14 days for second dose
Group 1	Intramuscularly Mice injected with 100 µl normal saline	Mice injected with 100 µl normal saline	
Group2			Orally infected with 100 µl of *shigella flexneri*
Group3	Intramuscularly mice injected with 100 µl Of LPS with complete free adjuvant	Intramuscularly mice injected with 100 µl Of LPS with complete free adjuvant	Orally infected with 100 µl of *shigella flexneri*

Group4	Intramuscularly mice injected with 100 µl Of liposome incorporated LPS	Intramuscularly mice injected with 100 µl Of liposome incorporated LPS	Orally infected with 100 µl of *shigella flexneri*
Group5	Intramuscularly mice injected with 100 µl Of LPS mixed with 100 µl of liposome	Intramuscularly mice injected with 100 µl Of LPS mixed with 100 µl of liposome	Orally infected with 100 µl of *shigella flexneri*

2.2.10 Laboratory Investigations

2.2.10.1 Total and Absolute Counts of Leukocytes

Both counts were carried out on blood obtained by heart puncture using insulin disposable syringe (3 ml) pre-coated with heparin after 10 days from infection .

- **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:

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

- **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:

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

2.2.10.2 Immunoassay for quantitative mouse IgG titer

Enzyme Principle

This ELISA kit is designed for use in measuring the mouse IgG antibody titers of the serum, plasma, ascites and supernatant samples. It is a peroxidase-based immune detection system. Provided as ready-to-use reagents, this kit contains all essential reagents required for ELISA procedure, and offers easy-to-use, time-saving and cost-effective benefits. Included reagents are blocking reagent, sample diluent, anti-mouse IgG HRP conjugate, ready-to-use TMB substrate reagent, and washing buffer. ELISA method is commonly used for determining antibody titers in serum, supernatant and ascites. An antibody titer is normally expressed as the greatest dilution ratio that still gives a positive detection for a particular epitope or antigen. With our sensitive anti-mouse IgG HRP conjugate and TMB solution, this reagents kit offers clean detection and reproducible performance.

› Assay Procedure

The procedure was done according to the instruction of General Bioscience Company.

- All reagent and microplate were brought to the room temperature and mixed for a few minutes prior to use. Fifty µl of diluted samples (at the concentration 10µg/ml) was added to each well with gently mixing, the plate was covered with a cardboard to prevent evaporation and put in incubator for 2 hrs at room temperature.
- Four hundreds µl of blocking reagent was added to each well and incubated for 2 hrs at room temperature.
- The plate was washed 3 times using a microtiter plate washer, and (1:50) dilution was performed. Then 100 µl was added to each well and the plate was incubated at room temperature for 2 hrs.
- The plate was washed 3 times using a microtiter plate washer.
- One hundred µl of anti-mouse HRP conjugate was added to each well. The plate was incubated for 1 hr at room temperature and washed 3 times.
- One hundred µl of TMP One Solution HRP substrate was added to each well and incubated at room temperature for 10 min.
- The reaction was stopped by addition 1N of sulfuric acid and the absorbance was read at 450nm or 405 nm.

2.2.10.3 Histopathological study

The liver, spleen and intestine 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.

2.2.11 Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA), least significant difference (LSD) and Duncan test, using the computer program IBM SPSS Statistics 19. The difference was considered significant when the probability value (P) ≥ 0.05 (Norman and Streiner, 2008).

A further estimation was also given; it was treatment efficiency (Perez-Serrano *et al*., 1997), which was calculated according to the following equation:

Treatment efficiency = A-B\B × 100

A=treated group.

B=control group.

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3.1 Isolation of *Shigella*

3.1.1 Stool samples:

One hundred stool samples were collected from children and infants (100cases: 63 males and 37 females) under the age of five years (40 days – 2.5 years old) with diarrhea from different hospitals in Baghdad city included Al-Alwia children hospital, Al-Yarmouk hospital, Al-Kathmia hospital and Central child hospital. Types of stool samples are mentioned in Table (3-1). Thirty stool samples were suspected to be *Shigella*. Results reveled that only 8 isolates were positive for *Shigella*.

Table 3-1: Distribution of *Shigella* occurance on clinical source.

consistent of sample	Number of samples suspected to be *shigella*	Positive *shigella%*
Bloody diarrhea	14	4 (13%)
Green diarrhea with mucous	6	3 (10%)
Brown diarrhea	10	1 (3%)
Total	30	8 (26%)

Specimens that cannot be cultured within two hours after collection should be placed in transport medium and refrigerated immediately. Unlike some organisms, *Shigella* will die, even in transport media, if they are not refrigerated (WHO, 2005).

Bacteria of the genus *Shigella* are capable of penetrating and multiplying within the epithelial cells of the colon. The infection produces ulcerative lesions in the colonic mucosa and results in the bloody mucous diarrhea characteristic of bacillary dysentery (Maurelli *et al*., 1984). Typically, after invasion and progression of the disease, erythrocytes,

leukocytes, cellular debris, and mucus appear in the lumen of the bowel. A stool containing such elements is defined as being dysenteric (Hirsh *et al*., 1980).

When patients develop bloody diarrhea or dysentery, *Shigella* must obviously be included in the list of possible causes. In some parts of the world, up to 50% of patients with bloody diarrhea or dysentery are culture positive for *Shigella*. In contrast, this diagnosis may escape consideration in the mild watery diarrhea caused by *S. sonnei*. The presence of leukocytes in diarrheal stool is a simple indicator of an invasive pathogen, and their detection provides immediately useful diagnostic information (Sansonetti, 2006).

3.1.2 Biochemical tests:

Biochemical tests were achieved on the suspected 8 isolates that showed morphological growth characteristics closely related to *shigella* sp. All expected *Shigella* isolates were Gram negative bacilli, in triple sugar iron agar they gave red (alkaline) slant so they do not ferment lactose nor sucrose, and yellow (acid) which means they ferment glucose with no production of gas (CO_2) or H_2S. On MacConkey agar they produced colorless colonies with fair to good growth which means they are lactose non-fermenter except one of the isolates gave positive reaction after 48 h of incubation. They are non-motile, Oxidase-negative, Urease-negative, Catalase-positive. Three of the isolates gave positive reaction for indole production. Also all isolates were positive for methyl red test, negative for Voges-Proskauer and citrate utilization tests. (Rowe and Gross, 1994; Kumar, 2007). Table (3-2) shows biochemical tests applied on isolated *shigella*.

Table 3-2: Biochemical characterization of *Shigella* isolates.

Isolation No.	TSI				IMViC				Lactose	Urease	Oxidase	Catalase
	Slant	Butt	H_2S	Gas	I	M	VP	C				
1	K	A	-	-	-	+	-	-	-	-	-	+
2	K	A	-	-	+	+	-	-	-	-	-	+
3	K	A	-	-	-	+	-	-	-	-	-	+
4	K	A	-	-	-	+	-	-	-	-	-	+
5	K	A	-	-	+	+	-	-	-	-	-	+
6	K	A	-	-	+	+	-	-	-	-	-	+
7	K	A	-	-	-	+	-	-	-	-	-	+
8	k	A	-	-	-	+	-	-	ɨ	-	-	+

K: alkaline; A: acid; +: positive; -: negative.

Microscopic examination of the stool is helpful in making the correct diagnosis for enteric disease caused by *Shigella*. Individuals with disease caused by this pathogen characteristically shed erythrocytes and leukocytes in their stool. Armed with this knowledge, the clinician can instigate rational therapy 24 to 48 h before the results of culture (Hirsh *et al*., 1980).

3.1.3 API 20E system results:

For confirmation of biochemical tests and further identification of the isolates, API 20E system was applied (Castillo and Bruckner, 1984; Neubauer *et al*., 1998). Table (3-3) and figure (3-1) shows results of Api 20E tests.

Table 3-3: Biochemical analysis results of API 20E system of *Shigella*.

No.	Biochemical tests	Results	Color
1	OPNG	-	Colorless
2	ADH	-	Yellow
3	LDC	-	Yellow
4	ODC	-	Yellow
5	CIT	-	Pale green/yellow
6	H_2S	-	Colorless/grayish
7	URE	-	Yellow
8	TDA	-	Yellow
9	IND	-	Colorless/ Pale green/ Yellow
10	VP	-	Colorless/ Pale pink
11	GEL	-	No diffusion of black pigment
12	GLU	+	Yellow
13	MAN	- or +	Blue green or Yellow
14	INO	-	Blue green
15	SOR	-	Blue green
16	RHA	-	Blue green
17	SAC	-	Blue green
18	MEL	- or +	Blue green or Yellow
19	AMY	-	Blue green
20	ARA	- or +	Blue green or Yellow

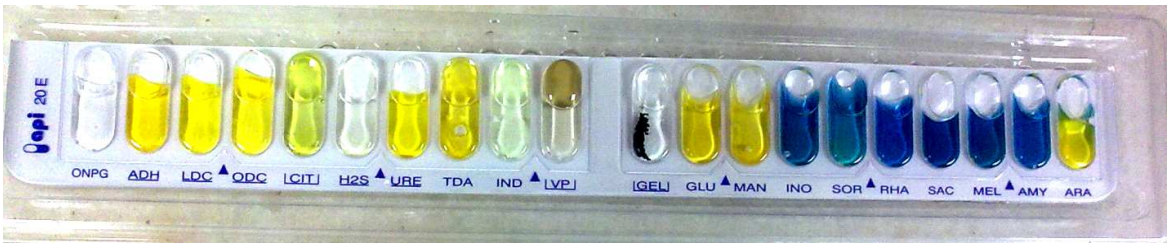

Figure 3-1: API 20E strip showing the results of one of *Shigella* spp.

3.1.4 *Shigella* serotyping:

Applying the *Shigella* somatic (O) antigen test. All isolates gave positive *Shigella* somatic (O) test which comprised the isolates belonged to

the well-known 4 species of this genus. The results of *Shigella* serotyping are shown in table (3-4).

Table 3-4: *Shigella* spp. isolated from clinical samples.

***Shigella* spp.**	**No. (%)**	**Source of isolation**
S. flexneri	4 (50%)	Bloody diarrhea or green diarrhea with mucus
S. boydii	2 (25%)	Bloody or brown diarrhea
S. dysenteriae	1(12.5%)	Bloody diarrhea
S. sonnei	1(12.5%)	Green diarrhea with mucus
Total	8(100%)	

According to the previous identification tests (biochemical tests, Api 20E systems and serotyping) it was stated that the most common *Shigella* species were four isolates of *S. flexneri*, followed by two isolates of *S. boydii* , while only one isolate belong to *S. dysenteriae* and *S. sonnei*. A study by Al-Khafaji (2002) in Al-Mustansiriya University revealed that from 25 isolates of *Shigella* 19(76%) were *S. flexneri*, 3(12%) *S. boydii*, 2(8%) *S. sonnei* and 1(4%) *S. dysenteriae*. A similar study by Taha (2002) revealed that among 74 isolates of *Shigella*, 25(33.7%) belonged to *S. flexneri*, 21(28.3%), *S. sonnei*, 16(21.6%) *S. boydii* and 12(16.2%) *S. dysenteriae*.

The 20th century has seen a steady and remarkable change in the relative frequency of the different *Shigella* species in the UK and other European countries. Infections due to *S. dysenteriae*, which were common before the first world war, are now rare. Between 1920 and 1930 both *S. flexneri* and *S. sonnei* were endemic and approximately equal in incidence, but by 1940 *S. sonnei* had become dominant, increasing in incidence annually to a peak of over 49,000 notifications in 1956. The incidence of *S. sonnei* then declined steadily in the UK to an annual average of about 3000 notified cases between 1970 and 1990. However, the number of cases rose sharply in 1991,

when there were several widespread community outbreaks, and continued to rise to peak of 17,000 cases in 1992, the highest level in the UK for more than 20 years. The incidence has since fallen, but there were still more than 4550 cases in 1995. Infections due to the other *Shigella*, usually imported, have remained fairly constant at about 800-900 a year (Lewis, 1997).

Similar changes have taken place in the USA although more slowly. Up to 1968, *S. flexneri* and *S. sonnei* were equally common, but *S. sonnei* now accounts for 65% of cases and *S. flexneri* for about 30%. Over the past several decades the other two *Shigella* spp. have been responsible for less than 5% of notifications, but there has been a recent increase in cases imported from Asia and South America (Lewis, 1997).

Almost all fatal cases of shigellosis occur in developing countries, and data on mortality are generally compiled from three sources: investigations of epidemics caused by *Shigella dysenteriae* type 1, surveillance of endemic diarrheal disease, and reports from hospitals. Attack rates during epidemics of dysentery due to infection with *S. dysenteriae* type 1 have ranged from 1% to 33%, and case-fatality rates have ranged from 1% to 7% (Bennish and Wojtyniak, 1991). The high incidence of *Shigella* in developing countries is generally attributed to the lack of clean water, poor sanitation, malnutrition and cost of antibiotic treatment (Jennison and Verma, 2004).

In developing countries where poor hygienic conditions prevail and contact with contaminated household surfaces occurs frequently, *S. dysenteriae type 1* in the viable but non-culturable state on fomites may be a potentiator in the transmission of shigellosis. A study by Islam *et al*. (2001) revealed that culturability of *S. flexneri* on different fomites with different survival times obtained from the fomites. Among the five materials, the

shortest and longest survival times during which culturable cells could be detected were: plastic, glass, aluminum, wood and cloth, respectively.

3.1.5 Antibiotic Sensitivity Test

The four isolates of *S. flexneri* were tested for their sensitivity to10 antibiotics, and the results showed that all *S. flexneri* isolate was resist to 4 antibiotics Ampiciln (Am), Streptomycin (S), Chloramphenicol (C) and Ceftriaxone (Cro), and sensitive to 4 antibiotics Tetracycline (Te), Ciprofloxacin (Cip), Nalidixic acid (Na) and Trimethoprime (Tmp). (Table 3-5).

Table 3-5: Antibiotic sensitivity of four *S. flexneri* isolates.

***Shigella* Spp.**	**Am (10)**	**S (10)**	**C (10)**	**TE (30)**	**CTX (30)**	**Cip (5)**	**Cro (30)**	**Na (30)**	**TMP (5)**	**AK (30)**
***S.flexneri* SH1**	**R**	**R**	**R**	**S**	**Int**	**S**	**R**	**S**	**S**	**Int**
***S.flexneri* SH2**	**R**	**R**	**R**	**S**	**Int**	**S**	**R**	**S**	**S**	**Int**
***S.flexneri* SH3**	**R**	**R**	**R**	**S**	**Int**	**S**	**R**	**S**	**S**	**Int**
***S.flexneri* SH4**	**R**	**R**	**R**	**S**	**Int**	**S**	**R**	**S**	**S**	**Int**

R: resistant, S: sensitive, Int: intermediate.

Qureishi *et al.* (2007) in Zahedan. Acta Medica Iranica found that 99.3% of *Shigella* isolates were resistant to ampicilin, 52% resistant to chloramphenicol, 1.3% resistant to nalidixic acid and there was no resistance to ciprofloxacin and ceftriaxone. While Khiyami *et al.* (2011) found that isolated *Shigella* spp. were resistant to ampicilin, trimethoprime and tetracycline and susceptible to amikacin, cefotaxime, ceftriaxone, chloramphenicol and streptomycin.

Haukka and Siitonen (2008) reported that 91% of *Shigella* strains were resistant to streptomycin, 85% resistant to tetracycline, 33% resistant to ampicilin, 21% resistant to chloramphenicol, 84% resistant to trimethoprime,

16% resistant to aalidixic acid, 1% resistant to ciprofloxacin but they found no resistant to ceftriaxone or cefotaxime.

3.2 Extraction of Lipopolysaccharide from *S. flexneri*

Lipopolysaccharide (endotoxin) is the major constituent of the outer membrane of Gram-negative bacteria (Morath *et al.*, 2002). 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, 2002). 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. flexneri* isolates was extracted according to phenol hot water method. The *S. flexneri* isolate was grown in brain heart agar at 37ºC, and in agreement with this, the present employed method yielded a bacterial mass of 20 grams dry weight bacteria.

Extraction of LPS by this method 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 5.45 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. Due to the amphipathic 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).

3.2.1 Chemical Characterization

Chemical characterizations of the crude endotoxin extracted from *S. flexneri* 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).

3.2.2 Partial Purification

Fifty 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 illustrated in (Figure 3-2).

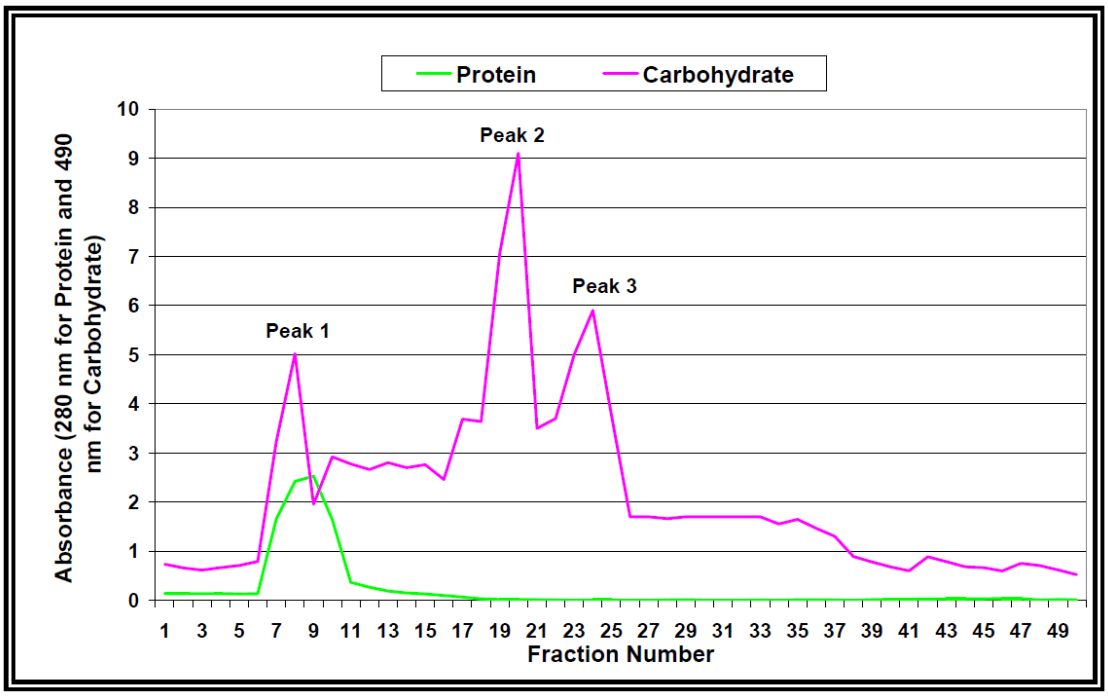

Figure 3-2: Gel-filtration chromatography for LPS partial purification from *S. flexneri* by using Sephacryl 200 S, 75×2cm column equilibrated and eluted with 0.025 M PBS pH 7.2 with a flow rate of 75 ml/hour.

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 14.0, 29.0 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 (29.0 vs. 18.1%) as shown in table 3-6.

Table 3-6: Carbohydrate and protein contents of crude and partially purified lipopolysaccharide of *S. flexneri* .

Lipopolysaccharide		**Carbohydrate (%)**	**Protein (%)**
Crude		18.1	5.2
PartiallyPurified	Peak 1	14.0	4.0
	Peak 2	29.0	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 29%) differs from that recorded by Horton *et al*. (1977), in which 12- 18% range was observed. However, an agreement was also reported 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 3-6, results showed that the protein percentage in partially purified LPS was 4% in peak 1 and 0.001% in peak 2 and 3.

Furthermore, Wilkinson and Galibraith (1975) reported that the differences in the methods used in extraction and purification of LPS and differences in the bacterial strains and their content of LPS may be related with the differences in the protein and carbohydrate percentages in the purified LPS.

It was also observed that the carbohydrate percentage in the partial purified LPS (29% in peak 2) was higher than that of the crude LPS (18.1%), and a similar observation was made for the protein (4.0 and 0.001 vs. 5.2%). Both observations suggest the efficiency of the applied method of purification by gel filtration. The partial purified LPS was analyzed by Sodium Dodecyl Sulphate Gel Electrophoresis (SDS-PAGE) and results reveled that two bands with molecular weight 100 KDa and 150 KDa were present and band with MW 100 kDa represented LPS (figure 3-3).

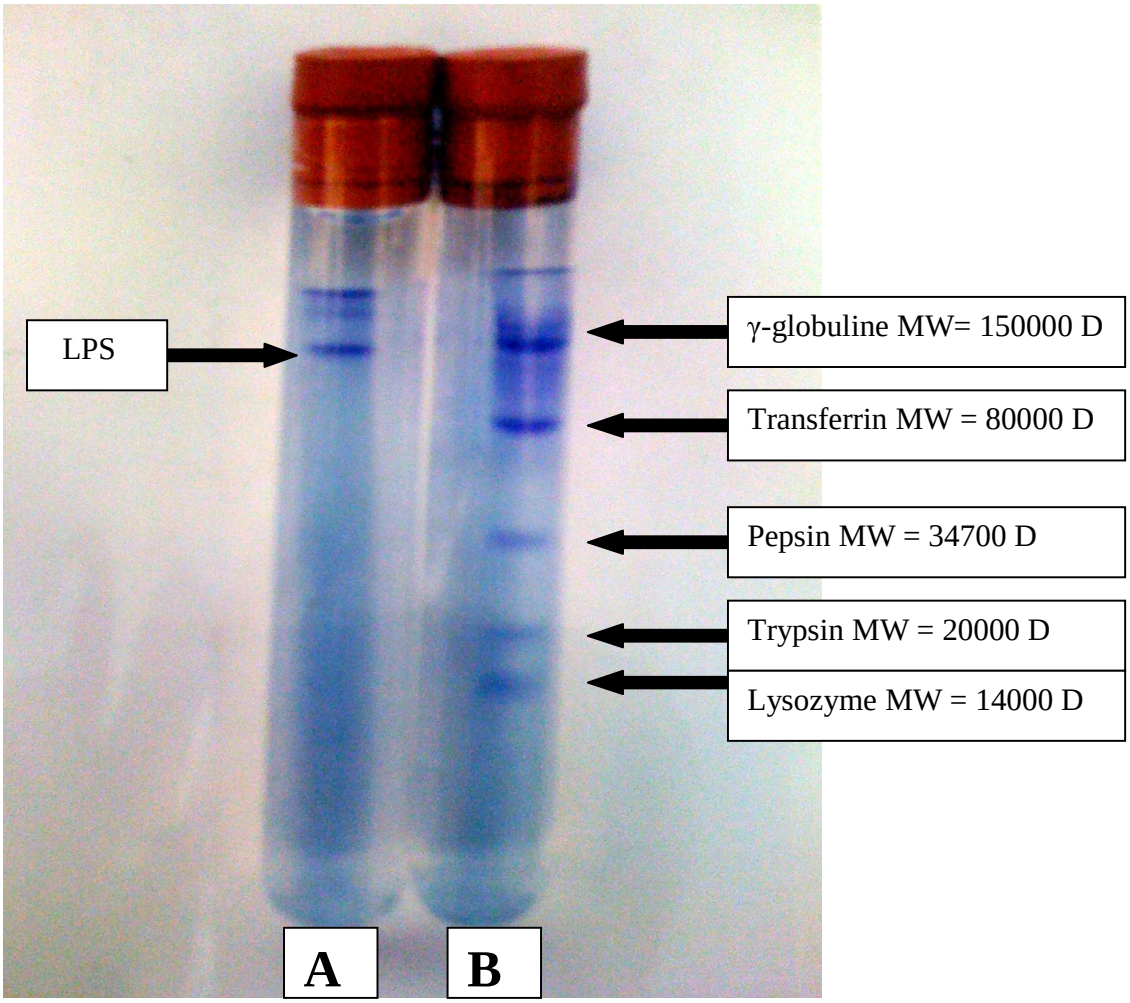

Figure (3-3) Electrophoresis by use Sodium Dodecyl Sulphate PolyAcryl amid, tube A act as LPS sample and, tube B act as marker tube.

3.3 Immunological Evaluation

3.3.1 Leukocyte Counts

• Total Leukocyte Count (TLC)

Results in table (3-7) indicated that mice infected with *S. flexneri* showed a significant increase in total leukocyte as compared with negative control $P \leq 0.05$ (7466 *vs.* 4633 cell/1ml.blood). A significant increase was recorded in mice treated with conjugate, mice coinjected with LPS and

liposome and mice treated with LPS as compared with negative control (7300, 4733 and 6700 vs. 4633 cell cell/1ml.blood). Moreover all treated groups recorded a significant decrease in total leukocyte as compared with positive control (7300, 4733 and 6700 *vs*.7466 cell/1ml.blood).

Table (3-7): Total leukocyte count in mice treated with LPS-liposome conjugate and infected with *Shigella flexneri*

Mice group	Mean±S.E. (Total Leukocyte Count: cells/cu. mm blood)
Negative control	**4633±987 b**
Mice infected with *Shigella* (Positive control)	**7466±450 a**
LPS-liposome conjugate and infected with *Shigella*	**7300±361 ab**
Mice with mixed LPS and liposome and infected with *Shigella*	**4733±985 ab**
Mice treated with LPS and infected with *Shigella*	**6700±361 ab**

***Different letters: Significant difference ($P \leq 0.05$) between means of column**

• Absolute Neutrophil Count (ANC)

Mice treated with conjugate, mice treated with LPS and treated with mixed LPS and liposome exhibited a significant increase in total neutrophils count as compared with negative control (2469,1690 and 1769 vs. 1249 cell/1ml.blood). A significant increase was recorded in mice treated with conjugate, mice treated with LPS and mice coinjected with LPS and liposome as compared with positive control (2469, 1690 and 1769 vs. 1476 cell/1ml.blood).

Table (3-8): Total neutrophils count in mice treated with LPS-liposome conjugate and infected with *Shigella flerneri*

Mice group	Mean ± S.E. (Absolute Neutrophil Count: cells/cu. mm blood)
Negative control	**1249±250 bc**
Mice infected with *Shigella* (Positive control)	**1476±803 bc**
LPS-liposome conjugate and infected with *Shigella*	**2469±107 a**
Mice with mixed LPS and liposome and infected with *Shigella*	**1769±309 b**
Mice treated with LPS and infected with *Shigella*	**1690±170 b**

***Different letters: Significant difference ($P \leq 0.05$) between means of column**

• Absolute Lymphocyte Count (ALC)

As shown in table (3-9) there were significant increase in lymphocyte count in mice infected with *S. flexneri*, mice treated with conjugate, mice coinjected with LPS and liposome and mice treated with LPS as compared with negative control(5941, 4818, 3964 and 4917 *vs*. 3310 cell/1ml.blood). Since a significant decrease was recorded in all treated groups as compared with positive control (4818, 3964 and 4917 *vs*. 5941 cell/1ml.blood).

Table (3-9): Total lymphocyte count in mice treated with LPS-liposome conjugate and infected with *Shigella flexneri*

Mice group	Mean ± S.E. (Absolute Lymphocyte Count: cells/cu. mm blood)
Negative control	**3310±861 c**
Mice infected with *Shigella* (Positive control)	**5941±1738 a**
LPS-liposome conjugate and infected with *Shigella*	**4818±238 bc**
Mice with mixed LPS and liposome and infected with *Shigella*	**3964±1171 bc**
Mice treated with LPS and infected with *Shigella*	**4971±864 bc**

***Different letters: Significant difference ($P \leq 0.05$) between means of column**

Treatment mice with LPS showed a significant increase in total and absolute count of leukocyte. This might be due to IL-1 and TNF secreted by macrophage as mentioned by Dijsktara *et.al* (1988) who reported that activation of mononuclear phagocyte by LPS results in the secretion of many factors interferons, prostaglandines, tumor necrosis factor (TNF) and interleukin-1 (IL-1). It is known that 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, 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*., 1990).

3.4 Serum IgG Level

As shown in table (3-10) the mean OD titers of IgG in mice infected with *S. flexneri* was significantly higher as compared with negative control (0.7890 vs. 0.5433) $P \leq 0.05$. The mean OD titer after treatment with conjugate and infected with *S. flexneri* , and mice treated with LPS and infected with *S. flexneri* were significantly higher as compared with negative control (0.9243 and 0.8420 *vs.* 0.5433). There was no significant difference between mice coinjected with LPS and liposome as compared with negative control (0.6293 *vs*.0.5433). There were no significant changes in all treated groups and positive control (0.6293and 0.8420 vs. 0.7890) except mice treated with conjugate and infected with *S. flexneri* (0.9243 *vs.* 0.7890).

Table (3-10): Serum IgG level in mice treated with LPS-liposome conjugate and infected with *Shigella flexneri*

Mice group	(Mean±SE *) OD titer
Negative control	**0.5433±0.0180 b**
Mice infected with *Shigella* (Positive control)	**0.7890±0.0330 a**
LPS-liposome conjugate and infected with *Shigella*	**0.9243±0.1094 a**
Mice coinjected with LPS and liposome and infected with *Shigella*	**0.6293±0.0666 b**
Mice treated with LPS and infected with *Shigella*	**0.8420±0.1196-5 a**

***Different letters: Significant difference ($P \leq 0.05$) between means of column**

experiments demonstrated that after the incorporation of LPS into liposomes induce humoral responses (Elkins *et al*., 1989). A liposomal complete core lipopolysaccharide vaccine induces humoral immunity toward lipopolysaccharide (LPS), while remaining non-pyrogenic should be beneficial, as high levels of antibodies against LPS are associated with a reduced risk of adverse outcome (Stewart *et.al*. 2002).

According to Cryz *et al.* (1984 b) LPS isolated from several strains of *P. aeruginosa* was derived either from the phenol or water phase, and was found to be highly immunogenic and protective in mice with doses as low as 0.001 μg; the level of protection correlated with anti-LPS antibody titers.

Dissanayake, *et al* (2010) found that LPS core specific IgG responses of chickens immunized with different doses of liposome encapsulated LPS

than those of non-immunized chickens as detected by ELISA which showed significantly high LPS core specific IgG responses.

3.5 Histopathological Evaluation of Liver , Spleen and Intestine

Different histopathological changes were observed in liver, spleen and intestine of groups of mice. For the ease of presentation, under each picture, the histopathological profile is given.

- Liver section of mice (negative control) showing normal looking appearance of parenchymal hepatic tissue, portal area and central vein (figure 3-4).
- Spleen section of mice (negative control) showing normal structure appearance with presence of white and red pulp (figure 3-5).
- Intestine section of mice (negative control) showing presence of payer's patch in the jejunum (figure 3-6).

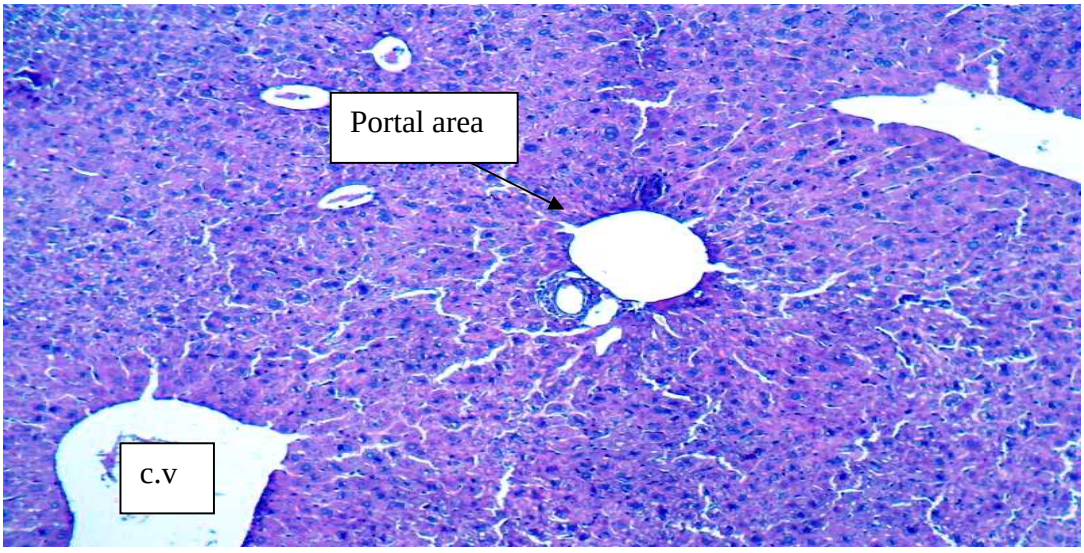

Figure 3-4: Liver section of mice (negative control) showing normal looking appearance of parenchymal hepatic tissue, portal area and central vein (H and E; 200X).

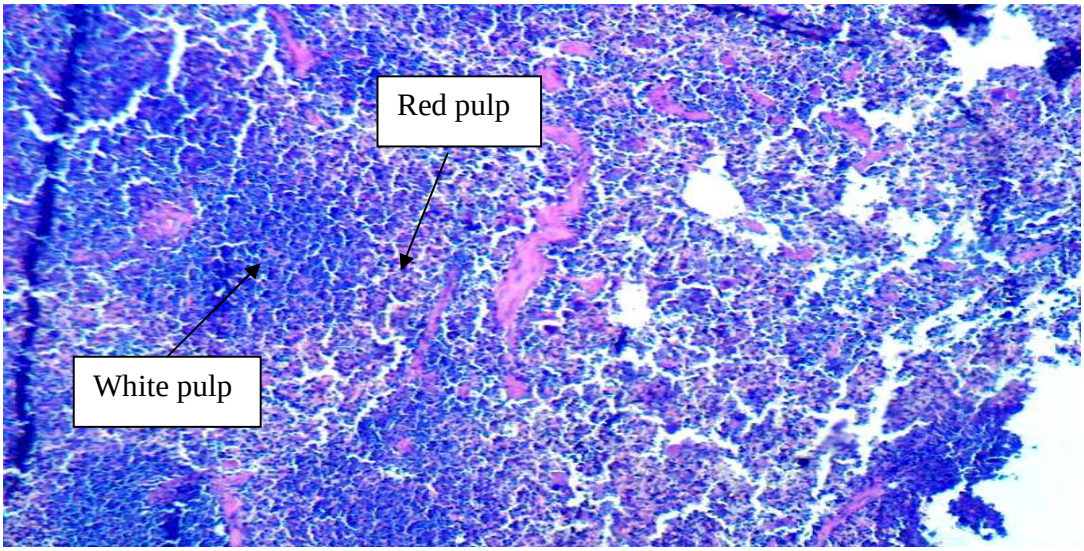

Figure 3-5: spleen section of mice (negative control) showing normal structure appearance with presence of white and red pulp (H and E; 200X).

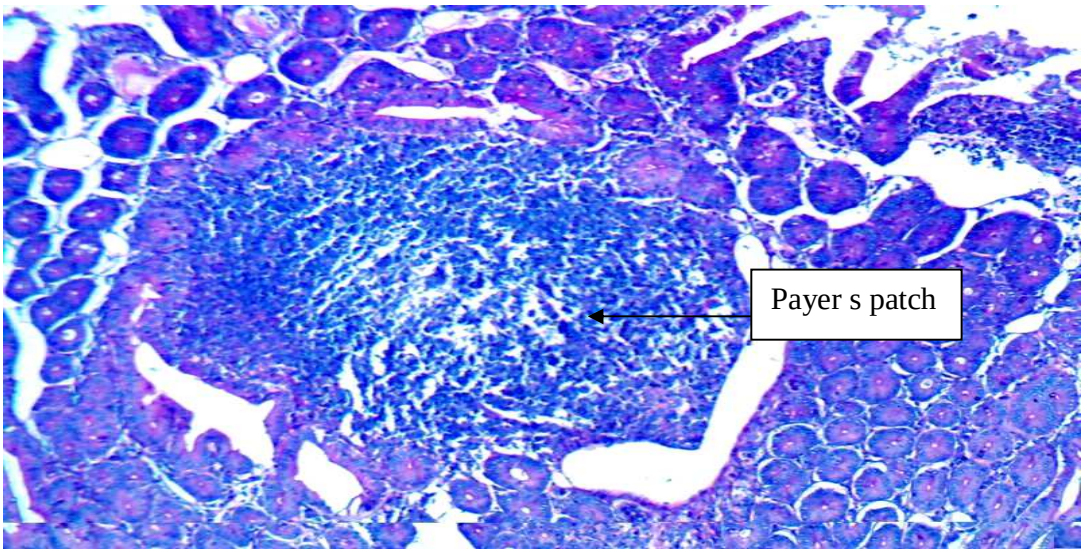

Figure 3-6: intestine section of mice (negative control) showing presence of payer's patch in the jejunum (H and E; 200X).

- Liver section of mice infected with *S.flexneri*, showing certain focal area necrosis with inflammatory cells infilterate especially at portal area (figure 3-7).
- Spleen section of mice infected with *S.flexneri*, showing mild necrosis of parenchymal tissue with widening and hyperplasia of white pulp (figure 3-8).
- Intestine section of mice infected with *S.flexneri*, showing normal look-like intestinal villi mucosa(figure 3-9).

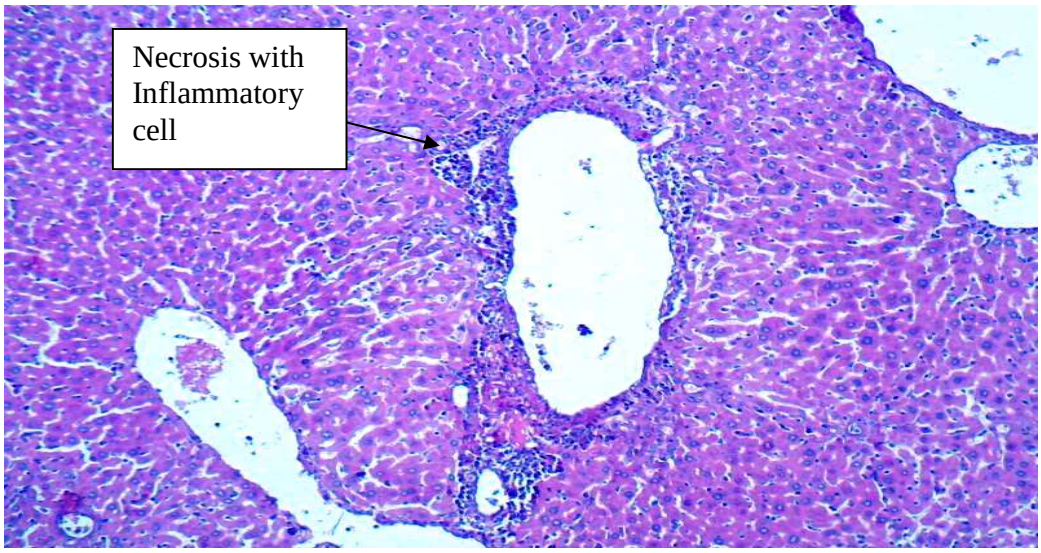

Figure 3-7: liver section of mice infected with *S.flexneri*, showing certain focal area necrosis with inflammatory cells infilterate especially at portal(H and E; 200X).

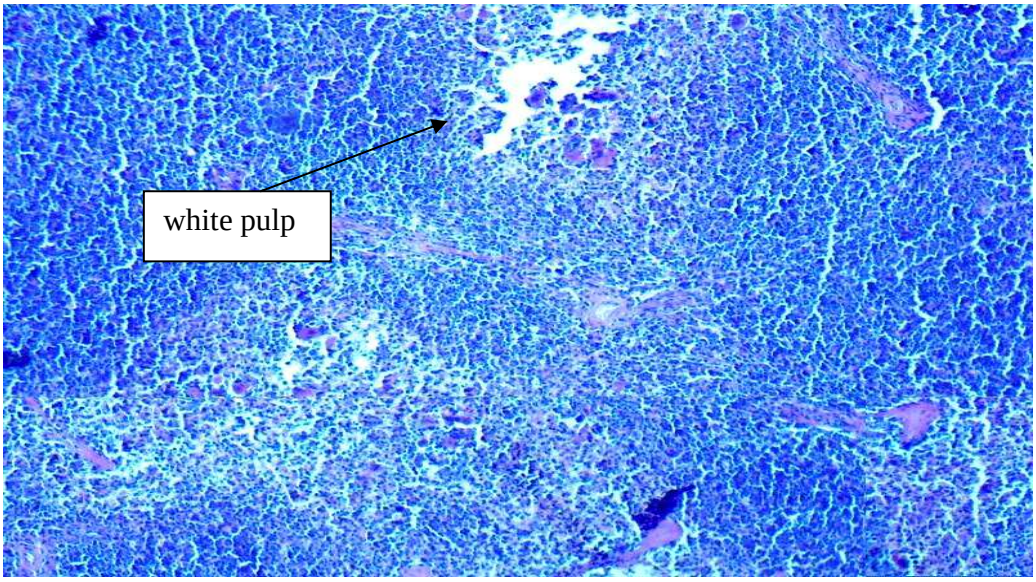

Figure 3-8: Spleen section of mice infected with *S.flexneri*, showing mild necrosis of parenchymal tissue with widening and hyperplasia of white pulp (H and E; 200X).

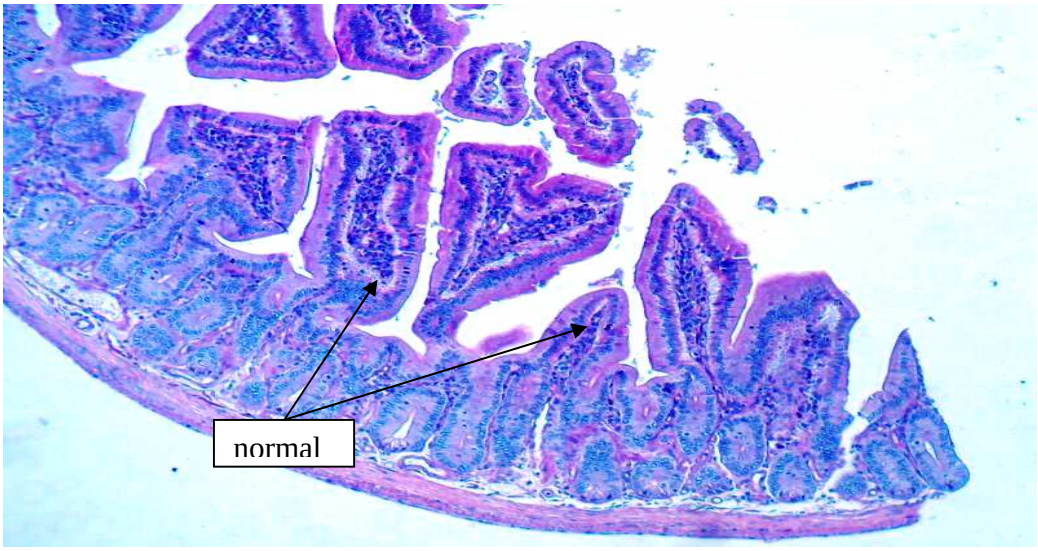

Figure 3-9: intestine section of mice infected with *S.flexneri*, showing normal look-like intestinal villi mucosa (H and E; 200X).

- Liver section of mice treated with (LPS-liposome conjugate) showing normal looking histological structure appearance(figure 3-10).
- Spleen section of mice treated with (LPS-liposome conjugate) showing diffuse spleening parenchymal lymphoid hyperplasia(figure 3-11).
- Intestine section of mice treated with (LPS-liposome conjugate) showing normal appearance of intestinal villi (figure 3-12).

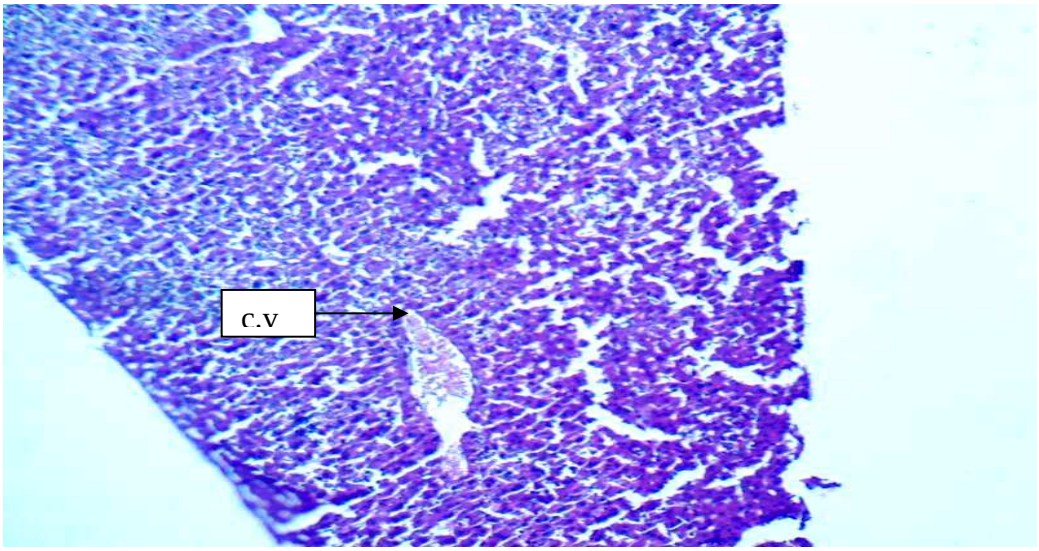

Figure 3-10: liver section of mice treated with (LPS-liposome conjugate) showing normal looking histological structure appearance (H and E; 200X).

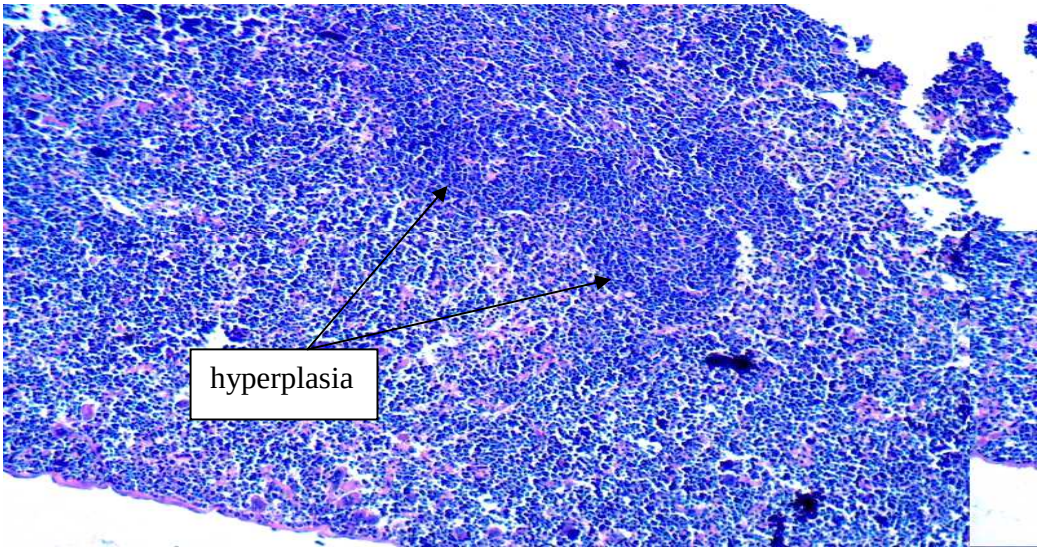

Figure 3-11: spleen section of mice treated with (LPS-liposome conjugate) showing diffuse spleening parenchymal lymphoid hyperplasia (H and E; 200X).

Figure 3-12: intestine section of mice treated with (LPS-liposome conjugate) showing normal appearance of intestinal villi (H and E; 200X).

- Liver section of mice treated with (LPS free+liposome free) showing normal looking appearance with mild inflammatory cells infiltrate (figure 3-13).
- Spleen section of mice treated with (LPS free+liposome free) showing diffuse hyperplasia of parenchymal tissue with reactive collagen fibers (figure 3-14).
- Intestine section of mice treated with (LPS free+liposome free)) showing intestinal mucosal hyperplasia (figure 3-15).

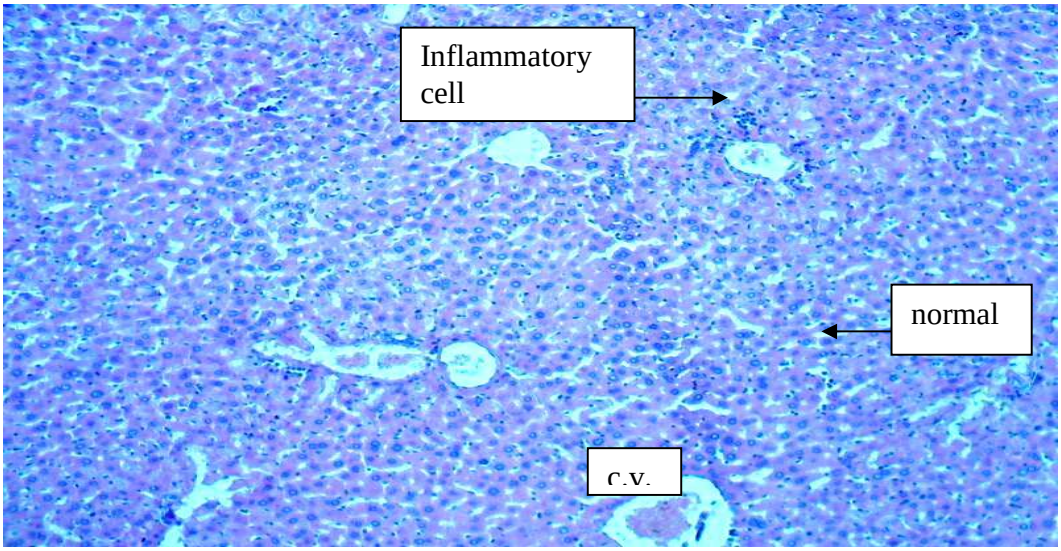

Figure 3-13: liver section of mice treated with (LPS free+liposome free) showing normal looking appearance with mild inflammatory cells infiltrate (H and E; 200X).

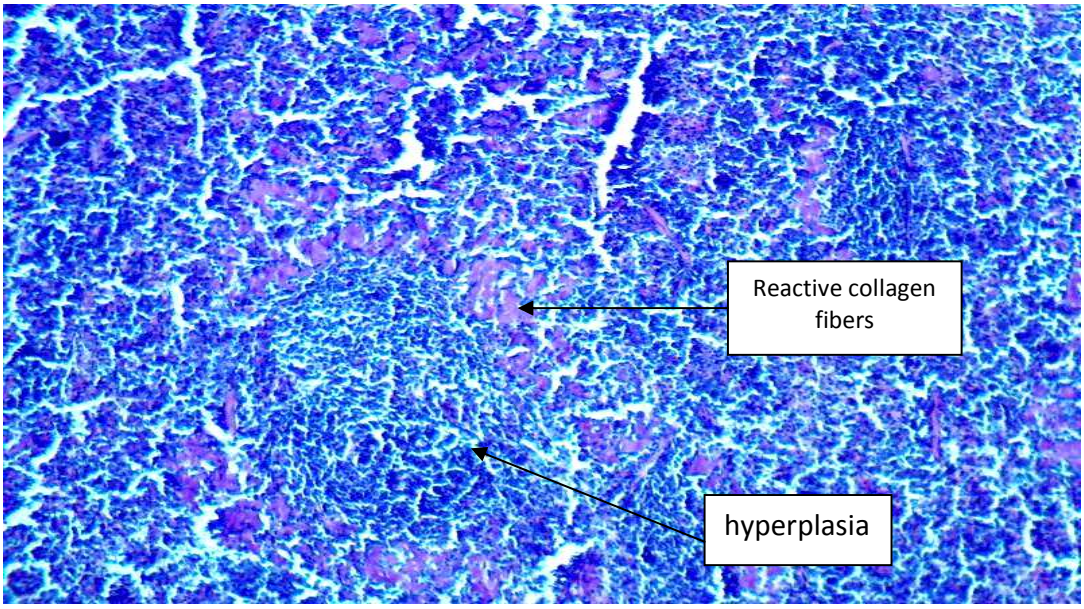

Figure 3-14: spleen section of mice treated with (LPS free+liposome free) showing diffuse hyperplasia of parenchymal tissue with reactive collagen fibers (H and E; 200X).

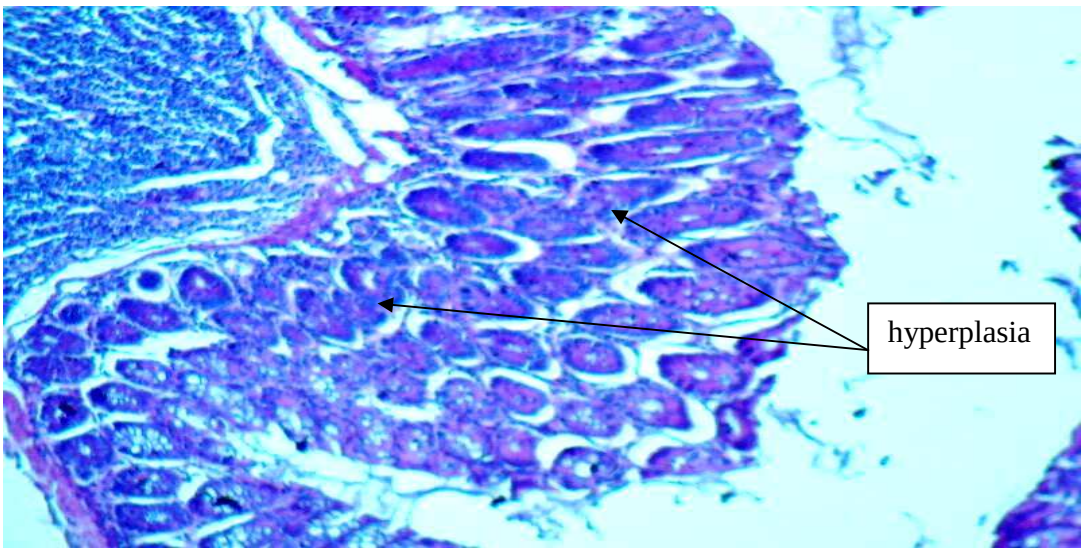

Figure3-15: intestine section of mice treated with (LPS free+liposome free) showing intestinal mucosal hyperplasia (H and E; 200X).

- Liver section of mice treated with (LPS) showing normal looking tissue structure with mild inflammatory cells infiltrate with dispersed cells necrosis (figure 3-16).
- Spleen section of mice treated with (LPS) showing widening of white pulp with diffuse hyperplasia of parechymal spleeninc tissue (figure 3-17).
- Intestine section of mice treated with (LPS) showing mucosal hyperplasia (figure 3-18).

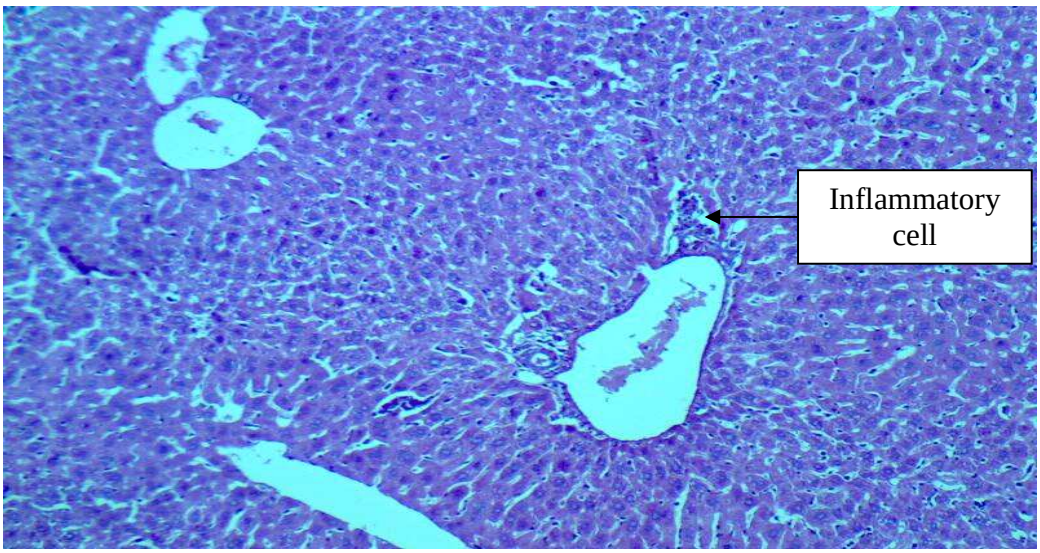

Figure 3-16: liver section of mice treated with (LPS) showing normal looking tissue structure with mild inflammatory cells infiltrate with dispersed cells necrosis (H and E; 200X).

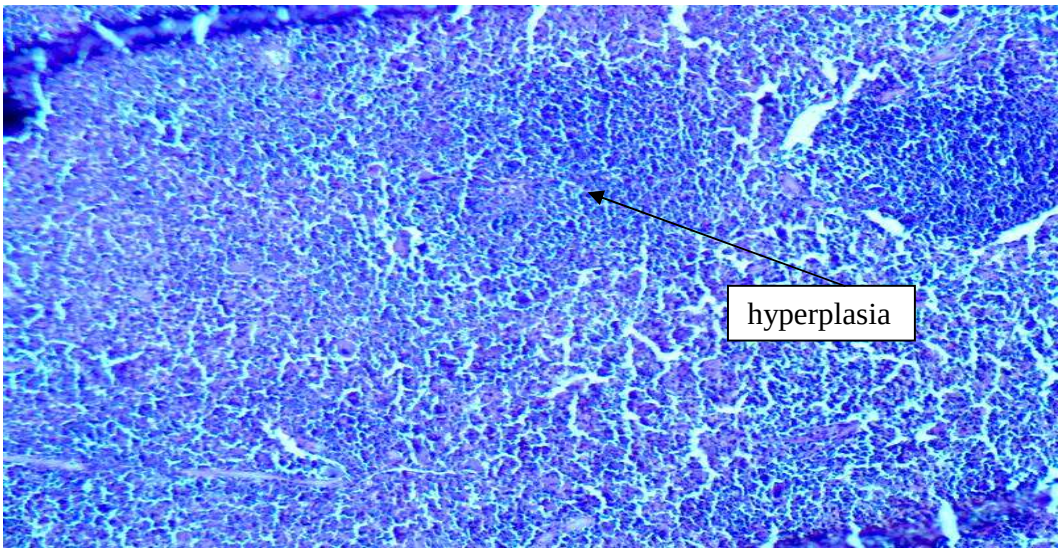

Figure 3-17: spleen section of mice treated with (LPS) showing widening of white pulp with diffuse hyperplasia of parechymal spleeninc tissue (H and E; 200X).

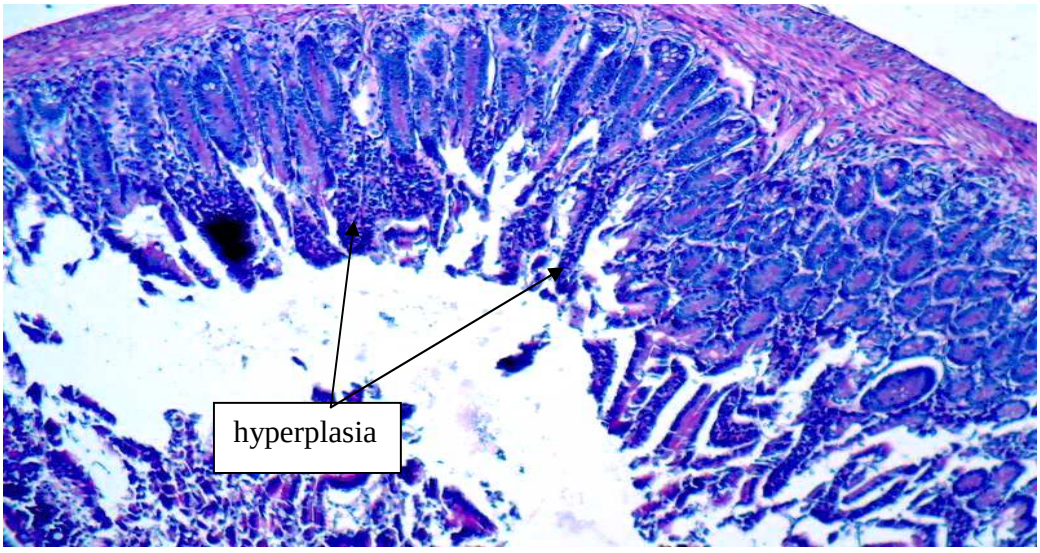

Figure 3-18: intestine section of mice treated with (LPS) showing mucosal hyperplasia (H and E; 200X).

The most important finding of evaluating histopatholigical changes in mice of different groups in the present study, is mice vaccinated with LPS+LIP, in which the liver histological section showed look-like normal hepatic tissue appearance especially near the portal area. 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. flexneri* might be responsible for the increase in mean survival time of mice by phagocytic and killing activity of neutrophils.

Dijkstra *et.al*.(1988) suggested that stable insertion of LPS into liposomal membrane accounts for its reduced toxicity, which thereby prevent a direct interaction of lipid A with appropriate plasma membrane components, which is necessary to efficiently trigger biological responses. After endocytosis of the liposome-LPS complex, it is assumed that disruption of the liposome 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. In turn, the outcome of the interaction between antigens, macrophages, and lymphocytes induce cell-mediated responses as opposed to humoral responses (Parish 1972; 1977). The ability of chemically modified antigens to induce CMI was

correlated with their hydrophobicity and to the affinity of the modified molecules for macrophages and immunocompetent cells. Additionally, lipid-modified antigens displayed a longer retention time in lymphatic tissue than did the unaltered antigen.

Summary

This study was conducted to determine the role of lipopolysaccahride-liposome conjugate (LPS-LIP) as a potential vaccine against shigellosis in mice. One hundred stool samples were collected from children and infants (100cases: 63 males and 37 females) under the age of five years (40 days – 2.5 years old) with diarrhea from different hospitals in Baghdad city during the period December 2011-May 2012. Thirty isolates were suspected to be *Shigella*. Four isolates of *Shigella flexneri* were obtained after performing some biochemical tests and Api System. Antibiotic sensitivity test was carried out and results revealed that all isolates were resist to antibiotics used in this study. One isolate was selected for LPS extraction by phenol hot water extraction method. Chemical characterization of the extracted LPS revealed that the carbohydrate content was 2.34 mg/ml, while the protein concentration was 0.52µg/ml. Partial purification of the extracted LPS was carried out by using gel- filtration chromatography on Sephacryl S-200 and results showed that three peaks were obtained and protein, carbohydrate concentration were estimated for each peak. The second peak observed to have the highest carbohydrate content (29%) and the lowest contaminated protein (0.001%). The partial purified LPS was analyzed by Sodium Dodecyl Sulphate Gel Electrophoresis (SDS-PAGE) and results reveled that two bands with molecular weight 100 KDa and 150 KDa were present and band with MW 100 kDa represented LPS. The role of LPS- liposome conjugate as a potential vaccine against *S. flexneri* was evaluated by determining total and absolute count of leukocyte, IgG titer and histopathological changes in liver, spleen and intestine.

Twenty five Mice treated with LPS at a concentration 100µg/ml, alone or in conjugation with commercially liposome were preformed as follows:

Group1: (negative control)

Group2: mice infected with *S. flexneri*

Group3: mice treated with conjugate (LPS+Liposome) and infected with *S. flexneri*

Group4: mice treated with LPS and liposome to infected with *S. flexneri*

Group5: mice treated with LPS and infected with *S. flexneri*

Each mouse was injected intramuscularly with 0.1 ml of the LPS and conjugate solution in day 1, and further dose in day 8, then these groups dosed with 0.1 ml of live bacteria in day 15, and they were dissected in the laboratory in day 30. The following results were obtained:

- Mice treated with conjugate and infected with *S. flexneri* showed a significant increase in total count of leucocytes (7300±361 ab), neutrophils (2469±107 a) and lymphocyte (4818±238 bc).
- A significant increase in IgG titer (0.9243±0.1094 a) was recorded in mice treated with conjugate after infection with *S. flexneri.*
- Results of histopathological study exhibited that sections of spleen, and liver of mice infected with *S.flexneri*. the spleen showed mild degenerative and loss its structure appearance while degeneration and necrosis of hepatocytes cells observed in the liver. While, sections of spleen, intestine and liver of mice treated with conjugate and infected with *S. flexneri* showed that the spleen intestinal and liver tissue near normal appearance.

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List of Abbreviations

Abbreviation	Meaning
ALC	Absolute Lymphocyte Count
AMC	Absolute monocyte count
ANC	Absolute Neutrophil Count
API	Analytical profile index
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
CFU	Colony forming unit
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
IFNγR	Interferon γ receptor
IFN-γ	Interferon-gamma
INT	Intermediate
IL	Interleukin
LPS	Lipopolysaccharide

Abbreviation	Meaning
LPS-LIP Conjugate	Lipopolysaccharide –Liposome Conjugate
LIP	Liposome
LB	Luria-Bertani
NC	Negative control
NCCLS	National committee for clinical laboratory standards
NF-κB	Nuclear factor kappa-light-chain B cell
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PC	Positive control
R	Resistant
S	Susceptible
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
WHO	World Health Organization

الَّذِينَ يُبَلِّغُونَ رِسَالاتِ اللهِ وَيَخشَونَهُ وَلا يَخشَونَ أحَداً إِلَّا اللهَ وَكَفَى بِاللهِ حَسِيبا

صدق الله العظيم

سورة الأحزاب

الآية 39

الخلاصه:

أجريت الدراسة الحالية لتحديد دور متعدد السكريد الشحمي المغروز في الجسم الدهني كلقاح ضد امراض الشيكلا في الفئران. جمعت ١٠٠ عينة براز من مرضى مصابين بالإسهال من مستشفيات مختلفة في مدينة بغداد خلال الفترة من ديسمبر ٢٠١١- مايو ٢٠١٢. تم الحصول على أربع عزلات من *Shigella flexneri* بعد أداء بعض الاختبارات الكيمو الحيوية ونظام API. وبعد اجراء اختبار الحساسية للمضادات الحيوية .أظهرت النتائج أن أربع عزلات من *Shigella flexneri* مقاومة للمضادات الحيوية المستخدمة في هذه الدراسة. اختيرت احد هذه العزلات لاستخلاص متعدد السكريد الشحمي باستحدام طريقة الفينول-الماء الساخن. اظهرت نتائج الكشف الكيمياوي لمتعدد السكريد الشحمي المستخلص أن محتوى الكربوهيدرات كان ٢,٣٤ ملغ / لتر، في حين أن تركيز البروتين كان ٠,٥٢ ملغ / لتر . أجريت تنقية جزئية لمتعدد السكريد الشحمي باستخدام الترشيح الهلامي على هلام الفصل Sephacryl S-200 اظهرت النتائج الحصول على ثلاث قمم وبعد تقدير تركيز البروتين والكربوهيدرات لكل قمة. اعطت القمة الثانية أعلى محتوى من الكربوهيدرات (٢٥٪). وأقل تركيز بروتيني (٠,٠٠١٪). تم التعرف على نقاوة متعدد السكريد المنقى باستخدام الترحيل الكهربائي اذ اظهرت النتائج الحصول على حزمتين ذات وزن جزيئي ١٠٠و ١٥٠ كيلو دالتون. قيم دور متعدد السكريد الشحمي المغروز في الجسم الدهني من خلال تحديد العدد الكلي والمطلق لكريات الدم البيض، والتغيرات النسيجية في الطحال والكبد والأمعاء من خلال معاملة خمسه وعشرون فأر بمتعدد السكريد الشحمي المغروز في الجسم الدهني بتركيز ١٠٠ ملغ / لتر على النحو التالي:

Group1: (negative control)

Group2: الفئران المصابة بـ *Shigella flexneri* .

Group3: الفئران التي عولجت بمتعدد السكريد الشحمي المقترن في الجسم الدهني واصيبت بـ *Shigella flexneri* .

Group4: الفئران التي عولجت مع متعدد السكريد الشحمي والجسم الدهني واصيبت بـ *Shigella flexneri* .

Group5: الفئران التي عولجت مع متعدد السكريد الشحمي واصيبت بـ *Shigella flexneri* .

حقنت العضل لكل فأر ب ٠,١ مل لمتعدد السكريد الشحمي المقترن في الجسم الدهني في اليوم الأول ، وجرعة أخرى في اليوم الثامن ، تم اصابة هذه المجموعات ب ٠,١ مل من البكتيريا الحية في اليوم الخامس عشر ، بعدها اجريت الفحوص التقييمية، يمكن تلخيص ماتم الحصول عليه من نتائج بالاتي:

- أظهرت الفئران التي عولجت بمتعدد السكريد الشحمي المغروز في الجسم الدهني بعد *اصابتها ب*Shigella flexneri زيادة كبيرة في إجمالي العدد الكلي لكريات الدم البيض و اللمفاويات.
- سجلت زيادة كبيرة في مستوى الاضداد نوع IgG في الفئران التي عولجت مع متعدد السكريد الشحمي المغروز في الجسم الدهني واصيبت بـ *Shigella flexneri*
- أظهرت نتائج الدراسة التشريحية المرضية التي أجريت على أجزاء من الأمعاء والطحال والكبد في الفئران المصابين S.flexneri، أظهرت ضمور الطحال وتحلل مع نخر في خلايا الكبد . في حين أظهرت مقاطع من الأمعاء والطحال والكبد في الفئران التي عولجت مع متعدد السكريد الشحمي المغروز في الجسم الدهني واصيبت ب S.flexneri أن أنسجة الطحال قريبة من مظهرها طبيعي، وكذلك الزغابات المعوية والكبد.

Chapter One

Introduction

and

Literature Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

Conclusions and Recommendations

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