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# Investigating the Adjuvanticity of *K. pneumoniae* Capsular Polysaccharide with Formalin-Killed *S. aureus* Against Live *S. aureus* Infection in Mice

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**Abstract**

*Klebsiella pneumoniae* capsular polysaccharide (CPS) antigen was evaluated for their capability to increase immune responses. And, CPS neutralizing antibodies were approved as the main response to vaccination in many disease. Therefore, killed *Stapthylococcus aureus* bacteria was employed to evaluate *K. pneumoniae* CPS adjuvanticity. The mice groups were immunized (orally, intra-peritoneally and by swab skin)with a dose of (25µl of formalin killed *S. aureus* ($1.5 \times 10^8$) with a CPS at dose 175µl/kg at a conc.50 µg/ml) vaccination occurred in first day then recurrent vaccination as booster dose beyond seven days. After first 7 days, the results revealed elevation of IL2,4,10,12 and IgG levels occurred mainly in oral and swab skin groups, and the obvious elevation seen in all treated groups especially oral and swab skin groups after booster dose compared with the negative control. On the other hand these results were in concordance with the challenge test which elucidated the treated groups were survived compared with the negative control one. Thus, the formalin killed- CPS vaccine induce immune response against live *S. aureus* compared to CPS alone as positive control and Freund's incomplete adjuvant, therefore this suggest that CPS have adjuvant effect on immune responses against *S. aureus* bacteria which is important in clinical treatment of *S. aureus* disease.

**Keywords:** *Klebsiella pneumoniae*, CPS as adjuvant, Capsule polysaccharide, *K. pneumoniae* capsule.

# التحقق من تأثير متعدد السكريد لكبسولة*Klebsiella pneumoniae* كمساعد مناعي مع *Stapthylococcus aureus* المقتولة بالفورمالين ضد الاصابه ببكتريا *S. aureus* المخمجه للفئران

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**الخلاصة**

قيمت مستضد كبسولة عديد السكريد *Klebsiella pneumoniae* حول قابليتها على زيادة الاستجابة المناعية . ومعادلة الأجسام المضادة للمستضدات تعد الدليل على الاستجابة لعملية التلقيح للعديد من الأمراض . لذلك ، تم توظيف بكتريا *Staphylococcus aureus* المقتولة لتقييم قابلية عديد السكريد كبسولة بكتريا *K. pneumoniae* للعمل كمساعد مناعي . مجاميع الفئران منعت عن طريق (الفم، داخل البريتون، وعن طريق المسحة للجلد) وبجرعة (25 مايكروليتر من معلق بكتريا *S. aureus* ($10^8$x1.5) المقتولة بالفورمالين مع الكبسولة بجرعة 175 مايكروليتر / كغم وبتركيز 50 مايكرو غرام/ مل)، عملية التلقيح تمت في اليوم الأول وتعاد كجرعة منشطة بعد سبعة أيام. أظهرت نتائج فحص مستويات الحركيات الخلوية 12،10،4،2 و الضد المناعي نوع جي زيادة بعد الأيام السبعة الأولى وبشكل خاص لمجاميع

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المجرعة عن طريق الفم والمسحة للجلد، إلا انه الزيادة الواضحة شوهدت في جميع مجاميع المعاملات المختلفة وكذلك بشكل خاص مجموعتي المجرعة عن طريق الفم والمسحة للجلد بعد الجرعة المنشطة وبالمقارنة بمجموعة السيطرة السالبة. ومن ناحية أخرى هذه النتائج تنسجم مع نتائج اختبار التحدي الذي أظهر بقاء مجاميع المعاملات على قيد الحياة بالمقارنة بمجموعة السيطرة السالبة. لذا فلقاح بكتريا *S. aureus* المقتول بالفورمالين مع CPS أدى إلى تحفيز الاستجابة المناعية ضد بكتريا *S. aureus* المنشطة بالمقارنة بمجاميع السيطرة السالبة والموجبة ومجموعة المساعد فروند غير الكامل ، لذا يمكن الذكر بان عديد السكريد المحفظة يمتلك فعل مساعد مناعي في أنتاج الاستجابة المناعية ضد بكتريا *S. aureus* والتي تعد مهمة في العلاج ألسريري للإمراض المتسببة عن هذه البكتريا.

## Introduction

*Klebsiella pneumoniae* is a gram negative bacterium. It is facultative anaerobic. It is rod-shaped and measures 2 µm by 0.5 µm. In 1882, Friedlander C. Uber first discovered *Klebsiella* to be a pathogen that caused pneumonia[1]. *K. pneumoniae* strains exhibit different virulence factors which give the bacteria the ability to invade the host. These are capsular polysaccharides, lipopolysaccharide, serum resistance, production of urea and enterotoxin, type 1 and type 3 adhesions, factors involved in aggregate adhesions and siderophores [2].The reason for its pathogenicity is the thick capsule layer surrounding the bacterium. It is 160 nm thick of fine fibers (capsule) that protrudes out from the outer membrane at right angles[3].Recently, the isolation, purification, and characterization of capsular polysaccharide (CPS) derived from *K. pneumoniae*. Anti-CPS antibody administered passively or elicited in response to immunization with purified antigen was found to provide a high degree of protection against fatal *K. pneumoniae* KP1-0 burn wound sepsis. Furthermore, this capsular antigen was found to be safe and immunogenic in human volunteers. The capsular polysaccharide (CPS) has the ability to stimulate antibody production [4].CPS–based vaccines should be multivalent against at least the 24 major K-types, in order to cover 70% of all bacteria isolates[5].In many cases, adjuvants are employed to evoke more powerful immune responses. The optimally formulated adjuvant must be safe, stable before administration, readily biodegraded and eliminated, able to promote an antigen-specific immune response, inexpensive to produce, and easy to use[6]. In addition, *Staphylococcus aureus* is a gram positive, non motile, non-flagellate, non-spore-forming cell wall contains peptidoglycan and teichoic acid. Usually un encapsulated or limited capsule formation. Staphylococcaceae are aerobic or facultative anaerobes grows well in medium containing 10% NaCl, poorly in 15% NaCl [7]. *S. aureus* is responsible for a variety of infections. *S. aureus* caused considerable morbidity and mortality as a nosocomial pathogen of hospitalized patients. Among the major human infections caused by this species are furuncles, carbuncles, impetigo, toxic epidermal necrolysis (scalded skin syndrome)[8], pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, enterocolitis, mastitis, cystitis, prostatitis, cervicitis, cerebritis, meningitis, bacteremia, toxic shock syndrome, and abscesses of the muscle, skin, urogenital tract, central nervous system, and various intra-abdominal organs. In addition, staphylococcal enterotoxin is involved in food poisoning[9]. Due to the limited adjuvantation effect of aluminum salts, constant mutation of the existing microbes, and identification of new disease-causing microbes, hence extensive search of more effective adjuvants has been the focus of many scientists for many years [10].The objective of this study was to develop a safe and novel immunoadjuvant to enhance the immunity and resistance of animals against *S. aureus* infections .The results were compared to, commercially available adjuvant, Freund's incomplete adjuvant.

## Materials and Methods

### Bacterial strains

The two isolates supplied locally by University of Baghdad/ College of Science/Biotechnology Department. *K. pneumoniae* initially isolated from sputum, characterized as K2 serotype, and used as a source for capsular extraction (prepared earlier)[11]. On the other hand *S. aureus* also previously identified from purulent wound. Both isolates were selected as the most resistant isolates to most locally used antimicrobial agents. The isolated *S. aureus* was activated on brain heart infusion broth(himedia) and incubated over night at 37 $^{o}$C.

**Preparation of formalin-killed*S. aureus* bacteria [12]**

In order to prepare killed *S. aureus* bacteria, the isolate was inoculated in tryptic soy broth (himedia) and incubated for 24h at 37°C. Formalin (40% w/v) was added to the broth culture at a final concentration of 0.5% (V/V) and left 48 hrs at room temperature in a sterile condition then centrifuge. After that, the bacterial suspension was checked for their sterility (free from the living cells) by streaking onto trypticase soy agar (himedia plate ), then prepared a culture at a concentration of 1.5X $10^8$ according to standard Macferland solution tube (No.0.5) to further use [13].

**Preparation of *S. aureus*-CPS Vaccine [14]**

To prepare 0.275 ml of vaccine, (25 µl) cell suspention of formalin-killed *S. aureus* and olive oil (75µl) were added to CPS at a dose of 175µl/kg (Conc.50 µg/ml ), which considered a dose for immunization. The prepared vaccine was mixed well and incubated overnight at 4°C with gentle stirring under high sterile conditions [14].

**Quantitative analysis**

Partial purified CPS extract [15] was investigated by measuring the amount of carbohydrates according to [16] using phenol-sulfuric acid method measured at 490 nm and compared to the standard curve of glucose. Moreover, the amount of protein that linked with partial purified CPS was estimated according to [17] using bovine serum albumin as a standard curve. The amount of lipid was estimated according to [18]**.** The concentration of nucleic acid was estimate a according to Nanodrop system.

***In vivo* test and schedule of immunization [14]**

Thirty two mice (average weigh 25g) were supplied from the animal house of National center for drug control. The mice were fed a commercial food and provided with fresh water. The animals were divided into eight groups (four mice each group):

Group A: control (negative control)
Group B: administered olive oil
Group C: administered incomplete Freund's adjuvant (Sigma /USA)
Group D: administered CPS act as positive control only
Group E: administered killed *S. aureus* only
Group F: administered the vaccine solution intra-peritoneally (i.p)
Group G: administered the vaccine solution orally
Group H: administered the vaccine solution skin swab (locally).

Mice were administered 0.275mlof prepared vaccine in the first day of experiment, booster dose (0.275 ml) was given at 7th day of experiment. The animals were monitored for another week, and then the blood samples were collected at day 14 by heart puncture, **c**lotted blood was centrifuged at 2000 rpm for 10 min. Serum samples stored at -20 ° C for further analysis. The experiment was performed in the animal house of the Biotechnology Center/ University of Al-Nahrain/Iraq.

**Determination of cytokine levels**

The serum levels of IL2, IL4,IL10 andIL12 were analyzed by ready-to-use mice different ELISA kits(Cloud-clone corp./ USA), and by comparing the O.D. of the samples to the standard curve, Figure-1. The cytokines were quantified according to manufacturer's protocol.

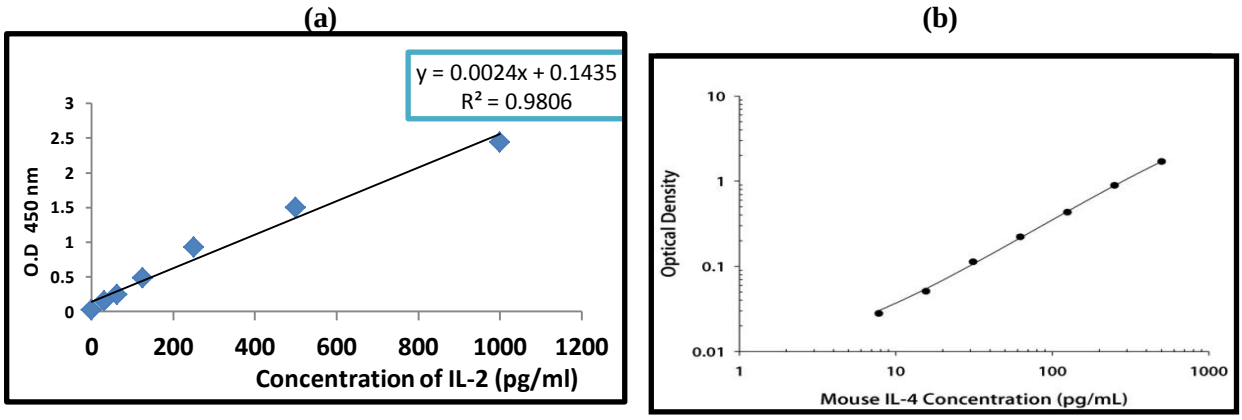


**Figure 1**- Standard curve of (a) IL2, (b) IL4

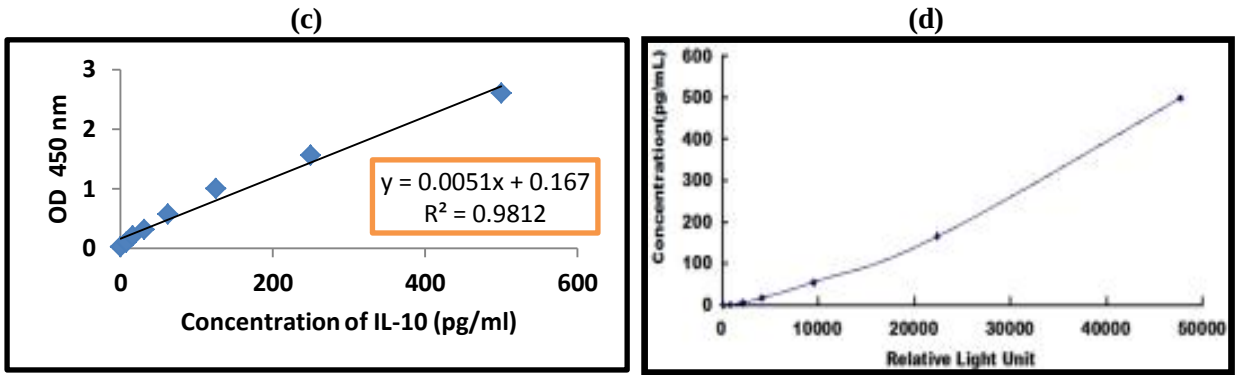


**Figure 1**- Standard curve of (c) IL10, (d) IL12

**Determination of neutralizing IgG antibody titers by Enzyme Linked Immunosorbent Assay (ELISA)**

Quantitative determination of mice IgG was performed using a competitive inhibition enzyme immunoassay technique (Cloud-clonecorp./USA)**.**

The color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of IgG in the samples were determined by comparing the O.D. of the samples to the standard curve, Figure-2.

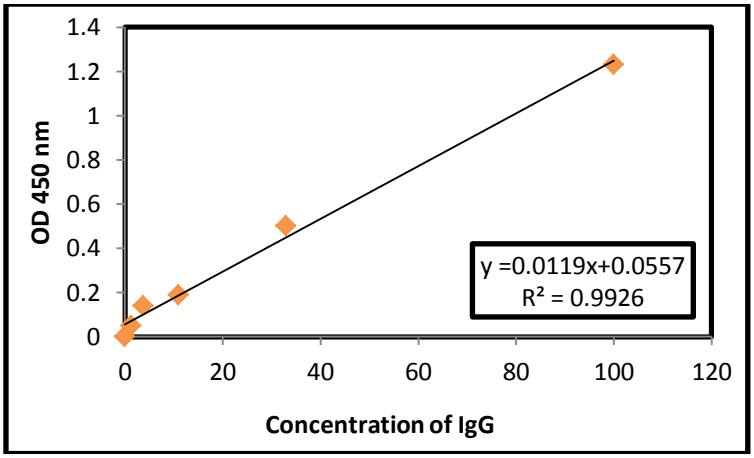


**Figure 2-**Standard curve of IgG

**Challenge test (Experimental Challenge)[19]**

All groups of (control and immunized (treated) mice) were exposed to challenge test for 2 weeks after the final dose, the mice were infected with virulent live *S. aureus* strain ($1.5\times10^{8}$CFU/mL) bacteria, by infected scratched dorsal skin, and monitoring the animals mortality and healing time were recorded, all survived mice were anatomized to check the pathological lesions of skin caused by the infection with virulent *S. aureus*.

**Statistical analysis**

Statistical analyses were performed by use of IBM SPSS computer program version 21. Differences between the groups were statistically analyzed by ANOVA table. Data are expressed as mean ± standard error (SE). A P value of≤0.05 was regarded as statistically significant.

**Results and discussion**

Partially purified CPS extract was chemically analyzed to check the concentration of carbohydrates, protein, lipids, and nucleic acid 285µg/ml (94%),1.2µg/ml (0.39%), 15µg/ml (4.9% ) and 0.7µg/ml(0.2%) respectively, Table-1.The results revealed highest content of carbohydrates and reflected the efficiency of extraction and purification methods.

**Table 1-** Amount and percentages of carbohydrates, proteins, lipids and nucleic acid in the partialLY purified *Klebsiella pneumoniae* CPS

| | **Carbohydrates µg/ml(%)** | **Protein µg/ml(%)** | **Lipids µg/ml(%)** | **Nucleic acid µg/ml(%)** |
|---|---|---|---|---|
| **After purification** | 285µg/ml (94%) | 1.2µg/ml (0.39%) | 15µg/ml (4.9%) | 0.7µg/ml (0.2%) |

**Quantitative determination of interleukins 2,4, 10 and IL 12 by ELISA**

**Interleukin 2:** The results Table-2 showed there were significant high elevation in the level of this interleukin specially in the oral and skin swab groups with significant slight elevation in the inter peritoneal group compared with the negative and positive control groups, and because IL2 is a Th1-type cytokines which is necessary in the proliferation of CD8 cytotoxic T cells and regulatory T cells, as well as it stimulates the natural killer cells to produce IFN-$_{\gamma}$ [20].On the other hand dendritic cell IL2 has been shown to be important for innate and adaptive immune responses [21]. It is vital for cellular activation and important for primary T-cell responses as and for secondary T-cell responses. Additionally, IL2 has the key function of down regulating immune responses, as well as has a specific role in promoting T-cell activation and proliferation of only those cells that have been stimulated by cognate antigenic interaction, down regulation of T-cell responses occurs non-specifically by facilitating a separate population of T regulation [22].Therefore the elevation of this interleukin acts as precursor modulation of innate and adaptive immune responses and, thus can suggested that there were increase in Th1,dendritic and killer cells which obviously increase in IL2.

**Interleukin 4:** It is a key regulator in humoral and cellular immunity. IL4 induces B-cell class switching to IgE, and up-regulates MHC class II production. IL-4 decreases the production of Th1 cells, macrophages, IFN-gamma, and dendritic cell IL-12.Overproduction of IL4 is associated with allergies [23].Thus this interleukin is related with hypersensitivity type 1 and phagocytosis ,and because CPS is avoluminous outer layer and is involved in protection against C3 deposition that occurs mainly in the inhibition of macrophage phagocytosis [24].The result Table-2 showed no significant increasing occurred in all groups unless oral group which elucidated slight significant elevation may be due to the initial interaction between bacteria and the host mucosal immune system probably occurred through recognition between these surface components and dendritic cells. Indeed, the mucosal immune response is orchestrated by a network of surveillance based on dendritic cells (DCs), which are professional antigen-presenting cells [25]. Immature DCs reside in peripheral tissues, especially in mucosal membranes, such as the gut or lung mucosae. They sense the microenvironment and have a great ability to detect, take up, and process antigens. Several studies have described the role of *K. pneumonia* surface polysaccharides in interactions with macrophages or human epithelial cells, in which CPS seemed to impede cell association and cellular activation [26].

**Interleukin 10**: IL10 is a cytokine with multiple, pleiotropic, effects in immune regulation and inflammation. It down regulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. The result of this interleukin Table-2 showed significant elevation in all treated groups and positive control in compared with negative control, the result suggest that the capsular polysaccharide may had synergistic effect to induce IL10 production, thereafter, these high IL-10 levels may serve to down-regulate the expression of pro-inflammatory cytokines such as IL4 thus, this result was confirmed to that concluded about IL4.

**Interleukin 12:** It's a bridges of innate and adaptive immunity. IL12 induces differentiation of naïve CD4T cells to Th1 cells and activates NK cells, and also protects CD4Th1 cells from antigen- induced apoptotic death, as well as play a role in T cell migration by inducing functional adhesion molecules such as P- and E- selectin ligand expression on Th1 only; therefore, these cells are selectively recruited to sites where Th1 immune responses are needed [27]. The interleukin is very important in inducing immune responses and produces mainly by antigen presenting cells, the present result of this study showed significant increasing specially in oral and swab groups after first and booster doses but slight significant increasing beyond booster dose, Table-2.We could concluded that the results of interleukin levels indicated that vaccine is characterized by slight significant increasing basal synthesis of IL4. These result suggest that there is a basal an increase of Th2. Enhanced IL2 levels in different treated groups are likely due to the necessity of T-cell proliferation stimulus rather than produced as

Th1 prevalent stimulation. Furthermore, it observed a significant increase in IL12 production in treated groups especially oral and swab groups from control ones. At the same time IL10 shows increased levels, which could cause a stimulation of Th2 differentiation. These observations showed imbalance between IL10 and IL12, these results strongly suggest that manipulation of interleukin network , i.e.IL10/IL12 balance, maintained by cells of the innate immune system.

**Table 2-**The mean ± standard error of IL2, IL4, IL-10 and IL12

| Groups | | Mean ± standard error (SE)pg/ml | | | |
|---|---|---|---|---|---|
| | | IL2 | IL4 | IL10 | IL12 |
| Negative control | | 12.13±0.14 | 10±0.14 | 12.9 ± 0.14 | 5.1±0.14 |
| Olive oil | | 14.3±0.17 | 12.6 ±0.11 | 13.8 ± 0.05 | 5.2 ±0.11 |
| Positive control(CPS) | | 12.5±0.23 | 11.8±0.18 | 16.7±0.11 | 6.9±0.29 |
| Incomplete adjuvant | | 27.2± 0.11 | 14.7±0.11 | 22.8±0.05 | 8.9±0.57 |
| *Killed S. aureus* | | 12 ± 0.14 | 11.1 ± 0.1 | 15.3 ± 0.12 | 6 ± 0.21 |
| Intraperitoneal | After first dose | 12.7±0.11 | 12.9± 0.05 | 18.9± 0.14 | 7.7± 0.63 |
| | After booster dose | 15.9±0.14 | 13.7± 0.11 | 20.2± 0.20 | 8.2± 0.05 |
| Oral | first dose | 31.2±0.11 | 15.3±0.11 | 21.2± 0.11 | 38.7± 0.11 |
| | After booster dose | 41.5±0.17 | 18.9±0.14 | 23.8 ± 0.06 | 50.5 ± 0.28 |
| Swab skin | first dose | 29.5±0.20 | 13.7±0.11 | 27.4± 0.11 | 31.5± 0.11 |
| | After booster dose | 30.8±0 .08 | 14.8±0.05 | 33.2 ± 0.11 | 41.6 ± 0.14 |

**Quantitative determination of IgG antibody by ELISA**

The efficacy of capsular polysaccharide (CP)was elicited by active immunization with vaccines composed of formalin killed *S. aureus.* A dose of (25 µl) of *S. aureus* ($1.5 \times 10^8$) with a CPS at dose 175µl/kg (Conc.50 µg/ml) per mouse administered intraperitoneally (i.p.), orally, and by swab skin. The results showed slight elevation of IgG level after first and second administered doses intraperitoneal, while other routes of administration appeared significant elevation beyond first and second doses when compared with the control groups, Table-3. These results consistence to that of interleukins, Table-2, and reflect the positive effects of interleukin 10 in stimulation of humoral immune response and in the same time counteract the effects of interleukin 12 about decreases the humoral immune response. Therefore can be concluded CPS that play important role in stimulation of IgG, and oral as well as swab skin routes were excellent in stimulation of humoral immune response than intra peritoneal rout. On the other hand, high IgG levels are usually associated with increased production of B lymphocytes.

**Table 3-**ELISA test to determine the titers of IgG in mice

| Negative control | Olive oil | Positive Control (CPS) | Killed *S. aureus* | Incomplete adjuvant | Intraperitoneal | | Oral | | Swab | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | One dose | Post dose | One dose | Post dose | One dose | Post dose |
| 4±0.05 | 5.5±0.11 | 7.1 ±0.08 | 5 ± 0.12 | 6.3 ± 0.11 | 4.8 ±0.11 | 5.9 ±0.14 | 7.3 ±0.11 | 12.3 ± 0.20 | 5.8 ± 0.05 | 7.2 ± 0.05 |

**Challenge test**

*S. aureus* can cause severe life threatening invasive diseases. The efficacy of capsular polysaccharide (CP)- elicited by active immunization with vaccines composed of *S. aureus* bacterial mouse challenge models at a dose of (25 µl) of *S. aureus* ($1.5 \times 10^8$) with a CPS at dose 175µl/kg (Conc.50 µg/ml)per mouse administered intra peritoneally (i.p.),oral, and swab skin groups, the results of challenge test showed, the treated animals were survived throughout the period of study more than ten days, whereas the negative control groups were found to cause 80 to 100% mortality in BALB/mice within 2 to 5 days following the bacterial challenge. Animals actively immunized with the monovalent type CP-killed *S .aureus* showed a survival rate of 95-100% compared with the

negative control groups, and the healing was occurred during the first fifth days of experiment and completed at the seventh day. These data suggest that *S. aureus*- CP polysaccharide administered by active immunization confer protection against *S. aureus* infections. This highlights the role of CP as an important immune evasion mechanism and supports the inclusion of capsular polysaccharide antigens in the formulation of prophylactic vaccines against *S. aureus.* On the other hand these results were consistent to the previously results of interleukins 2, 4,10,12 and IgG elevation that led to protect animals from infection, thus CPS (K-antigen) for *K. pneumoniae*, has been found to exert as adjuvant effect with inactivated *S. aureus* bacteria to evaluate AgCPS adjuvanticity. This results was agreed with [28] and [29] who reported that when mice immunized with vaccine of *K. pneumoniae* subspecies, they were protected against virulent challenge with strains**.** Also the CPS may be important for the establishment of pneumonia, because active immunization with purified CPS protects experimental rats against lethal pneumonia caused by *K. pneumoniae* [30].

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