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Assessment of *K. pneumoniae* capsular antigens isolated from different clinical specimens in stimulation several immune parameters

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ABSTRACT

Capsule of *K. pneumoniae* is a polysaccharide in structure, it's one of bacterial virulent factors. In this study we focused to finding the best capsular antigen from different isolates led to stimulate immune response, throughout measuring several biochemical and immunological parameters. Thus six *K. pneumoniae* isolates from different specimens (urine, wound and burn swabs, sputum, blood, stool) were used to extract and partially purified the capsules by organic solvents (ethanol, chloroform, butanol) and strong alkaline. The results of chemical analysis showed that the carbohydrates concentration of the partially purified CPS extracts were 115(87.1%), 140(90.7%), 150(89.8%), 163(88.3%), 135(88.8%), 130(90.9%)µg/ml, whereas the concentrations of protein in the same extracts were5(3.7%),3(1.94%),4(2.39%),5(2.7%),2(1.31%),1 (0.69%) µg/ml, while the concentration of lipids 12(9.09%), 11.2(7.2%), 13(7.7%), 16.4 (8.8%), 15(9.8%), 12(8.3%)µg/ml ,with(0.6, 0.8, 0.6,0.7, 0.5, 0.7) ng/µl nucleic acid respectively . *In vivo* study revealed that, the concentration of anti *K. pneumoniae* CPSs (IgG) in serum of rabbits were elevated significantly (P ≤0.05) in all groups (108.64±5.38 , 168.52±3.20, 129.63±4.27, 196.30±7.70, 102.47±10.10 ,and 121.23± 5.58)ng/ml, as well as the level of IL-4, IL-10 and TNF-α ,were (48±9.16, 59.66±8.83, 58±7.50,64± 1.52, 47±5.50 and 56±7), (42.33±4.40, 41.33±1.85, 42±1.73, 35.3±2.02 ,43±5.50and 42.33±0.88) and (307±1.73, 366.3±6.43, 501.66±6, 616.6±2.18, 766.3±13.73 and 835±22.91) pg/ml respectively for that *K. pneumoniae* CPSs extracted from urine, wound, burn, sputum, blood and stool respectively, in comparison with control. But IL-2 level shown a significant elevation (P ≤0.05) in some groups which were (74± 6.24, 68±17, 54±2.08, 53.66±15.38) pg/ml for that CPSs of sputum, wound, burn and stool respectively, while the level of IL-6 showed a significant elevation (P ≤0.05) in groups of(sputum, burns, blood and stool)were(417±3.71, 294±22.27, 263.3±21.85 and 150±16.07)pg/mlrespectively. The positively increasing of WBC counts, which effect on the phagocytosis by increasing the PI compared with control. Thus the challenge test with virulent *K. pneumoniae*, showed the ability of CPSs extracts to protect the treated rabbits against *K. pneumoniae* infection.

Keywords: *K. pneumoniae*, Capsular polysaccharide, *K. pneumoniae* capsule.

1. INTRODUCTION

The genus *Klebsiella* belongs to the tribe Klebsiellae, a member of the family *Enterobacteriaceae* (1). *K. pneumonia* is a gram-negative bacterium, most commonly encountered by physicians worldwide as a community-acquired and a hospital-acquired pathogen (2). *K. pneumoniae* strains exhibit different virulence factors one of them is capsular polysaccharides (3), which is related with its pathogenicity (4). *Klebsiella* is composed of 63% capsular polysaccharide (CPS; the K antigen), 30% lipopolysaccharide, and 7% protein. The outer surfaces of *K. pneumonia* is covered with a layer filled with fibrous material. This layer definitely represents the capsular layer of these bacteria. The thickness of the layer is approximately 160 nm in *K. pneumoniae*. The capsule of *K. pneumoniae* was seen to consist of a heavily packed accumulation of fine fibers

which represented a polymer of capsular polysaccharide. The capsular layer has two layers, an inner layer and an outer layer. The fibers were arranged differently in these layers. In the inner half, the fibers formed a dense layer which probably consists of an assembly of thick bundles, which were seen to be standing at right angles to the surface of the outer membrane. In contrast, the outer half of the capsule consisted of a net-like assembly of the fine fibers. Thick bundles of fibers were rarely seen in this layer. The direction of the arrangement of the fibers was random, and some of the fibers were running parallel to the cell surface (5). The capsule is a complex acidic polysaccharide (6) composed of repeating subunits of four to six sugars (thick hydrophilic polysaccharide such as glucose, galactose, mannose, fructose and rhamnose) and acids such as: glucouronic acid, galacturonic acid, pyruvic acid and commonly contain uronic acids (as negatively charged components) (7). The capsular polysaccharide (CPS) has the ability to stimulate antibody production, and stimulates other immune aspects (8-9)**.** Because of the importance of this antigen, therefore this study was aimed to assess the best *K. pneumonia* capsular extracts from different clinical specimens, to stimulate immune response.

2. MATERIALS AND METHODS

2.1 Bacterial strain

From previous study (10) *Klebsiella pneumoniae* isolates from different clinical specimens were collected, only six clinical isolates from urine, wound, burn, sputum, blood and stool were selected and represented the most resistant isolates to usable antimicrobial agents.

2.2 Extraction and partial purification of capsular polysaccharides:

The capsule of *K. pneumoniae* (CPS) isolates was extracted according to as light modification of the method described by (11-12).The selected isolates were reactivated on brain heart infusion broth(himedia/India), then transfer to petri dishes containing trypticase Soya agar(himedia/India), and incubated overnight at 37ºC. The growing isolates were harvested by suspending the bacterial growth with sterile (3ml) phosphate buffer saline(0.01M)(pH=7.2)Kallested/U.S.A, the harvested bacteria was collected in sterile tube, the plate was rewashed with 2ml of same PBS, then centrifugation at 10,000 rpm for 15 min., the pellet was suspended with 5 ml distilled water and mixed well by magnetic stirrer. And then amount (0.125 gm) of cetavlon(SCRC/China) was added to suspended bacteria with stirring for 30 min at room temperature, and centrifugation at 10,000 rpm for 30 min. was done. The pellet was collected again and dissolved in $CaCl_2$ (1M)) (CCL/USA), ethanol (BDH/England) was added to equal 25%(vol/vol). After stirring for 60 min at room temperature, centrifugation at 10,000 rpm for 30min was done, the supernatant was collected and equal amount of 80% ethanol absolute (Fluka /England) was added. By centrifugation 10,000 rpm at 30 min., the capsular material are precipitated, and dissolved with (2ml) distilled water. Then chloroform: butanol (5:1) (Fluka /England)mixture was added with the same amount of distilled water, The previous step was repeated three time. Finally the aqueous phase suggested as containing CPS was collected for further step.

2.3 Partial purification of crude extracts (Detoxification of LPS)

According to (13) method, the aqueous phase of previous prepared products were partially purified by adding 2 ml NaOH (Fluka /England)solution(0.1N),and 95% ethanol for 30 min at 37ºC with stirring. The mixture was neutralized by adding (2N) acetic acid (Fluka /England). The precipitant was represented as the capsular material which dissolved in distilled water. Then, the suspension was dialyzed for 24 hr. in distilled water, and concentrated in sucrose at 4°C, until the final volume was reduced to 5 ml.

2.4 Quantitative analysis

Partial purified CPS extracts were investigated by measuring the amount of carbohydrates according to (14) method ,and the amount of protein that linked with partial purified CPS was estimated according to (15) method, both materials were compared with the standard curve of glucose and bovine serum albumin respectively. Moreover the amount of lipid was estimated according to (16), while the concentration of nucleic acid was estimate by Nanodrop system.

2.5 Preparation of antigen –adjuvant emulsion

According to the adjuvant type, the capsular suspensions were prepared at conc. 50µg/ml (complete Freund's adjuvant and incomplete Freund's adjuvant) which were represented as stock suspensions. The prepared suspension must be mixed well until converted to a milky emulsion, which represented as a vaccine.

2.6 *In vivo* tests

2.6.1 Rabbit

Twenty-one healthy white rabbits, weighing (1) kg used. The rabbits were housed in an individual cages in a room with controlled temperature,12 hr. light/ dark cycle for 7 days to adaptation for environmental condition (Biotechnology Center/ University of Al-Nahrain). The rabbits were daily fed a standard diet, provided with fresh water. In this experiment the lab. animals, were divided into seven groups each group contains three rabbits

2.6.2 The schedule of immunization

Immunization was carried out according to the (11) method (table 1). All six groups were interval injected (subcutaneously) with two types of prepared vaccine (50µg/kg) of extracted CPS from *K. pneumoniae* isolate (urine, wound, burn, sputum, blood, and stool) respectively, while the control group injected with (complete or incomplete) Freund's adjuvant only. The experiment was carried out for 14 days.

Table 1: The schedule ofimmunizatio (Sikarwar and Batra, 2011).

Day	Type of vaccine
1	CPS+ complete Freund's adjuvant
2	CPS+ complete Freund's adjuvant
3	CPS+ incomplete Freund's adjuvant
4	CPS+ complete Freund's adjuvant
5	CPS+ complete Freund's adjuvant
6	CPS+ incomplete Freund's adjuvant
7	CPS+ complete Freund's adjuvant

All the groups were monitored during this period. Blood samples were pooled from the heart using 5ml syringe at the end of the experimental period, and transferred into EDTA tubes for WBC counting and phagocytosis index tests. On the other hand, another blood samples were used to study some immunological parameters by transferring the blood samples to centrifuge at 2000 rpm for 10 min. to collect serum. Serum samples were preserved in sterile tubes and stored at a temperature of -20ºC. For further tests such as antibody titers of IgG and some cytokines (IL2, IL4, IL6, IL10, TNF-α)level measurement by ELISA technique.

2.6.3 Enzyme Linked Immunosorbent Assay (ELISA)
ELISA(Biotek/USA) for serological examination were performed using Rabbit IgG ELISA Kit (PIERCE company/ USA) for detection of anti-capsular polysaccharide antibody(IgG), and rabbit Interleukins 2,4,6,10 and TNF-α ELISA Kits (EIA-ab company/china) for quantitative determination of rabbit cytokines. The immunoassay procedure was done according to the manufacturer's instructions.

2.7 Microscopic examination
2.7.1 Phagocytosis index
It has been done according to (17) as follows:
Each *K. pneumoniae* isolates which were used are reactivated in nutrient agar. Bacterial colonies are suspended in normal saline and adjusted to1.5 x 10^8 CFU/ml according to McFarland standards(0.5). From immunized mice (different groups) 1 ml of blood was pooled from the heart were transmitted to tubes contain EDTA, then (0.5) ml of blood were mixed gently with (0.05)ml of *K. pneumoniae* suspension (10^8/ml) in sterile test tube, thereafter incubated at 37ºC for 1.5 hours. Smear had been prepared by taking a drop from the mixture on the cleaned slide; duplicate slides were made for each tube, then air dried, and stained with Giemsa stain for 10 min. subsequently, washed by D.W. The slides had been examined microscopically at (100X) to calculate the phagocytic index according to the following equation.

$$\text{The percentage of phagocytosis \%} = \frac{\text{No. of phagocytic cells}}{\text{No. (phagocytic + non phagocytic) cells}} \times 100$$

2.7.2 Differentia leukocyte count (18)
The leukocytes differential count determines the number of each type of white blood cell, present in the blood, according the following protocol:

A drop of each tested animal blood was placed on the clean slide to produce a smear. The blood film should be dried, giemsa stain was poured over the smear for 8-10 minutes. Wash off with water and dried. Microscopically examination was performed at (40X).

2.8 Challenge test
All groups of rabbits(control and immunized) were exposed to challenge test after 2 weeks from the final dose, which were infected by scratched the skin with activated *K. pneumoniae* (1.5×10^8) CFU/ml (19). The experiment period was 10 days after exposing to virulent bacterial challenge.

2.9 Statistical analysis
Statistical analyses were performed by using 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 $\leq$0.05 was regarded as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Extraction and partial purified of CPS (K-antigen)
The percentage of carbohydrates, proteins and lipids in the crude extracts were range (60.6%-64%), (5.3%-11%) and (25.6%-31.9%) respectively (table 1). The highly content of carbohydrates that were obtained could be related to the efficiency of extraction method in which the crude extracts were treated with many solvents (chloroform and butanol) for lysis proteins and lipids. In addition the high concentration of salt ($CaCl_2$) had a role in separation nucleic acid from proteins, therefore its result reported a very little amount in the samples, due to the efficiency of ethanol solvent in removing nucleic acid from the extracts, which increases the precipitation of nucleic acid, and then neglected, hence elevated the efficiency of this method (20). In addition the ability of ethanol 70% to remove salt (21), meanwhile, cetavlon have an important role in the CPS extraction, thus the extraction steps of this method considered as the extraction and partial purification as (12-11) were mentioned. This

result agreed with (22) who found, the percentage of capsular polysaccharide was 63%, with 30% lipopolysaccharide, and 7% protein. From the previous results, the solvent method is simpler, safer and more efficient in extracting CPS. Moreover purification was done for the crudes CPSs and detoxified via de acylation by alkaline treatment (13) ,this method greatly reduced the pyrogenicity of CPS, and may prove to be a useful technique for producing a safe and immunogenic *Klebsiella* polysaccharide vaccine, but did not markedly alter its antigenicity. The chemical analysis of partial purified CPSs was done, and the amount of carbohydrates, proteins, nucleic acids and lipids of CPS extracts were estimated in the (table 1).The results revealed the highest content of carbohydrates(87.1%-90.9%) obtained may be due to the alkaline treatment and its neutralization, led to detoxification and removing some impurities. On the other hand, the results recorded the percentage of protein (0.69%-3.7%) and lipids (7.2%-9.8%). These results agreed with (23) who found, the capsular vaccines had a carbohydrate content of 40-89%, with protein content (less than 1 to 16%), for different serotypes. Another study by (12) reported, the percentage of carbohydrates reached to 86.3%, while protein's 1.03%, when using gel filtration for partial purification of CPSs.

Table 1: Percentages, amount of carbohydrates, proteins, lipids and nucleic acid in the crude extracts and partial purification CPSs.

Extracts of selected isolates	Carbohydrates µg/ml (%)		Proteins µg/ml (%)		Lipids µg/ml (%)		Nucleic acid ng/µl	
	Before purification	After Purification	Before purification	After purification	Before purification	After Purification	Before purification	After purification
Urine (2)	85(62.9%)	115(87.1%)	8 (5.9%)	5(3.7%)	42(31.1%)	12(9.09%)	0.7	0.6
Wound (4)	69(63.3%)	140(90.7%)	12(11%)	3(1.94%)	28(25.6%)	11.2(7.2%)	0.9	0.8
Burn (5)	52.5(63.2%)	150(89.8%)	5.5(6.6%)	4(2.39%)	25(30.1%)	13 (7.7%)	0.8	0.6
Sputum(10)	96(64%)	163(88.3%)	8(5.3%)	5(2.7%)	46(30.6%)	16.4(8.8%)	0.9	0.7
Blood (14)	36(60.6%)	135(88.8%)	3(5.05%)	2(1.31%)	17.4(29.2%)	15(9.8%)	0.7	0.5
Stool (17)	30(63.8%)	130(90.9%)	2(4.25%)	1(0.69%)	15(31.9%)	12(8.3%)	0.9	0.7

3.2 *In vivo* test

3.2.1 Effect of partial purified CPS extracts on the differential leukocyte count

For detection the effect of CPS on different blood leukocytes, the results of subcutaneous injection of partial purified CPS material showed, the number of Lymphocytes, Monocytes and Neutrophils significantly increased after 14 days from injection, a significant increasing were observed obviously when compared with the control. But Basophil and Eosinophil did not reach to a significant level (table 2). Leukocytes are considered as the active cells in carrying out the functions of the immune system, both specifically and non-specific, their count may give a general picture about the function of the immune system and the results demonstrated that the treatment with CPSs extracted from *K. pneumoniae* had an effect on a differential count of leukocytes especially the Lymphocytes, Neutrophils and Monocytes, the Neutrophils are mainly involved in the innate immune system to carry out phagocytosis, while lymphocytes represent the humeral 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 (24). These results are agreed with (25), who elucidated the number of PMN increasing after injection of smaller doses of CPS-*K. pneumoniae* and mononuclear leukocytes can be activated through the release of cytokines, such as tumor necrosis factor-α (TNF-α).

Table 2: Count leukocytes of rabbit blood samples treated with partial purified CPS extracted of *K. pneumoniae* isolated from varies clinical sample compared with control.

Groups	Lymphocytes Cell/field	Monocytes Cell/field	Neutrophils Cell/field	Eosinophils Cell/field	Basophils Cell/field
Control	14	1	3	1	0
Urine	20	4	5	2	2
Wound	53	21	18	0	0
Burn	59	14	28	1	1
Sputum	56	3	11	0	0
Blood	26	0	9	0	0
Stool	39	3	9	0	0

3.2.2 Quantitative determination of Interleukins 2,4, 6, 10and TNF-α by ELISA

The serum levels of five cytokines, including IL-2, IL-4, IL-6, IL-10 and TNF-α were assessed in the various groups and compared with the control group. The level of IL-2 showed significant elevation (P ≤0.05) in some groups which were (74±6.24,68±17, 54±2.08, 53.66±15.38) for CPSs extracted from *K. pneumoniae* of sputum, wound, burn and stool, respectively, in compared with the control (20 ±1.15) pg/ml (table3). These results confirmed with that obtained of leukocytes counting specially the increasing number of lymphocytes, IL2 production is related mainly with lymphocytes and its function effects on the T lymphocyte proliferation and regulation of cellular immunity (26). While the level of IL-4 showed significant elevation (P≤0.05) occurred in all groups (64±1.52, 59.66±8.83,58±7.50, 56±7, 48±9.16 and 47±5.50) pg/ml for that *K. pneumoniae* CPSs extracted from sputum, wound, burn, stool, urine and blood respectively in comparison with control (20.33±0.33) pg/ml (table3). Also this result reflected the increasing number of lymphocytes, because one of the cellular sources of this interleukin from lymphocytes, as (27) reported. The level of IL-6 revealed significant elevation (P≤0.05) in some groups which were (417±3.71, 294±22.27, 263.3±21.85 and 150±16.07) pg/ml for CPSs extracted from *K. pneumoniae* of sputum, burn, blood and stool respectively, compared with control (44±0.57) pg/ml (table3). This interleukin secretes from lymphocytes and macrophage and its function on hematopoiesis, differentiation and inflammation, as (28) mentioned, thus both IL 4 and 6 described the leukocytes counting increase. Meanwhile ,the level of IL-10 also revealed a significant elevation (P≤0.05) in all groups (43±5.50, 42.33±4.40, 42.33±0.88, 42±1.73, 41.33±1.85 and 35.3± 2.02) pg/ml for that *K. pneumoniae* CPSs extracted from blood, urine, stool, burn, wound and sputum respectively compared with control (26±1.4) pg/ml (table3).This interleukin is secreted from cytotoxic, Th2, B lymphocytes, and macrophage, hence its function are inhibition INF γ, TNF-β, interleukin 2, that related with Th1 cell, and DTH (Delayed type hypersensitivity), but stimulate Th2 (29). Therefore this interleukin deemed as regulator for immune response due to its suppression the main arms of immune system. Finally, the level of TNF- α revealed a significant elevation (P ≤0.05) in all groups which were(835±22.91, 766.3±13.73, 616.6±2.18, 501.66±6, 366.3±6.43 and 307±1.73) pg/ml for CPSs extracted from *K. pneumoniae* of stool, blood, sputum, burn, wound and urine, respectively compared with control (230±6.42) pg/ml (table3), CPS induces secretion of tumor necrosis factor-α (TNF-α) by macrophages through Toll-like receptor 4 (TLR4), as (30,31-32) reported. We can concluded, bacterial CPS seemed to be a good stimulator of immune system, and the high level that were observed in all groups specially that deal with TNF-α in comparison with control, may assist the defense mechanism against *K. pneumoniae*, and simultaneously, the variation that were declared in the results of different interleukins may be due to the variation of bacterial serotypes, which may reflect the genetic and environmental factors may contributing in the interleukins production.

Table 3: The mean ± standards error of IL-2, IL-4, IL-6, IL-10and TNF-α

Group	Cytokines pg/ml Mean± SE				
	IL-2	IL-4	IL-6	IL-10	TNF-α
Control	20±1.15	20.3±0.33	44±0.57	26±1.4	230±6.42
Urine	46.6±11.56	48±9.16	65±1.73	42.33±4.40	307±1.73
Wound	68±17	59.66±8.83	51±0.57	41.33±1.85	366.3±6.43
Burn	54±2.08	58±7.50	294±22.27	42±1.73	501.66±6
Sputum	74±6.24	64±1.52	417±3.71	35.3±2.02	616.6±2.18
Blood	46±15.01	47±5.50	263.3±21.85	43±5.50	766.3±13.73
Stool	53.66±15.38	56±7	150±16.07	42.33±0.88	835±22.91

SE: Standard Error

3.2.3 Effect of partial purified CPS extracts on phagocytosis

The results of phagocytic index (PI)were measured for rabbits blood samples treated with partial purified CPS extracted from *K. pneumoniae* after 14 days of injection, showed a significant increase in PI of treated rabbit's blood 25 (26.3%), 25 (21.3%), 24 (18.8%), 22 (38.5%), 19 (27.1%) and 18 (35.2%) for sputum, wound, burns, blood, stool and urine respectively in compared with control 4 (17.3%) (table 4).These results indicated that the CPS extracted from *K. pneumoniae* has a positive effect on phagocytosis by increasing the PI; and confirms with the findings of (33) who claimed that role of *K. pneumoniae* CPS (K2) contributes to enhance the phagocytic cells by alveolar macrophages *in vitro*. In spite of the role of *K. pneumoniae* capsule is antiphagocytic activity to protect the bacterium from destruction and elimination (34). But our study improves reversibly results through increasing the number of phagocytic ability, this result may be due to some capsule components are removed from the capsule structure during the extraction and purification methods, as a result of solvents treatment that effect on capsule structure (35), lead to an elevation of sugar moiety, and increases the phagocytes binding ability throughout lectin binding receptor (including mannose) as (36) mentioned about the attachment of the pathogen to the mannose receptors (MR) on phagocytic cells can trigger the production of chemokines and cytokines.

Table 4: Phagocytosis index

Groups	Total No. (Non phagocyte+ phagocyte cell)	No. phagocytes and Efficiency (%)
Control	23	4 (17.3%)
Urine	51	18 (35.2%)
Wound	117	25 (21.3%)
Burn	127	24 (18.8%)
Sputum	95	25 (26.3%)
Blood	57	22 (38.5%)
Stool	70	19 (27.1%)

3.2.4 Effect of partial purified CPS extracts on antibody level using enzyme immunoassay for quantitative determination of IgG antibody

ELISA technique was performed for the quantitative determination of anti *K. pneumoniae* CPS (IgG antibodies) in the serum rabbits produced throughout immunization of rabbits. The results of the IgG concentration in the rabbits serum of treated groups showed a significant elevation (P ≤0.05) (196.30±7.70, 168.52±3.20, 129.63±4.27, 121.23±5.58, 108.64± 5.38and 102.47±10.10) ng/ml for (sputum, wound, burn, stool, urine and blood) groups respectively when compared with control (46.91±3.26)ng/ml (table5). These results were confirmed with (37) who referred that, the capsular polysaccharide (CPS) has the ability to stimulate antibody production against *K. pneumoniae.* Different factors effect on the IgG serum levels, such as genetic makeup, immune statue and environmental factors. High IgG levels are usually associated with increased production of lymphocytes.

Table 5: IgG titer in rabbits serum measured by ElISA technique at 450nm.

Group	Concentration IgGng/ml Mean±SE
Control	46.91±3.26
Urine	108.64±5.38
Wound	168.52±3.20
Burn	129.63±4.27
Sputum	196.30±7.70
Blood	102.47±10.10
Stool	121.23±5.58

We can conclude that different *K. pneumoniae* isolates from different specimens elucidated variation in immune response intensity, whether in its ability to produce IgG or different and important cytokines that play a role in immune response. But in general K antigen of *K. pneumoniae* is immunogenic; therefore these results reflect ability of this antigen to use it as vaccine or adjuvant. In addition the highly elevation of TNF-α level, makes this antigen suitable as anticancer.

Challenge test

Challenging the rabbits immunized with CPSs *K. pneumoniae* with freshly grown *K. pneumoniae* pathogenic bacteria isolated from different patients. All rabbits didn't infected with the pathogenic isolates or died but with the grossly symptoms included slight increasing in body temperature and indolence through two days after infection, and then all are healed during one week in comparison with the control group which are infected and dying within the same period. These results indicated the stimulation of immune responses were occurred due to the immunization by *K. pneumoniae* capsule, and these results also agree with the previous results of interleukins 2, 4, 6, 10, TNF-α and IgG elevation that led to protect animals from infection. The IgG antibodies neutralized the CPSs and protected rabbits (probably by opsonisation), thus CPS (K antigen) of*K. pneumoniae*, had been exert a strong adjuvanicity effect due to antibody responses, this results was agreed with (12) and (38) results, they were reported, the immunized mice with CPSs of *Klebsiella pneumoniae* subspecies, they were protected against virulent challenge with different 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* (39). IgG isolated from the serum was found to be highly protective against fatal experimental *Klebsiella* K2 burn, wound, sepsis indicating that the functional antibody is elicited following vaccination. The vaccine was nontoxic and non-pyrogenic for animals, an immunizing dose of 50µg elicited a fourfold or greater immunoglobulin G (IgG) response to all vaccine antigens (40).

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