

Klebsiella pneumoniae Ghosts as Vaccine Using Sponge Like Reduced Protocol

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Abstract

Recently, different microbial ghosts (BGs) were prepared using a strategy based on the critical chemical concentrations. In the original protocol, it was given the name "Sponge Like" (SL) protocol. The protocol was reduced to be more reliable and given the name "Sponge Like Reduced Protocol" (SLRP). In this study, using SLRP we succeeded to prepare *Klebsiella pneumoniae* (KPGs) with correct 3D structure and surface antigens. The study has included oral, S/C, inhalation, I/P and I/M vaccination with KPGs and controlled with challenge test. Results of both the SLRP and the animal experiments prove that we have KPGs able to immunize rats subjected to different routes of administration against viable *K. pneumoniae*. The evaluation of the immune response include humoral and cellular immune responses, IFN- γ production, phagocytic activity, NBT reduction activity, serum agglutination titer and the bacterial load in the various tissues, was performed in BG-vaccinated animals subsequently challenged with *K. pneumoniae*. The results were compared with animals that were not immunized with the KPGs. KPGs not only are able to promote the generation of high titer antibodies and IFN- γ production but also are able to stimulate a significant increase in phagocytic activity, NBT reduction, a marked decrease in the bacterial load in the various tissues, indicated that the vaccine was able to induce clearance of intracellular *K. pneumoniae*. The protective effects of BG vaccination in rats against virulent *K. pneumoniae* were a result of the induction of a more effective immune response in vaccinated animals than that observed with the non-vaccinated animals. These findings demonstrate the potential of *K. pneumoniae* ghosts to be used as effective vaccines.

Keywords: Microbial ghosts; *K. pneumoniae*; DNA; Vaccine

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Abbreviations

BG: Bacterial Ghosts; BGsQ: BGs Quality; MGC: Minimum Growth Concentration;

MIC: Minimum Inhibition Concentration; MIF: Mean Intensity of Fluorescence; OMP: Outer Membrane Proteins; SDS: Sodium Dodecyl Sulfate; SL: Sponge Like; SLRP: Sponge Like Reduced Protocol; SGPT: Serum Glutamic Pyruvic Transaminase; SGOT: Serum Glutamic Oxaloacetic Transaminase

Introduction

Bacterial ghosts (BGs) are empty non-deformed bacterial envelopes of gram-negative bacteria produced by controlled expression of cloned gene *E* from bacteriophage, forming a lysis

tunnel structure within the envelope of the living bacteria. BGs are devoid of cytoplasmic contents and possess all bacterial bio-adhesive surface properties in their original state. Using cloned or genetically modified elements (*E* lysis gene) for BGs preparation could have some risk factors. Foreign genetic elements could interact with our macromolecules. That pose a risk when any of

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them entered into our bodies [1]. BGs preparation was recently established using methods other than the *E* lysis gene, such as using the critical concentrations of some chemical compounds able to convert viable cells to BGs [2]. Sodium dodecyl sulfate (SDS) and sodium hydroxide (NaOH) were reported for their ability to interfere with the bacterial cell wall. H_2O_2 is well known for its ability (as an oxidant) to degrade the DNA and the other genetic elements [2]. There is no reliable *K. pneumoniae* vaccine produced so far, although many attempts were made [3,4].

In 1982 LPS vaccines have a relatively high incidence of side effects, as well as the problem of requiring a mixture of various LPS serotypes to make the vaccine effective in the treatment of *K. pneumoniae* infections [5,6]. Using the porin of *K. pneumoniae* as a protective vaccine in mice [5] induced good protective activity by immunizing mice with capsular polysaccharide of *K. pneumoniae* [7]. However, this activity was not observed in man. Lee et al. in 2000 reported that outer membrane proteins (OMPs) elicit antibodies with protective efficacy against *K. pneumoniae* infection in burned patients only [8]. Heat-killed *K. pneumoniae* was used as a vaccine against the infection, but this immunogen caused serious adverse effects [9]. The use of killed pathogens as substitutes for the living infectious agents was widely used as a principle for vaccine development. BGs can be used as vaccine candidates with intrinsic adjuvant properties based on well-known immune-stimulating compounds such as lipopolysaccharides, lipid A, and peptidoglycan, to potentiate non-specifically the immune response to a target antigen [10].

Materials and Methods

Bacterial strain

K. pneumoniae strain was used in this study was kindly obtained from the Bacteriology Department at the Faculty of Medicine, Tanta University by Prof., Mohamed Zakaria on MacConkey plates from Egyptian patient suffering from pneumonia.

Bacterial cultivation

K. pneumoniae was cultivated in 500 mL NB (in one-liter flask) under shaking condition at 37°C for 72 hrs.

Determination of the minimum inhibition concentration (MIC) and minimum growth concentration (MGC) for NaOH, SDS, and H_2O_2

The MIC and MGC for each of NaOH, SDS, and H_2O_2 were determined using standard criteria [11, 12]. 10% of each of NaOH and SDS as well as 30% H_2O_2 stock solutions were prepared. Standard serial dilution method was used to determine the MIC and the MGC values, where 0.5 mL of the above solutions were added (each) to 4.5 mL of the medium in 10 mL test tube (to gain the final volume 5 mL and the 1:10 dilution), and so on. 200 μ L of an overnight *K. pneumoniae* culture was added to each tube in the serial dilution experiment. In case of $CaCO_3$ the used amount of +1 value was 1.05 μ g/mL, while -1 value was 0.35 μ g/mL. The test tubes were then incubated overnight at 37°C under static condition [11].

Sponge like reduced protocol for preparing the BGs

Two experiments with different parameters were conducted. The two experiments were selected from the best results obtained by Amara et al. as in 2013. They are mainly experiments numbers one and eleven in the original SL protocol [2].

Determination of the DNA and protein concentrations

The concentrations of the DNA and the protein were derived from the spectrophotometer measurement (Thermo Scientific-VISIONpro Software V4.30) and determined according to Amara et al. as in 2013.

KPGs evaluation using light microscope (Optika)

Bacterial smears from the both preparations were stained using crystal violet for 30 seconds. The cells were examined using the light microscope. The BGs quality (BGsQ) was determined for each preparation based on the quality of the 3D structure as either being correct or deformed. The overall BGsQ was given as a %.

Transmission electron microscope for *K. pneumoniae* Gs evaluation

Transmission electron microscope (JEOL TEM 100 CX) was used to evaluate the *K. pneumoniae* Gs.

Viability test

The prepared KPGs were evaluated for the existence of any still viable cells, where samples were taken from each preparation and grown in MacConkey agar plates. The plates then were incubated at 37°C for two days.

Animals and experimental design

Fifty-two male Sprague-Dawley rats weighing 110 ± 10 g were purchased from the animal house of the Institute of Graduate Studies and Research, Alexandria University, Egypt. After two weeks of acclimatization, rats were randomly assigned to six groups. The animals were divided to groups according to the treatment type and the route of administration. A: control group included 13 rats, which are not inoculated with bacterial ghost and five residual groups included B: 5 rats I/M, C: 5 rats I/P, D: 5 rats inhalation, E: 12 rats oral, F: 12 rats S/C which inoculated with bacterial ghost. Each rate received 100 μ L (10^7 CFU) of KPGs in sterile saline. Initial dose of KPGs were administered at week 1 and followed by two booster doses at week 3 and week 5.

Challenge test

At one week from the 1st booster, rats were challenged on the IP, inhalation and vagina by 100 μ L sterile saline containing (10^8) viable *K. pneumoniae*. The clinical signs and mortalities were monitored for 7 days post challenge (8 animals/12 oral and 7 animals/12 S/C).

Challenged rats were received one dose for I/P challenge and three doses for virginal and inhalation challenge on three successive days.

After one week from the 2nd booster 18 animals (5 control, 3 oral, 2 S/C, 2 I/M, 2 I/P, 1 inhalation, 1 I/P + Adjuvant, 1 oral + adjuvant, 1 inhalation + adjuvant) were received 10⁸ CFU of viable *K. pneumoniae* in the same routs of administration as above. The used adjuvant is Freund's adjuvant where 1800 µl saline is mixed with 750 µl concentrated Freund's adjuvant and each rate received 100 µl. The clinical signs and mortalities were monitored for seven days post challenge.

Phagocytic activity

Phagocytic activity was investigated using viable *K. pneumoniae* and KPGs, and determined as the percentage of phagocytizing neutrophils and as the mean intensity of fluorescence (MIF) value, which equaled the mean number of bacteria phagocytized by the cells. Cells were visualized using light microscope "LM" and immuno-fluorescent microscope "IFM" with the aid of acridine orange and determined as either live or killed bacteria.

IFN-γ level

IFN-γ levels in different treatments were measured according to manufacturers' instructions using Rat IFN-γ platinum ELISA (Ready- to -use Sandwich ELISA, eBioscience, North America).

Slide agglutination test for KPGs

Antibody titers were measured by slide agglutination titer. Glass slide was cleaned well, wiped free of water. One drop of each sample was placed with antiserum of KPGs vaccinated animals. The content of each circle was mixed using separate wooden applicator or stick and spread to fill the whole area of individual circle. The slide was shacked for one to three minutes and observed for agglutination [13].

Nitroblue Tetrazolium Test (NBT)

Heparinized blood samples are incubated with a buffered solution of NBT under carefully controlled conditions [14]. Smears are then prepared, stained and examined microscopically to determine the percentage of neutrophils showing intracytoplasmic deposits of reduced NBT insoluble formazan.

Isolation of LPS

Lipopolysaccharide (LPS) was extracted from *K. pneumoniae* cells, cultured overnight on NB medium, by the phenol–water method of Westphal and Jann in 1965. The preparations were purified by treating them with 2% cetavlon before being dialyzed extensively against distilled water and then lyophilized.

SDS-PAGE analysis

SDS-PAGE was performed on 12% (w/v) polyacrylamide gels as previously described [15]. Lanes were loaded with either freshly

grown bacteria or KPGs in saline or LPS (300 ng) preparations. All samples were solubilized in sample buffer containing 2% (w/v) SDS and 5% (w/v) β-mercaptoethanol and heated at 100°C for 3 min prior to loading. Gels were electrophoresed at 100 V through the stacking gel and then at 150 V for about 3 hrs. Molecular weight markers (Pharmacia) and the LPS gels were subjected to Ammonia Silver staining using the Phast GelTH (Pharmacia) kit. Other gels were fixed and stained with Coomassie blue R250 (Sigma).

Bacteriological analysis for measurement of bacterial load in tissues

Infected rats Lungs, liver, spleen, gut and vagina were harvested and equal wet tissue weights were homogenized and briefly centrifuged to remove gross particulate matter. Serial ten-fold dilutions of tissue homogenates were applied to selective agar plates and incubated at 37°C for 24 hrs. under aerobic conditions. Plates were subsequently analyzed by colony counts and expressed as CFU/g tissue weight [16].

Statistical analysis of the data

All data is expressed as mean ± standard error of the mean using IBM SPSS software version 20.0. [17]. For normally distributed data, comparison between more than two independent populations was done using F-test (ANOVA) to be used and Post Hoc test (LSD). Significance of the obtained results was judged at the 5% level.

Results and Discussion

Recently a new protocol for BGs preparation was introduced. The protocol is able to evacuate different microbes including gram positive, negative, spore former, capsulated etc., bacteria. Even it is succeeded to prepare ghosts from eukaryotic cells as well as viruses. The protocol find acceptance worldwide particularly due to its reliability, cost effective and applicability with different microbes. The first step is based on determining the MIC and the MGC of the used chemical compounds. In case of NaOH; the MIC and the MGC were 0.8 mg/ml and 0.08 mg/ml, respectively. In case of SDS; the MIC and the MGC were 0.1 mg/ml and 0.01 mg/ml, respectively. In case of H₂O₂, the MIC and the MGC were 12 mg/ml and 2.4 mg/ml, respectively. In case of CaCO₃, the used amount of +1 value was 1.05 µg/ml while -1 values was 0.35 µg/ml according to Amara et al. in 2013.

The results show correct release of both of DNA and protein during the preparation steps. The amount of the protein is greater than that of DNA (Table 1). That is logic, particularly at the H₂O₂ step. None of the cells upon cultivation on NA medium for two days at 37°C has shown any growth.

Table 1 The amount of the DNA and Protein realized during different ghost's preparation steps.

Experiment* No.	NaOH step		SDS+CaCO ₃ step		H ₂ O ₂ Step		Ethanol step		(BGQ)%
	Protein µg ml ⁻¹	DNA µg ml ⁻¹	Protein µg ml ⁻¹	DNA µg ml ⁻¹	Protein µg ml ⁻¹	DNA µg ml ⁻¹	Protein µg ml ⁻¹	DNA µg ml ⁻¹	
1	1425	186.35	171.7	101.3	89.5	33.25	22.61	19.45	100

*Experiment no 2 is not shown

Chemically induced KPGs were produced for the first time by the critical chemical concentration method as described by Amara et al. in 2013. In the present study the MIC determination is critical step in the BGs preparation. For that and by using several steps in the protocol the bacterial cell loss their content of DNA and protein. The first point is to expose the bacterial cells to the chemical compounds, either alone or in correct combination (did not enable chemical interactions), and the second point is to enable several centrifugations and washing steps to remove the DNA and the protein content completely. The gentle centrifugation enables fine pressing to the cells to enable them to get rid of their cytoplasm constituents. The quality of the prepared BGs (QBGs) was monitored using the light, fluorescent and electron microscope. The correct 3D structure of the cells is presented in **Figures 1-3**. Treated bacterial ghost cells and non-treated control cells were examined for the presence of any remaining protein by SDS-PAGE analysis. Non-treated control cells showed strong protein bands (data note tissues). This electrophoretic profile indicates that the protein extract contains several antigenic proteins from various parts of the bacterial cell, as expected. While the chemically treated cells show weaker protein bands. This is due to the lack of cytoplasmic contents in BGs cells [18]. In addition, the complete degradation of bacterial genomic DNA was determined using agarose gel electrophoresis (**Figure 4**). This result indicates that DNA free KPGs are successfully induced by critical chemical concentration of NaOH, SDS and H₂O₂. Also, Amara et al. in 2013a showed similar results that the genomic DNA of *E. coli* JM 10⁹ bacterial ghost was completely degraded using the SLRP.

Serum Agglutination titer is highly positive in all vaccinated groups when compared to control groups (**Figure 5**). This indicates that KPGs vaccine stimulate humoral immune response. After

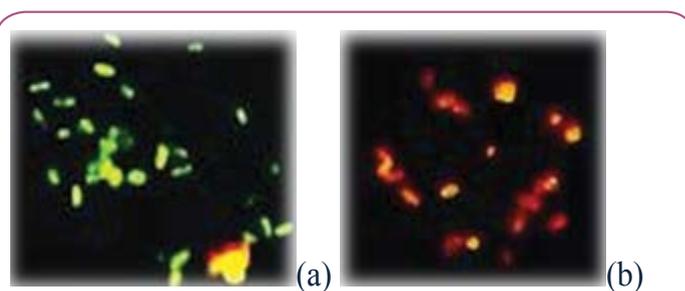


Figure 2 Acridine orange stained smears under florescent microscope for (a) live *K. pneumoniae* green, (b) *K. pneumoniae* ghost orange.

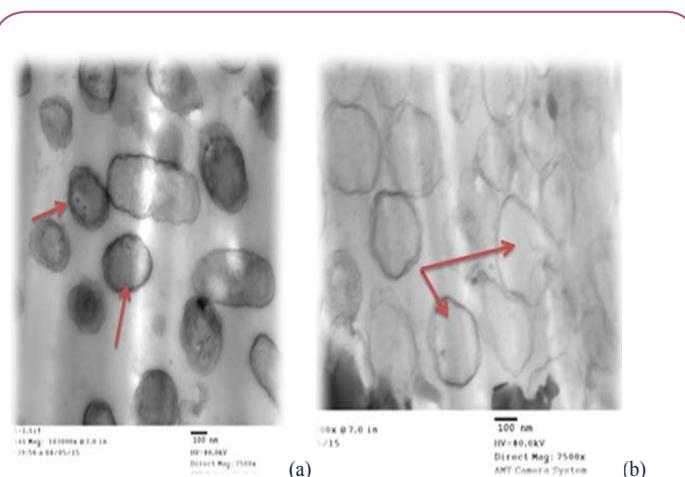


Figure 3 Transmission electron micrographs at different magnification power. (a) *K. pneumoniae* whole cell (before treatment) as a control, arrow showed a complete cell with all its cytoplasmic content. (b) Ghosts of *K. pneumoniae*. The empty areas in the bacterial cells which marked by the arrows indicated that all cytoplasmic contents were removed.

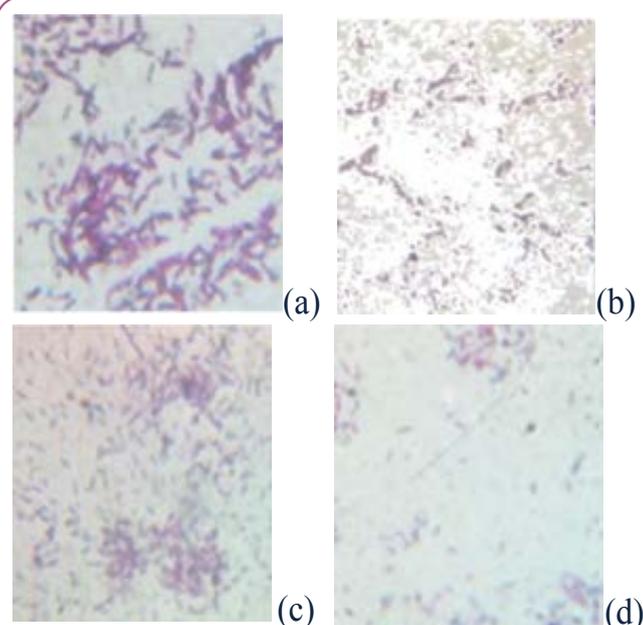


Figure 1 Crystal violet staining (a) live *K. pneumoniae*, (b) cells after NaOH step, (c) cells after SDS, CaCO₃, and H₂O₂, (d) *K. pneumoniae* ghost.

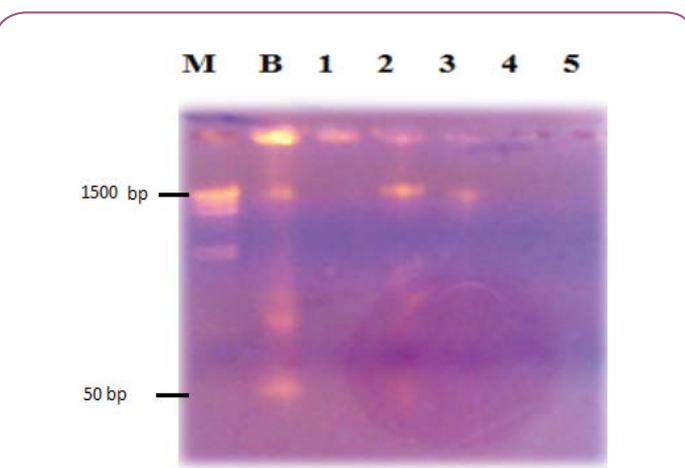


Figure 4 Agarose gel electrophoresis for *K. pneumoniae* (lane B) show DNA bands DNA marker (lane M), *K. pneumoniae* ghost (lane 1-5).

challenge, there was marked decrease in agglutination titer in vaccinated group when compared to an increase in agglutination

titer in control group. This is due to the consumption of specific Abs in neutralize of *K. pneumoniae* used in the challenge, thus mild positive reaction occurs, so our vaccine gives long lasting effect. Control group gives highly positive reaction due to first exposure and absence of specific Abs in serum. We noticed marked increase in agglutination titer after each booster due to secondary humoral immune response against each booster that leads to increase the amount of the specific Abs.

The concentrations of IFN- γ in the different groups are summarized in **Figure 6**. After booster with *K. pneumoniae* ghost (KPGs), there was statistically significant increase ($p \leq 0.05$) in IFN- γ levels in groups vaccinated by inhalation and S/C routes when compared to the control group. This indicates a significant activation of T_H cells; so KPG is able to activate the specific immunity in vaccinated rats. INF- γ levels were high in inhalation and S/C vaccinated rats, there was a significant increase in the control group after challenge when compared with results obtained before the challenge, in the same time there was an insignificant decrease in S/C vaccinated rats after challenge when compared to before challenge. This may be due to neutralization of *K. pneumoniae* used in the challenge by specific Abs in the sera of vaccinated rats so mild activation of T_H cells occurs. This indicates that KPG is able to induce specific humoral immune response among vaccinated rats especially when given subcutaneously.

Our results revealed that the bacterial load from the internal organs was significantly reduced in all vaccinated groups when compared with control non-vaccinated group ($p \leq 0.05$) (**Figure 7**), but is still high. This may be due to lower efficacy of dose used, either initial or booster dose, so we recommend increasing the initial and the two booster doses to overcome bacterial load. Rats from S/C group show higher protection than orally vaccinated group. So, efficacy of KPGs is more prominent when given subcutaneous. Some studies on BG vaccines have reported that BG-vaccinated animals not only exhibit a good immune response but are also resistant to infection by highly virulent strains, indicating the protective effect of the BG vaccine [19-22]. Results of bacteriological examination indicate that the chemically induced KPGs are an effective vaccine candidate against virulent *K. pneumoniae* challenge.

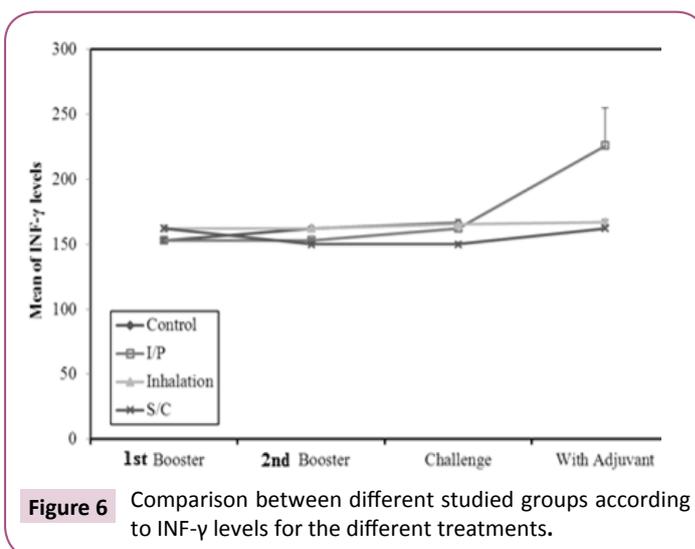


Figure 6 Comparison between different studied groups according to INF- γ levels for the different treatments.

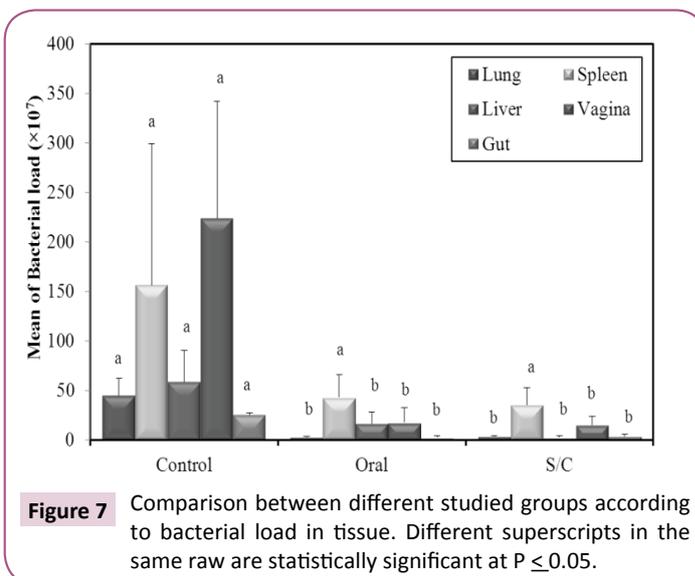


Figure 7 Comparison between different studied groups according to bacterial load in tissue. Different superscripts in the same raw are statistically significant at $P \leq 0.05$.

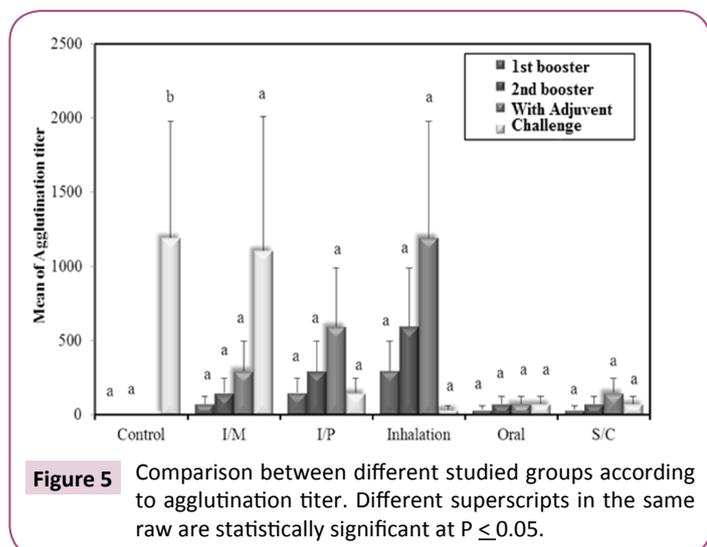


Figure 5 Comparison between different studied groups according to agglutination titer. Different superscripts in the same raw are statistically significant at $P \leq 0.05$.

Our results showed that KPGs are potent antigens causing activation of phagocytic cells and stimulation of non-specific innate immunity that results in rapid elimination of the bacteria and presentation of foreign antigen to T and B lymphocytes to start the specific, long lasting immune response [23]. All groups showed significant increase when compared with control (**Figure 8**). The percent of active phagocytic cells was the highest increase after the second booster in (inhalation + adjuvant) group. Freund's adjuvant was used (1800 μ l saline+750 μ l concentrated Freund's adjuvant). This indicated that the antibodies exhibited a strong opsonization effect. The phagocytic process is the principal mechanism for destruction of extracellular bacterial pathogens in mammals [24].

After second booster schedule, there was a significant increase in the percentage of positive NBT neutrophils (**Figure 9**) in all the vaccinated groups when compared with control group ($p \leq 0.05$). After challenge, there was a significant increase in number of positive cells in all vaccinated groups when compared with non-vaccinated group (control) ($p \leq 0.05$). This indicates that

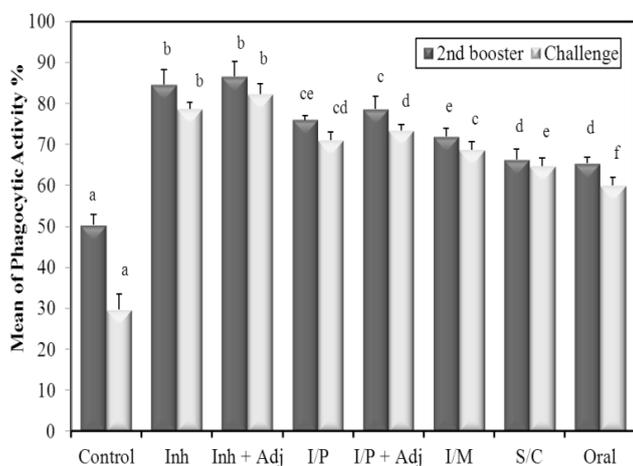


Figure 8 Comparison between 2nd booster and challenge according to phagocytic activity %. Different superscripts in the same row are statistically significant at $P \leq 0.05$.

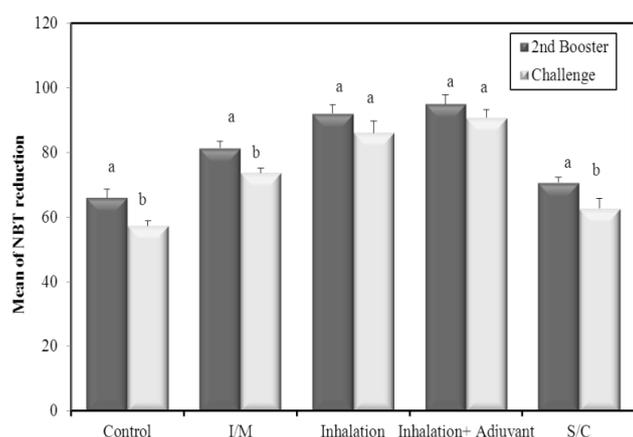


Figure 9 Comparison between 2nd booster and challenge according to NBT reduction activity. Different superscripts in the same row are statistically significant at $P \leq 0.05$.

KPGs caused increase in the killing activity of phagocytic cells especially when given by subcutaneous rout. The NBT test shows that numbers of neutrophils are influenced by the vaccination with KPGs. Some recent experiments have shown that the immunostimulants can be given alone to induce the *in vitro* and *in vivo* responses [25]. It is well known that these agents stimulate the non-specific immune response (also called innate or natural protection) and boost the specific immune response. Therefore, immune-stimulants can also be used as adjuvants, to heighten the specific immune response. We can conclude that according to the results of the present study; the NBT is a useful tool for the follow up of the stimulating effects KPGs as a vaccine.

After second booster; there was significant decrease in serum glutamic oxaloacetic transaminase (SGPT) among immunized groups when compared with control group (Figure 10). In serum, glutamic oxaloacetic transaminase (SGOT) there was significant decrease in oral group when compared with control and S/C (p

≤ 0.05) (Figure 11). These results indicate that KPGs as a vaccine provided complete protection for liver cells against highly virulent *K. pneumoniae* strain.

Conclusion

In conclusion, our study concern with developing KPGs using the SLRP, that the first step was by determining both of the MIC and MGC of (NaOH), (H₂O₂), (SDS) and (CaCO₃). By following the SLRP we succeeded to prepare KPGs with correct 3D structure and surface antigen, empty and dead. KPGs could contribute in the vaccine-induced protection against viable *K. pneumoniae* infection. The obtained results prove the role of humoral and cell-mediated immune responses in KPGs vaccinated rats upon

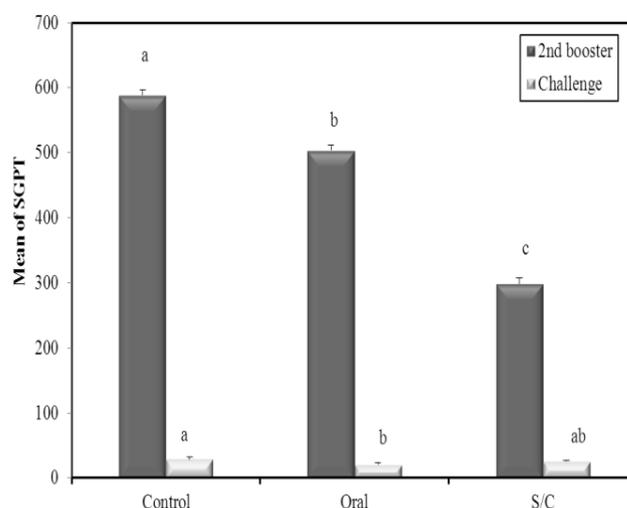


Figure 10 Comparison between different studied groups after 2nd booster and after challenge according to SGPT. Different superscripts in the same row are statistically significant at $P \leq 0.05$.

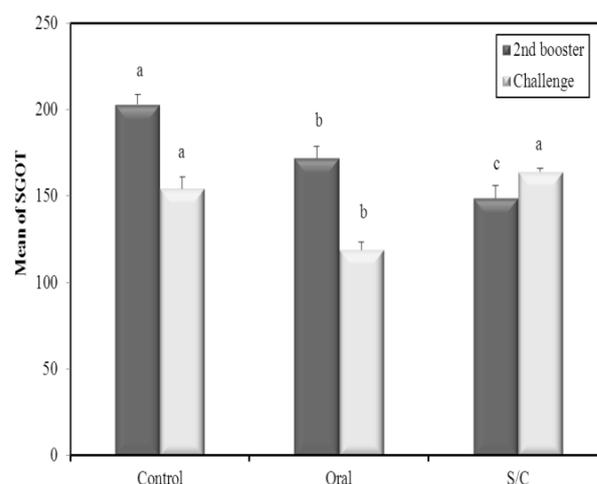


Figure 11 Comparison between different studied groups after 2nd booster and after challenge according to SGOT. Different superscripts in the same row are statistically significant at $P \leq 0.05$.

infection with *K. pneumoniae*. The BG vaccine not only induced powerful humoral and cell-mediated immune responses in vaccinated rats, but it also provided complete protection against virulent *K. pneumoniae*. Our findings have a promising implication for future *Klebsiella sp* vaccine research. The present study, demonstrated that subcutaneous immunization of KPGs can induce strong immune responses which leads to a significant eradication of infection as well as reduction in bacterial colonization of the infected rats.

Ethical Approval

This study was carried out in strict accordance with the

recommendations in the guide for the Care and Use of Laboratory Animals of the College of Medicine, Alexandria University which is prepared and approved by the Committee of Postgraduate Studies and Research on the Ethics of Animal Experiments of the University of Alexandria.

Conflict of Interest

The authors have no conflict of interests to declare.

Financial Disclosure

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