

## Development of a new trend conjugate vaccine for the prevention of *Klebsiella pneumoniae*

Tarek A. Ahmad,<sup>1</sup> Medhat Haroun,<sup>1</sup> Ahmad A. Hussein,<sup>1</sup> El Sayed H. El Ashry,<sup>2</sup> Laila H. El-Sayed<sup>3</sup>

<sup>1</sup>Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University; <sup>2</sup>Department of Chemistry, Faculty of Science, Alexandria University; <sup>3</sup>Department of Immunology, Institute of Medical Research, Alexandria University, Alexandria, Egypt

### Abstract

*Klebsiella pneumoniae* is a major cause of nosocomial pneumonia, septicemia and urinary tract infections, especially in newborns, blood cancer patients, and other immunocompromised candidates. The control of *K. pneumoniae* is a complicated issue due to its tight pathogenesis. Immuno-prophylactic preparations, especially those directed toward the bacterium O-antigen, showed to be the most successful way to prevent the infection incidence. However, all previously proposed preparations were either of limited spectrum or non-maternal, and hence not targeting the main *Klebsiella* patients. Moreover, all preparations were directed only to prevent the respiratory diseases due to that pathogen. This article addresses the development of a method originally used to purify the non-capsular bacterial-endotoxins, as a new and easy method for vaccine production against *K. pneumoniae*. The application of this method was preceded by a biotechnological control of capsular polysaccharide production in *K. pneumoniae*. The new produced natural conjugate between the bacterial O-antigen and its outer membrane proteins was evaluated by physicochemical and immunological methods to investigate its purity, integrity, safety and immunogenicity. It showed to be pure, stable, safe for use, and able to elicit a protective immunoglobulin titer against different *Klebsiella* infections. This immune-response proved to be transferable to the offspring of the vaccinated experimental rabbits via placenta.

### Introduction

*Klebsiella pneumoniae* is a major cause of nosocomial pneumonia, septicemia and urinary tract infections,<sup>1,2</sup> especially in newborns,

blood cancer patients, and other immunocompromised candidates.<sup>3</sup> Despite usage of appropriate antibiotic therapy, morbidity and mortality due to *Klebsiella* bacteraemia and pneumonia exceeded 50%.<sup>4-6</sup> These data were consolidated by our epidemiological investigation throughout three successive years in Alexandrian university and public hospitals.<sup>7</sup> Many studies worldwide proved the increasing resistance of such pathogen resulting in an average rate of 1.63 outbreak every year.<sup>8</sup> Furthermore, the control of *K. pneumoniae* has been a complicated issue due to increasing resistance toward antibiotics, production of endotoxins that induce septic shock,<sup>3</sup> in addition to the bacterium capsular polysaccharide (CPS) that can cause immuno-paralysis by inhibiting phagocytosis,<sup>9</sup> and resistance to complement-mediated killing.<sup>10</sup> These risk-factors together point to the need for immunoprophylactic agents to prevent such uncontrollable disease and propose immunizations and immunotherapy treatments as a potential powerful tool to manage *K. pneumoniae* infections.<sup>11</sup>

The development of immuno-prophylactic trials against *K. pneumoniae* over the past 40 years proved the superiority of lipopolysaccharide (LPS)-based vaccines to protect from the infection incidence.<sup>7,12-14</sup> New approaches to detoxify the LPS, either by alkaline/acidic hydrolysis or incorporation with liposomes, enhanced their use as vaccine core-targets. The design of the vaccine mainly depends on the targeted patients;<sup>15</sup> for *K. pneumoniae* those are chronic patients and neonates, that should profit from safe, broad spectrum maternal vaccines. However, all up-to-date LPS-based vaccines against *K. pneumoniae* are only mono-valent, of limited LPS spectrum and might be of non-maternal potency.<sup>12,14,16,17</sup> The only trial that provoked a maternal broader spectrum immune-response vaccine was based on the iron-regulated cell-surface-protein (IRCSP) as a carrier for the detoxified LPS. The preparation was found to be protective against lobar pneumonia;<sup>13</sup> however, this vaccine production was complicated, depended on many purification steps, and hence never reached the phase of the clinical trials since its introduction in 1995.<sup>18</sup> The epitope mapping of *K. pneumoniae* proteins revealed the involvement of common outer membrane proteins (OMP) other than the IRCSP in the immune-response against different *K. pneumoniae* infections.<sup>7,19</sup> Therefore, the development of a conjugate between such proteins and the most propagated O-antigen may result in a broad spectrum maternal vaccine intended to prevent blood stream infection, pneumonia and urinary tract infections due to *K. pneumoniae*; and this is the aim of our study.

Correspondence: Tarek Adnan Ahmad, Biotechnology Department, Institute of Graduate Studies and Research (IGSR), Alexandria University, 34 Memphis St., Alexandria 21526, Egypt. Tel. +2.0100.1701579 - Fax: +2.03.4285792. E-mail: tarekadnan@yahoo.com

Key words: conjugate vaccines, *K. pneumoniae*, isobutyric acid, lipo-polysaccharides.

Acknowledgments: the authors express their finest appreciation to Dr. Wafa Habbal, Damascus University for the true support she offered to edit this review.

Conflict of interests: the authors report no potential conflict of interests.

Received for publication: 27 October 2011.

Revision received: 23 March 2012.

Accepted for publication: 28 April 2012.

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Infectious Disease Reports 2012; 4:e33  
doi:10.4081/idr.2012.e33

## Materials and Methods

### Bacterial strain and production of capsule-reduced biomass

Being the most propagated O-serotype,<sup>20</sup> O1-antigen (K41:O1) *K. pneumoniae* acquired from Institut Pasteur (CIP 53.16; Paris, France) was used as the standard isolate for the investigations. The strain was grown on brain heart infusion (BHI; Oxoid, UK) broth. Different concentrations of sodium salicylate (0, 10, 20, 30, 40 and 50 g/mL medium) were used to inhibit the capsule production, in the presence of 50 mm ethylenediaminetetraacetic acid to chelate calcium and magnesium;<sup>21</sup> at different growth temperatures (26, 30, 34, 38 and 42°C), agitations (100, 150, 200, 250 and 300 rpm), and harvesting times (12, 16, 20, 24, and 28 hours). Dry biomass was monitored in all experiments. Four-way ANOVA (analysis of variance) was used to analyze the data obtained from the capsule-reduced production process.

### Quality control of the produced biomass

#### Molecular quality control

Constant amounts of biomass (30 µg/well) were lysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) lysis buffer and electrophoresed on 12% separating

gel at 120 V for 150 min, then stained by Alcian blue to detect the presence of acidic polysaccharides.<sup>22,23</sup>

### Instrumental quality control

Samples were sonicated (Ultrasonic Homogenizer 4710 series, Cole-Palmer Instrument Co., USA) for three continuous min on an ice-bath. A sample of known carbohydrate concentration using the phenol sulfuric acid method was applied to high performance liquid chromatography (HPLC: Agilent Technologies 1200 series, USA),<sup>24</sup> using C-18 Column (Eclipse XDB-C-18, USA), and positive polarity refractive index (RI) detector operated at 35°C.<sup>25</sup> Pure acetonitrile (BDH, UK) was used as eluent at a flow rate of 1 mL/min<sup>-1</sup>.

### Glycopeptide conjugate extraction

A modification for the method of Caroff and Karibian's was used to extract the glycopeptides.<sup>26,27</sup> In brief, the bacterial cells were washed three times with phosphate buffer saline pH 7.2, and centrifuged each time at 4000 rpm for 15 min. The cells were mixed with isobutyric acid/1M-ammonium hydroxide (5:3; v/v), stirred for 1 hour, and centrifuged again at 4000 rpm for 15 min. The supernatant was added drop-wise to ten times its volume of cold 96% ethanol, stirred, and freeze-dried (-15°C) over night. The precipitate was collected by centrifugation at 5000 rpm and -5°C for 10 min. The pellets were dissolved in minute amounts of distilled water and added again to 10 times their volumes of cold 96% ethanol for washing, then centrifuged, and lyophilized for further usage.

### Quality control of the produced glycopeptide

#### Functional groups assessment and conjugation monitoring

Dry samples of the different glycopeptide production steps (before and after alcohol washing; then after lyophilization) were tested by Fourier transformation infra red device (FTIR: TENSOR 37, Bruker Optics, USA) using KBr discs. Standard polystyrene was used to calibrate the device.

Glycopeptide conjugation control was monitored by dissolving the lyophilized glycopeptide in pyrogen-free water, then applying it to HPLC (Agilent Technologies 1200 series, USA), using C-18 Column (Eclipse XDB-C-18, USA) connected either to positive polarity RI detector operated at 35°C, or ultra-violet (UV) detector operated at 280 nm. The applied eluent was pure acetonitrile (BDH, UK) with a flow rate of 1 mL min<sup>-1</sup>. The collected fractions corresponding to a peak were analyzed for carbohydrates content by the phenol sulfuric acid method,<sup>24</sup> and the developing pink color was measured at

490 nm micro-plate reader absorbance (BioTek, USA). The protein content was measured by colorimetric method using Olympus AU400 autoanalyzer (Olympus, Germany).<sup>28</sup>

### Lethal toxicity, pyrogenicity, and allergic reaction

For each antigenic preparation, a group of five white New Zealand rabbits (body weight 2.7±0.1 kg; kept at Institute of Graduate Studies and Research, Alexandria, Egypt, official animal house facility) were injected subcutaneously with different concentrations (50, 75 and 100 g/Kg) of filter-sterilized (0.22 µm, PES membrane, TPP®, Switzerland) glycopeptide, while the control group were synchronously injected with 1 mL saline. Animals were observed up to one week for toxicity manifestation as previously described.<sup>14</sup> Anticipated death was prevented by a single subcutaneous injection of 8 mg dexamethasone followed by warming the rabbit and massaging its chest to improve respiration and circulation;<sup>29</sup> physiologic support and antibiotics administration were provided whenever needed.

For pyrogenicity assessment, rectal temperature was recorded before and at 1, 2 and 3 hours intervals after injections of 50, 100 and 150 g/Kg for the previous rabbits. A preparation was considered to be pyrogenic when temperature increased more than 1.15°C above the baseline.<sup>14</sup> The allergic (Local Schwartzman) reaction was assayed by injecting 100 g of the vaccine suspended in 0.1 mL 0.9% saline intra-dermally at different sites on the shaved back of three healthy non-vaccinated rabbits (2.7 Kg). After 18 h, the rabbits were given a provocative intra-venous injection including 100 g of the vaccine. The intensity of local skin reaction was determined 5 hours after the provocative injection.<sup>13</sup>

### Protective role of the glycopeptide conjugate

#### Antibody titer and statistics

Rabbits involved in the toxicity assessment tests above were re-injected with conjugate concentration of 50, 100, and 150 µg/kg (hence abbreviated GP.2.50, GP.2.100 and GP.2.150) in 2 boosting doses on two weeks intervals each (2, and 4 week doses), while the control group (GP.1) were synchronously injected with 1 mL saline. Blood samples were collected from the injected rabbits groups at 0, 1, 2, 3, 4, 5 and 7 weeks intervals and centrifuged. Sera of the three groups of vaccinated rabbits were preserved at -80°C for further use, as primary antibodies for enzyme-linked immunosorbent assay (ELISA). Anti-glycopeptide antibody titers were assayed using horse radish peroxidase-labeled polyvalent goat anti-rabbit immunoglobulin (H+L) (Sigma-Aldrich, USA)

as secondary antibody.<sup>20</sup> The developing color was read at 450 nm on a micro-plate reader (BioTek, USA). Control and blank wells missing the primary antibody and the conjugate-coating; respectively, were set up. The obtained data were analyzed by three-ways ANOVA.

### Maternal antibodies investigations

To assay the antibody transfer to the offspring, three virgin female white New Zealand rabbits representing each group previously injected were allowed to mate with an adult male for 2 days, at a schedule interval before and after the first injection in order to test the effect of the number of doses given to the mother before delivery on the offspring antibody titer. Blood sample were drawn from the 3, 5, and 7 weeks aged offspring and tested for antibody titer by ELISA as previously described.<sup>20</sup> Orphan bunnies were let to lactate from the injected females and antibody titer was assayed in their sera at ages of 3, 5, and 7 weeks.

### Challenge trials

Challenge trials were applied after the last serum collection on the previously immunized non-pregnant or lactating rabbits. Three sound vaccinated rabbits of each group (control and different vaccine concentrations) were used. The standard bacterial suspension was monitored by McFarland method to prepare a concentration of 10<sup>8</sup> colony forming unit (CFU) per mL.<sup>30</sup> Three different animal models were injected with 1 mL of the bacterial suspension as follows: i) sepsis/peritonitis models;<sup>17</sup> animals were challenged by an intra-peritoneal injection of >10<sup>7</sup> CFU of the standard *Klebsiella*. Twenty-four and seventy-two hours later, two blood specimens were drawn, cultured on thioglycolate, and investigated for bacterial growth. Death or pathological manifestations were always recorded and prevented as previously described;<sup>29</sup> ii) pneumonia models;<sup>13</sup> rabbits were challenged via-trachea by more than 10<sup>7</sup> CFU, then pneumonia symptoms development was inspected over 2 weeks; iii) urethritis models, female rabbits were challenged via-vagina by more than 10<sup>7</sup> CFU.<sup>31</sup> Vaginal mucus was then quantitatively cultured on blood agar (Oxoid, UK) before and 3, 6 and 9 days after the challenge. All inflammatory manifestations were recorded over the investigation period.

## Results and Discussion

### Production of the capsule-reduced biomass

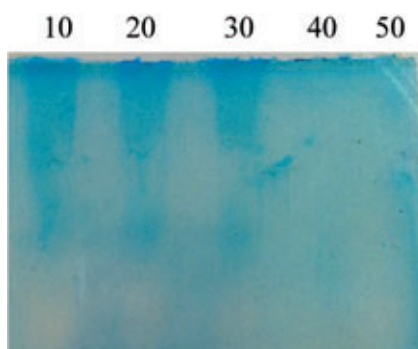
It has been confirmed that the produced glycopeptide or LPS by Caroff's method contains

high amount of CPS impurities unless using capsule-free bacterium.<sup>7,26</sup> Therefore, the optimization of CPS-reduced biomass production by sodium salicylate was followed in our study.<sup>32</sup> The standard bacterial isolate was used for such purpose as a reference strain for the desired O-antigen. The remarked best growth, estimated as dry weight, was obtained by using the BHI (over 3 g/400 mL of medium), then the Luria-Bertani and Nutrient Broth that recorded 1.2 and 1.5 g/400 mL medium; respectively. Furthermore, pH 7-7.5 showed to be the optimum for biomass production. The four-way ANOVA confirmed that the variation of results is accepted ( $P < 0.01$ ), while the data showed an inverse relationship between biomass production and salicylate concentration.

### Quality control of the produced biomass

Wells were optimized to contain 30  $\mu\text{g}$ /well of the lysed biomass. The electrophoresed samples were stained by Alcian blue to detect the presence of CPS as shown in Figure 1. The PAGE plate manifested a decrease in CPS intensity from the lowest salicylate concentration (10 g/mL medium) at the left-hand side toward the highest salicylate concentration (50 g/mL medium) at the right-hand side. The detection limit of Alcian blue was shown at 40  $\mu\text{g}$ /mL of salicylate since CPS was undetectable at equal or higher salicylate concentrations.

The integration of all findings above indicates that the best concentration of sodium salicylate that delivers the best results of CPS reduction and biomass production might be 40  $\mu\text{g}$ /mL. This preliminary result was subjected to confirmation by more accurate biophysical methods. The 20  $\mu\text{g}$  sonicated biomass sample



**Figure 1.** Twelve percent sulfate-polyacrylamide gel electrophoresis stained by Alcian blue for the biomass; in the presence of 10, 20, 30, 40 and 50  $\mu\text{g}$ /mL medium, sodium salicylate (from left to right). The blue smear indicates the presence of capsular polysaccharide. The gel was run at 120 V for 150 minutes.

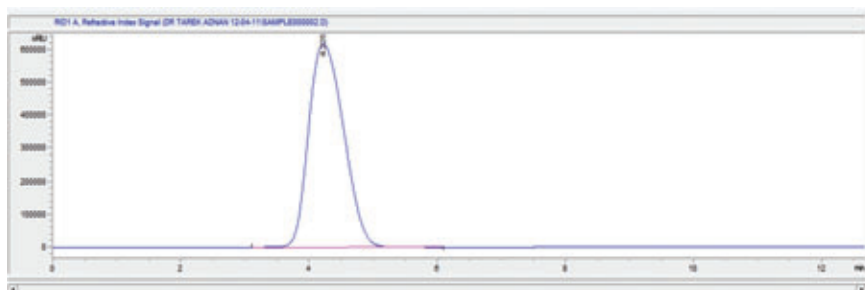
was passed through a 0.45  $\mu\text{m}$  filter, and applied to HPLC separation using C-18 column and pure acetonitrile (BDH, UK) as eluent (this was optimized to give the best distinctive peaks). Two main peaks were detected and their corresponding fractions were further investigated by SDS-PAGE. The major peak of the higher concentration and 96.45% area percentage was CPS-free while the smaller peak representing 3.55% contained the CPS impurities. Therefore, the relatively CPS full-removal while keeping the highest biomass production was observed at salicylate concentration of 40  $\mu\text{g}$ /mL medium at 200 rpm, 38°C and 20 hours of incubation; this fits what was mentioned before by Dominico *et al.*<sup>32</sup>

### Quality control of the extracted glycopeptide

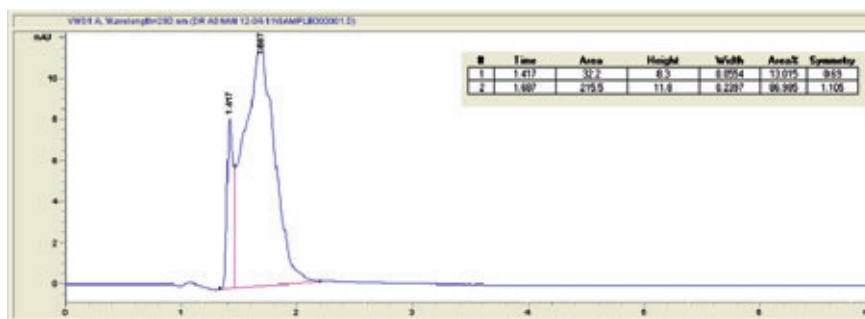
The functional groups of the compound were investigated by FTIR after each step of production (before/after washing, and after lyophilization) in order to evaluate the structural changes in the polysaccharide. They all showed to have major bands at wavelengths of 3450, 2964, 2926, 1645, 1540, 1400, 1246, 1085, and 860  $\text{cm}^{-1}$ . Visual comparison of the FTIR charts did not show any major modification all along the production steps. This indicates that

the production steps are endurable by the chemical structure of the glycopeptide macromolecule. The only noted change was the disappearance of one band previously detected at transmittance 1745  $\text{cm}^{-1}$  in the original LPS corresponding to the ester group in the molecule.<sup>7</sup> This disappearance was noted along all the investigated glycopeptide production steps. It is well-known that only one ester group exists in the LPS molecule linking between fatty acid tails and sugar backbone.<sup>33</sup> Moreover, the toxicity of the LPS reduces remarkably when the ester linked fatty acids are removed even if the amide linked fatty acids remains.<sup>34</sup> This fact confirms that the isobutyric/ammonia mixture used for the extraction of the glycopeptide macromolecule hydrolyses that bond, a property previously defined as ester hydrolysis of weak acids by a salt (like ammonium).<sup>35</sup> The functional group that resulted from such ester hydrolysis was a carboxylic group detected at transmittance of 1645  $\text{cm}^{-1}$ . This hydrolysis step attacked only the ester linkage, but neither the glycosidic bonds of the sugar, nor the amide bond that links the less toxic fatty acids which appeared on the FTIR chart at the transmittance values of 3450, 1246 and 1085  $\text{cm}^{-1}$ .

The most critical and challenging chemical



**Figure 2.** Refractive index chart of the glycopeptide, on high performance liquid chromatography using pure acetonitrile at flow rate of 1  $\text{mL min}^{-1}$ .



**Figure 3.** Ultra-violet 280 nm chart of the glycopeptide, on high performance liquid chromatography using pure acetonitrile at flow rate of 1  $\text{mL min}^{-1}$ . The two main peaks description is provided and shows their relative proportion.



test were to investigate how intact the glycopeptide macromolecule was, and how much contaminated with non-intact matters. To fulfill this task, a known concentration of the glycopeptide was subjected to HPLC analysis, using a dual detector system of RI and UV-280 nm that were able to detect polysaccharides and proteins in the running sample. The RI (Figure 2) showed that the glycopeptide consisted of one pure polysaccharide component at retention time of 4.225 min with no traces of CPS. This may be attributed to that the combination of the LPS to the protein forms big macromolecules, and the minor amount of CPS impurities (3.55%) previously detected may be neglected. The appearance of one single distinct band confirmed that the polysaccharide was not damaged or degraded along the preparation steps.

The collected fraction corresponding to that peak was analyzed for protein content by the auto-analyzer,<sup>28</sup> and for carbohydrate by the phenol sulfuric acid method against a standard curve of galactose, which is found to be the main sugar in the LPS.<sup>24,33</sup> The analysis showed that the percentage proportion of the proteins to the carbohydrate was 48.34:51.67, which means an almost equal amount of the two components in the preparation. Interestingly, the carbohydrate peak was the one containing the proteins ensuring that those two molecules were intact together.

The presence of other protein impurities in the sample may refute such intactness to some extent. However, the UV-280 nm detector confirmed that the proteins were present as two major overlapped sharp bands, both of which showed to contain minor bands (Figure 3). This form proved that there were no free proteins other than those conjugated to the polysaccharide. The splitting of the bands is an indication of the two major OMP, previously identified to have a molecular weights of 17 and 32-kDa,<sup>7</sup> while the minor peaks are attributed to the other minor OMPs. The HPLC did not detect any bands at the 260 nm spectral region, confirming that the preparation was free from detectable nucleic acids impurities.

### Safety and immunogenicity of the glycopeptide conjugate

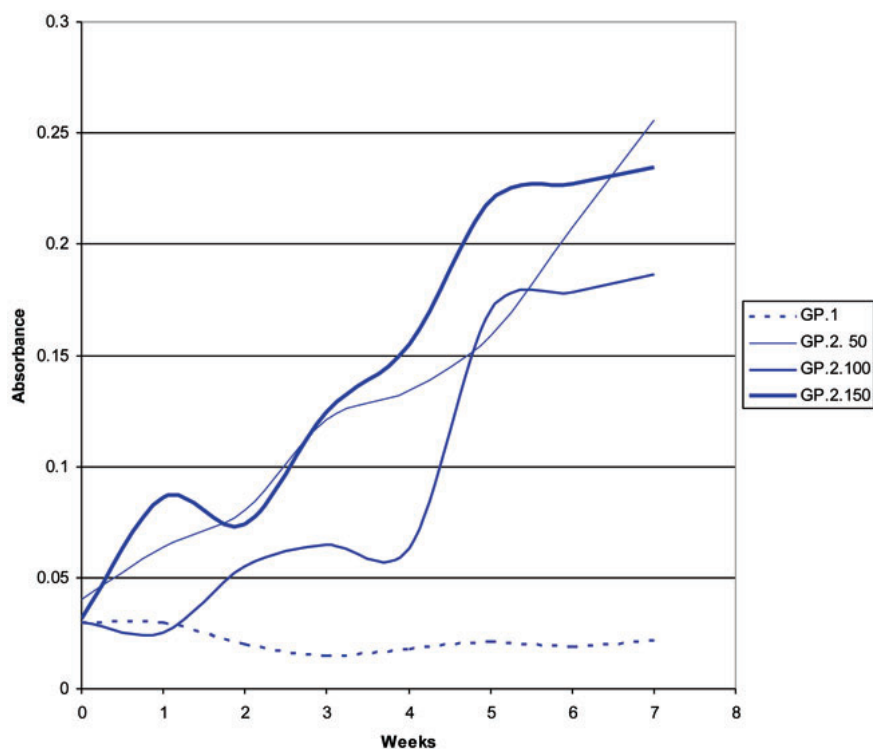
Slight temperature increase of less than 0.7°C was noticed after 4 hours of conjugate injection in all rabbits except those given the lowest dose (50 µg/Kg). Appetite loss was recorded in all the rabbits expressing temperature increase along the first day of injection. All other physiological and behavioral parameters showed to be normal. However, vaccines safety usually depends more on their toxicity and pyrogenicity in experimental animals. No septic toxicity or increase in body temperature more than 0.7°C were recorded in all rabbits which is an additional confirmatory proof that

the LPS in the conjugate was detoxified enough to be administrated safely in different doses.<sup>13</sup> Simultaneously, the conjugate proteins conferred no *in vivo* toxicity to the biological system. Vaccines safety measures also consider the local allergic reaction they may cause at the injection site. Such reaction, if vigorous, may be a remarked unpleasant side effect causing discomfort for using such preparation. Cheerfully, no dermal allergic reaction was detected.

The key immunogenic test that evaluates the potency of a vaccine is its ability to raise the immunity represented by antibody titer for a period of time against that vaccine. Antibodies bind to bacteria and act as direct opsonins or result in complement activation.<sup>13</sup> ELISA was used to detect the antibody titer in the sera of the different groups. The primary optimization experiments confirmed that adopting anti-serum concentration of 1/10; coating wells with 25 µg/mL of antigen; reading at 450 nm; and using horse radish peroxidase as secondary antibody substrate are the most optimum condition that showed the most significant reading variation and sensitivity. Different concentrations of saline and conjugate were administrated to white New Zealand

rabbits. Figure 4 shows the antibody titer development over 7 weeks since the first injection. The saline-injected control group rabbits (GP.1) did not show any antiserum titer rising against the conjugate. The absorbance reading was always below 0.05. The rabbits injected with different concentrations of the conjugate (GP.2) showed an antibody titer increase at the 7<sup>th</sup> week represented by average absorbance readings of 0.256, 0.186, and 0.233 for the 50, 100 and 150 µg/Kg injections; respectively. The relative antibody fold-increase of the groups was 0.733333, 6.395833, 6.222222, and 7.449735 for GP.1, GP.2.50, GP.2.100, and GP.2.150; respectively. In general all groups injected with the conjugate showed a step-wise increase in antibody titer after each injection, which is a natural raise of antibody after each boosting dose.<sup>36</sup>

The capacity of the preparation to induce transferable immune response to offspring via placenta was another target aim of our investigation. Sera of the offspring and orphan rabbits were tested for antibodies against our conjugate at the age of 3, 5 and 7 weeks. The controlling factor for the antibody concentration in the offspring showed to be the number of vaccine doses the mother's received before deliv-



**Figure 4.** Enzyme-linked immuno-sorbent assay antibody titer monitored all over 7 weeks for rabbits groups: GP.1 control group, GP.2 study group with different concentrations of glycopeptide injection (50, 100, and 150 µg/kg). The absorbance of the developing color due to horse-radish peroxidase labeled polyvalent goat-anti-rabbit immunoglobulin substrate (Sigma-Aldrich, USA) was read at 450 nm, data were analyzed by three-ways analysis of variance statistics that revealed a P-value of less than 0.005 for the read absorbance replica.

ery, since only offspring of mothers that received the full 3 doses, regardless of conjugate concentration, manifested a remarkable antibody titer at the age of 7 weeks, that equaled to 92% of its mother's. This indicates that these antibodies transferred from the mother to the offspring via the placenta, and hence as the longer the fetus persists in the presence of mother's antibody; the more likely it gets the antibody. The tested orphans group allowed to suckle from immunized mothers, but were not in contact with their placenta, did not show any antibody increase which ensures the trans-placental route of antibody transfer. These facts confirmed that our prepared vaccine is a maternal vaccine, which confers protecting antibodies in neonates. Obviously the vaccine was totally safe to be given to the pregnant females, as being physiologically immuno-compromised models.

### Protective role of the glycopeptide conjugate

Septicemia, whether primary or secondary consequently to urinary tract infection (UTI) or lungs infections, is correlated with the highest mortality rate due to septic shock.<sup>3-6</sup> Nevertheless, the majority of *Klebsiella* immunotherapy research studies and all conjugate vaccines were directed toward the protection from *Klebsiella*-caused pneumonia. Therefore, the aim of our research was to evaluate the potency of the vaccine for the prevention of UTI, pneumonia and septicemia due to *K. pneumoniae*. Three different rabbit models were designed to show the protective potency of the vaccine, toward septicemia, pneumonia or urethritis due to *K. pneumoniae*. Three different rabbits previously injected by different doses (GP2.50, GP2.100, and GP2.150) of the vaccine were challenged by the standard *K. pneumoniae* peritoneally (sepsis model), intra-tracheally (pneumonia model) and intra-vaginally (urethritis model). Cheerfully, they all overcame the infection, a clear experimental proof that the prepared vaccine generates specific polyclonal antibody titer, which can prevent the manifestation of *K. pneumoniae* infection in lungs, kidneys and septicemia. Although all challenged rabbits expressed an average elevation of body temperature of 1.38°C, this was referred to the normal immune response toward the pathogen components.<sup>37</sup> In the pneumonia model, no physiological or pathological manifestations were recorded, except coughing for the first two days after injections, accompanied by loss of appetite that persisted for 5 days. The two-day coughing are probably due to the bacterial suspension dropped in the lungs and due to local irritation that induced the lungs reflex action to expel it mechanically.<sup>38</sup> The correlated loss of appetite, as previously noted, is related to temperature increase or discomfort after the

intra-tracheal catheter surgery. In the urethritis model, a vaginal discharge was noticed in 2/3 of the tested animals for 3-4 days after challenge; no other physiological or pathological manifestations were noticed. The vaginal swabs culture in all replica, after 6 and 9 days, did not differ from those done before the challenge or for the control group. However, few colonies of *Klebsiella* appeared 3 days after challenge in that model. This observation shows a relative delay in clearance of the infection in the vagina that was shortly restored before the 6<sup>th</sup> day with no manifestation of infection.

### Conclusions

The produced conjugate proved its purity, integrity of its components, stability, safety, and efficacy in eliciting a protective immunetiter toward septicemia, pneumonia and urethritis in experimental models; as well as, being transferable via placenta to the offspring's. The conjugate proved to be adjuvant independent, as the antibodies elicited by the optimum dose were enough to protect the experimental rabbit's models from different modes of infections by *Klebsiella*. This may be due to the presence of the OMP-moiety in the conjugate, that allows their carried polysaccharides to produce protective immunoglobulin titers without the need of an adjuvant, as have been noted previously.<sup>39</sup> The benefit from using propolis, as an immune-booster safe adjuvant was evaluated. The only remarked profit from its use was to reduce the required dose of the conjugate to produce the highest level of antibodies.<sup>7</sup>

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