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Preparation and Characterization of a *Staphylococcus aureus* Capsular Polysaccharide-Protein Conjugate Prepared by a Low Cost Technique: a Proof-of-Concept Study

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Abstract *Staphylococcus aureus* is a worldwide distributed pathogen that produces several diseases in many species and is the major cause of mastitis in dairy cows. *S. aureus* capsular polysaccharide 5 (CP5) has been widely proposed as a vaccine candidate since it is expressed in a high proportion of isolates from intramammary infections and is able to induce opsonophagocytic antibodies. However, to reach immunological properties, polysaccharides need to be coupled to carrier proteins. The aim of this study was to evaluate a conjugation method employing *p*-benzoquinone (PBQ), which was not previously reported for the

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development of vaccine components. Purified *S. aureus* CP5 was coupled to human serum albumin (HSA) with high efficiency, reaching a rate PS/protein of 0.5. Mice groups were immunized at days 0, 14, 28, and 42, with the conjugate (CP5-HSA_{PBQ}), free CP5, or PBS, formulated with incomplete Freund adjuvant, and after 3 months, they were challenged with free CP5 to evaluate the memory response. IgG and IgM isotypes were measured on serum samples all along the experiment, and IgG subclasses were determined to analyze the humoral profile. In contrast to the response obtained with free CP5, CP5-HSA_{PBQ} induced IgG titers of 1/238,900 after three doses and a memory response was observed after the challenge. Results indicate that immunization with CP5-HSA_{PBQ} effectively induce a T-dependent immune response against CP5. Moreover, besides IgG2a was the main subtype obtained, the joint production of specific IgG1, IgG2b, and IgG3 types indicated a balanced humoral response. As *p*-benzoquinone conjugation of CPs to proteins is far less expensive and straightforward than other methods commonly used in vaccine preparations, the robust humoral response obtained using this method points out that this can be an interesting alternative to prepare *S. aureus* CP5 conjugate vaccines.

Keywords Capsular polysaccharides · *Staphylococcus aureus* · Conjugate · Antibody · Humoral response

Introduction

Staphylococcus aureus is the most frequently isolated pathogen from bovine intramammary infections [1, 2] worldwide. Control of *S. aureus* intramammary infection (IMI) is based on milking-time hygiene, antibiotic therapy, and culling of chronically infected cows. However, despite the application of these practices, the chronic nature of most *S. aureus* IMI and the limited cure rate following antibiotic therapy make this disease difficult to control [2]. Hence, other approaches to control *S. aureus* IMI, such as vaccination, have been proposed to complement classical measures. As *S. aureus* infections have been shown to be multifactorial, a general consensus is that an efficient vaccine against this pathogen has to include distinct bacterial molecules selected according to a rational design [3]. Capsular polysaccharides (CPs) have been widely proposed as vaccine candidates since they have been shown to play a role in staphylococcal virulence and to generate opsonophagocytic antibodies [4–6]. Among the 11 *S. aureus* CP serotypes identified, the capsular polysaccharide 5 (CP5) is the most prevalent type isolated from bovine IMI in many countries [7–9]. Therefore, it is considered an important target for staphylococcal vaccines development [10–12].

The comprehension of the mechanism involved to mount a B cell response gave rise to the development of vaccines using polysaccharide-protein conjugates to achieve high effective anti-polysaccharide antibodies and immunological memory. Indeed, many meningococcal serogroup C (MenC) glycoconjugates are commercially available for human use, such as Menjugate®, Meningitec®, and NeisVac-C® (monovalent MenC conjugate vaccines); Menactra®, Menveo® and Nimenrix® (quadrivalent conjugate vaccines containing serogroups A, C, Y, and W135), and Menitorix (combination of two conjugates based on *Haemophilus influenzae* b and MenC polysaccharides) [13]. Regarding *S. aureus*, Fattom et al. have developed a conjugated vaccine for human use that reached the phase III clinical trials [14]. Extensive data employing this strategy have shown efficacy of the conjugates obtained to generate humoral responses and in some cases to protect against infections caused by the target pathogen in different animal models [15–18].

Studies aimed to obtain immunogenic conjugates have focused on different conjugation methods [19–23]. All these techniques are complex, very laborious, and involve the use of expensive reagents. Hence, simpler, less expensive, and easy to scale alternative methods are required for conjugate vaccines development [24]. In the present work, we prepared a *S. aureus* CP5-human bovine serum albumin conjugate (CP5-HSA_{PBQ}) employing *p*-benzoquinone (PBQ) as coupling reagent and assessed its potential avail for immunogenic preparations. This method has been widely used to obtain protein-protein or protein-polysaccharide conjugate with different applications in diagnosis and different immunoassays but, to the best of our knowledge, was not applied for immunogenic formulations. We chose PBQ method since it has been described as very easy to perform, reproducible and economical [25], which makes it profitable for commercial vaccine production.

Materials and Methods

Bacterial Strains and Culture Conditions

Staphylococcus aureus strain Reynolds is a human CP5 isolate. The strain was kept as frozen stock and cultured overnight in tryptic soy agar (TSA) (Britania) at 37 °C. An isolated colony was inoculated in tryptic soy broth (TSB) and cultured for 18 h at 37 °C. The growth medium was then plated on T-bottles containing TSA supplemented with 2.5 % NaCl. After overnight incubation, bacterial growth was suspended in phosphate buffer saline (PBS) and this suspension was used for polysaccharide purification.

S. aureus Capsular Polysaccharide Type 5 Purification

Ethanol precipitation method previously described by Fattom et al. [21] was carried out with some modifications. Briefly, the bacterial suspension was magnetically stirred for 90 min and then centrifuged (Hermle Z32HK, LaborTechnik, Germany) at 1,500 g for 30 min at 4 °C. The supernatant was treated overnight with 80 % ethanol (*v*/*v*) at −20 °C, and the precipitated product was separated by centrifugation at 1,500 g for 30 min at 4 °C. The pellet was washed twice with 80 % ethanol and finally resuspended in PBS. This solution was digested with proteinase K following the manufacturer's instructions (Biolabs), and the teichoic acids were then eliminated by peryodate treatment (Sigma-Aldrich) for 45 min in darkness. The fraction containing the purified polysaccharide was obtained by centrifugation followed by a 48 h dialysis against 0.05 M sodium acetate (0.1 NaCl, pH 6). Then an ion-exchange purification in a DEAE-Sephacel column (GE Healthcare, Life Sciences) was performed as previously described [21]. The fractions containing polysaccharide, detected by SDS-PAGE, silver staining, and Western blot assays (see below), were pooled and conserved lyophilized at −20 °C until used. Neither proteins nor DNA impurities were detected by Qubit™ Quantitation Platform (Molecular Probes, Invitrogen).

CP5 Derivatization

Ten milligrams of the lyophilized CP5 were dissolved in NaOH 0.1 M and shaken 18 h at 37 °C. The solution was neutralized with HCl and dialyzed against 20 volumes of PBS.

Conjugation Reaction with *p*-Benzoquinone

The CP5 was bound to human serum albumin (HSA) using a conjugation method employing PBQ, previously described [26]. Briefly, 100 μl of a 30 mg/ml of PBQ ethanol solution was mixed with 400 μl of 10 mg/ml of HSA in phosphate buffer (pH 6) and incubated 1 h at room temperature in the dark. A 48 h dialysis against PBS was carried out with a buffer exchange after 24 h. The solution pH was raised to 8 with NaOH 10 M and 2 mg of de-acetylated CP5 was added. Then 1 M $NaHCO_3$ (pH 8) followed by 1 M lysine (pH 7) were added in a final concentration of 0.1 M. Finally, ultrafiltration with 100 kDa cut off membranes (Vivaspin 6, 100,000 MWCO, Life Sciences, GE Healthcare) was carried out to separate the conjugated product from low weight molecules not conjugated. The conjugate was defined as CP5-HSA_{PBQ}. The protein content in the conjugate was determined by bicinchoninic acid technique (BCA Protein kit assay, Pierce). For polysaccharide determination, the phenol-sulfuric acid method was employed [27], using a serial dilution of a CP5 solution as standard curve.

SDS-PAGE

Samples of the CP5-HSA_{PBQ}, the non-conjugated CP5 and HSA, were run in triplicate in 12 % SDS-PAGE. One of the copies was stained with Coomassie brilliant blue [28] followed by silver staining. This last technique was carried out according to the protocol described by Chevallet et al [29]. Briefly, the Coomassie-stained gel was swamped in fixation solution for 1 h. After two 10 min washes in ethanol 20 % and other two in water, gel was sensitized by submerging it 1 min in a sodium thiosulfate solution (0.8 mM). Gel was washed twice in distilled water and then incubated 45 min in silver nitrate solution (12 mM). A fast 10 s wash with distilled water was carried out and immediately after the gel was swamped in developing solution (sodium carbonate 0.28 M, formalin 0.025 % (*v*/*v*), sodium thiosulfate 0.05 mM) until polysaccharide appears. At that moment, the color reaction was stopped with a stopper solution (Tris 0.33 M, acetic acid 2 % (*v*/*v*)) and then an exhaustively wash with distilled water was done.

Western Blot Assays

The other replicates from the SDS-PAGE run were transferred to nitrocellulose membrane (Hybond ECL, Amersham) using the Mini Trans-Blot® Electrophoretic transfer cell system (Bio-Rad). Western blot analysis was carried out according to standard methodology using specific serums to detect the conjugate components. To detect specific CP5 in nitrocellulose membranes, a serum of a rabbit with antibodies against capsular polysaccharide was prepared as described in previous works where the phenotypic detection of capsular polysaccharide in *S. aureus* strains was done [9, 18, 30, 31]. Briefly, rabbits were immunized with an inactivated CP5 producer *S. aureus* bacteria. To eliminate all those antibodies raised against any other molecules different from CP5, serum was adsorbed with an inactivated *S. aureus* strain which does not produce capsular polysaccharides. To detect HSA, a mouse serum was obtained by immunizing mice with two doses (biweekly) containing 5 μg of HSA and formulated with ISCOMATRIX™ (Isconova). The specificity of these serum samples was confirmed by enzyme-linked immunosorbent assay (ELISA) using HSA as coating antigen (data not shown). The assay was developed with anti-rabbit IgG/HRP or anti-mouse IgG/HRP, correspondingly, and using 3,3′-diaminobenzidine (Sigma-Aldrich) as substrate.

UV-Visible Spectrometry

UV-Vis spectra were obtained using the PerkinElmer Lambda 20 UV/Vis spectrometer. The 260–700 nm UV-Vis spectrums were obtained for the following solutions: free CP5, free HSA, HSA coupled to PBQ (HSA-PBQ, which corresponds to the first step product of the conjugation reaction) and conjugate (CP5-HSA_{PBQ}). Each solution was prepared and measured in PBS buffer, pH 7, at proportional concentrations to the ones used for conjugation reaction. The spectrums features were analyzed in order to determine changes caused during the conjugate formation. Concentration of PBQ present in the conjugate was also measure by a UV-Vis experiment. As detailed above, once HSA-PBQ product is obtained, a dialysis step is performed. The quantity of PBQ, corresponding to the unbounded reagent, was determined in the eluted fraction. A curve of twofold dilutions of PBQ (in PBS buffer, pH 7) was prepared, and the UV-Vis spectrums were capture at 289 nm. Absorbance versus PBQ concentration was plotted, and the free PBQ quantities in the eluted fraction were determined by interpolation. PBQ attached to the protein was estimated by subtracting that value to the initial quantity used for the conjugation reaction. This procedure was done for five conjugation batches in order to determine an average percentage of PBQ which binds protein.

Animals and Immunization Schedule

Female 6 weeks old CF1 mice were used for the immunogenicity assays. All procedures requiring animals were approved by ethical committee from Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral. Groups of six mice each received four intraperitoneal doses at days 0, 14, 28, and 42, containing 7 μg of conjugated CP5 (CP5-ASH_{PBQ} group), 7 μg of free CP5 (CP5 group), or PBS (control group). In all cases, the immunogens were prepared with Freund's incomplete adjuvant. Seven days after each dose, blood samples from the mice tail vein were taken, allowed clotting and sera collected via centrifugation. To assess the memory response, all groups were challenged with 7 μg of free CP5 at day 132, and serum samples were taken at days 132, 134, 136, and 139.

Antibody Responses

Specific-IgG_{total} and IgM kinetics along the experimental days were determined by ELISA. Briefly, 96-well plates (Microlon 600, high binding, Greiner Bio One) were coated with 5 μg of native CP5 (neither conjugated nor derivatized) as previously described for polysaccharide antigens [32]. Then, serum samples, belonging to different time points (days 7, 14, 21, 35, 49, 132, 134, 136, and 139), were added by duplicate in 1/200 dilution. A dilution of 1/5000 anti-mouse IgG/HRP (Jackson Immunoresearch) or 1/2500 anti-mouse IgM/HRP (Sigma-Aldrich) were added wherever applicable. The medium optical density obtained at every time point for each antibody isotype was used to build the kinetic curve for IgG and IgM. The IgG titers were measured in serum samples taken 7 days after the third and fourth doses. The assay was performed in the same conditions described above, but using half diluted serum samples from 1/400 to 1/409,600. The titer was defined as the last dilution at which the O.D. was higher than the 1/200 preimmune serum dilution. ELISA to characterize the IgG1, IgG2a, IgG2b, and IgG3 subclasses after the third dose was carried out using the Mouse Typer® Sub-isotyping Kit (Bio-Rad), and levels of the IgGs subclasses were expressed as O.Ds

Statistics

To analyze the kinetics of specific IgG and IgM antibodies, areas under the curve were determined between day 0 and 49. Then differences between areas were analyzed by ANOVA-Kruskal-Wallis followed by Dunn's post test for multiple comparisons. Statistical significance of IgG increment after each vaccine dose was analyzed by ANOVA-Friedman test and Dunn's post test. Comparisons between IgG subclass levels were also analyzed using ANOVA-Kruskal-Wallis followed by Dunn. The software MedCalc.12.7.5 for Windows was used in all the cases.

All graphics were performed using GraphPad Prism 5.0 and figures were edited with Power Point, Microsoft Office 2007.

Results

Conjugate Characterization

The coupling reaction consisted in two successive addition oxidation reactions: first, the carrier protein is treated with PBQ and thereby converted in a reactive intermediate and, in the second step, polysaccharide is coupled to the activated carrier. The reaction product is then a 2,5-substituted hydroquinone (Fig. 1a) [25, 26]. Although some quinone derivatives are formed during conjugation reaction, they were all referred to as PBQ to simplify explanation of the results. *S. aureus* CPs vary in the degree of O-acetylation among different strains, so we performed a de-acetylation to ensure availability of OH groups and improve the conjugation reaction yield (Fig. 1b). In order to confirm the successful CP-protein conjugation, a SDS-PAGE followed by a Western blot assay was performed. Staining with Coomassie of the SDS-PAGE is shown in Fig. 1. Only products corresponding to the HSA and the CP5-HSA_{PBQ} lanes could be seen, since free polysaccharide requires a further silver staining to show up

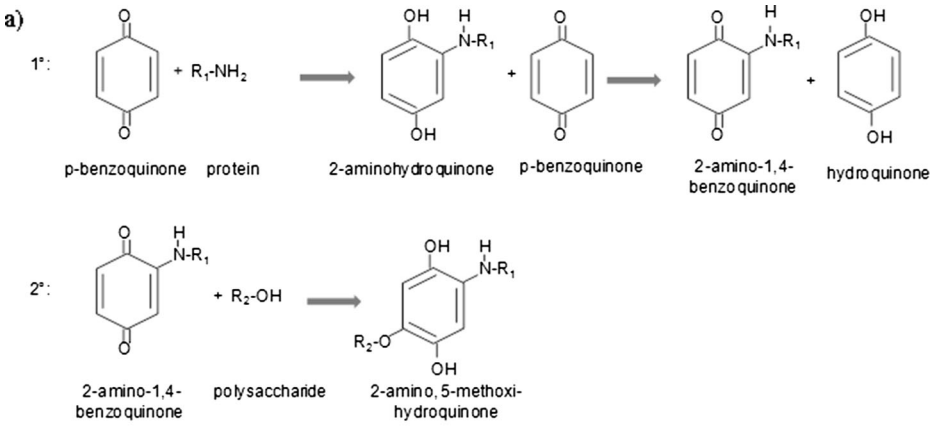

Fig. 1 **a** *p*-Benzoquinone conjugation reaction scheme, adapted from mechanism proposed by Brandt et al. and Tijssen [25, 26]. **b** Chemical structure of capsular polysaccharide type 5 from *Staphylococcus aureus*

(Fig. 2a). In lane 4, the expected banding of the CP was obtained corresponding to a heterogeneous molecular weight range (~30–1000 kDa) [33]. Conjugate sample showed the same electrophoretic pattern than free CP5, but with higher molecular weights. The presence of both CP5 and HSA molecules in the conjugate sample was evidenced in the Western blot assay (Fig. 2b). CP5-HSA_{PBQ} lane gave positive reaction with both anti-CP5 and anti-HSA serum, all along the run pattern of the sample.

The results from the UV-Vis experiments are shown in Fig. 3. Different spectrums were obtained for each solution. Free HSA and PBQ gave peaks of absorbance at 280 and 289 nm, respectively. No peak could be detected for CP5 through the wavelengths used. In HSA-PBQ and CP5-HSA_{PBQ} spectrums, a new strong absorption is evident at 350 nm which suggest the formation of a new chromophore compound.

For further preparation of the immunogens for the immunization schedule, protein, polysaccharide, and PBQ contents in the CP5-HSA_{PBQ} sample were determined. The calculated weight relative proportion HSA:PBQ:CP5 was 2:0.9:1. PBQ was quantified in five batches of conjugation reaction, and a 60 % average of the initial PBQ was determined to couple to the HSA.

Kinetics of Antibody Production

To compare the humoral response raised by the conjugate with the one raised by non-conjugated CP, kinetics of IgM and IgG_{total} responses were assessed all along the experimental schedule (Fig. 4). Immunization with both immunogens induced IgM but only free CP5 gave significant levels compared with control (ANOVA-Kruskal-Wallis, $p=0.0037$; Dunn's $p<0.05$). Nonetheless, this group failed to achieve an IgG response since IgG_{total} levels were as low as in the control group. Inoculation of CP5-HSA_{PBQ} induced significantly high levels of IgG_{total} antibodies compared to control and CP5 group, which lasted at least for 3 months (ANOVA-Kruskal-Wallis $p=0.0030$, Dunn's $p<0.05$).

Despite two doses of conjugate were able to attain high absorbance measurement at 1/200 serum dilution, optical densities obtained with the third dose were significantly higher (ANOVA-Friedman, $p=0.0001$; Dunn's $p<0.05$). Moreover, no differences were found between the third and fourth dose, indicating that a plateau of antibody levels was reached with only three doses. At that time point, mean titer was 1/238,900.

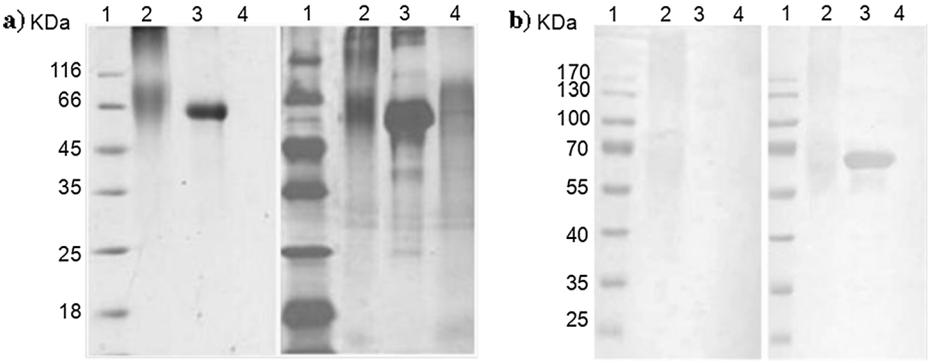

Fig. 2 Characterization of the conjugate prepared with *S. aureus* type 5 capsular polysaccharide, CP5-HSA_{PBQ}. **a** SDS-PAGE in 15 % polyacrylamide gels. *Left*: Coomassie staining, *right*: silver staining. **b** Western blot assay. Membranes were revealed with *left*: anti-CP5 rabbit serum, *right*: an anti-HSA mouse serum. Lanes *1*: molecular weight markers, lanes *2*: CP5-HSA_{PBQ}, lanes *3*: HSA, lanes *4*: CP5

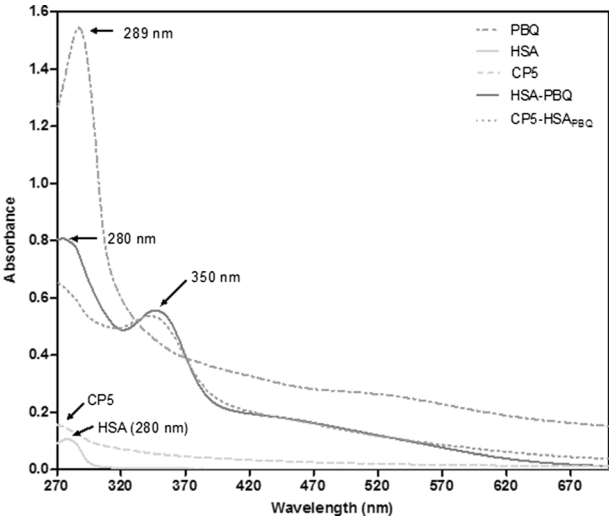

Fig 3 UV-Vis spectrums. Initial compounds, intermediate, and final products from the conjugation reaction were monitored by UV-Vis spectrometry. All the solutions were prepared in PBS buffer, pH 7, at same proportions as those used for conjugate preparation

In order to determine if immunological memory was established, at day 132, we performed a challenge with purified free CP5 and compared IgM and IgG_{total} specific responses in all groups during the first week after challenge (Fig. 2). Control and CP5 groups responded only with IgM isotype suggesting lack of immune memory. Conversely, mice immunized with CP5-HSA_{PBQ} yielded rapid stimulation of specific IgG_{total}, indicating a memory T-dependent response development. Furthermore, comparison of IgM and IgG_{total} curves showed that IgM declination at days 21 and 134 was accompanied by a raise of IgG_{total}. In addition, the IgG_{total} achieved the maximal levels (at day 49) as IgM reached the minimal.

IgG Subclasses

To further analyze the immune response profile, IgG subclasses were determined in serum samples from CP5-HSA_{PBQ} group after the third dose, at day 35 (Fig. 5). Figure 3 shows equivalent IgG1, IgG2b, and IgG3 levels, while IgG2a was the prevalent subtype (ANOVA-Kruskal-Wallis $p=0.0041$; Dunn's $p<0.05$).

Discussion

CP5 from *S. aureus* is an important component to be included in the design of a vaccine to protect dairy cattle from this microorganism; however, it has to be conjugated to generate effective long lasting humoral response [17, 18]. PBQ has been previously used to conjugate protein to polysaccharides but, to the best of our knowledge, only aimed to obtain reagents for in vitro use. In the present study, the technique was assessed to achieve a T cell-dependent antigen based in CP5 polysaccharide from *S. aureus*.

The conjugation process was optimized to bind purified CP5 to human albumin, a protein previously used for this purpose [17]. To bear out the conjugation success, the rationale of our work was based on two features. First, Coomassie does not stain sugars but CP5 development

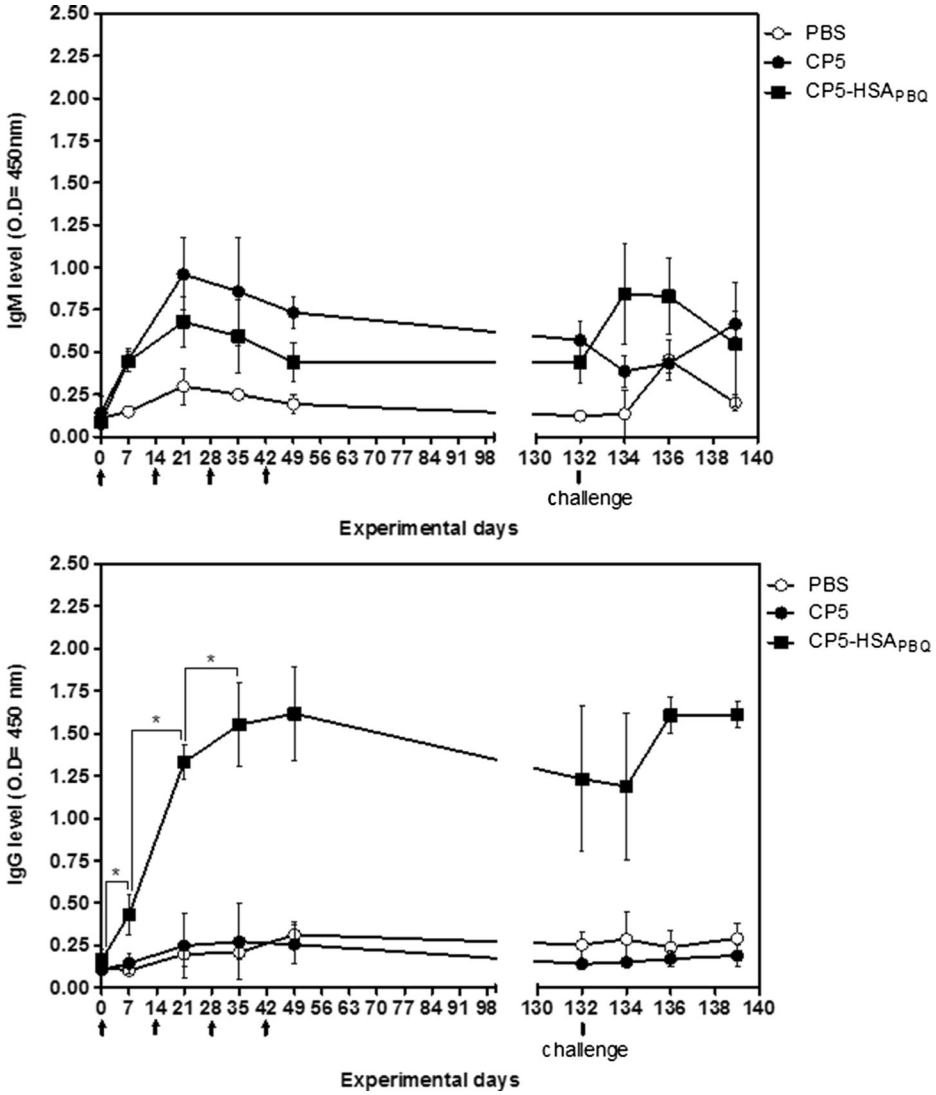

Fig. 4 Kinetics of antibody production. Mice were immunized with four biweekly doses of CP5-HSA$_{PBQ}$, free CP5, or PBS, and then challenged with free CP5 at day 132. IgM (**a**) and IgG$_{total}$ (**b**) were determined by ELISA all along experimental days. Mean±SD (*bars*) are shown. *Arrows* on X-axis represent the four doses. At day 132, the challenge is indicated. *Asterisk* represents differences between doses (ANOVA-Friedman, $F=74.0278$, $p<0.00001$; Dunn's post test, $p<0.05$). The graph is representative of three repeated experiments

can be achieved by a further silver staining. This strategy of using a cationic dye (like Alcian blue or Coomassie blue) followed by silver nitrate enhances sensitivity of staining and is currently used for stain acid molecules as polysaccharides and highly negatively charged proteins, poorly detected otherwise [34–36]. Second, CP5 fails to bind to nitrocellulose membrane. We confirmed the last characteristic by a dot blot assay, in which CP5-HSA$_{PBQ}$ was recognized by specific antibodies, but not free CP5 (data not shown). The analysis allowed us to discard transferences troubleshoots. On one hand, the SDS-PAGE analysis

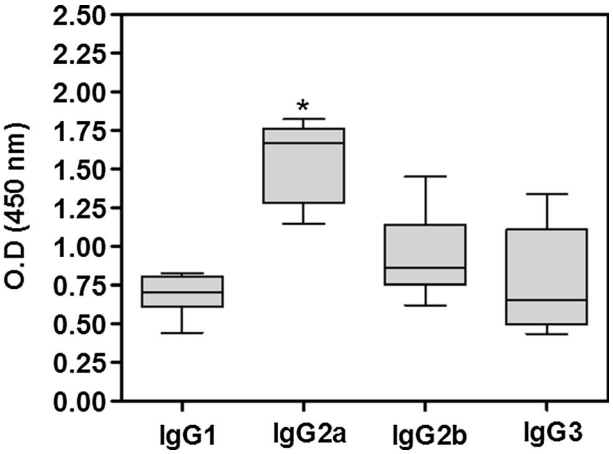

Fig. 5 IgG subclasses. IgG1, IgG2a, IgG2b, and IgG3 subclasses were determined on serum samples taken after the third dose (day 35), in mice immunized with CP5-HSA_{PBQ}. Whiskers show the minimum and maximum of the data. *Asterisk* represents differences between subclass levels (ANOVA-Kruskal-Wallis, $p=0.0041$; Dunn's post test $p<0.05$)

showed that the CP5-HSA_{PBQ} obtained gave a diffuse banding comparable with CP5, but shifted towards higher molecular weights and observable with Coomassie staining. This suggests a link-up between protein and polysaccharide. At the same time, Western blot assay also confirmed the presence of CP5 and HSA along the conjugate pattern. It is noteworthy that the low reactivity obtained in lane 2 was due to the serum used, which had been prepared by immunizing rabbits with inactivated bacteria. Because of that, the immune response was raised against native CP5 being a low and short lasting humoral response.

The UV-visible spectrometry experiment showed the characteristic absorption at 280 nm for HSA, while any peak was detected for CP5 along the wavelengths assayed. PBQ solution gave a peak at 289 nm and broad but weaker absorption between 360 and 600 nm. This spectrum feature corresponds to hydroquinone and hydroxyquinone which are the main products formed from the electrochemical reduction of PBQ in aqueous solution [37]. Absorption at 290 nm has been attributed to the hydroquinones compounds [38, 39] while hydroxylated products give the wide absorbance [37, 39]. Spectrum recorded for HAS-PBQ resulted different from those obtained for the free protein and reagent solutions. The peak at 280 nm corresponds to protein absorbance itself, but the maxima absorbance at 350 nm suggests the formation of a new chromophore. The same peak has also been obtained in recent works where authors studied interactions between other quinones and proteins [40, 41] and it was specifically attributed to the reaction of PBQ with lysine residues of proteins [41]. Then, the absorption changes produced in HAS-PBQ sample indicate that the protein is covalently linked to PBQ. Finally, CP5-HSA_{PBQ} gave the same two peaks than HSA-PBQ but there was a decrease in the absorbance at 280 nm. The fact that addition of polysaccharide interferes with the protein absorption denotes that it is attached to HAS-PBQ.

The combined results from SDS-PAGE, Western blot, and UV-VIS experiments indicate that HSA, CP5, and PBQ were coupled together establishing the conjugate molecules and corroborating conjugation success.

PBQ method enabled conjugation of all the proteins from the reaction mixture to CP5 in only two steps, resulting in a PS:protein rate of 0.5 (*w*/*w*). This proportion is the same obtained

by other authors [25]. It is noteworthy that this rate is encompassed within the range 0. 5 to 1 currently used in approved vaccines [13].

It is important to remark that non-toxicity effects were observed in the inoculated mice with the dose of conjugate used here. Indeed, after the chemical reaction, the PBQ is oxidized to give a hydroquinone in the final conjugated product. The Food and Drug Administration (FDA) has classified hydroquinone currently as a safe product, since experiments with rats and mice show that toxic effects are visible at high concentrations ranged between 50 and 300 mg/Kg/day [42]. In the present work, mice were immunized with 7 μg of CP5, then, taking into account the ratio PBQ:CP=0.9:1, the dose of PBQ (or substituted hydroquinone derivative) was 6.3 μg, which was present considering an average mouse weight of 20 g the quantities of inoculated reagent results 0.32 mg/kg/dose. In relation to the unsuitability of using PBQ during the conjugation process due to the risk of having PBQ traces in the formulation, it is noteworthy that despite exposure to high levels of *p*-benzoquinone results in irritation of the eyes, visual disturbances, ulceration, and dermatitis [43, 44], no information is available about reference concentrations or doses [45]. Moreover, the Environmental Protection Agency [46] and the International Agency for Research on Cancer (IARC) have not classified *p*-benzoquinone for carcinogenicity [45, 47].

Through conjugating the polysaccharide to HSA by PBQ method, we achieved a specific IgG response to CP5. The IgG increment obtained after each booster in mice immunized with CP5-HSA_{PBQ} indicated a secondary T-dependent memory response. As reported in a previous work [48], non-IgG response against non-conjugate free *S. aureus* CP was obtained. To compare CP5-HSA_{PBQ} versus CP5 response, the kinetics of specific IgM antibodies were also analyzed. This isotype is developed in the primary response to T-independent or T-dependent antigens but, in the latter, a switch to IgG is obtained. As expected, both groups immunized with CP or conjugated CP responded with specific IgM but only the conjugated group switched to IgG isotype. This response was quickly mounted since maximum absorbance measurements were obtained after three doses. At that point, the mean titer obtained was 1/238,900. This result is important considering the practical significance of achieving an optimal response with a minimum dosage application.

One important point to assess was the immunological memory reached by immunizing with the conjugate. As CP5 is not conjugated in the microorganism, a challenge with free CP5 was performed to analyze the immune response against the native polysaccharide as it is released in a natural infection. Plain CP5 was used instead of bacteria to assure that the amount of polysaccharide used for challenge was as previously described for other CP-conjugated immunogens developed [13]. Mice previously inoculated with CP5-HSA_{PBQ} were able to quickly respond to the antigen before a week, indicating that immunity evoked with the vaccination schedule induced an immunological memory response, which suggests that during a natural *S. aureus* infection, vaccinated individuals might be able to react against native CP5.

Notably, in PBQ method, the polysaccharide requires to be de-acetylated to release OH groups needed for coupling reaction to PBQ. In spite of this chemical modification, immunological properties were not altered. In fact, although all immunoassays were performed with native CP5, antibodies raised against the conjugate were able to recognize this antigen. Furthermore, when mice were challenged with native CP5, an increased response was obtained in this group. This response indicates that native CP5 can activate B memory cells, previously developed against de-acetylated CP5. Some authors have also reported that de-acetylation did not affect the immunological properties of *S. aureus* polysaccharides, since antibodies raised to the backbone of the molecules are highly opsonic and can protect even against strains with different degree of acetylation [23, 49].

An additional analysis was performed to characterize the profile of the immunological response obtained with the conjugate. After the third immunization, specific IgG subtypes were determined. High and equivalent levels were obtained for IgG1, IgG2b, and IgG3, while the highest levels were obtained for IgG2a. Interestingly, this subtype is more protective than the others as it can bind complement, enhance phagocytosis, and promote Antibody Dependent Cell Cytotoxicity (ADCC) [50]. Indeed, this antibody class was described to be more protective against encapsulated bacteria [51] and, particularly for *S. aureus*, it has been determined that mice IgG2a and IgG2b are needed to opsonize the microorganism [52]. Furthermore, the ratio IgG2a/IgG1>1 obtained is consistent with a Th1 response [53]. This profile of cellular response is highly protective in infections with microorganism like *S. aureus* that are capable of invading cells [12]. Regarding IgG3, this subtype, which is normally the less expressed, has been shown to be protective against bacteria expressing CPs [50]. Although IgG3 anti-polysaccharide antibodies have been associated with a T-independent response [54], it is noteworthy that we obtained this subtype in high levels with the conjugate, but not with the non-conjugated CP5.

We can conclude that conjugation by PBQ method is highly efficient to prepare low cost *S. aureus* CP5-protein conjugates. The technique is easy to perform; conjugates obtained are very stable, high amounts of fixed protein are achieved and can be carried out over a broad pH range [25]. These characteristics make the method versatile, easy to scaling up, and profitable for commercial vaccine production. More importantly, the obtained immunogen enabled achievement of high IgG responses in few doses, reaching higher antibody titers than those previously described. In addition, the obtained IgG subtype profile denotes a potentially protective response against CP5-expressing *S. aureus*. This immunogen is therefore a promising candidate to be incorporated in multicomponent vaccines against *S. aureus* IMI in dairy cattle. Further studies are in progress to evaluate the immune response and protective effect in dairy cattle of prototype vaccines including CP conjugated by this methodology.

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