EVALUATION OF THE IMMUNOGENICITY OF REVERSE-VACCINOLOGY DERIVED *STAPHYLOCOCCUS AUREUS* PROTEINS ON EXPERIMENTAL ENDOCARDITIS MODEL

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ABSTRACT

Staphylococcus aureus has an increasing prevalence in infective endocarditis (IE), with high morbidity and mortality rates. Therefore, the interest in developing strategies for generating protective immunity against *S. aureus* has increased. In this study, the immunogenicity and protective capacity of four *S. aureus* surface antigens expressed as recombinant proteins (rEbpS, rCna, rFnbA, rSraP), containing presumptive immunogenic epitopes predicted by reverse-vaccinology, were evaluated. Immunogenicity assessment on mice revealed a strong antibody response against all proteins.

An experimental infective endocarditis rabbit model was used to evaluate the protection capacity of these antigens. Three groups of rabbits were included in the study: one primed and boosted with a mix of recombinant proteins, the second one primed with a mix of recombinant proteins and boosted with heat-inactivated *S. aureus*, and a control group. Survival rate after *S. aureus* infection proved to be 100% for the rabbits boosted with heat-inactivated bacteria, in contrast with the lack of protection identified for rabbits boosted with recombinant proteins. Also, the bacterial load of the endocarditis-specific vegetations was lower for the rabbits boosted with heat-inactivated bacteria, than in the other two groups.

This approach confirms the immunogenicity of reverse-vaccinology predicted epitopes and may represent a strategy to be considered in developing an efficacious anti-staphylococcal vaccine.

Keywords: *Staphylococcus aureus*, endocarditis, vaccine, reverse-vaccinology, immunogenicity

REZUMAT

Staphylococcus aureus are o prevalenţă crescută în endocardita infecţioasă, cu rate mari de morbiditate şi mortalitate. Astfel, a crescut interesul în dezvoltarea unor strategii de imunizare faţă de *S. aureus*. În acest studiu au fost evaluate imunogenitatea şi capacitatea protectoare a patru antigene de suprafaţă specifice pentru *S. aureus*, exprimate ca proteine recombinante (rEbpS, rCna, rFnbA, rSraP), pentru care s-a realizat predicţia potenţialilor epitopi imunogeni prin vaccinologie inversă.

Testarea imunogenităţii pe şoareci a indicat un răspuns semnificativ, demonstrat prin sinteza de anticorpi cu specificitate faţă de cele patru proteine testate. Pentru evaluarea capacităţii protectoare a acestor antigene, a fost utilizat un model experimental de endocardită infecţioasă la iepuri. În studiu au fost incluse trei grupuri de iepuri: un grup imunizat iniţial şi la rapel cu proteine recombinante, al doilea care a fost imunizat la rapel cu celule de *S. aureus* inactivate termic şi un grup control. Rata de supravieţuire post-infecţie pentru iepurii inoculaţi cu celule inactivate termic a fost de 100%, contrastând cu lipsa de protecţie observată pentru iepurii inoculaţi doar cu proteine recombinante. De asemenea, încărcătura bacteriană a vegetaţiilor specifice endocarditei a fost mai mică pentru iepurii inoculaţi cu celule bacteriene inactivate termic faţă de celelalte două grupuri. Aceasta abordare confirmă imunogenitatea epitopilor prezişi prin vaccinologie inversă şi poate constitui o strategie viabilă pentru dezvoltarea unui vaccin anti-stafilococic eficient.

Cuvinte-cheie: *Staphylococcus aureus*, endocardită, vaccin, vaccinologie inversă, imunogenitate

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INTRODUCTION

Infective endocarditis (IE) is a life-threatening infection of the heart endothelium. *Staphylococcus aureus* has an increasing prevalence in IE, with high morbidity and mortality rates [1]. Inducing protective immunity against *S. aureus* has been a goal since the discovery of this species, but all the attempts to develop an efficacious vaccine have failed [2]. The focus on vaccines providing humoral immunity, instead of vaccines stimulating both humoral and cellular immunity, is considered a potential cause for these failures [3]. However, the development of vaccines inducing both immunity types requires large experimental efforts.

Reverse-vaccinology is a new concept for vaccine development that exploits genomic information to find potential vaccine candidates with the help of adequate bioinformatics instruments [4]. One success example is a registered vaccine for *Neisseria meningitidis* infections, which was the first developed by using this concept [5].

The same approach was used to identify potential epitope candidates for a staphylococcal vaccine which can stimulate both B and T lymphocytes [6], leading to the identification of four *S. aureus* surface associated proteins, containing presumptive immunogenic epitopes. The four proteins were: collagen adhesin (Cna), fibronectin binding protein A (FnbPA), serine-rich adhesin for platelets (SraP) and elastin binding protein (EbpS). These adhesins are broadly distributed among the human staphylococcal strains within ranges of 32 to 93.75% for Cna, 57.7 to 87.5% for FnbA, 43.9 to 93.75% for EbpS and 85% reported for SraP [7-12]. Involvement of some of them in IE, i.e., FnbA, SraP or Cna, was previously documented [12-17]. Also, previous studies have shown that vaccines consisting of several components may induce better protective immunity against infective *S. aureus* [18].

The aim of this study was to evaluate the presumptive immunogenicity of the four surface antigens, expressed as recombinant proteins, and to assess the protective capacity, using an experimental infective endocarditis model on rabbits, as a pilot study.

MATERIAL AND METHODS

Bacterial strain

Staphylococcus aureus MW2 (GenBank: NC_003923.1), a highly pathogenic strain with a great potential to produce both sepsis and vegetations in an IE experiment [19], was used in this study as genetic material for the cloning experiment and also for the infection of vaccinated rabbits.

Cloning, expression and purification of recombinant proteins

The surface-associated proteins cloned in this study were: elastin binding protein (EbpS), collagen adhesin (Cna), fibronectin binding protein A (FnbPA) and serine-rich adhesin for platelets (SraP), which were previously found to bear antigenic epitopes [6].

The amplification of targeted gene fragments from the genetic material of *S. aureus* MW2, consisted in an initial amplification of the entire sequence of the genes (PCR 1), followed by a second nested PCR (PCR 2), targeting internal sequences to introduce restriction sites for cloning: *Nde*I for forward primers and *Xho*I/*Bam*HI for reverse primers. These sequences bear the antigenic epitopes. The oligonucleotides (Table 1) used for both PCRs were designed using PerlPrimer software [20].

PCR FastStart High Fidelity system, dNTPack (Roche Diagnostics GmbH Mannheim, Germany) enzyme was used, as recommended by manufacturer.

Two μl PCR product from the first amplifications were used as matrices for the second PCR. The final products were gel agarose purified, using NucleoSpin PCR and Gel Clean-up Kit (Macheray Nagel GmbH & Co.KG, Germany). Amplicons were designated: $ebpS_{13\text{-}197}$, $cna_{30\text{-}344}$, $fnbA_{36\text{-}607}$ and $sraP_{90\text{-}753}$, depending on the encoded amino-acid positions for *S. aureus* MW2.

The cloning and expression were performed in pET 28(a) vector (Novagen, Madison, WI). The PCR products were inserted between *NdeI*/*XhoI* or *NdeI*/*BamHI* restriction sites. Vectors dephosphorylation was performed with Shrimp Alkaline Phosphatase (Fermentas, Vilnius, Lithuania). Vectors and inserts were

Table 1. Oligonucleotides used for PCRs

Gene		Primer	Sequence 5′-3′	Annealing temp.	Amplicon
ebpS	PCR 1	ebpSFf	ACCGGCACAAGATCGATG	55°C	1854 bp
		ebpSRf	TATGCCAATATTCGAGACAAC		
	PCR 2	ebpSFi	GGGAATTCCAT**ATG**CAATCGATAGACAC[a]	60°C	552 bp
		ebpSRi	CGCCTCGAG**TTA**AGATTCATCTTTGTCTTG[b]		
cna	PCR 1	cnaFf	TATGACATAGAGTATAAGGAGG	60°C	3864 bp
		cnaRf	TACATACAGATTAAAAGTGACC		
	PCR 2	cnaFc	GGGAATTCCAT**ATG**GCACGAGATATTTCATC[a]	60°C	945 bp
		cnaRi	CCGCTCGAG**TTA**AGCCTTGGTATCTTTATC[b]		
fnbA	PCR 1	fnbAFf	AACCGAACAATATAGACTTGC	60°C	3262 bp
		fnbARf	GTTCGTTATCAATCATCAGAC		
	PCR 2	fnbAFc	GCGAATTCCAT**ATG**GCATCAGAACAAAAGAC[a]	60°C	1713 bp
		fnbARi	GGCCTCGAG**TTA**CTCAGAGGACTCAG[b]		
sraP	PCR 1	sraPFf	AGATTATACGCATAAACGAG	60°C	7167 bp
		sraPRf	CTAGTGATTGGTGCCTCAG		
	PCR 2	sraPFi	GGGAATTCCAT**ATG**GCTTCTGATGCACC[a]	60°C	1990 bp
		sraPRi	GCGGATCC**TTA**TGTTGATACGGAATCACTC[c]		

[a] *Nde*I restriction site; [b] *Xho*I restriction site; [c] *Bam*HI restriction site; Bold – START and STOP codons

ligated with Rapid DNA Ligation kit (Roche Diagnostics GmbH Mannheim, Germany).

The bacterial strains used for propagation and expression of inserts were *Escherichia coli* Mack1-T1, respectively *E. coli* BL21 (DL3) (Invitrogen, Oregon, SUA). Due to an additional *Nde*I restriction site, $sraP_{90\text{-}753}$ was cloned in two steps (data not shown). Sanger nucleic acid sequencing was used to confirm that the insert-vector constructs created are the expected ones and the inserts are in the right frame. Expression was induced with 1mM of isopropyl β-D-1-thiogalactopyranoside added in the growth medium. Recombinant proteins containing a fused His - tag were purified using nickel–nitrilotriacetic acid affinity chromatography.

Immunization protocol

In order to verify the immunogenic potential hypothesis of the recombinant proteins, three groups of BALB/c female mice, 6-8 weeks old, were used. Mice from test groups were primed with recombinant proteins (10μg of each protein, with Sigma Adjuvant Systems, Sigma Aldrich Co, LLC) and busted 21 days later with either recombinant proteins (Group 1) or heat inactivated *S. aureus* MW2 (1h at 80°C), 2.4 x 10^8 CFU per mouse (Group 2). Control group received only reconstituted adjuvant. The recombinant proteins mix and the inoculation protocol followed the adjuvant producer recommendations. Sera were harvested every 2 weeks after each inoculation, for immunological assays.

For experimental endocarditis procedure, New Zealand female white rabbits, 2-2.5 kg, were used. Three groups of rabbits were randomly created, with six rabbits per group. Two test groups (Group 1 and Group 2) received a mix of recombinant proteins (50 μg of each protein), along with Sigma Adjuvant

System (Sigma-Aldrich Co, LLC). At boost time (21 days later), the rabbits from Group 1 received the same combination proteins-adjuvant, as for the priming, but rabbits from the other test group (Group 2) were inoculated with heat inactivated *S. aureus* MW2 (1h at 80°C), 1.2 x 10^9 CFU per rabbit. Sera were harvested every 2 weeks after each inoculation, for immunological assays.

ELISA assays

The presence of specific antibodies raised in mice/rabbits was checked by enzyme-linked immunosorbent assays (ELISAs). Briefly, flat-bottomed 96-well stripes (NUNC Maxisorp, Portsmouth, NH) were coated overnight at 4°C with 500 ng/well of purified recombinant proteins and then blocked with BSA 1% (wt/vol) in PBS pH 7.4 for 1h at 37°C.

Different dilutions of the sera and of the HRP-IgG conjugate (Pierce Antibody Goat Anti-Antigen: Rabbit IgG F(ab)2 – HRP – Thermo Scientific, Rockford, USA) were tested in order to optimize the antigen-antibody reaction. Finally, hyperimmune sera were diluted 500-fold for the sera obtained after the first inoculation and 5000-fold for the sera obtained after the boost. Plates were incubated with the sera, for 1 hr at room temperature. After washing, HRP-IgG conjugate (500-fold diluted) and 3,3′,5,5′-Tetramethylbenzidine (TMB) as horseradish peroxidase substrate (Liquid Substrate System for ELISA from Sigma-Aldrich Co., Missouri, USA) were added to each well. Colorimetric reactions were stopped by the addition of 50 µl of 2N sulfuric acid solution. Three washes with PBS–0.05% Tween 20 were performed after each step. Plates were read for absorbance at 450 nm using a spectrophotometer (Thermo Scientific Multiskan EX", ThermoLabSystems)

An ELISA procedure using cell wall extract was performed for each rabbit serum. The cell wall extract was obtained following a protocol previously described [21]. The stripes were coated with 50 µl from 1/100 dilution cell wall extract and the sera were diluted 1/50000. The other reaction parameters were the same as described above.

Western Blot assays

Specificity of antibodies raised in mice/rabbits was verified by Western blot assays. Three µg from each recombinant protein were separated in SDS-PAGE and semi-dry transferred to PVDF membrane (Trans Blot SD Semi-dry Transfer Cell - Bio-Rad Laboratories, Hercules, CA, USA). For membrane blocking, buffer with 5% skim milk powder (wt/vol) was used, at 4ºC overnight. Sera diluted to 1/10000 and the secondary antibody - Anti-Rabbit IgG, whole molecule, alkaline phosphatase conjugate, developed in goat (Sigma-Aldrich Co., Missouri, USA) (1/10000 dilution) were used. The reaction time for the primary and secondary antibody was 1 h at room temperature. The antigen-antibody reaction was developed with NBT-BCIP substrate (Roche Diagnostics GmbH, Mannheim, Germany), and the specific immune complexes were visualized and compared with a standard molecular mass marker mixture (PageRuler Plus Prestained Protein Ladder, Thermo Scientific).

Opsonophagocytosis assay

An ideal vaccine to prevent *S. aureus* IE should not only induce synthesis of antibodies able to prevent bacterial adherence, but also to promote opsonophagocytic uptake. To evaluate this, an opsonophagocytosis test was performed.

Whole blood was collected from non-immunized New Zealand rabbit by ear venous puncture in heparinized tubes. Fifty microliters of *S. aureus* MW2 (5 × 10^5 CFU) suspension in PBS pH 7.4 were mixed with 950 µl of fresh rabbit blood in the presence of 10 µl rabbit serum sample (one serum per group).

Samples were incubated at 37°C with slow rotation for 1h. Serial dilutions were performed and, from each dilution, 100µl were plated on blood agar medium for colony formation, five plates for every dilution.

Experimental endocarditis procedure

The working protocols with laboratory animals respect EU Directive 2010/63/EU for animal experiments and were approved by the

Ethics Committee in Cantacuzino NMMIRD (CE/30/10.02.2014; CE/32/26.05.2014).

An already published protocol, with slight modifications, was used [22]. Briefly, induction of endocardial lesions required surgery, during which, a polyurethane catheter was placed through the right carotid artery into the left side of the heart and left in place, to damage the endocardium. Twenty-four hours after the surgery, rabbits were challenged with 1-1.5 x 10^6 CFU/rabbit *S. aureus* MW2 through the ear vein and were monitored for 4 days. At the time of impending death or at the end of the 4 days, rabbits were euthanized. Hearts were removed, opened by incision along the aorta, and examined for catheter position and vegetations. When vegetations were observed, they were aseptically excised, weighted, homogenized and the mixture was serially diluted in PBS pH 7.4, to determine CFUs after plating on agar surfaces.

Statistics

In this study all the data were statistically analyzed with non-parametric Wilcoxon-Mann-Whitney test. A *p*-value less than 0.05 was considered statistically significant and when *p*-value was below 0.01 the results were considered highly significant.

RESULTS

Cloning, expression and purification of recombinant proteins

The selected surface antigens corresponding amplicons were successfully cloned in pET-28(a). The nucleic acid sequencing confirmed that the right sequence, in the correct frame, was cloned. The recombinant proteins, expressed in *E. coli* BL21(DE3), were recovered from cellular extract, as soluble proteins, purified, then verified on SDS-PAGE. The expected molecular mass for recombinant proteins, based on theoretical data are: rEbpS – 21kDa, rFnbA – 63 kDa, rCna – 34 kDa, rSraP – 69 kDa. However, rEbpS, rFnbA and rSraP migrated slower than the predicted molecular mass. Recombinant EbpS migrated at ~ 40kDa, together with 2 other forms, one at ~ 21 kDa, as the predicted molecular mass, and the other at ~ 30kDa. Similar aberrant migration was observed for rFnbA and rSraP, which migrated slower than predicted, the migration pattern corresponding to approx. 100 kDa proteins (data not shown). The shift in the apparent molecular mass is characteristic to some Gram-positive surface proteins, including EbpS, FnbA and SraP, that have an abnormal migration, due to a particular acidic structure or to multiple repeats as already pointed out in the literature [23, 24].

Immunogenicity of recombinant proteins

ELISA assays using hyperimmune mice sera confirmed the immunogenic potential of recombinant proteins. Specific antibodies were detected in sera collected after both the first and the second immunizations. Median optical densities following boost were significantly higher for both test groups sera, as compared to the control group sera, for all four recombinant proteins (*p*-value <0.01) (Fig. 1). These findings suggested that all recombinant proteins are suitable to be tested in an experimental infective endocarditis procedure.

Rabbit sera tested for specific antibodies in ELISA assays exhibited, similar to mice, higher optical densities (O.D.'s) for the two test groups than for the control group for all four recombinant proteins. rEbpS was less immunogenic, optical densities values being lower comparing to the O.D. obtained for the other three proteins. Following the boost, a higher titer of specific antibodies for the recombinant proteins was observed for the Group 1 sera (recombinant proteins boost), comparing to Group 2 sera (bacterial boost) (Fig. 2). This result positively correlates with the dose of recombinant proteins received by the rabbits. However, the titer of specific antibodies increased in Group 2 sera, comparing with Group 3 sera (control), following the boost with the heat-inactivated *S. aureus*, suggesting a re-stimulation of the rabbits with native antigens. The same conclusion was generated from the ELISA results using cell wall extract, when native antigens coating the well were recognized by antibodies from both test groups, including the hyperimmune sera obtained only with recombinant proteins (Fig. 3).

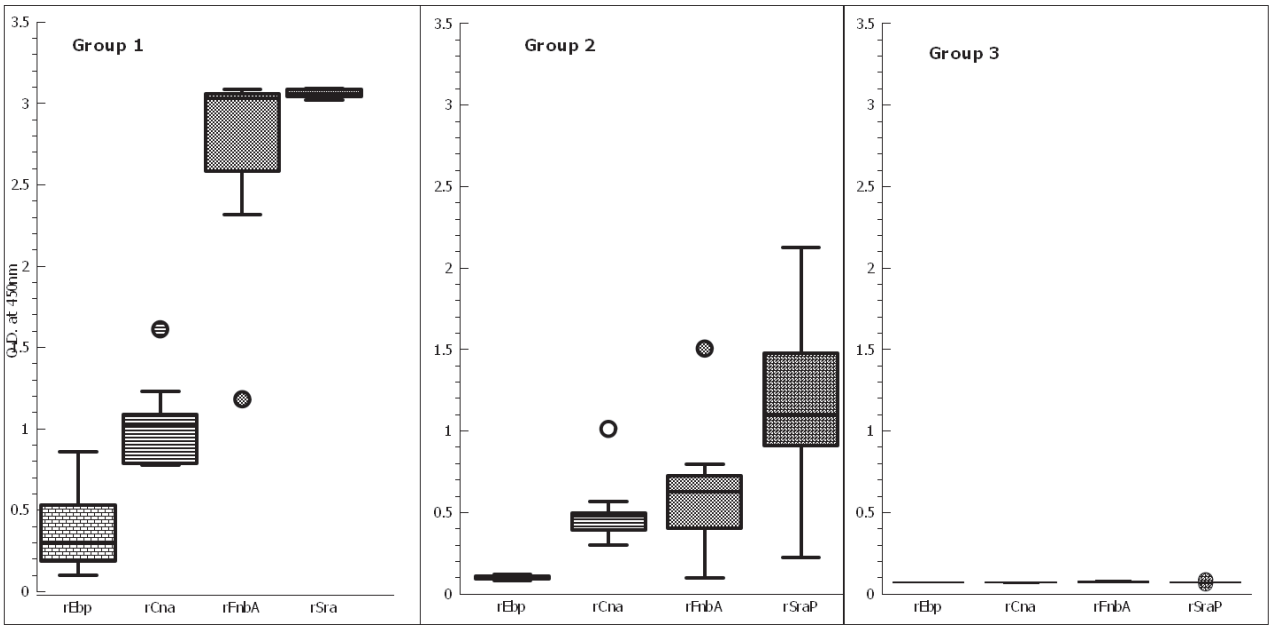

Fig. 1. Optical densities at 450 nm for *mice* antibodies raised against the four recombinant proteins following the boost.
Group 1 – recombinant protein boost; Group 2 – bacterial boost; Group 3 – control

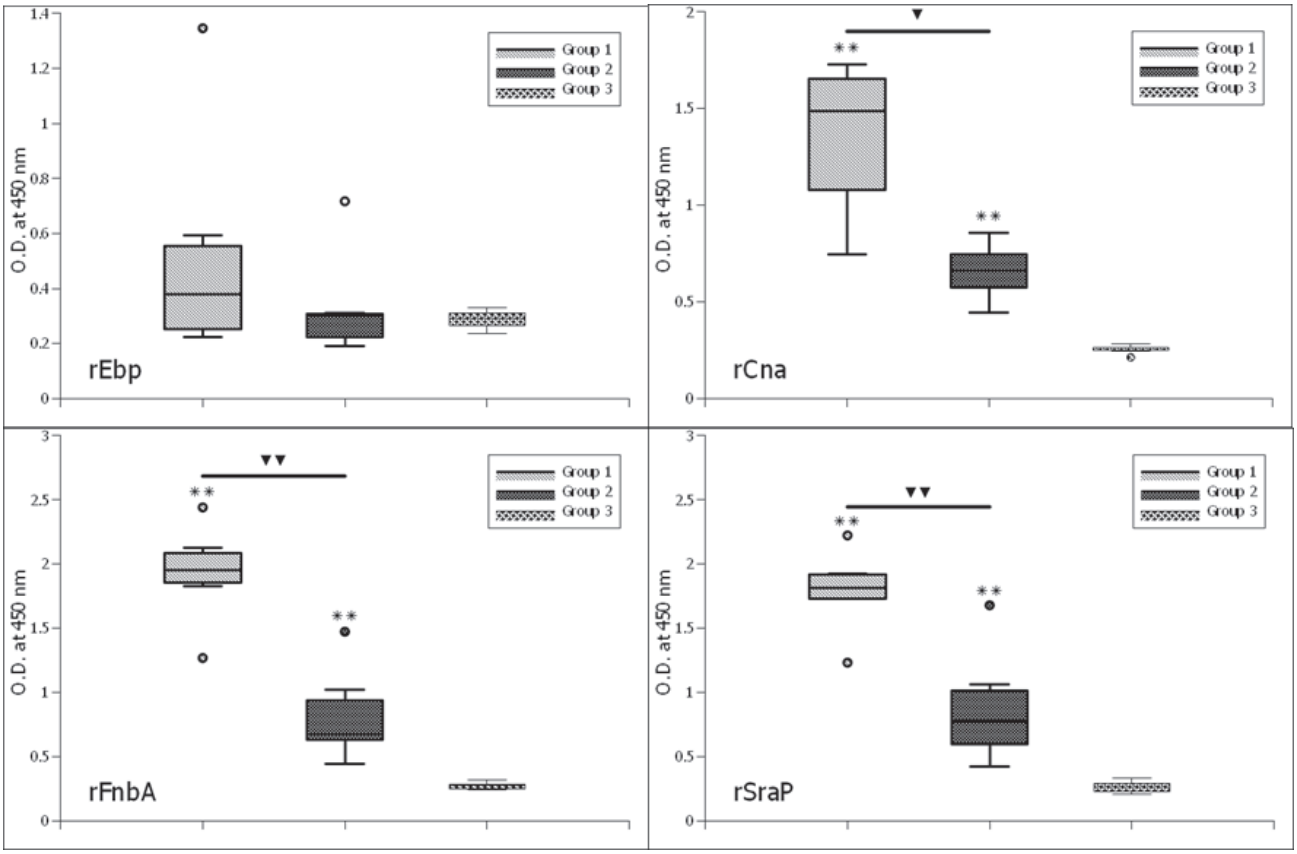

Fig. 2. Specific *rabbit* antibodies levels for the recombinant proteins, following the boost.
Group 1 – recombinant protein boost; Group 2 – bacterial boost; Group 3 – control
**p*-value < 0.05, test group versus control group; ***p*-value < 0.01, test group versus control group
▼ *p*-value < 0.05, test group versus test group; ▼▼ *p*-value < 0.01, test group versus test group

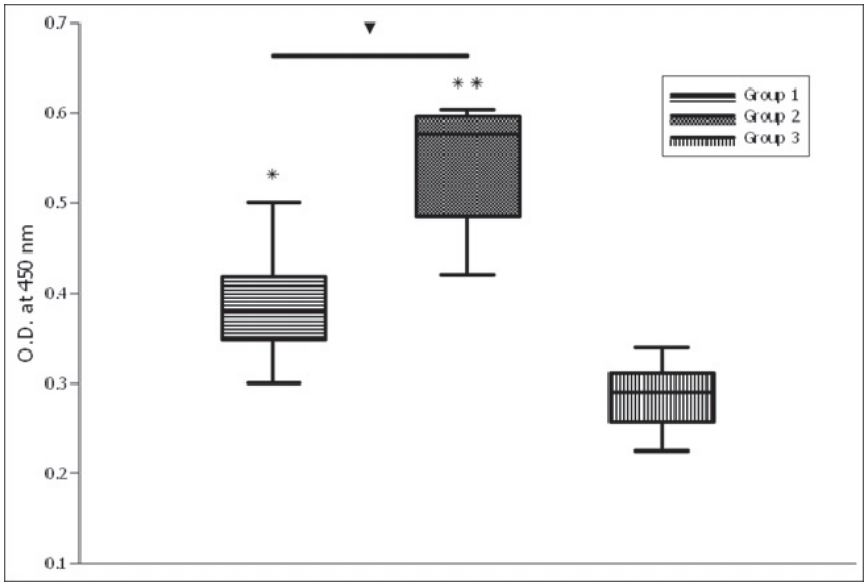

Fig. 3. ELISA assay with cell wall extract of *S. aureus* MW2 coating, and hyperimmune *rabbit* sera.
Group 1 – recombinant protein boost; Group 2 – bacterial boost; Group 3 – control
**p*-value < 0.05, test group versus control group; ***p*-value < 0.01, test group versus control group
▼ *p*-value < 0.05, test group versus test group; ▼▼ *p*-value < 0.01, test group versus test group

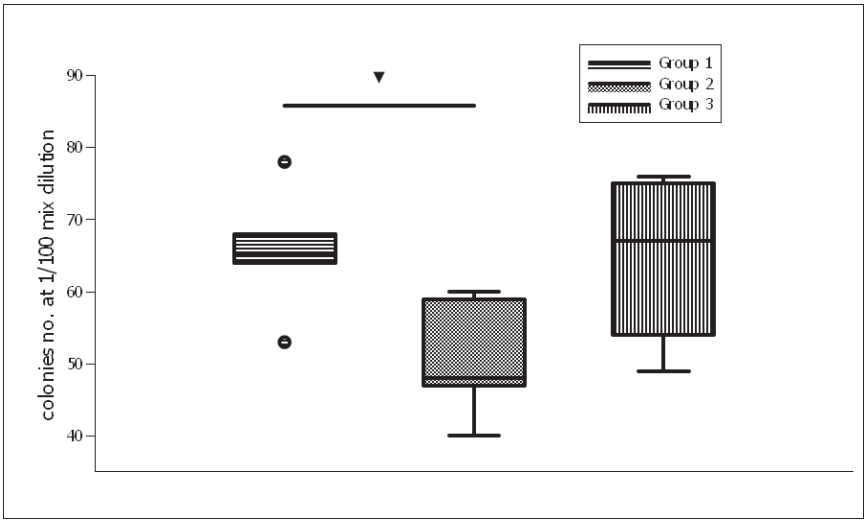

Fig. 4. Number of colonies recovered in opsonophagocytosis assay.
Group 1 – recombinant protein boost; Group 2 – bacterial boost; Group 3 – control
▼ *p*-value < 0.05, test group versus test group

Specific antigen - antibody interactions were demonstrated in Western blot assays. For rCna, rFnbA and rSraP specific bands were visible for all the test groups sera, while for rEbpS they were visible only for two Group 1 sera, probably due to the lower titer of specific antibodies generated. No bands were obtained when control group sera were tested (data not shown).

***In vitro* protection - opsonophagocytosis assay**

The results of opsonophagocytosis assay showed an increased capacity of Group 2 sera (with bacterial boost) to participate in the process of opsonization, with statistical significance compared to Group 1 sera (with recombinant proteins boost) (Fig. 4).

Experimental endocarditis procedure

The induced endocarditis experiment had a success rate of 73%. Final analysis was performed using four rabbits from Group 1, four rabbits from Group 2 and five rabbis from control group. The other animals were excluded because of the non-aortic valve position of the catheter or due to the fact that the rabbits died during the night.

Following surgery and infection, all the rabbits that received recombinant proteins for boost (Group 1) included in the final analysis, died before the end of the follow-up period. In contrast, all the rabbits that received heat-inactivated bacteria for boost (Group 2), survived until day 4. Regarding the control group, only 3 out of 5 rabbits managed to survive 4 days (Fig. 5).

Excised vegetation weighted 20 - 97.7 mg for Group 1, 38.8 - 67.4 mg for Group 2 rabbits and, respectively, 24.2 - 54.9 mg for rabbits included in the control group. Wilcoxon-Mann-Whitney test showed no statistically significant difference between the three groups in terms of weight of excised vegetations (p value > 0.05).

Regarding the bacterial load, after counting the colonies in various dilutions and application of a correction adjustment, a trend of lowering bacterial load of the vegetations in Group 2 of rabbits was observed as compared to the other two groups (Fig. 6). The calculated value of $p = 0.08$ by the statistic Wilcoxon-Mann-Whitney test was just below the significance threshold.

DISCUSSION

Prevention of bacterial infective endocarditis remains an issue of major concern in the context of severity of this disease, especially if it is caused by *S. aureus*. An efficient and safe vaccination of persons included in risk groups could bring significant benefits both for the patients and the health care system, by reducing costs of hospitalization and therapy [25]. Such an anti-staphylococcal vaccine is not yet available, in spite of many attempts. Multiple vaccine candidates, such as the polysaccharide capsule, extracellular polysaccharides or cell wall associated attachment proteins presented promising results in pre-clinical testing, using animal models, but those that entered in clinical human studies failed to demonstrate efficacy [26]. The reasons for failure of abandoned vaccines are related to the versatility of this bacterium, but also to vaccine developers' tendency to consider and evaluate mainly the humoral response of the host [2, 3, 27]. Some studies pointed out the fact that innate immunity is also very important in anti-staphylococcal response and this should be taken into account for vaccine development [28, 29].

Along with the progress in the genomic sequencing, reverse-vaccinology appeared as a new approach for vaccine development. This method, already successfully used, involves the genomic information analyses *in silico* with dedicated software for the selection of the best candidates for immunization, without culturing microorganism. Reverse-vaccinology has many advantages comparing with classic vaccinology. For instance, by reverse-vaccinology approach epitopes that elicit both innate and adaptive immune response can be selected [30, 31].

In a previously published study, ten *S. aureus* surface proteins, involved in IE vegetations attachment, were tested for the presence of immunogenic epitopes using reverse vaccinology, and five epitopes, belonging to four proteins were found to be immunogenic, stimulating both T and B cells [6].

In the present study, the four recombinant proteins containing presumptive epitopes were generated and used within a prophylactic vaccine, in order to test their immunogenicity and protective capacity in an experimental IE due to *S. aureus.* A rabbit experimental model was used, as this animal is suitable for experimental endocarditis procedure [32].

In order to refine the experimental setup, mice were primed with recombinant proteins and busted at 14 days with either recombinant proteins or killed bacteria. This experimental approach was used to identify whether the response to any antigen can be enhanced when whole bacterium is used for boosts. The results revealed that recombinant proteins rCna,

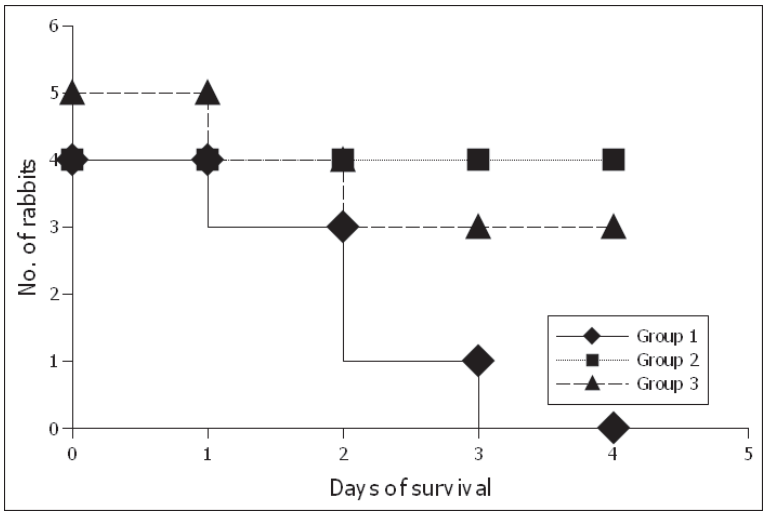

Fig. 5. Days of survival for challenged rabbits.
Group 1- recombinant protein boost; Group 2 – bacterial boost; Group 3 – control

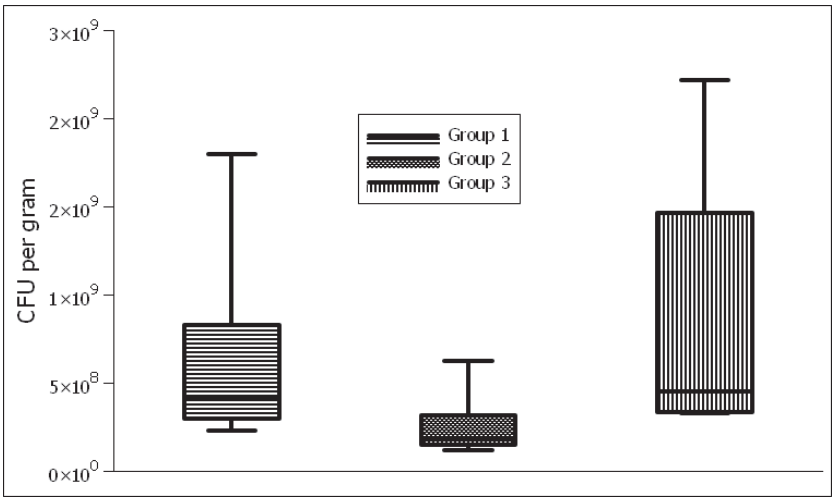

Fig. 6. Bacterial load of excised vegetations.
Group 1- recombinant protein boost; Group 2 – bacterial boost; Group 3 – control

rFnbA and rSraP induced a strong antibody response, while rEbpS only moderate one. The results were similar in mice and rabbits. There was a strong response when whole bacterial cells are used for boosts, but however, lower than the one induced by recombinant antigen cocktail. The response to collagen adhesin is quite similar in the two variants, although slightly reduced in Group 2.

Regarding protection, a rather good survival rate was detected in the Group 2, where vaccination was based on boosting with heat-inactivated *S. aureus* cells. At the same time, a tendency in lowering bacterial load was detected in the endocardial vegetations for this group. By contrast, although the titers of specific antibodies were higher in the group that received two times recombinant proteins, the animals had a very bad evolution. The high specific antibody titers did not only fail, but worsened the animal condition, probably due to an inhibitory or toxic effect.

The precise mechanism by which the immunization scheme with heat inactivated *S. aureus* cells boost seems to provide protection against *S. aureus* infection in experimental infective endocarditis is not completely elucidated. There are several studies that

demonstrate the importance of Th17 cell response, along with antibody response in achieving efficient protection in infective endocarditis [33-36]. Also, it is equally demonstrated that this protection requires opsonic activity of the antibodies [37-39]. In this study, it was also found that the protection observed for Group 2 was correlated with the opsonic activity of the antibodies. According to this study and previous ones [38-40], opsonophagocytosis assay can be a valuable tool in order to avoid or reduce high severity of animal tests during the development of a vaccine.

The approach presented here confirms the immunogenic capacity of epitopes selected by reverse-vaccinology and may represent a new strategy to be considered in developing anti-staphylococcal vaccine. However, although attractive, the methodology does not assure by itself the entire development of a new vaccine.

Further studies should be carried out for improving the understanding of the mechanisms addressed in this study. Some of them should address the importance of the recombinant proteins priming approach presented in this study by comparison with a bacterial cell priming approach.

Conflict of interests: There is nothing to declare.

Acknowledgements

This research was financially supported by the Ministry of National Education - PN 09-22 01 01. We thank the colleagues from The Advanced Studies Department in Cantacuzino NMMIRD, for their support, especially to bioch. Cătălin Ţucureanu, who helped with statistics. Also, we are greatly indebted to The Faculty of Medicine and Pharmacy "Târgu Mureş" for the good collaboration in performing experimental rabbit model, with warm thanks to dr. Adrian Man for facilitating the experiment.

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