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Investigation of Recombinant SagA Antigen as A Potential Vaccine Candidate Against *Enterococcus faecium* Infection

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ABSTRACT

Background: *Enterococcus faecium* is a Gram-positive bacterium with evolving multi-drug resistance. It is one of the primary causes of urinary tract infections and endocarditis, among other opportunistic bacteria. The rise of antibiotic resistance in *E. faecium* makes treating its illnesses a significant therapeutic challenge. **Objectives:** As a result, there is an urgent need for alternative treatments as well as preventive measures like vaccination. Previous research studies have suggested that the secreted antigen A (SagA) protein of *E. faecium* is a promising vaccine candidate. However, it was reported that capsular polysaccharides prevented the phagocytic activity of PMN to several *E. faecium* strains. Moreover, the surface accessibility of protein antigens was reported to be variable from one encapsulated strain to another. **Methods:** In this study, recombinant SagA was produced and investigated as a potential vaccine candidate against an encapsulated *E. faecium* isolate. Antibodies against SagA were raised in vivo and tested against *E. faecium* using ELISA and dot blot assay. Active immunization of mice using the recombinant SagA was performed, which was followed by a challenge with *E. faecium*. **Results:** Even though the ELISA and dot blot assay showed promising results, antibodies against SagA failed to be opsonic in the opsonophagocytic assay and failed to produce significant protection in an experimental mouse model against encapsulated *E. faecium*. **Conclusion:** The absence of protection despite the presence of significant antibody titer could be attributed to the existence of the capsule which might limit the surface exposure of the protein antigens and prevent the opsonophagocytosis in vivo.

Keywords: *Enterococcus faecium; SagA; recombinant vaccine; Capsule; Opsonophagocytosis*

INTRODUCTION

For many years, several opportunistic pathogens that cause life-threatening nosocomial diseases have emerged. The ESKAPE (***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa* and ***E****nterobacter* species) bacteria are the most notorious of these pathogens[1]. As the multiple antibiotic resistance of these pathogens increases, morbidity and mortality dramatically rise. In addition to their fatal effect, these infections raised treatment cost, extended hospital stays and increased the possibility of further infections. Several infection control programs, treatments, and immunization approaches are urgently needed to limit the infection with those pathogens [2].

Enterococcus faecium is a Gram-positive bacterium that has a rising multi-drug resistance (MDR)

profile. It causes several opportunistic infections such as bacteremia, endocarditis and urinary tract infections[3]. The infections due to *E. faecium* are arduous to treat owing to the increase of MDR traits. The number of enterococcal infections caused by *E. faecium* has steadily risen compared to other *Enterococcus* species, like *Enterococcus faecalis* [4]. *E. faecium* specifically has prominent adaptation ways that help explain its environmental persistence in healthcare settings [5]. The urinary tract is the most prevalent entry for enterococci into the bloodstream, leading to bacteremia that causes *E. faecium* morbidity and mortality [6]. Secondary to bloodstream infections, a high percentage of bacterial endocarditis is caused by enterococci [7].

In modern medicine, immunization plays a remarkable role in the prevention of diseases [4]. There are several trials to develop protective vaccines or anti-sera for the prevention of infections by enterococci. Some of these trials were successful but needed further investigation, while others proved to be unsatisfactory for protection against enterococcal infections. Targets for developing immunotherapies include capsular polysaccharides, cell wall polysaccharides, and cell-surface-associated protein antigens. However, the use of surface proteins as vaccine candidates has the advantages of low production costs, high homology across strains, and high immunogenicity compared to polysaccharide antigens [8].

There is no vaccine available currently for human use against *E. faecium* infections. The current study focused on assessing Secreted antigen A (SagA) as a potential vaccine candidate against infection caused by encapsulated *E. faecium*.

SagA protein is a notable antigen that binds to the extracellular matrix (ECM) proteins and is critical for *E. faecium* proliferation and growth. The ECM proteins fibrinogen, collagen type I, collagen type IV, fibronectin, and laminin are among those to which the SagA protein exhibited broad-spectrum binding [9]. The adherence to the ECM confers biofilm formation and, subsequently, potentiates the virulence of *E. faecium* [10]. An extensively produced protein of approximately 72 kDa was recognized by the opsonic serum against heat-killed whole *E. faecium*. According to LC-ES-MS/MS, this protein was SagA. Serum antibodies from rabbits against SagA statistically lowered *E. faecium* count in the mice blood[11]. Promising outcomes were also observed with monoclonal antibodies (mAbs) directed against *Enterococcus faecalis* well-characterized capsular polysaccharide, diheteroglycan (DHG), and the immunogenic protein, SagA, from *E. faecium*. These mAbs, as well as rabbit antibodies against the DHG-SagA glycoconjugate, exhibited good opsonic killing against *E. faecalis* and *E. faecium* [12, 13]. Several studies have indicated the potential of anti-SagA for protection from *E. faecium* infections[11-13]. However, all these studies were done without investigating the presence of capsule polysaccharides. The presence of a capsule was reported to affect complement activation, hence protecting the bacteria from opsonophagocytosis [14]. Moreover, capsule serotypes can significantly alter the response to a vaccine [15]. Given these two limitations, the potential of SagA as a vaccine candidate in the presence of a capsular polysaccharide was assessed in this study.

MATERIALS AND METHODS

Bacterial strains and plasmids

An *Enterococcus faecium* clinical strain was isolated from a urine specimen at the routine analysis laboratory of the Faculty of Medicine, Alexandria University. The intermediate cloning host, *Escherichia coli* (*E. coli*) DH5α, was obtained from the Microbiology and Immunology department laboratory stock, Faculty of Pharmacy, Alexandria University. pQE31 (Qiagen, Germany) was used as a plasmid vector for cloning and expression of SagA. The expression host, *E. coli* M15 containing pREP4 (Qiagen, Germany), was used to express SagA.

Culture media, antibiotics, and incubation conditions

E. faecium isolate was grown in brain heart infusion (BHI) broth at 37°C. *E. coli* strains were grown in Luria Bertani (LB) broth at 37°C in a shaker incubator at 180-200 rpm. Solid media were prepared with the same composition of liquid media with added agar agar (2% w/v). For *E. coli* DH5α containing pQE31 plasmid, growth media containing ampicillin (Epico, Egypt) at a final concentration of 100 µg/ml were used. For *E. coli* M15 (pREP4), growth media containing kanamycin (Sigma Aldrich, USA) at a final concentration of 25 µg/ml were prepared.

Antony capsule stain

Antony capsule stain was done, as mentioned previously, to detect the presence of capsular polysaccharide around the used *E. faecium* isolate [16]. Briefly, the smear was prepared by mixing twenty microliters of *E. faecium* suspension with an equal amount of 5% skimmed milk. The mixture was placed and spread over a clean microscope slide and left to air dry. Crystal violet was used to stain the smear, and the excess dye was rinsed with a copper sulfate solution (20%). The slide was observed using a light microscope under oil immersion.

In silico analyses and Polymerase Chain Reaction (PCR)

The subcellular location of the SagA protein was confirmed to be extracellular using the online PSORTdb v3.00 tool (http://www.psort.org/psortb/). The conservation of *sagA* among different strains of *E. faecium* was confirmed using the BLAST-N tool of the

NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers for specific amplification and cloning of *sagA* were designed based on the GenBank accession number AFK60183.1. The forward primer was "TATAGGATCCGATGACCTTGACTGCCGTAGC", while the reverse primer was "TATAGGTACCTTACATGCTGACAGCAAAGT". Restriction sites of BamHI and KpnI (underlined) were added to the forward and reverse primers, respectively.

Colony PCR was done using Q5® High-Fidelity DNA Polymerase (# M0491, NEB, England). Briefly, one colony of the *E. faecium* clinical isolate was suspended in 5 µl of nuclease-free water and heated at 95°C for 10 min to lyse the cell. Then, the reaction was completed to 25 µl using 6 µl of nuclease-free water, 0.75 µl of each primer, and 12.5 µl of 2X Master-mix. The PCR annealing and extension conditions were adjusted at 70°C/30 sec and 72°C/1min, respectively. Successful amplification was confirmed using agarose gel electrophoresis.

Cloning of sagA

Amplified *sagA* was purified using Zymo™ DNA cleanup and concentrator kit (ZYMO research, USA). Both *sagA* and pQE31 were double digested using BamHI and KpnI (NEB, England). Ligation of the digested construct was done using T4 DNA Ligase (NEB, England) following manufacturer's instructions. Chemically competent *E. coli* DH5α and *E. coli* M15 (pREP4) were prepared using the calcium chloride protocol [17]. The transformation was done by cooling the competent cells/plasmid mixture on ice followed by heat shock at 42°C for 90 sec. Successful transformation of the intermediate host (*E. coli* DH5α) was confirmed by growing cells on LB agar containing ampicillin, while the transformation of the expression host, *E. coli* M15 (pREP4), was confirmed by growing cells on LB agar containing ampicillin and kanamycin. Further confirmation using PCR with insert-specific primers was also carried out on pQE31-*sagA* as a template.

The pQE31-*sagA* plasmid construct was subjected to extraction using the QIAprep spin plasmid Miniprep Kit (QIAGEN, Germany). SagA DNA sequence was confirmed using the DNA Sequencing services offered by the LGC group, UK. The result obtained from sequencing was analyzed using DNA lasergene software. The *sagA* nucleotide sequence was searched for homology against the GenBank database using the Nucleotide BLAST search tool (BLAST-N).

SagA expression and purification

The QiaExpressionistTM manual of Qiagen (Germany) was the guide used in protein induction and purification. Expression of SagA in *E. coli* M15 (pREP4) was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG, Melford, UK). Briefly, 100 ml of LB broth containing ampicillin and kanamycin was inoculated with a fresh 10 ml overnight LB culture of *E. coli* M15 (pREP4) (pQE31-*sagA*). The culture was subsequently grown at 37 °C in a shaking incubator at 200 rpm till reaching OD600 in the range of 0.4–0.6. Afterward, 1 mM IPTG was added to the grown culture and incubated at the same conditions for 3 h to allow protein expression. Pelleting of bacterial cells was performed by centrifugation at 3200 xg for 20 min at 4°C. The supernatant was decanted, and the pellet was washed once with 20 ml of sterile saline (0.9% w/v) and then centrifuged at 3200 xg for 10 min at 4°C. The supernatant was discarded, and the pellets were frozen at -80°C until cell lysis and protein purification.

E. coli M15 (pREP4) (pQE31-*sagA*) cell lysis and SagA protein purification were done under denaturing conditions. Briefly, *E. coli* pelleted cells were resuspended in 5 ml of denaturing lysis buffer containing 8 M urea. Lysis was performed by shaking at room temperature for 60 min until the solution became translucent. The lysate was centrifuged at 4000 xg for 20 min at 4°C to pellet the cell debris and supernatant was collected. The supernatant was passed through Nickel-Nitrilotriacetic Acid (Ni-NTA, QIAGEN, USA) resin to capture the His-tagged SagA protein. This was followed by washing and elution of bound SagA. Refolding of denatured SagA in phosphate-buffered saline (PBS) was done using a PD-10 column containing Sephadex G25 (GE Healthcare, UK). After protein refolding, the absence of urea from denaturing buffers was confirmed using a urease test kit (Diamond Diagnostics, Egypt). To check for successful protein expression, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as mentioned previously [18]. In SDS-PAGE, the used acrylamide/Bis-acrylamide 40% (Noragen Biotek Corp., Canada) was 12% in the separating gel and 4% in the stacking gel. The gel was stained using Coomassie brilliant blue R-250 dye (MP Biomedicals, USA) and destained using a solution composed of glacial acetic acid, methanol, and distilled water with a ratio of 2:1:7, respectively.

Assessing the immunogenicity of SagA recombinant antigen

Mice used in this study were kept under standard housing conditions, with water and food provided without restrictions. All animal handling procedures had been approved by the Animal Care and Use Committee (ACUC) of the faculty of pharmacy at Alexandria University (Approval number: 062024193217).

Dot blot assay

Dot blot assay was performed as mentioned previously [18]. This dot blot assay was to ensure the immunogenicity of the purified recombinant SagA by

testing its interaction with mice antibodies raised against *E. faecium* clinical isolate. SagA was applied onto the surface of a nitrocellulose membrane by spotting 15µl of SagA solution (195µg/ml) using a micropipette. An irrelevant *Klebsiella pneumoniae* His-tagged antigen was used as a negative control to ensure the absence of non-specific interactions. The primary antibody, diluted 1:250, was a serum collected from mice previously immunized with *E. faecium*. In the latter immunization, *E. faecium* (2×10^7 CFU) had been injected into mice in three consecutive injections, separated by two-week intervals. The two booster injections were a mixture of *E. faecium* cells and incomplete Freund's adjuvant (IFA, Becton & Dickinson, USA). In the dot blot assay, the antigen-antibody complex was identified by adding horseradish peroxidase (HRP)-labeled antibody to mouse IgG (KPL, USA) at a dilution of 1/500. The chromogenic substrate, 3, 3'-diaminobenzidine tetrahydrochloride (DAB, BioBasic Inc., Canada) was then used to visualize the activity.

Evaluation of SagA recombinant antigen through active immunization of mice

The *in vivo* study was carried out using female Swiss albino mice (6–8 weeks old) as an animal model to evaluate the protective efficiency of the SagA recombinant antigen. Mice were divided into groups of ten mice each. The immunization regimen was divided into four consecutive doses, each 200µl, injected subcutaneously, and at two-week intervals. The priming dose was a mixture of SagA and Immune BCG-T® (VacSera co., Egypt) as an adjuvant, followed by three doses of SagA with IFA. On the other hand, the negative control group was given the adjuvant alone at each dose. SagA was injected at a final dose concentration of 15 µg/mouse. Immune BCG-T® (3×10^8 CFU/ml) was diluted to reach a final count of 10^6 CFU of mycobacteria/mouse. In the booster doses, a mixture of SagA (15µg /mouse) and IFA (100 µl/mouse) was injected.

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood samples were collected from the submandibular vein of the immunized mice before the challenge with *E. faecium*. Serum samples were tested with Enzyme-Linked Immunosorbent Assay (ELISA) to compare the response against SagA in the immunized group and the negative control group as described previously [19]. Briefly, the wells of the ELISA plate were coated with 100µl of 10µg/ml recombinant SagA. The primary antibody was serum samples from immunized and control groups, whereas HRP-conjugated anti-mouse total IgG (KPL, USA), anti-mouse IgG1 (Abcam, USA), and anti-mouse IgG2a (Abcam, USA) antibodies were used as the secondary antibodies. Tetramethylbenzidine (TMB) ELISA Substrate System (KPL, USA) was used as a substrate.

Indirect ELISA was also done to test the interactions between the raised antibodies and the *E. faecium* whole cell. In this ELISA, wells were coated with 100 µl / well of whole *E. faecium* cells (10^7 CFU). The primary antibody was serum samples from immunized and control groups while HRP-conjugated anti-mouse total IgG was used as the secondary antibody.

Another ELISA was done to compare the interactions between mice sera and the *E. faecium* whole cell before and after immunization. The wells of the ELISA plate were coated with *E. faecium* whole cells. The primary antibody was serum samples from mice before and after immunization with SagA and anti-mouse total IgG was used as the secondary antibody.To test the presence of antibodies against the His-tag of the recombinant SagA, indirect ELISA was performed. The wells of the ELISA plate were coated with different irrelevant His-tagged recombinant proteins of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and the SagA recombinant antigen. The primary antibody was serum samples from immunized and control groups while anti-mouse total IgG was used as the secondary antibody.

Mice challenge with E. faecium

Two weeks after the last dose, both immunized and control groups were challenged intraperitoneally with 2×10^9 CFU/mouse (200µl) of *E. faecium* clinical isolate. The bacterial count in both the kidneys and heart was calculated after 48 h where mice were sacrificed by cervical dislocation. Mice dissection was done under strict aseptic conditions to isolate both the kidneys and the heart. Organs were homogenized in 2 ml sterile saline (0.9% w/v), serially diluted and plated onto BHI agar. The plates were incubated at 37°C for 24 h, and *E. faecium* count/organ was calculated for immunized and negative control groups.

Opsonophagocytic assay

Opsonophagocytic assay (OPA) was carried out as previously described [19]. In this assay, polymorphonuclear neutrophil (PMN) were isolated from 7 ml of fresh human blood using Lymphosep™ (Biowest, France). The reaction mixture of the OPA was composed of 5 µl *E. faecium* suspension (500 CFU), 20 µl heat-inactivated serum from immunized or non-immunized mice, 15 µl PMN (7500 cells), and 20 µl active complement (human serum). The reaction mixture was incubated in a shaking incubator at 200 rpm, 37°C, for 40 min. Serial dilution and plating of the reaction mixture were done to estimate and compare the count of *E. faecium*.

Statistical analysis

ELISA, *E. faecium* organ count, and

opsonophagocytic assay results were analyzed by the standard parametric method using one-way analysis of variance (ANOVA) followed by a Tukey test to compare different groups. The statistical analysis tests were done using GraphPad Instat 3. All the data in this study are expressed as means, and the displayed error bars represent the standard error values. The P-value was considered significant if it was equal to or less than 0.05 (*), 0.01 (**), and 0.001 (***).

RESULTS

Antony capsule stain

The staining of the *E. faecium* clinical isolate using the Antony staining technique ensured the presence of a clear or very faint blue halo (capsule) around purple cells with a purple background as shown in **Figure 1**.

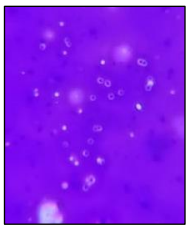

Figure 1. Capsule stain of *E. faecium* clinical isolate. A clear or very faint blue halo (capsule) is present around purple cells with a purple background.

Cloning of SagA gene, SagA expression and purification

PCR amplification of *sagA* resulted in a pure band at the expected size on agarose gel, as shown in **Figure 2**. Successful cloning was confirmed by colony PCR using vector-specific primers, followed by agarose gel electrophoresis (**Figure 3**). The sequence of the recombinant *sagA* was identified and searched for homology against the GenBank database using BLAST-N. The cloned *sagA* gene sequence was found to be conserved among different E. faecium strains. The SDS-PAGE of recombinant SagA (~70kDa) is shown in **Figure 4**. The ultimate overall yield of the recombinant SagA was 1.95 mg per one liter of induced culture.

Assessing the immunogenicity of SagA recombinant antigen

In dot blot, mice serum against *E. faecium* was reactive with recombinant SagA, as shown in **Figure 5.** For ELISA, as shown in **Figure 6**, the total IgG and IgG1 levels of the immunized group were significantly higher than the negative control group. Unfortunately, the IgG2a response was not statistically significant when compared to the control group. Sera of SagA-immunized group interacted significantly with *E. faecium* whole cells as compared to the control group or the sera before immunization (**Figure 7** and **8**). The result shown in **Figure 9** indicated a significant antibody response against the SagA itself, not the His-tag. When the mice immunized with the recombinant SagA as well as the non-immunized mice were challenged with *E. faecium*, the bacterial count in immunized mice organs showed no significant difference compared to its count in the negative control group (**Figure 10).** In addition, OPA results showed no significant difference between the immunized and the negative control groups (**Figure 11**).

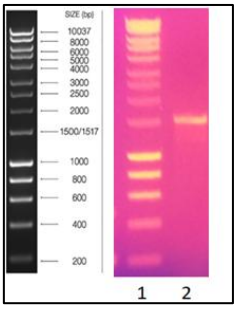

Figure 2. Agarose gel electrophoresis for PCR amplification of sagA. Lane 1: 1kb DNA ladder; Lane 2: sagA (1536 bp).

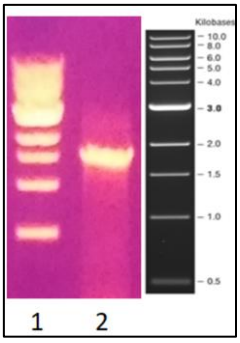

Figure 3. Agarose gel electrophoresis for colony PCR product of cloned sagA in pQE31-*sagA* using vector specific primers. Lane 1: 1kb DNA ladder; Lane 2: amplified *sagA* (~ 1769 bp) using vector-specific primers.

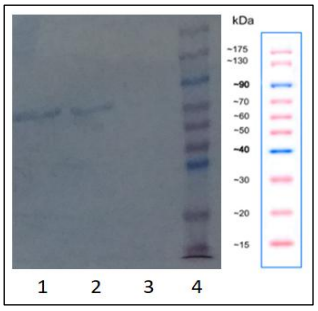

Figure 4. SDS-PAGE of SagA purified under denaturing conditions. Lane 1 and 2: Purified protein bands of SagA (~70kDa). Lane 3: Empty well. Lane 4: Pre-stained protein ladder.

Figure 5. Dot blot assay of purified recombinant SagA. SagA (left panel) or a negative control *Klebsiella pneumoniae* His-tagged antigen (right panel) was placed on the surface of a nitrocellulose membrane by spotting using a micropipette. The primary antibody, diluted 1:250, was pooled sera collected from mice previously immunized with sublethal *E. faecium*.

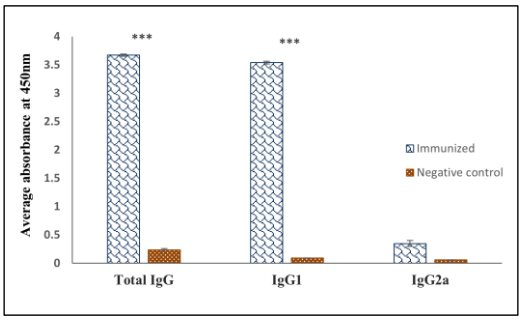

Figure 6. SagA-specific total IgG, IgG1, IgG2a response. ELISA plates were coated with recombinant SagA antigen and sera from immunized group and non-immunized control group were used as primary antibodies. Absorbance readings represent mean +/- standard error of the mean.

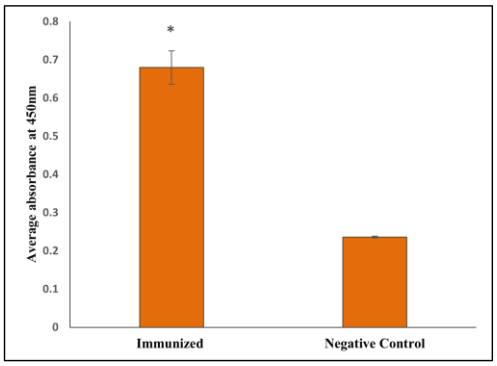

Figure 7. *E. faecium* whole cell-specific total IgG antibody response. ELISA plates were coated with *E. faecium* whole cells and sera from immunized group and non-immunized control group were used as primary antibodies. Absorbance readings represent mean +/- standard error of the mean.

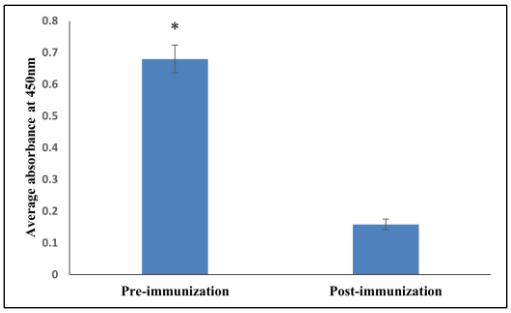

Figure 8. *E. faecium* whole cell-specific total IgG antibody response in mice before and after immunization. ELISA plates were coated with *E. faecium* whole cells and sera from mice before and after immunization were used as primary antibodies. Absorbance readings represent mean +/- standard error of the mean.

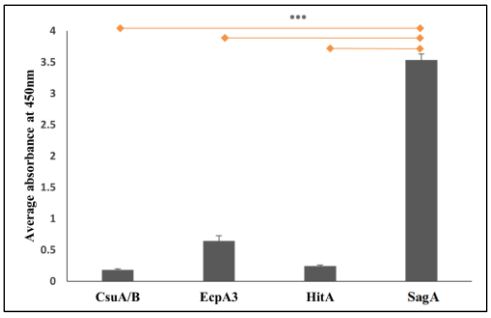

Figure 9. Indirect ELISA results of mice sera against His-tagged antigens. ELISA plates were coated with His-tagged antigens CsuA/B, EcpA3, HitA of *A. baumnnaii*, *K. pneumoniae*, and *P. aeruginosa*, respectively. As well as SagA of E. faecium in order to ensure reactivity of sera from SagA-immunized mice with recombinant His-tagged SagA was antigen-specific and not due to reactivity with the His-tag.

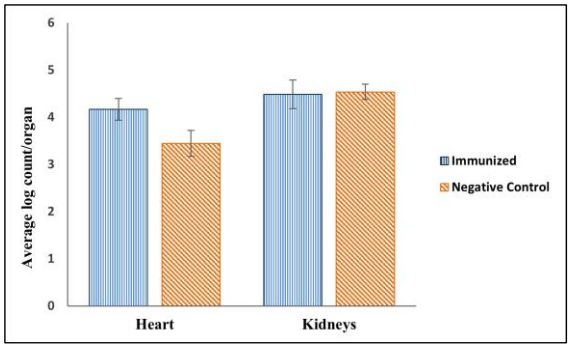

Figure 10. Organ bioburden in hearts and kidneys of mice challenged with *E. faecium*. Mice were immunized with four doses of recombinant SagA and challenged with *E. faecium* two weeks after the last booster.

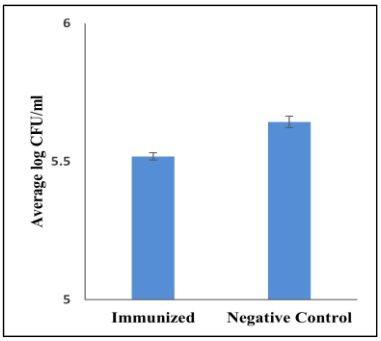

Figure 11. Opsonophagocytic assay result. In vitro opsonophagocytic activity of serum from SagA-immunized mice and from non- immunized control mice was assessed against *E. faecium* clinical strain as described in the Methods section.

DISCUSSION

Enterococcus faecium is known for its increasing multi-drug resistance, making it a major cause of urinary tract infections and endocarditis. The rise in antibiotic resistance highlights the need for alternative measures, such as vaccines[1]. It was found that capsular polysaccharides in some enterococcal strains could hinder the phagocytic activity, and protein antigen accessibility [14, 20]. Previous studies identified the secreted antigen A (SagA) protein as a potential vaccine candidate[11-13]. In the current study, recombinant SagA was produced and tested as a vaccine against an encapsulated *E. faecium* isolate. The used *E. faecium* clinical isolate was confirmed to be encapsulated**.** Successful cloning and expression of *sagA* was achieved. The BLAST of the recombinant *sagA* sequence confirmed the gene is conserved among different *E. faecium* strains and its potential as vaccine against various *E. faecium* strains. Interestingly, the reactivity of SagA protein against anti-*E. faecium* serum demonstrated the expression and immunogenicity of SagA during *E. faecium* infection as proven by the elicited anti-SagA antibodies in the serum. This confirms what other reports found about the fundamentality of SagA for *E. faecium* growth and virulence. Garcon *et al.* [21] concluded from the generation of a mutant strain that SagA appeared to be essential for *E. faecium* growth, while Paganelli *et al.* [22] confirmed its contribution to bacterial adhesion and biofilm formation. The high level of total IgG and IgG1 in SagA immunized mice indicates significant immunogenicity of the recombinant antigen. Unfortunately, the IgG2a response was not statistically significant when compared to the control group. IgG2a antibodies are opsonizing antibodies and are used as an important indicator for T helper type 1 (Th1) cell-mediated immune response[23, 24] . The significant response of total IgG in comparison to the non-immunized groups or pre-immunization was confirmed with ELISA. To ensure the absence of a high antibody response against the His-tag of the recombinant SagA, an indirect ELISA using irrelevant His-tagged proteins was done. This ELISA excluded the probability that the raised antibody was mainly against His-tag. Since the heart and kidneys are the main sites of *E. faecium* infections, they were subjected to *E. faecium* count determination after challenge. These infections are mainly infective endocarditis and UTI [6, 7]. Surprisingly, no significant differences of *E. faecium* count were found between immunized and non-immunized groups. This outcome apparently indicates the lack of protection against *E. faecium* in immunized mice. OPA was done to find out whether the SagA-induced antibodies could induce efficient complement deposition and subsequent opsonophagocytic killing of the encapsulated *E. faecium*. The result was found to be consistent with the *E. faecium* organ count where there was no significant difference between the immunized and the negative control groups.

The presence of capsules could explain the absence of a significant reduction in *E. faecium* count for both bacterial organ count and OPA. Even though the raised antibodies were specifically induced against SagA, these antibodies were not able to protect against *E. faecium* infection.

Anti-SagA was demonstrated by Kropec *et al.* [11], Kalfopoulou *et al.* [12], and Romero *et al.* [13] to be reactive with *E. faecium* and protective against its infections. However, in our study, the protective effectiveness of antibodies against recombinant SagA was not observed in a mouse infection model. The reason for this could be the presence of a polysaccharide capsule in the used *E. faecium* isolate, which may conceal protein antigens and reduce their ability to bind as well as their accessibility to phagocytes or complement machineries. This interpretation was also used by Romero *et al.* [25] to explain the minor opsonophagocytic killing of antigen-specific antibodies against some *E. faecium* strains.

The surface exposure of protein antigens in encapsulated bacteria is different among strains [26]. Hence, the significance of bacterial capsules in masking antibody recognition and preventing opsonization has been reported in many studies. The effective phagocytosis of encapsulated Gram-positive bacteria, such as enterococci, depends on the cooperation of two processes. Both complement and antibodies are necessary for the effective phagocytosis of enterococci by PMN in a protective immunological response [25]. Hyams *et al.* [27] confirmed that the capsule inhibits complement activity against *Streptococcus pneumoniae*. In addition, different enterococcal species were shown to differ in their sensitivity to PMN phagocytosis. In a research by Arduino *et al.*[20], a reduced PMN internalization and resistance to phagocytosis were found

in 13 of the 26 *E. faecium* studied strains. This event was found to be caused by a non-sialic acid carbohydrate structure of the capsule. Additionally, instances of phagocytic cells failing to eliminate enterococci have been documented. This could turn them into conduits for the transfer of enterococci to other organs. The systemic spread of intracellular enterococci may result from the immune system's incapacity to eradicate them. Furthermore, Ali *et al.* [14] showed that *E. faecalis* capsular polysaccharides possess the capacity to conceal the complement protein C3b that has been deposited on the microbial surface, thereby shielding the bacteria from opsonophagocytosis. In the current study, we demonstrated the in-vitro binding of serum antibodies from SagA-immunized mice with whole *E. faecium* cells **Figure 7** and **8**. Nevertheless, it seems that proper complement fixation and facilitation of phagocytosis were impeded by the organism's capsule. Hence, to address the effectiveness of enterococcal vaccinations, the opsonophagocytic assay, which combines these three elements, is a valid proxy of the protective immune response [28].

CONCLUSION

In conclusion, antibodies to SagA were naturally induced in an *E. faecium* mouse infection model and were able to bind SagA as shown by dot blot assay, suggesting its credibility as a promising vaccine candidate. Recombinant SagA is a potential immunogen that can induce a high level of IgG in mice. Despite the presence of a high antibody response, there was no protection when comparing the *E. faecium* count in both the heart and kidneys of the SagA-immunized and negative control groups. The presence of a polysaccharide capsule in the used *E. faecium* isolate might have contributed to the absence of protection by inhibiting opsonophagocytosis. The assessment of SagA as well as other antigens as vaccine candidates using both capsulated and non-capsulated *E. faecium* strains is highly encouraged. The use of SagA in conjugation with other capsular components as vaccine immunogens might provide the desired protection against a wide range of *E. faecium* strains.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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