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ORIGINAL ARTICLE

Impact of *sarA* Mutation on Immune System Evasion and Stress Response in *Staphylococcus aureus*

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ABSTRACT

Background: *Staphylococcus aureus is a major human pathogen responsible for a large number of infections. In S. aureus, SarA is an important global locus responsible for the regulation of virulence factors, as well as biofilm formation.* ***Objectives:*** *The aim of this work is to clarify the impact of SarA on biofilm formation, immune system evasion, as well as the survival of S. aureus under stress conditions.* ***Methodology:*** *A comparative study between S. aureus wild type strain, sarA mutant and complemented strains was established addressing the biofilm formation, opsonization, phagocytosis, as well as ability of the bacterium to survive in stressful environments including acidic pH, hyperosmotic and oxidative stress. The in vitro experiments were confirmed by challenging of mice via intraperitoneal injection with the wild type strain, sarA mutant and complemented strains.* ***Results:*** *Mutation of sarA diminished significantly biofilm formation. Moreover, this mutation resulted in a slight decrease in the deposition of the most important opsonin in complement-mediated immunity, named C3 on S. aureus cells. However, this mutation was associated with a significant enhancement of bacterial phagocytosis and killing by human neutrophils. Furthermore, this mutation altered bacterial survival in stressful conditions. It is also noteworthy that sarA mutation resulted in a significant higher survival rates during the challenging of mice.* ***Conclusion:*** *SarA plays a role as a key regulator of biofilm formation, which in turn has a great impact on immune system evasion through affecting opsonization and phagocytosis. In addition, SarA improves the ability of S. aureus to survive in stressful conditions.*

INTRODUCTION

Staphylococcus aureus is a Gram-positive commensal bacterium living in the nostrils, skin and hair of humans as well as other warm-blooded animals [1]. On the other hand, this bacterium is highly pathogenic leading to localized lesions such as skin abscesses and wound infections, in addition to wide range of systemic infections including osteomyelitis, endocarditis, pneumonia, bacteremia and toxemic syndromes including toxic shock syndrome (TSS), staphylococcal scarlet fever, staphylococcal scalded-skin syndrome (SSSS), and staphylococcal food poisoning [2, 3].

Virulence of *S. aureus* is a multifactorial, and the bacterium possesses many types of toxic factors including diverse range of extracellular proteins (e.g. proteases, hemolysins, toxic shock syndrome toxin 1, enterotoxins, exfoliatins, coagulase, staphylokinase, Panton-Valentine leukocidin) and cell wall-associated proteins (e.g., protein A and fibronectin binding protein)[4,5]. Moreover, the potential disease spectrum is coupled with the ability of the bacterium to form biofilm, which is considered to be one of the most relevant virulence factors that assists both colonization of the bacterium and immune system evasion [5, 6]. The regulation of virulence determinant production, including biofilm formation, in *S. aureus* involves several global regulatory loci; of these, SarA, a regulatory DNA binding protein involved in virulence gene expression, is relatively important [7, 8].

Indeed, the immune system is divided into the innate immunity (natural immunity) and adaptive immunity (acquired immunity) [9]. The complement system, a major humoral non-specific component of the innate immunity consisting of more than 40 proteins, plays a pivotal role in the immunity [10]. It recognizes foreign cells, removes cellular debris and is considered a key factor in the adaptive immunity activation [11]. Complement can be activated through three different cascades: classical, lectin and alternative pathways. These three pathways differ only in the way they get activated, but all lead to the activation of the central component named, C3 which is the most important opsonin deposited on foreign materials to aid in their phagocytosis [10, 11].

In the present study, we outlined the impact of *sarA* deletion mutation on immune system evasion in *S. aureus*. The effects of the mutation on biofilm

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formation was investigated. Moreover, we examined the effect of *sarA* mutation on complement C3 deposition, neutrophil-mediated phagocytosis and killing and virulence of the bacterium following *in vivo* mice challenge. In addition, we clarified the effect of *sarA* mutation on survival of the bacterium under different stress conditions.

METHODOLOGY

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich, USA.

Bacterial strains and culture conditions

In the present study, the *S. aureus* standard strain UAMS-1, its *sarA* deletion mutant strain, and the *sarA* gene complemented strain (kindly provided as a gift from Prof. Dr., Mark S Smeltzer, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences | UAMS, USA) were used. The strains were either cultured in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7) or Tryptic soy broth (TSB) medium (Difco™, USA). LB agar or TSB agar, as well as Mannitol salt agar (Oxoid, Thermo Fisher, UK), were used when required for streaking the strains to get pure colonies.

Culturing conditions and measurement of bacterial growth

S. aureus strains were cultured in either LB broth or TSB (5 ml) and incubated overnight at 37°C with shaking at 200 rpm. Then, an aliquot of the overnight culture was re-cultured in fresh LB or TSB broth and incubated at 37°C with shaking at 200 rpm.

Biofilm assay

Quantitative assay of biofilm formation in *S. aureus* standard strain UAMS-1, its *sarA* mutant and complemented strains were accomplished as described previously by Deka [13] in 96-well flat-bottomed polystyrene Microtitre plates (Grienerbione, Germany). The bound crystal violet with biofilm-forming cells was resolubilized by adding 150 µl of 33% (v/v) glacial acetic acid to each well and then the OD of the tested isolates was measured using a microtitre plate reader (Biotek instruments inc., USA) at 630 nm. In these experiments, wells receiving LB broth only or TSB only were used as negative controls. For each strain the mean of OD (ODT) of three wells was calculated and compared with cut-off OD (ODC) which is defined as three standard deviations (SD) above the mean of the negative control (3SD + mean). The amount of biofilm formed was scored as non-adherent (ODT ≤ ODC), weakly adherent (ODC < ODT ≤ 2 ODC)**,** moderately adherent (2 ODC < ODT ≤ 4 ODC), or strongly adherent (4 ODC < ODT).

C3 deposition assay

C3 deposition was evaluated as described previously by Kenawy et al. [14] on formalin fixed overnight culture of *S. aureus* strains. Bacterial suspensions of OD_{600} of 0.5 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) were used for coating wells of microtitre plate (Maxisorp, Nunc, Sigma-Aldrich, USA). Zymosan at a concentration of 1 µg per well was used as a positive control. A blocking solution of 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS; 10 mM Tris, 140 mM NaCl, pH 7.4) and washing buffer of TBS containing 0.05% Tween 20 and 5 mM $CaCl_2$ were used in the assay. Blocking of the Fc receptors of *S. aureus* was done by adding 100 µl of 1/2000 heat-inactivated sheep serum for 1 h at room temperature. Thereafter, two-fold serial dilutions of human serum were prepared in TBS starting with 1/80 dilution. Serum dilutions were added in duplicates to the corresponding wells. Negative control wells of formalin fixed *S. aureus* were used, where serum was replaced by the buffer used for serum dilution. Rabbit anti-human C3b antibody (Dako, Bloomberg, USA; 1/5000) and alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma Aldrich; 1/10000) served as primary and secondary antibodies, respectively. After washing the plates, the substrate p-nitrophenyl phosphate (pNPP, Sigma fast p-nitrophenyl phosphate tablets) was added and absorbance at 405 nm was measured using Microtitre plate reader (Biotek instruments inc., USA).

Opsono-phagocytosis and killing assays

Phagocytosis assay was performed according to Kenawy et al. [14]. In brief, human peripheral blood polymorphonuclear leukocytes (PMNs) were isolated in accordance to Sigma-Aldrich protocol using histopaque-1119 and histopaque-1077. The layer containing polymorphonuclear leukocytes (PMNs) was carefully withdrawn and PMNs were washed twice in DMEM (Dulbecco's Modified Eagle's medium). Residual RBCs were lysed using hypotonic conditions, where cell pellet was resuspended in 2 volumes of sterile distilled water and 1 volume of PBS and incubated for 5 min. One volume of sterile 2.7% NaCl solution was then added. PMNs were separated by centrifugation at 200 xg for 10 min, washed once in DMEM and then resuspended in DMEM to a final concentration of 8×10^6 cell/ml.

In each phagocytosis and killing assay, 250 µl of prepared PMNs ($2x10^6$ cell) were incubated at 37°C with 250 µl of pre-opsonised microbe ($2x10^6$ CFU) in 24-well plate on an orbital shaker at 80 rpm. Wells containing pre-opsonised microbe only were used as negative control, where PMNs were replaced with DMEM only. Samples from each phagocytosis reaction were taken at time points 0, 30, 60 minutes for analysis. Phagocytosis was stopped by incubating samples on ice and assessed through viable count determination of surviving microbe in each phagocytosis reaction following incubation with PMNs. Ten-fold serial dilutions of each sample were prepared in normal saline and then plated onto nutrient agar plates. Viable count was determined in triplicate using surface drop method.

Survival of *S. aureus* strains under different stress conditions

Survival assays were established as described previously by Elgaml and Miyoshi [15]. Bacterial suspensions of 1×10^8 CFU/ml of overnight culture in LB broth or TSB were exposed to different stress conditions while incubated at 37°C. At predefined time points, aliquots were taken from each tube, to determine the survival through viable count determination of surviving microbes. The test stress conditions included acidic (pH 4.0), hyperosmotic (2.7 M NaCl) or oxidative (2 mM H_2O_2) conditions. In these experiments, pH 7.5, 0.35 M NaCl and 0 mM H_2O_2 were used as non-stress conditions, respectively.

***In vivo* survival experiments**

All animal experiments were approved by the local institutional ethics committee and licensed by Faculty of Pharmacy, Mansoura University. *In vivo* experiments were performed as described previously by Sunagar et al. [16]. Seventy BALB/c female mice aged 6–8 weeks old (body weight, 20-25 gm) were divided into 7 experimental groups each experimental group included 10 mice. *S. aureus* strains were cultured overnight in 10 mL LB broth or TSB media at 37°C, washed twice in saline and finally resuspended in saline to yield a bacterial suspension of 5×10^8 CFU/ml. Each mouse was injected i.p. with 0.2 ml of the prepared bacterial suspension. One group was injected with saline only as a negative control. The test animals were observed for 72 h.

Statistical analysis

Statistical analysis was performed using GraphPad prism version 5 software, where unpaired t-test was used in all experiments except for the survival experiment, where Log-rank (Mantel–Cox) test was used. The *P* values less than 0.05 were considered significantly different.

RESULTS

Impact of culturing media and *sarA* mutation on biofilm formation

The standard strain UAMS-1, its *sarA* mutant and the complemented strain were assayed for the biofilm formation after culturing in LB broth. All strains were found to be weak biofilm producers or even non adherent. Changing the culturing medium into TSB was associated with a significant increase in biofilm production (Fig. 1).

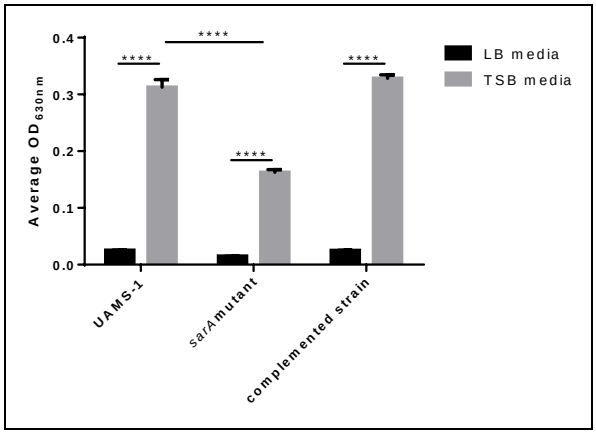

Fig. 1: Impact of culturing media and *sarA* mutation on the biofilm formation in *S. aureus*.

Biofilm formation was quantitatively assayed in the *S. aureus* standard strain UAMS-1, its *sarA* mutant and complemented strains. The strains were assayed for their biofilm formation after culturing in both LB broth (black bar) and TSB (grey bar). Using TSB as the culturing medium was associated with a significant increase in biofilm production, where, *sarA* mutation resulted in a significant decrease in the biofilm formation in the *sarA* mutant strain compared to both the wild type UAMS-1 and the *sarA* complemented strains. In these experiments LB or TSB only was used as negative controls. The data is the mean ± S.D. of three experiments. **** $P < 0.00001$.

The mean OD (ODT) of the three wells was calculated for each strain and compared with the cut-off OD (ODC), which is calculated to be 0.106, then; the strains were categorized as strong, moderate, and weak biofilm producers after culturing in TSB. As seen in Fig. 1, *sarA* mutation resulted in a significant decrease in biofilm formation in the *sarA* mutant strain, which was classified as weak biofilm producer, compared to the wild type UAMS-1, which was classified as moderate biofilm producer. The reduction of the biofilm formation in the mutant strain was restored in the *sarA* complemented strain.

Impact of *sarA* mutation on the C3 deposition on *S. aureus* strains

C3 deposition is a crucial step to mark the pathogens before phagocytosis. The standard strain UAMS-1, its *sarA* mutant and the complemented strain were assayed for the C3 deposition after culturing in LB broth or TSB media. In all strains, using TSB as culturing media significantly enhanced C3 deposition when compared to LB broth (Fig. 2).

Mutation of *sarA* resulted in a slight decrease in the C3 deposition in case of *sarA* mutant strain compared to the wild type UAMS-1. This decrease in the C3 deposition in the mutant strain was restored in the *sarA* complemented strain (Fig. 2).

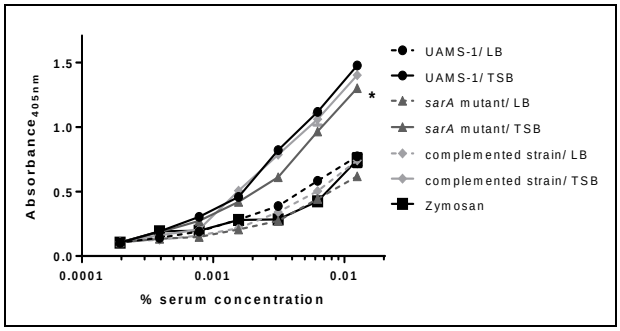

Fig. 2: Impact of culturing media and *sarA* mutation on the C3 deposition on *S. aureus*.

The strains UAMS-1, its *sarA* mutant and its complemented strain were assayed for C3 deposition following culturing in both LB broth and TSB media. Dilutions of human serum under buffer conditions that allow complement activation through both the classical and lectin pathways were used. The *sarA* mutation resulted in a slight, however, significant decrease in the C3 deposition when compared to both the wild type UAMS-1 and the *sarA* complemented strain. Using TSB as culturing media significantly enhanced C3 deposition on the surface of all tested strains compared to LB broth. Zymosan was used as a positive control. Negative control wells of formalin fixed strains were used as a negative control, where the serum was replaced by the buffer used for serum dilution. The results are means of duplicates for each strain and are expressed as means ± SD. * $P < 0.05$.

Impact of *sarA* mutation on phagocytosis and killing of *S. aureus* strains

The effect of *sarA* mutation on the opsonophagocytosis and killing of the UAMS-1, its *sarA* mutant and the complemented strain were assayed after culturing of the strains in LB broth or TSB. In contrast to biofilm formation and C3 deposition, culturing all of the tested strains in LB broth induced their phagocytosis and killing by human neutrophils (Fig. 3).

In case of *sarA* mutant strain, the mutation significantly enhanced the susceptibility of bacterial cells to phagocytosis compared to both the wild type UAMS-1 and the *sarA* complemented strain when cultured in TSB (Fig. 3).

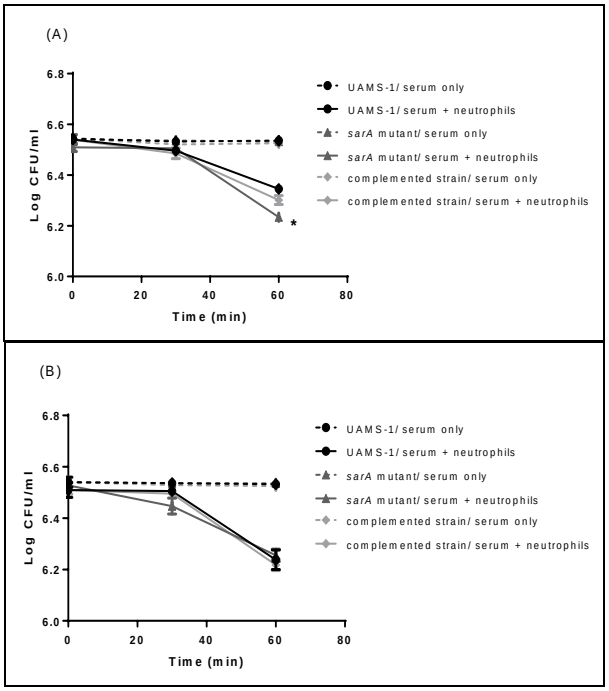

Fig. 3: Impact of culturing media and *sarA* mutation on the phagocytosis and killing of *S. aureus.*

Viable counts of bacterial strains were determined following opsonophagocytosis by human neutrophils. **(A)** shows a significant decrease in the viable count of the *sarA* mutant compared to both the standard UAMS-1 and the *sarA* complemented strain when using TSB as a culturing medium. In **(B)**, viable counts of all tested strains were similar following the opsonophagocytosis, when bacteria were cultured in LB broth. Pre-opsonised bacteria only were used as negative controls, where PMNs were replaced with DMEM only. The results are expressed as means ± SD of 3 independent experiments. * $P < 0.05$.

Impact of *sarA* mutation on the survival of *S. aureus* under different stress conditions.

The impact of SarA on the survival of *S. aureus* under various environmental stresses was established by exposing the wild type strain, *sarA* mutant and complemented strains to diverse stresses, then determining their survival rates (Fig. 4). The *sarA* mutant strain was significantly more sensitive to the acidic condition, pH 4.5. In the hyperosmotic condition, 2.7 M NaCl, SarA enhanced the bacterial survival remarkably. The viable count of *sarA* mutant rapidly decreased, while the wild type strain and the complemented persisted at high levels. Moreover, the mutant strain was also much more sensitive to 2 mM H_2O_2. These results may suggest that SarA is vital for *S. aureus* to survive under the stressful conditions.

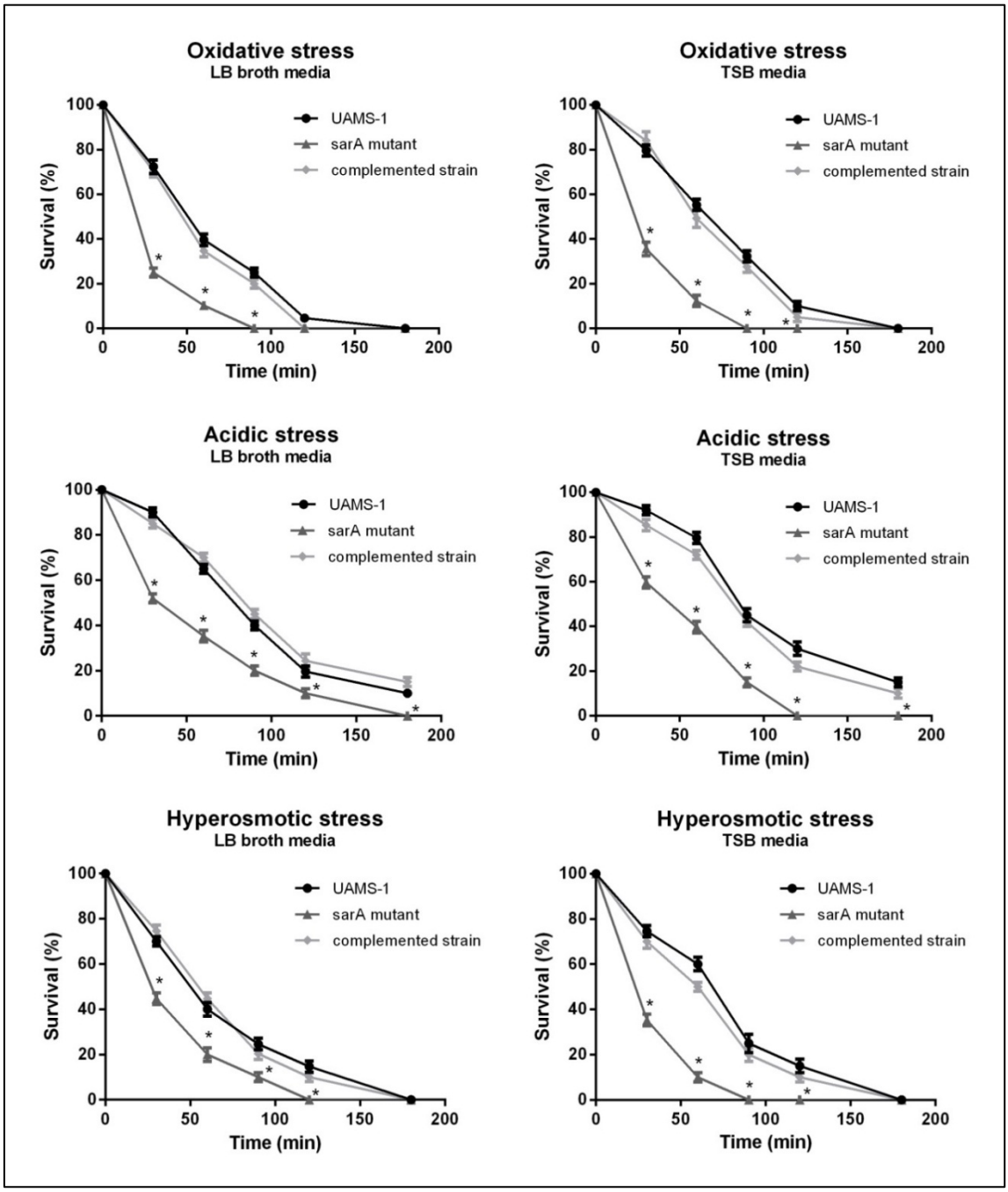

Fig. 4: Survival of *S. aureus* to acidic, hyperosmotic or oxidative stress

S. aureus strain UAMS-1, its *sarA* mutant and its complemented strain were subjected to the acidic (pH 4.5), hyperosmotic (2.7 M NaCl), or oxidative (2 mM H_2O_2) conditions after culturing in LB broth or TSB. Aliquots were taken, at predefined time points, to determine the survival rates. The *sarA* mutant strain was significantly more sensitive to all applied stresses than the wild type and complemented strains. All strains showed a significant higher resistance to the applied stresses, when cultured in TSB than in LB broth. The data is the mean ± SD of three independent experiments. Non-stress conditions; pH 7.5, 0.35 M NaCl and 0 mM H_2O_2, after culturing in either TSB or LB broth, showed no decrease in viable counts of all bacterial strains. * $P < 0.05$.

Impact of *sarA* mutation on survival of mice following the challenge with *S. aureus*

As shown in Fig. 5, *sarA* mutation significantly altered the survival rates in the challenged mice when bacteria were cultured in TSB, where there was a significant drop in survival rates in case of wild and complemented strain compared to *sarA* mutant strain starting from 12 hours till 72 hours following the challenge. By the end of the experiment the survival rates were 100%, 60%, 70% in case of *sarA* mutant strain, standard UAMS-1 strain, and complemented strain, respectively. On the other hand, using LB broth as a culturing media for the tested bacterial strains altered the survival rates dramatically, where survival of mice after challenge was 100% in all of the tested strains.

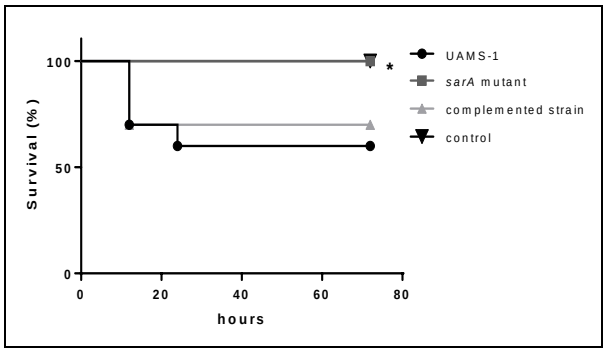

Fig. 5: Impact of *sarA* mutation on survival rates of mice following the challenge with *S. aureus*

Following the challenge of mice (10 mice per group) with *S. aureus* strains cultured in TSB (i.p. injection of 1×10^8 CFU/mouse), the survival rates of mice in each group was recorded. Mice challenged with the wild type bacterial strain UAMS-1, showed the lowest survival rate with only six mice surviving the bacterial challenge following 24 h post-infection. The *sarA* mutant strain was the least virulent strain for mice, where all the mice survived the bacterial challenge. The complemented strain showed similar virulence to the wild type strain, where seven mice only survived the bacterial challenge. For the negative control experiment, mice received saline only. * $P < 0.05$.

DISCUSSION

S. aureus may cause severe infections utilizing extracellular and cell wall associated virulence factors, among those, biofilm formation is relatively important during distinct stages of infection [2, 3, 17]. Regulation of virulence gene expression is crucial to successful infection [18]. This regulation, at both transcription and translation levels, is mediated by global regulatory elements that switch on selective sets of genes as a consequence to variable host environments [18, 19, 20]. Among these regulatory elements, the DNA binding protein SarA is relatively important [21]. SarA plays vital roles in regulating target genes either directly by binding to their promoters or indirectly through downstream effects on regulons (e.g. binding to the *agr* promoter) or by stabilizing mRNA over the log phase [21, 22].

In agreement with a previous finding [23], our data shows that culturing conditions and media have a great impact on *S. aureus* ability to form biofilm, where using TSB has significantly increased biofilm formation in all of the tested strains compared to LB broth. Our results confirm a previous study [12] and show that *sarA* mutation greatly decreased the ability of the bacterium to form biofilm. In addition, reduction of biofilm formation was reported by using a novel small molecule inhibitor targeted to interact with *sarA*, thus hindering its positive control towards genes that up-regulate biofilm formation [24].

Phagocytosis and killing of bacteria require opsonization [10]. Previous studies have supported that C3 protein is the principal opsonin in complement-mediated immunity [10, 11]. Biofilm formation in bacteria has been shown to interfere with this process, where previous studies showed that sera containing complement opsonins are able to effectively kill bacteria in planktonic state but not in the biofilm state [25]. Our data shows that *sarA* mutation significantly affected phagocytosis and killing of *S. aureus* by human neutrophils using TSB culturing medium. Interestingly, *sarA* mutation slightly decreased the C3 deposition levels on the outer surface of bacterial cells, however, it significantly increased their phagocytosis and killing by human neutrophils compared to UAMS-1 and *sarA* complemented strains. This reduction in C3 deposition, could be explained by the negative effect of *sarA* mutation on biofilm formation capabilities compared to both the wild type UAMS-1 and *sarA* complemented strains that alters cell surface structure. The slightly higher C3 deposition observed in the wild type and complemented strains could be attributed to the entrapment of more C3 fragments within the dense extra-cellular polysaccharides (EPS) matrix [26, 27]. Thus, the entrapment of the deposited C3 fragments within the EPS matrix make them inaccessible to C3 receptors of phagocytes (complement receptor 3, CR3) that lead to the increased resistance of the wild type and complemented strains to phagocytosis compared to the mutant strain [26, 27]. Culturing of bacteria in LB (basal medium) was associated with lowered C3 deposition level compared to TSB as a result of not only reduced biofilm formation, but also change in bacterial surface composition. However, the lower level of C3 deposition on bacteria was sufficient to drive efficient phagocytosis and killing, with no observed significant difference among all of the tested strains.

Furthermore, our results show that SarA affected the survival of *S. aureus* under various stressful conditions. Specifically, *sarA* mutant showed higher sensitivity towards the acidic, high osmotic, or oxidative condition than the wild type and its complemented strains. Mostly important was its higher sensitivity to the oxidative stress. During infection, reactive oxygen species may be produced due to the oxidative burst of host macrophages; consequently, the persistence and survival of pathogens in the human host may be highly attributed to the oxidative resistance [28]. Additionally, these results may indicate the effect of SarA in the ability of *S. aureus* to endure the higher osmolarity conditions during skin infections [29] or higher acidic conditions in human stomach before enteric infections [30].

Our results obtained from the *in vivo* survival experiments in mice with these test bacterial strains support results obtained from the *in vitro* experiments. *SarA* mutation significantly reduced the virulence of *S. aureus* (cultured in TBS), when injected into the peritoneal cavities of mice compared to the wild type (UAMS-1) and complemented strains. These results are in accordance with a previous study [12], where UAMS-1 showed increased biofilm formation capacity than the *sarA* mutant using a murine model of catheter-associated biofilm formation. Moreover, our results confirmed the results of another study [31], where *sarA* mutation significantly attenuated virulence of *S. aureus* in a murine bacteremia model. Additionally, our results agreed with another study [32], that outlined the impact of *sarA* mutation in limiting virulence of *S. aureus* in a posttraumatic bone infection model. Interestingly, preparing *S. aureus* in LB broth had greatly diminished virulence all of the three bacterial strains, where all of the challenged mice survived, which may indicate that under these condition bacteria were susceptible to host immune destruction. These results were in consistence with a previous study [33] that outlined the importance of using nutrient-rich media, such as TSB, in all *in vitro* and *in vivo* assays. This study also confirmed the absence of *sarA* role in biofilm production upon using nutrient-deficit media leading to reduced virulence of *S. aureus*.

Overall, it could be concluded that SarA in *S. aureus* is a key regulator of the biofilm formation which plays important role in immune system evasion through affecting opsonisation and phagocytosis-mediated killing by human neutrophils. Additionally, as a global regulator, SarA may be involved in the regulation of many processes required for enduring many environmental stresses. This would open up new perspectives for the antimicrobial chemotherapy in *S. aureus* using key inhibitors of SarA to overcome the problems of antimicrobial resistance and immune system evasion of this pathogen.

Conflicts of interest:

The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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