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ORIGINAL ARTICLE

Antimicrobial Effect of Platelet Rich Plasma and Platelet Gel against *Staphylococcus aureus* Isolated from Surgical Site Infections: An *In Vitro* Study.

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ABSTRACT

Background: *There is growing interest in platelets concentrates contribution to antimicrobial host defense functions.* ***Objective:*** *This study aims to investigate the in vitro antimicrobial and antibiofilm properties of platelet rich plasma (PRP) and platelet gel (PG) against S. aureus isolates recovered from Surgical Site Infections (SSIs).* ***Methodology:*** *A total of 200 SSI specimens were collected. S. aureus isolates were identified and biofilm producers were detected by modified tissue culture plate method. The isolates were checked for antibiotic susceptibility by Kirby-Bauer disc-diffusion method. Alamar blue (AB) susceptibility assay was applied to test the killing effect against planktonic and biofilm cultures of S. aureus. Time kill assay was performed during 24 hours period.* ***Results:*** *Out of 40 S. aureus isolates, 80% were biofilm producers which show more resistance to antibiotics than non-biofilm producers. However, there was non-significant difference between the effect of platelet poor plasma (PPP), PRP and PG between biofilm and non-biofilm producing planktonic S. aureus. Using AB assay, the viability of S. aureus within biofilm was reduced by 28%, 29% and 46% after 24 hours of treatment with PPP, PRP, and PG respectively. In time kill assay, bacterial count was reduced after 2 and 4 hours and was increased again after 24 hours. PG and PRP had more significant antimicrobial effect than PPP. PG had more significant antimicrobial effect than PRP.* ***Conclusion:*** *Antimicrobial effect of PG is more potent than PRP against S. aureus and both components have similar antimicrobial effect on biofilm and non-biofilm producing S. aureus.*

INTRODUCTION

Surgical site infections (SSIs) are one of the most important causes of health-care associated infections (HAIs), which are responsible for the increasing costs, morbidity and extended hospital stay with difficulty in treating multi-drug resistant microorganisms' infections[1].

In a previous study performed in Egypt [2], Staphylococcal species represented 64.8% among collected isolates; from whom 61% were *Staphylococcus aureus (S. aureus)* which is a virulent strain that resists most of the conventionally prescribed antibiotics and it is considered to be one of the most clinically significant pathogens involved in biofilm-associated infections [3]. There is an association between biofilm production with persistent infection and antibiotic therapy failure. So, identification of infection caused by biofilm producing *S. aureus* might help to modify the antibiotic therapy and prevent infection [4].

Platelet rich plasma (PRP) is a potent and effective strategy used to prevent SSIs due to its useful properties in wound healing process and its antimicrobial efficacy against many SSIs causing organisms[5]. It is believed that the use of PRP is more advantageous than conventional antibiotic treatments because PRP is less likely to induce antibiotic resistance and its antimicrobial and healing-promoting properties may have a synergistic effect on infection prevention [6]. PRP antimicrobial properties is attributed to its content of multiple antimicrobial peptides which possess broad-spectrum antimicrobial activity against both Gram positive and Gram negative bacteria [7].

PRP is a hemoconcentrate enriched in platelets and contains a high concentration of viable growth factors and neutrophilic polymorphonuclear leukocytes which play a crucial role in innate immune defense against infections[8]. Platelet rich gel is not only a platelet concentrate but also an immune node able to stimulate defense mechanisms [9]. Platelets have multiple functions include navigation toward the inflammatory chemoattractant, expression of Fc and complement $C3_a$/$C5_a$ receptors, and generation of antimicrobial oxygen metabolites including superoxide, hydrogen

peroxide, and hydroxyl free radicals. In addition, platelets participate in antibody-dependent cell cytotoxicity against microbial pathogens [10].

PRP usage as a prophylactic therapy will reduce wound complication which will reduce SSIs incidence, as well as provide improved healing benefits [11].

The aim of this study was to determine the *in vitro* antimicrobial and antibiofilm properties of PRP and platelet gel against *S. aureus* isolates recovered from SSIs.

METHODOLOGY

Study type:

This is a comparative cross sectional analytical study.

A total of 200 specimens (1 specimen per patient) were collected from patients with clinical SSIs in Suez Canal University Hospital, Ismailia, Egypt; from September 2013 to September 2014. All age groups and both sexes were included in the study.

SSI was defined as the development of general and local signs and symptoms indicating infection within 30 days after surgical intervention if no implant is left in place or within one year if implant is in place and the infection appears to be related to the operation [12].

Data were collected from each patient after obtaining their written consent to participate in the study. Data included age, sex, length of hospital stay, use of invasive medical devices, receiving antibiotics and the general surgical or medical problem necessitating admission.

A study approval was obtained from the ethics committee of the Faculty of Medicine, Suez Canal University, Egypt and the Hospital Management Board.

Specimen collection:

Wound swabs:

Representative samples were collected according to Baranoski and Ayello[13]. Swabs were transferred to the laboratory on trypticase soya broth transport medium.

Wound fluid sampling:

When a copious volume of wound fluid exists, sampling by needle aspiration was employed according to wound culture protocols [14].

Wound specimens were inoculated onto mannitol salt agar and blood agar (Becton Dickinson, Cockeysville, Maryland, USA) and then incubated aerobically at 37^{o}C for 24-48 hours.

Typical colonies of *S. aureus* were selected and then subjected to the conventional biochemical analysis and gram stain [15].

Detection of biofilm producing S. aureus isolates using Modified Tissue Culture Plate (TCP) method:

TCP assay was done according to method described by Stepanovic and colleagues. [16]

PRP preparation:

Eight healthy donors were recruited from Suez Canal University Hospital Blood Bank and consents prior to blood collection to use the platelet concentrates for research purposes of this study were obtained. Their ages were more than 18 years, both sexes were included. They were unmedicated. Their platelets counts were more than 150,000/µl.

Platelet concentrates were prepared from the whole blood within two hours of collection. The whole blood was centrifuged at soft spin 750 x *g* for 7 minutes at 22°C (Centrifuge: Thermo Sorvall TM RC 12BP, Thermo Fisher Scientific, USA) to separate it into a phase of red blood cells and a clouded phase containing buffy-coat, platelets and plasma. Red blood cells were separated in the original bag and all accepted packed red cell units were used in the blood bank center and the resulting plasma and buffy-coat were transferred to a separate bag through a closed circuit. A second centrifugation step was done as a hard spin at 5,300 x *g* for 10 minutes at 22°C. All supernatant platelet poor plasma (PPP) was transferred to the third satellite bag and the remaining platelets concentrate (PRP) was left with 20–30 mL of plasma for suspension.

Determination of platelets and leukocytes count:

Platelets count, and white blood cell counts were assessed in whole blood, PPP and PRP with a fully automated hematology analyzer (Sysmex KX-21 cell counter, Kobe, Japan), on two mL sample taken under sterile conditions.

Thrombin preparation:

Thrombin was obtained by collecting 10 mL of whole blood from the donor into two plain vacutainer tubes, centrifuged at 3,000 x g for 10 minutes; the serum was separated under a flow hood.

Activation of PRP

Ten minutes before use, PRP was activated by 10 % calcium gluconate in a ratio (5:1) for 10 minutes.

Preparation of platelet gel (PG)

PG was prepared 20 minutes before use. A 0.8 mL of 10 % calcium gluconate and 2 mL of thrombin were added to 4 mL of PRP in a sterile petridish and then incubated at 37°C for 10 minutes.

Determination of antimicrobial and anti-biofilm activity of PRP and PG:

1- Antibiotic susceptibility testing by Disk Diffusion method:

Antimicrobial susceptibility of *S. aureus* isolates was performed using disc diffusion method according to modified Kirby-Bauer technique [17] on Muller Hinton agar (MHA) (Becton Dickinson, Cockeysville, Maryland, USA) and interpretation of zone diameters was performed as recommended by CLSI [18], antibiotics tested were chosen to represent different classes for typing of isolated strains according to susceptibility profiles.

2- Alamar Blue (AB) biofilm susceptibility assay:

AB assay was performed according to the method described by Petit et al [19], briefly, isolated colonies from MHA plates were used to prepare inocula. The organism was transferred from four to five colonies to a 5 ml Cation Adjusted Muller Hinton Broth medium (CAMHB) (Becton Dickinson, Cockeysville, Maryland, USA) to obtain suspension with an optical density equal to 0.5 McFarland (1×10^8 CFU/mL). Bacterial suspensions were diluted 1:200 in CAMHB to target an inoculum concentration of approximately 5×10^5 CFU/ml.

AB (Trek Diagnostic System) Assays were performed in flat bottom, polystyrene, non-tissue culture treated microtiter plates containing 100 µl of 5×10^5 CFU/ ml CAMHB. Plates were covered and incubated at 37°C without shaking. After 24 hours, PPP, activated PRP and PG in CAMHB were prepared external to the plates. One hundred µl of the appropriate PPP, PRP and PG were added, and then incubated at 37°C without shaking. After 20 hours, 10 µl AB (0.67 g/100 ml distilled water) was added to the wells (210 µl total volumes) and the plates were shaken gently, covered and incubated for 1 hour at 37°C. Plates were gently shaken again, and absorbance at 540 nm and 630 nm were obtained in microplate reader. Controls included media alone, media plus AB, media plus AB plus PPP, media plus AB plus PRP, media plus AB plus PG, and bacterial cells plus media plus AB. Percent reduction of AB were calculated [19]**.**

The AB minimum biofilm inhibitory concentration (MBIC) was defined as the lowest PG or PRP concentration resulting in ≤ 50% reduction of AB with purple/blue well 60 minutes after the addition of AB. Assays were performed twice and the average percent reduction used to determine the AB MBIC

Alamar blue planktonic susceptibility assay:

Planktonic susceptibility testing of *S. aureus* isolates was performed by the CLSI reference broth microdilution assay [20]. Assays were performed twice, and the average percent reduction was used to determine the MIC.

Time kill assay:

After seeding on Blood Agar medium a suspension of CAMHB was prepared for each strain, with an optical density equal to 0.5 McFarland (1×10^8 CFU/mL). Bacterial suspensions were diluted 1:100 in MHB to target an inoculum concentration of approximately 1×10^6 CFU/ml. One hundred µl of each suspension were inoculated in a round-bottom, polystyrene, non-tissue culture-treated microtiter plates containing 100µl of PPP, PRP, PG and thrombin to obtain final concentration equal to 5×10^5 CFU/ ml.

For each strain, a positive control was performed, which consists of an inoculum of the bacterial suspension in CAMHB without treatment and negative controls consists of CAMHB medium alone, CAMHB plus PPP, CAMHB plus PRP and CAMHB plus PG (to ensure the sterility). After 0, 2, 4, 6 and 24 hrs incubation at 37°C, a 10 µl sample was taken from each well. Serial 10-fold dilutions of each sample were made, and 10 µL samples were plated on MHA plates. (Thrombin containing wells were only cultured after 24hrs). After 24 hrs incubation at 37°C, the number of viable bacteria was determined [21].

Initial inoculums size at time zero were determined to confirm that appropriate initial bacterial density was tested ($5x\ 10^5$ CFU/ml). Colonies on each of serial dilution bacterial count plates were counted. The count was converted to actual CFU/ml by multiplying average of bacterial counts by dilution factors**.** The assay was performed in duplicate for each strain and, Results were expressed as mean ± standard deviation.

Statistical analysis

Data were reported as mean ±standard deviation. Statistical analysis was performed using SPSS Statistics for Windows (Version 17.0. Chicago: SPSS Inc) using repeated measures analysis for antibacterial activity and one-way ANOVA for platelet count. P values ≤ 0.05 were considered statistically significant.

RESULTS

From a total of 200 collected specimens, forty *S. aureus* isolates (20%) were identified, of which MRSA was the most common, constituting 95% of isolates. Age distribution of the 40 SSI patients with *S. aureus* was 17 (42.5%) from the middle age group (36 – 60 years), 12 (30%) from 18 -35 years, 4 (10%) from 2 -17 years and 4 (10%) less than 2 years while the elderly group (> 60 year) was 3 (7.5%). Male to female ratio was 1.2:1

About 57.5% of patients with *S. aureus* were from surgery department while 12.5, 12.5, 7.5, 5 and 5% were from orthopedics, burn, neonatal ICU, neurosurgery and ICU departments.

Platelets and White Blood Cells Count:

The mean platelets count was $239.37 \pm 58.62 \times10^9$/L in whole blood, and significantly increased to $1147.89 \pm 296.08 \times10^9$/L in PRP with an average 4.7 fold enrichment of platelet concentration after processing. The mean white blood cell count of whole blood was $5.93 \pm 1.4 \times10^9$/L, and was markedly reduced to $0.84 \pm 0.43 \times 10^9$/L in PRP. In PPP, the mean platelets count was $45.25 \pm 25.5 \times10^9$/L and the mean white blood cell count was $0.27 \pm 0.15 \times 10^9$/L.

Biofilm production of S. aureus isolates by Modified TCP method:

Thirty-two (80%) were found to be biofilm producers, from whom 15 (37.5%) were interpreted as strongly adherent and 17 (42.5%) were moderately adherent. Eight strains (20%) were found to be non-biofilm producers. The two reference strains, ATCC

35984 and ATCC 12228, were found to be positive and negative respectively.

In vitro antibiotic susceptibility results for biofilm and non-biofilm producing S. aureus.

Antibiogram studies were done as described by Kirby Bauer Disc Diffusion technique[17]. The antibiotic resistance exhibited by biofilm producers and non-producers against selected antibiotic agents was analyzed and presented in **Table 1**. All biofilm producers and non-biofilm producers were sensitive to Linezolid. It was found that biofilm producing isolates showed more resistance than non-biofilm producing ones to the following antibiotics; chloramphenicol (12.5% vs 0%), clindamycin (18.75% vs 0%), rifampin (25% vs 0%), and gentamycin (43.75% vs 37.5%).

Table 1: Antibiotic susceptibility results (%) of biofilm producing and non-biofilm producing *S. aureus* isolates.

Antimicrobial Agent	Non-biofilm producers (n=8)			Biofilm producers (n=32)		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Cefoxitin	0	0	100	25	0	75
Tetracycline	53.2	0	46.8	37.5	12.5	50
Chloramphenicol	84.38	3.12	12.5	87.5	12.5	0
Gentamycin	50	6.25	43.75	37.5	25	37.5
Erythromycin	0	62.5	37.5	25	37.5	37.5
Clindamycin	78.13	3.12	18.75	75	25	0
Ciprofloxacin	71.88	3.12	25	75	0	25
Trimethoprime/ sulphamethoxazole	59.37	18.75	21.88	37.5	25	37.5
Rifampin	75	0	25	100	0	0
Linezolide	100	0	0	100	0	0

Antimicrobial effect of PPP, PRP, and PG against biofilm vs. non-biofilm producing planktonic S. aureus (after 24 hours) using colony count method.

There was non-significant difference between the effect of PPP, PRP and PG between biofilm and non-biofilm producing planktonic *S. aureus* (p value > 0.05) (Table 2).

Table 2: Results of antimicrobial effect of PPP, PRP, and PG against biofilm and non-biofilm producing planktonic *S. aureus* using colony count method.

Component	Biofilm producers (n=32)		Non-biofilm producers (n=8)		*P value*
	Bacterial count (CFU/ml) ($\times 10^8$)	% Reduction	Bacterial count (CFU/ml) ($\times 10^8$)	% Reduction	
Control	28 ± 2.4		28 ± 2.2		**0.54**
PPP	14 ± 1.6	46.4	15 ± 1.9	50	**0.36**
PRP	8.9 ± 0.20	67.8	9 ± 0.22	68.2	**0.17**
PG	6.6 ± 0.14	76.7	6.5 ± 0.24	76.4	**0.68**

PPP: platelet poor plasma, PRP: platelet rich plasma, PG: platelet gel. CFU: Colony forming units

The effect of PPP, PRP, and PG on S. aureus biofilm using AB reduction:

There was a significant effect of PPP, PRP and PG on *S. aureus* biofilm (p value ≤ 0.001), as the AB reduction percentage were 72% ± 8.5, 71% ± 13.9, and 54% ± 11.4 respectively compared to control well.

The metabolic activities of living cells resulted in AB reduction, so the increasing of AB reduction percentage means more existence of living cells and less effect of antimicrobial agent used. This indicates that the viability of *S. aureus* within biofilm was reduced by 28%, 29% and 46% after 24 hours of treatment with PPP, PRP, and PG respectively. Both PPP and PRP had similar antimicrobial effect on *S. aureus* biofilm (p value > 0.05), on the other hand PG had a significant difference in reduction percentage when compared with PPP and PRP (p value < 0.001).

The effect of PPP, PRP, and PG on S. aureus planktonic cells using AB reduction:

After 24 hours, AB reduction percentage for PPP, PRP, and PG were 61% ±7.37, 33% ± 10.59, and 30% ± 8.165 respectively. These results indicate that bacterial count of viable cells was reduced by 39%, 67%, and 70% for PPP, PRP and PG respectively.

Time kill of S. aureus during 24 hours period

Significant differences were found when comparing PPP, PRP, and PG with control after 2, 4, 6 and 24 hours. Bacterial count was reduced after 2 and 4 hours and was increased again after 24 hours. There was a significant difference when comparing PRP and PG with PPP group, and by comparing PG with PRP after 2, 4, 6, 24 hours (table 3).

Table 3: Time kill of *S. aureus* during 24 hrs period using plate count technique.

Time intervals (hours)		Bacterial count CFU/ml (Mean ± SD)				*P* value
		Control	PPP	PRP	PG	
0	$\times 10^5$	5	5	5	5	
2	$\times 10^5$	8.5±0.2	4.5 ± 0.2	4.3± 0.2	3.1 ±0.2	<0.001*
4	$\times 10^7$	3.2±0.2	1.3 ±0.2	0.7 ±0.02	0.5 ±0.02	<0.001*
6	$\times 10^8$	1.0±0.2	0.49 ±0.02	0.3 ±0.02	0.2 ±0.02	<0.001*
24	$\times 10^9$	2.3 ±0.2	1.5* ±0.2	0.9* ±0.02	0.7* ±0.02	<0.001*

PPP: platelet poor plasma, PRP: platelet rich plasma, PG: platelet gel. CFU: Colony forming units

PRP has a slower and less strong decrease in the absolute number of bacteria than PG. The strong antimicrobial effect of PG seems to be limited to the first hours after application. (Table 4, Figure 1).

Table 4: Percentage reduction of the absolute number of bacteria compared to control after 2, 4, 6, 24 hours using colony count.

Time intervals (hours)	Reduction percentage (%)		
	PPP	PRP	PG
2	47.3%	49.4%	63.2%
4	58.1%	78.5%	84.2%
6	50.2%	70.9%	79.1%
24	46%	67.7%	76.3%

PPP: platelet-poor plasma; PRP: platelet-rich plasma; PG: platelet gel.

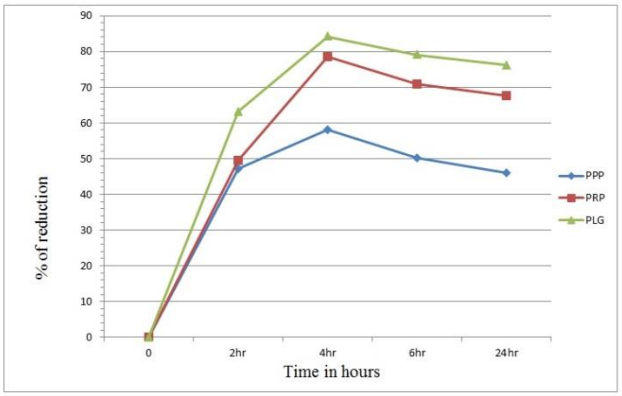

Fig. 1: Percentage reduction of the absolute number of bacteria compared to the control after 2, 4, 6, and 24 hours using colony count method.

PPP: platelet-poor plasma; PRP: platelet-rich plasma; PG: platelet gel.

Effect of thrombin on S. aureus bacterial count after 24 hours incubation:

A significant difference in the mean of bacterial count has been demonestrated in the microplate wells containing thrombin alone compared with the control ones. The mean of bacterial count for thrombin containing wells was $0.025\times10^9 \pm 0.004$ CFU/ml, while in the control ones was $2.3\times10^9 \pm 0.2$ CFU/ml. This result indicates that thrombin reduced bacterial count after 24 hours by 98.9% (p value ≤ 0.001).

DISCUSSION

S. aureus is a major human pathogen that is capable of causing diseases ranging from superficial skin infections to life-threatening sepsis. It has different mechanisms to colonize and evade the host immune response. Of these mechanisms, biofilms production is particularly problematic [22]. Biofilms are complex structured architecture protect bacteria from host-defense mechanisms and killing by antimicrobials [23].

In the present study, among 200 clinical specimens collected from patients with SSIs, 40 (20%) *S. aureus* isolates were identified. This matches with the results of [24-26] who found that *S. aureus* were the most common organisms isolated from SSIs and represented 24.3%, 29.5% and 20% respectively. In our study, MRSA were the most common (95%) of *S. aureus* isolates. Other studies [24,25,27] reported that MRSA were the predominant *S. aureus* isolates from SSI representing 68%, 60.7% and 65% respectively.

In the current study, 32 strains (80%) were biofilm producers by modified TCP method. Mathur and coauthors [28] stated that modified TCP method was sensitive and specific with high accuracy in terms of discriminating between biofilm producers and non-producers.

In the present study, both biofilm producers and non-biofilm producers were sensitive to linezolid. These results were consisted with those of Juayang et al.[29],

who reported that 100% of MRSA strains were susceptible to linezolid making it the drug of choice. In another study[30] which was done on 870 *S. aureus* isolates; 80.5% were methicillin-resistant (MR) and only 1.7% of MR strains were resistant to linezolid and all staphylococci tested were susceptible to vancomycin. PRP has major advantages when compared to conventional antibiotic treatment for infection prevention as PRP is less to induce bacterial resistance and in addition, PRP not only reduces infections but also promote wound healing [31].

In the current study, by using AB reduction method, there was a significant antimicrobial effect of PG, PRP and PPP against *S. aureus* planktonic cells when compared with control (p value ≤ 0.001).

We found that both PG and PRP have an antibacterial effect against *S. aureus*, but in PRP a slower and less strong decrease in the absolute number of bacteria was observed. The strong antimicrobial effect of PG seems to be limited to the first hours after application. After 4 hours the antimicrobial effect of PG reached the maximum with 84.2% reduction in bacterial number, this reduction percentage was decreased reaching 76.3% after 24 hours.

Recent studies reported the PRP is effective in suppressing the infected wounds experimentally [32,33]. Also Cetinkaya et al.[32] reported that PRP had a synergetic effect for MRSA when combined with vacomycin[32].

In a study by Moojen and colleagues [34], the authors found a strong antimicrobial effect of PG which was limited to the first hours after application. Although a proportional reduction of bacteria of about 99% compared to the control could be maintained up to 8 hours. In another study [33] investigated *in vitro* susceptibility to PRP by disc diffusion test and demonstrated effective inhibition of some gram negative bacteria.

Bielecki and coauthors[5] analyzed the *in vitro* antibacterial effect of PG by the Kirby-Bauer disc-diffusion method. PG inhibited the growth of *S. aureus* and was also active against Escherichia coli.

Tang and coauthors [34] identified seven thrombin-releasable antimicrobial peptides from human platelets: fibrinopeptide A, fibrinopeptide B, thymosin β-4, platelet basic protein, connective tissue activating peptide 3, RANTES, and platelet factor 4 and the authors suggested that antimicrobial activities are related to platelet concentration.

Interestingly, thrombin alone in the current study had reduced the bacterial count of *S. aureus* by 98.9%. This result may explain the stronger antimicrobial effect of PG than PRP which had been activated with calcium glocunate. This may be explained by a previous study[37] showed that C-terminal peptides of thrombin constitute a class of host defense peptides, released upon proteolysis of thrombin in vitro, and detected in human wounds in vivo. These peptides exert antimicrobial effects against Gram-positive and Gram-negative bacteria, mediated by membrane lysis, as well as immunomodulatory functions, by inhibiting macrophage responses to bacterial lipopolysaccharide.

There are some limitations of our study. We could not correlate the clinical outcomes with a fixed reduction in bacterial count. In addition, the *in vitro* behavior of *S. aureus* may not resemble the *in vivo* condition in case of SSIs. And finally, an optimum platelets concentration remains controversial and their leucocytes content could have an impact on PRP's antimicrobial effects.

CONCLUSION

Antimicrobial effect of PG is more potent than PRP against *S. aureus* and both components have similar antimicrobial effect on biofilm and non-biofilm producing *S. aureus* recovered from SSIs.

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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