ORIGINAL ARTICLE

Incomplete Hemolytic MRSA Strains Associated with Hemolysin and Panton-Valentine Leucocidin Virulence Genes as a Cause of Blood Stream Infections

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ABSTRACT

Background: *Staphylococcus aureus (S. aureus) with the incomplete hemolytic phenotype (SIHP) is known for its dark hemolytic ring, which differs from the transparent S. aureus with complete hemolytic phenotype (SCHP). SIHP is recently linked to severe infections and antimicrobial resistance. Panton-Valentine leucocidin (PVL) and hemolysin are documented virulence factors for S. aureus infection.* ***Objectives****: We conducted this study to recognize methicillin-resistant S. aureus (MRSA) strains with SIHP phenotype and evaluate its association with the PVL and hemolysin in patients with bloodstream infections (BSIs).* ***Methodology:*** *Ninety-Three S. aureus isolates were recognized during the study period, and they were evaluated for resistance to thirteen antibiotics. Blood agar plates were used to culture these isolates, and they underwent ten passages for identification of their hemolytic phenotype. PVL and hemolysin genes were tested via polymerase chain reaction (PCR).* ***Results:*** *SIHP constituted 34.4% of the collected isolates. PVL positivity was significantly increased in SIHP (34.4% vs. 16.4% in SCHP). Also, SIHP was strongly associated with MRSA (62.5% vs. 39.3% of SCHP). In SIHP isolates, PVL positivity was associated with MRSA (55% vs. 0 % in MSSA) and increased resistance to Augmentin, cefoxitin, and gentamycin.* ***Conclusion:*** *The prevalence of SIHP is increasing among S. aureus. The prevalence of the PVL gene is higher in SIHP, and it is associated with the presence of MRSA.*

INTRODUCTION

S. aureus is one of the most important bacterial pathogens that mediates multiple infectious human diseases ranging from biofilm-associated infections to life-threatening ones [1]. This organism is incriminated in both community and hospital-acquired infections [2]. In addition, the emergence of MRSA has made such organisms a global threat [3]. These drug-resistant strains increase the potential dangers of that organism on both economic and healthcare levels, and it also poses a great challenge for the treating physician [4-5].

In contrast to the classic complete hemolytic ring characteristic for SCHP phenotype, some *S. aureus* strains were observed to have a darker incomplete hemolytic ring, and they are called SIHP phenotype. It was reported that most SIHP organisms were linked to severe infections with high virulence and antimicrobial resistance [2].

Multiple enzymes and toxins mediate the virulence of *S. aureus* and make them amenable to surviving inside the human macrophages [6]. PVL and hemolysin are examples of these virulence factors [2]. The former negatively affects the phagocytic activity of human leukocytes [7], whereas the latter damages the membrane of circulating red blood cells [8].

PVL was first described in 1932 by Panton and Valentine as a virulence cytotoxin[9]. It acts through synergistic action of two secretory proteins, lukF-PV and lukS- PV [10]. That toxin induced pore formation in the cell membrane of different leukocytes, including macrophages, neutrophils, and monocytes [7,11]. Epidemiological studies showed that MRSA virulence had been strongly related to the presence of *pvl* genes [12-13]. MRSA with positive PVL was isolated from patients with soft tissue infections, BSIs, and necrotizing pneumonia [3,14].

Although earlier reports stated that less than 5% of *S. aureus* carry the *pvl* gene [15], it could be transmitted from bacteria to another via plasmids or bacteriophages, which could explain the increased prevalence of PVL-positive organisms [16].

S. aureus can also induce hemolysis of red blood cells by different hemolysins, alpha, beta, gamma, and delta types. The alpha type is a 33-kDa toxin encoded by *hla* gene, and mainly secreted by *S. aureus* incriminated in BSIs, pneumonia, and brain abscess [17-18]. The beta type is a 35-kDa, encoded by *hlb* gene, and

secreted by *S. aureus* causing eye and lung infections [19-20]. The gamma type is encoded by *hlg* gene and composed of slow and fast subunits [21-22]. In addition, the delta type is a 26-aminoacid polypeptide and encoded by *hld* gene [23].

We conducted the current study to recognize MRSA strains with SIHP phenotype, evaluate its antimicrobial resistance pattern, and study its association with the PVL and hemolysin in patients with BSIs.

METHODOLOGY

Study plan:

During the time period extending from March 2021 and July 2022, the current descriptive cross-sectional study was conducted on patients admitted to Inpatient Wards of Mansoura University Hospital and diagnosed to have *S. aureus* bloodstream infection (SA-BSI). Patients were enrolled in our study if they expressed the clinical manifestations suggesting BSIs (like fever, chills, malaise, hypotension ± localizing signs) along with positive blood cultures for *S aureus*. Duplicate isolates from the same patient and those on antibiotic therapy were excluded [7].

We scored the severity of the disease (sepsis, severe sepsis and septic shock) according to 2001 International Sepsis Conference definition [24]. Sepsis was defined as a systemic inflammatory response to BSIs with temperature ≥38.3°C/ <36°C, heart rate >90 beats/minute, respiratory rate >20/min, leucocytes >12 × 10^9/l / < 4× 10^9/l, high CRP or procalcitonin level. Severe sepsis was defined as sepsis with organ dysfunction; and septic shock was defined as sepsis with systolic blood pressure <90 mmHg and evidence of organ failure.

Ethical considerations:

Our research was approved by Mansoura Faculty of Medicine Ethical Committee (R.22.08.1795), and written consent was taken from all patients in agreement with the Declaration of Helsinki.

Collection and processing of the blood samples:

Ten mL of blood were collected from each patient under aseptic conditions. Blood samples were immediately infused into the blood culture bottles (Egyptian Diagnostic Media, Egypt) and transferred to the Microbiology Diagnostics and Infection Control Unit located at Microbiology Department, Mansoura Faculty of Medicine.

The collected blood culture bottles were incubated for one week at 37°C. Specimens from the bottles that showing signs of bacterial growth were sub cultured on MacConkey agar (HiMedia, India) and sheep blood agar (Oxoid, UK) and incubated at the same temperature for 24 hours. Blind subculture was done 24 hours after collection and before reporting the samples as negative, in case of absence of growth signs [2].

Identification of the *S aureus* and antibiotic sensitivity testing:

Ninety-Three *S. aureus* isolates were identified based on their criteria on the sheep blood agar, Gram staining, slide catalase, tube coagulase and mannitol fermentation testing [25].

The Kirby-Bauer disk diffusion method was used to test the isolated *S. aureus* for antimicrobial sensitivity according to the Clinical and Laboratory Standards Institute (CLSI) criteria [26]. Preparation of the isolates was performed with a concentration of McFarland (0.5) along with Muller-Hinton broth (MHB). Then, the isolates were spread over Muller-Hinton agar (Oxoid-UK) and incubated at 37°C for 24 hours after spreading the antibiotic discs. The isolates were tested for 13 antibiotics (Oxoid/ Bioanalyse), including ampicillin (AMP), Augmentin (AMC), linezolid (LZD), vancomycin (VA), erythromycin (E), cefoxitin (FOX), ceftriaxone (CRO), imipenem (IPM), gentamycin (CN), trimethoprim/ sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LEV), and clindamycin (DA). Methicillin resistance was established via the measurement of cefoxitin disc zone (≤ 21 mm).

Detection of *S. aureus* isolates with SIHP phenotype:

The sheep blood agar was used to culture all *S. aureus* isolates. The culture was incubated in 5% CO2 at 37°C for 24 hours. One or two colonies were taken from one agar to the next one. This was repeated for 10 serial passages, and the hemolytic phenotype was observed. If the growth has a donut-like shape with a central lighter hemolytic ring surrounded by a darker opaque ring that was stable on the 10 passages, this was typical for the SIHP strains, otherwise, the colony surrounded with complete ß-hemolytic ring was identified as SCHP. ATCC25923 was taken as a reference for the SCHP [2].

Molecular detection of PVL and hemolysin virulence genes:

DNA extraction:

Inoculation of all *S. aureus* isolates was done on blood agar plates. We selected three to four pure colonies that were suspended in MHB (8 ml) and cultured at 37°C for 24 hours. One ml of the suspension was moved to a sterile tube containing 200 µL lysostaphin (Sigma- Aldrich, USA) and incubated at 37°C for two hours. Then, DNA extraction was completed via QIAamp DNA Mini Kit (QIAGEN, Netherlands), according to the instruction of the manufacturer. Purity and concentration of the DNA was assessed by the NanoDrop One (Thermo Scientific, USA). Storage of the extracted DNA was done at −20°C until the PCR procedure [16].

PCR amplification:

The specific primers used for the detection of *pvl* and hemolysin genes are shown in table 1. 25 µL PCR reaction mixture consisted of 2x Taq master mix (12.5µL) (Cosmo Red, Willowfort, UK), DNA sample

(5 µL), forward and reverse primers of the examined gene (1 µL for each), in addition to nucleases free water (5.5 µL). For *pvl* gene, PCR program was; initial denaturation of 94◦C for three-minutes followed by thirty-five cycles of denaturation at 94◦C for half a minute, annealing at 57◦C for half a minute and an extension at 72◦C for one minute [27-28].

Alpha and delta hemolysin gene amplification was done as follows; five-minute desaturation at 94°C, followed by 35 cycles of 94°C for half a minute, 59°C for one minute, and 72°C for one minute. For beta and gamma hemolysin genes, five-minute denaturation was performed at 94°C, followed by 45 cycles of 94°C for half a minute, 65°C for half a minute, and 72°C for half a minute. Finally, the extension step (all genes) was done at 72°C for ten minutes. PCR amplification was done in the Thermal Cycler (Bio Rad Laboratories, USA). The PCR products were subjected to 2.5% Agarose gel electrophoresis [21].

Statistical analysis:

The collected data were processed via the SPSS 22 software. Numerical data were expressed as mean, standard deviation, and range, while categorical data were expressed as numbers and percentages. For the latter, the Chi-Square or Fischer Exact tests were applied when comparing two groups. Any p-value <0.05 was significant.

Table 1: Primers used to detect *pvl* and hemolysin genes in the *S. aureus* isolates.

Gene	Primer sequences (5' to 3')	product size (bp)	References
pvl	F: ATCATTAGGTAAAATGTCTGGACATGATCC R: GCATCAAGTGTATTGGATAGCAAAAGC	433	**27**
Hla	F: CTGATTACTATCCAAGAAATTCGATTG R: CTTTCCAGCCTACTTTTTTATCAGT	209	**28**
Hlb	F: GTGCACTTACTGACAATAGTGC R: GTTGATGAGTAGCTACCTTCAGT	309	**28**
Hld	F: AAGAATTTTTATCTTAATTAAGGAAGGAGTG R: TTAGTGAATTTGTTCACTGTGTCGA	111	**28**
Hlg	F: GTCAYAGAGTCCATAATGCATTTAA R: CACCAAATGTATAGCCTAAAGTG	535	**28**

RESULTS

Demographic, clinical, and descriptive data of patients with SA-BSI:

We encountered 93 non-duplicate *S. aureus* isolates detected from patients having BSIs during the study period. According to the severity of the disease, 58 patients (62.3%) had sepsis, 32 patients (34.4%) had severe sepsis and only 3 patients (3.3%) had septic shock. The age of those patients had a mean value of 53.11 ± 10.3 years (range, 16 – 62). Regarding their gender, 55 patients (59.1%) were men, and the remaining 40.9% were women.

According to the hemolytic phenotype of *S aureus* isolates, SCHP was identified in 61 isolates (65.6%), whereas interestingly the remaining 32 ones (34.4%) were SIHP. MRSA formed 47.3% of our isolates. Otherwise, 52.7% of the isolates were formed by methicillin-sensitive *S. aureus* (MSSA) (table 2).

Amplification of *pvl* gene showed positivity in 21 isolates (22.6%). Regarding hemolysin gene status, alpha, beta, gamma, and delta hemolysin genes were positive in 7.5%, 25.8%, 7.5%, and 9.7% of isolates, respectively (table 2). Nine *S aureus* isolates (9.7%) showed Concurrent positivity of *pvl* and hemolysin genes.

Table 2: Descriptive data of all *S aureus* isolates included in our study

Descriptive data	*S aureus* = 93	
	No	%
Hemolytic phenotype		
• SCHP	61	65.6
• SIHP	32	34.4
Methicillin resistance		
• MRSA	44	47.3
• MSSA	49	52.7
Panton-Valentine Leucocidin		
• Negative	72	77.4
• Positive	21	22.6
Alpha- Hemolysin		
• Negative	86	92.5
• Positive	7	7.5
Beta- Hemolysin		
• Negative	69	74.2
• Positive	24	25.8
Gamma- Hemolysin		
• Negative	86	92.5
• Positive	7	7.5
Delta- Hemolysin		
• Negative	84	90.3
• Positive	9	9.7

Virulence and antimicrobial characteristics of SIHP and SCHP phenotypes of *S aureus* isolates:

As shown in table 3, the positivity of the *pvl* gene showed a significant increase in association with the SIHP (34.2% vs. 16.4% in SCHP – p = 0.049). Likewise, the alpha-hemolysin gene showed an increased positivity with the same incomplete hemolytic phenotype (15.6% vs. 3.3% in SCHP– p=0.032). Nonetheless, beta-hemolysin showed more positivity in the SCHP (39.3% vs. 0% in the SIHP – p < 0.001). The gamma- and delta- hemolysin genes were statistically comparable between the two groups.

MRSA strains were detected in 62.5% of the SIHP isolates, compared to 39.3% of SCHP isolates, which was significantly different (p = 0.034). Studying the antimicrobial resistance pattern, showed increased resistance of the SIHP isolates to 8 antibiotics with no significant difference, apart from ceftriaxone, cefoxitin, and gentamycin, which showed statistically increased resistance with the SIHP (p < 0.05). On the other hand, vancomycin, imipenem and linezolid still exhibited the lowest resistance pattern in both groups (table 3).

Table 3: Comparison between SCHP and SIHP phenotypes of *S aureus* in relation to virulence factors and resistance to antibiotics

Virulence and antibiotic resistance	SCHP (n=61)		SIHP (n=32)		P Value
	N	%	N	%	
Positive Panton-Valentine Leucocidin	**10**	**16.4**	**11**	**34.4**	**0.049***
Positive Alpha-Hemolysin	**2**	**3.3**	**5**	**15.6**	**0.032***
Positive Beta-Hemolysin	**24**	**39.3**	**0**	**0**	**<0.001***
Positive Gamma-Hemolysin	**5**	**8.2**	**2**	**6.2**	**0.735**
Positive Delta-Hemolysin	**4**	**6.6**	**5**	**15.6**	**0.160**
Methicillin resistance					
• **MRSA**	**24**	**39.3**	**20**	**62.5**	**0.034***
• **MSSA**	**37**	**60.7**	**12**	**37.5**	
Ampicillin	**53**	**86.9**	**31**	**96.9**	**0.122**
Augmentin	**42**	**68.9**	**26**	**81.2**	**0.200**
Linezolid	**8**	**13.1**	**8**	**25**	**0.149**
Vancomycin	**0**	**0**	**0**	**0**	**_____**
Erythromycin	**47**	**77**	**23**	**71.9**	**0.583**
Cefoxitin	**24**	**39.3**	**20**	**62.5**	**0.034***
Ceftriaxone	**30**	**49.2**	**25**	**78.1**	**0.007***
Imipenem	**10**	**16.4**	**2**	**6.2**	**0.166**
Gentamycin	**14**	**23**	**15**	**46.9**	**0.018***
Trimethoprim/sulfamethoxazole	**30**	**49.2**	**22**	**68.8**	**0.071**
Ciprofloxacin	**30**	**49.2**	**14**	**43.8**	**0.618**
Levofloxacin	**22**	**36.1**	**8**	**25**	**0.278**
Clindamycin	**27**	**44.3**	**19**	**59.4**	**0.166**

** p-value < 0.05 was significant*

When SIHP was subdivided into MRSA and MSSA subgroups, and correlated to the virulence factors, 55% of MRSA isolates were PVL positive compared to 0% in MSSA subgroup which was statistically significant (p = 0.001). All hemolysin genes showed no significant difference between SIHP MRSA and MSSA subgroups (p > 0.05) (table 4).

Table 4: Characteristics of MRSA and MSSA SIHP isolates as regard the presence of the virulence factors.

Virulence factors	SIHP (n=32)				
	MRSA (n=20)		MSSA (n=12)		P value
	N	%	N	%	
Positive Panton-Valentine Leucocidin	**11**	**55**	**0**	**0**	**0.001***
Positive Alpha-Hemolysin	**4**	**20**	**1**	**8.3**	**0.366**
Positive Beta Hemolysin	**0**	**0**	**0**	**0**	**----___**
Positive Gamma Hemolysin	**1**	**5**	**1**	**8.3**	**0.617**
Positive Delta Hemolysin	**2**	**10**	**3**	**25**	**0.261**

** p-value < 0.05 was significant*

Antimicrobial resistance of PVL- positive and -negative strains:

Regarding PVL-positivity in SIHP *S aureus* isolates and its association with antimicrobial resistance, PVL-positive strains revealed higher resistance pattern for most of the tested antibiotics, with the resistance to Augmentin, cefoxitin, and gentamycin showed a significant increase in the PVL-positive vs PVL-negative subgroup (figure 1).

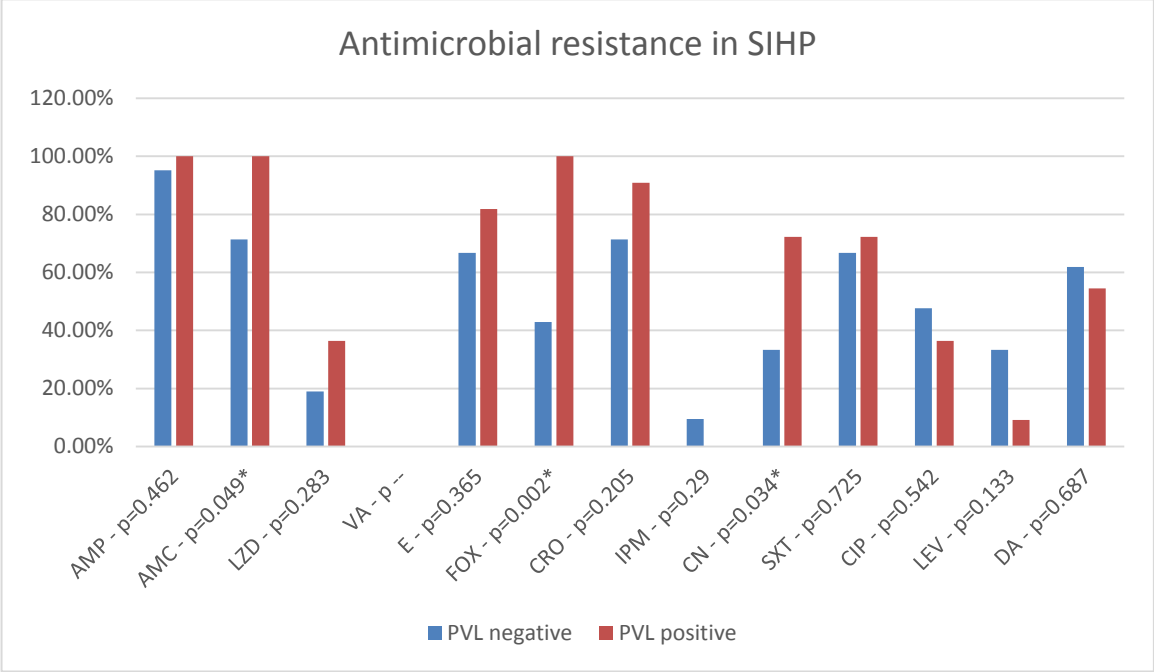

Figure 1: Antimicrobial resistance in SIHP isolates according to PVL gene positivity and negativity.
** p-value < 0.05 was significant*
Note: Ampicillin (AMP), Augmentin (AMC), linezolid (LZD), vancomycin (VA), erythromycin (E), cefoxitin (FOX), ceftriaxone (CRO), imipenem (IPM), gentamycin (CN), trimethoprim/ sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LEV), and clindamycin (DA).

DISCUSSION

S aureus is one major pathogen that could easily acquire resistance as MRSA and VRSA along with other multi resistant pathogens as *Klebsiella pneumoniae*, *Enterococcus species*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*, and they are classified as ESKAPE high alert organisms [29]. Taken together, SIHP could be a new variant of MRSA with high virulence, which needs more attention in prevention and treatment of these strains in hospitals [2]. Herein, we studied the prevalence of SIHP, its antimicrobial resistance, as well as the relation to virulence factors.

First of all, according to the hemolytic phenotype, SCHP was identified in 65.6% of the isolates, whereas 34.4% were SIHP in the current study. Similarity, Gao et al. [16] observed that fifty-two (24.6%) of 211 hospital-acquired *S. aureus* were SIHP strains.

The prevalence of the *pvl* gene in all of our *S. aureus* isolates was 22.6% (21/93). Darboe et al. [7] reported a far higher prevalence of PVL (61.4%), whereas Gao et al. [16] reported a lower prevalence of the same gene (7.11%). Additionally, Melles et al. [30] reported a prevalence of 38.9% of PVL in the *S. aureus* isolated from patients with abscesses and arthritis. This heterogenicity in the prevalence of such a gene could be due to different types of patients, types of infection specimens and the application of effective infection control program, as PVL could be easily transmitted from environmental or hospital agents.

Our findings showed an increased prevalence of the *pvl* gene in association with SIHP compared to SCHP (34.4% vs. 16.4% respectively – p = 0.04). This is in accordance with Gao et al. [16] (53.33% SIHP vs. 3.57% SCHP respectively – p < 0.05). Zheng et al. [31] also reported that SIHP is of high virulence and carrying the *pvl* gene is one of the etiologies of this high virulence.

In the same context, Darboe et al. [7] reported that a higher prevalence of *pvl* gene was detected in invasive *S. aureus* samples compared to the non-invasive ones (72.9% vs. 57.9% respectively). Other studies confirmed the relationship between the *pvl* gene and severe invasive *S. aureus* infections like BSIs and necrotizing pneumonia [15, 32, 33]. This needs the implementation of effective infection prevention care bundles.

In the current study, the alpha-hemolysin gene was significantly increased with SIHP (p = 0.032), while a significant increase of beta-hemolysin was noted with SCHP (p < 0.001). The other two hemolysin genes did not differ between SIHP and SCHP. Zhang et al. [2] contradicted our findings, as they reported that the expression of the alpha-hemolysin gene showed a significant decline in SIHP. Gamma and delta hemolysin genes also decreased in SIHP. Additionally,

the same study showed an increased expression of the beta hemolysin gene in SIHP. Zheng et al. [31] also reported an increased expression of beta hemolysin in SIHP. These results suggest that the expression of the four hemolysins in the SIHP phenotype is most likely different compared to the SCHP strains. Also, this variation could be related to different clinical samples, sample size and epidemiology of the study area.

In our study, MRSA formed 47.3% in all of *S. aureus* isolates (44/93). In addition, the prevalence of MRSA strains showed a significant increase in our SIHP group (62.5% vs. 39.3% in SCHP - p = 0.034). Similarly, a previous study reported that MRSA strains formed 61.54% of SIHP compared to 39.62% of SCHP, which was statistically significant [16]. Additionally, Zhang and his colleagues reported that all of the isolated 60 SIHP strains turned out to be MRSA [2]. A recent study in Egypt showed a high prevalence of MRSA detected from ICU patients (73.9%) [34]. These dissimilarities of the results between various studies may be due to the differences in type of the patients, the bacterial detection methods, the culture of drug use and the implementation of antimicrobial stewardship that could limit the MRSA and other resistant strain in hospitalized patients.

In our study, SIHP expressed more antimicrobial resistance to cefoxitin, ceftriaxone and gentamycin compared to SCHP. Likewise, Gao et al. [16] reported similar findings. That should highlight the association between SIHP and drug resistance by means of certain regulatory genes. In addition, this may be due to the frequent use of those antibiotics as empirical therapy in suspected BSIs.

Our findings revealed that the PVL gene is associated with a significant increase in antimicrobial resistance in SIHP (Augmentin, gentamycin, and cefoxitin). A previous study confirmed our findings but with different antibiotics (ciprofloxacin, levofloxacin and rifampicin) [16]. This may be due to carriage of *pvl* a virulence gene together with resistance genes on genetic elements transmitted between bacteria.

On the other hand, another study reported no association between the *pvl* gene and antimicrobial resistance in *S. aureus* isolates [7]. Bhatta et al. [3] found that PVL-positive *S. aureus* had less antimicrobial resistance, and that was statistically evident when erythromycin was applied (65.8% vs. 83.3% respectively – p = 0.02). That should highlight the debate regarding PVL and antimicrobial resistance between literatures

The current study has some limitations. First of all, the included sample size was relatively small. Additionally, the prevalence of hemolysin genes was too small and not sufficient to perform a correlation between each gene's positivity with hemolytic phenotype and antimicrobial resistance.

CONCLUSION

The prevalence of SIHP was 34.4% among *S. aureus* isolates. The SIHP had a high frequency of *pvl* gene, and it was associated with presence of MRSA and antimicrobial resistance to Augmentin, cefoxitin, and gentamycin. Reporting could be useful for right selection of antibiotics in patients with SIHP- associated infections

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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