



Phenotypic and Molecular Characterization of Heme Release in Selected Clinical Isolates of *Staphylococcus aureus*: Expression Analysis of PSMA and HLD Genes by RT-PCR and Quantification by HPLC

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Abstract This study evaluated whether the expression of *psmA* and *hld* is associated with HPLC-detected heme release from human erythrocytes after exposure to clinical *S. aureus* isolates. A total of 90 human clinical samples were examined, including wound swabs, pus aspirates, blood-culture isolates and catheter-tip isolates. *S. aureus* was identified using culture, Gram staining, catalase, coagulase, mannitol fermentation and automated biochemical confirmation. Thirty confirmed *S. aureus* isolates were selected for the erythrocyte-exposure experiment. Bacterial isolates were incubated with washed human erythrocytes for 2 h at 37°C. Total RNA was extracted from bacterial pellets, converted into cDNA and analyzed by RT-qPCR for *psmA* and *hld*, using *gyrB* as the housekeeping gene. Free heme released into the supernatant was quantified by reverse-phase HPLC using a hemin chloride calibration curve. *S. aureus* was recovered from 42/90 samples, giving an isolation rate of 46.7%. Among the 30 selected isolates, 15 were classified as high heme-release isolates and 15 as low heme-release isolates. High heme-release isolates showed significantly higher *psmA* expression than low heme-release isolates, with mean fold expression of 3.41 ± 1.02 versus 1.48 ± 0.51 , respectively. The *hld* gene was also significantly upregulated in high heme-release isolates, with mean fold expression of 2.87 ± 0.94 versus 1.26 ± 0.42 . HPLC analysis showed significantly higher free heme concentration in the high heme-release group than in the low heme-release group, 38.6 ± 9.4 μ M versus 12.4 ± 5.1 μ M. *psmA* expression correlated positively with HPLC-detected heme concentration, while *hld* expression also showed a significant positive correlation. Clinical *S. aureus* isolates with increased *psmA* and *hld* expression showed greater HPLC-detected erythrocyte heme release.

Key Words *Staphylococcus aureus*, Phenol-Soluble Modulins, Clinical Microbiology

INTRODUCTION

Staphylococcus aureus is a primary bacterial pathogen in humans and a leading cause of numerous infections that includes abscesses and infections of skin, tissues and bones, as well as infections of the bloodstream, pneumonia and infections caused by the use of medical devices [1]. The ability of *S. aureus* to produce a diverse range of virulence factors such as adhesins and evasion and immune factors, along with biofilm and cytolytic toxins, contributes to its pathogenicity [2]. Hemolysis is a crucial aspect of *S. aureus* pathogenicity. Destruction of erythrocytes results in the release of hemoglobin and heme, both of which have the potential to serve as iron nutrients during the course of an

infection [3]. The presence of hemolytic activity is routinely tested in clinical laboratories in the presence of blood on agar. This method may, however, miss the presence of hemolysis that is weak, delayed and/or dependent on the conditions [4].

The phenol-soluble modulins are a group of small amphipathic toxins important for *S. aureus* virulence that are produced by staphylococci [5]. Among the phenol-soluble modulins, *psmA* is associated with cytolytic and inflammatory activities as well as biofilm and invasive infection manifestation [6]. Delta hemolysin or phenol-soluble modulin γ , is a component of the accessory gene regulator system and is embedded in RNAMII, which is the

main effector RNA [7]. Given that the genes *psm* and *hld* code for small toxic peptides, as opposed to large classical pore-forming proteins, they are relevant substitutes for the unproven cryptic names of hemolysin such as *conv* and *Fysin* [8].

Recent studies show that phenol-soluble modulins (PSMs) dominate the hemolysis of human erythrocytes by methicillin-resistant *S. aureus* [9]. Other studies show that some *S. aureus* clinical isolates produce phenol-soluble modulins and delta toxins in different amounts and that this production varies depending on the strain and certain regulators [10]. The *agr* system is the main regulator of *psm* and *hld*. Changes in *agr* can affect the production of hemolysins and extracellular toxins as well as the clinical manifestations of the bacteria [11]. Thus, measuring the expression of *psm* and *hld* using RT-qPCR is a convenient way to study non-classical hemolysis-associated activities in clinical *S. aureus* isolates [12].

Applicable to the study of erythrocyte damage, HPLC has the potential to quantify heme and heme-related by-products present in supernatants of cultured erythrocytes [13]. Quantifying heme is difficult because free heme, heme that is bound to proteins and heme that is derived from haemoglobin may have different behaviours in the course of extraction and analysis [14]. Recent studies have analysed heme and haemoglobin-related by-products in biological samples [15]. Therefore, the combination of RT-qPCR analysis of *psm* and *hld* along with HPLC heme-release measurement may provide an excellent model to assess small peptide-related lysis of erythrocytes in a clinical microbiology context.

METHODS

Study Design

This study incorporated a cross-sectional experimental design to determine how hemolysis-associated gene expression links to heme release in clinical *Staphylococcus aureus* isolates, as detected using HPLC. The study components include isolation and identification of *S. aureus* from human clinical samples, preparation of bacterial cultures, erythrocyte-exposure experiment, RT-qPCR for *psm* and *hld* and HPLC to measure heme levels in culture supernatants.

Clinical Sample Collection

Ninety human clinical samples were obtained from hospital of Diyala microbiology submissions. Samples were collected over approximately one month. The clinical samples from different patient comprised 35 wound swabs, 25 pus aspirates, 20 subcultures of blood cultures that were positive and 10 catheter tips. All samples were transported to the microbiology laboratory in sterile containers or transport swabs and were processed within 2-4 h of collection. Patient-identifying information was excluded. Labels on samples included sample type, date and the source department and duplicate isolates were excluded.

Isolation and Identification of *Staphylococcus aureus*

Clinical samples were inoculated onto blood agar and mannitol salt agar. Blood agar was prepared by mixing Oxoid Blood Agar Base (Thermo Fisher Scientific, UK, Cat. No. CM0055) with 5% defibrinated sheep blood. Mannitol Salt Agar was purchased from HiMedia Laboratories, India, Cat. No. M118. The prepared agar media were incubated at 37°C for 24 hours. Following the incubation period, colonies were selected based on the following criteria: colony morphology, Gram stain reaction, catalase test, slide and tube coagulase test and mannitol salt agar test. The final biochemical identification was done using the VITEK 2 Compact system (bioMérieux, France) according to the manufacturer's instructions.

Preparation of Bacterial Inoculum

The confirmed *S. aureus* isolates were sub-cultured on Tryptic Soy Agar (TSA). TSA was purchased from Oxoid (Thermo Fisher Scientific, UK, Cat. No. CM0131). A single colony was inoculated into 5 mL of Tryptic Soy Broth (TSB) (Oxoid, UK, Cat. No. CM0129) and incubated at 37°C for 18 hours. The suspension was modified to a McFarland standard of 0.5, which is approximately 1.5×10^8 CFU/mL, using sterile phosphate buffered saline (PBS). The suspension was also used to prepare the erythrocyte exposure experiment and was diluted 1:100 in TSB.

Preparation of Human Erythrocytes

Anonymized blood bank donations served as the source of human erythrocytes. Samples were collected from several individuals. Blood was centrifuged (1000×g for 10 minutes, 4°C). Plasma and the buffy coat were removed. Erythrocytes were pelleted at the bottom of the tube and washed three times with sterile PBS, pH 7.4. A 2% erythrocyte suspension was prepared in sterile PBS. Erythrocytes were used immediately after preparation for bacterial exposure.

Erythrocyte Exposure Experiment

For each *S. aureus* isolate, 900 µL of bacterial suspension was mixed with 100 µL of 2% washed human erythrocytes in sterile microcentrifuge tubes. Control tubes contained erythrocytes without bacteria. The tubes were incubated for 2 hours at 37°C and were gently mixed every 20 minutes. After incubation, tubes were centrifuged (5000×g for 10 minutes at 4°C). The bacterial pellets were used immediately for RNA extraction. Supernatants were collected and stored at -20°C until HPLC analysis after being filtered with 0.22 µm syringe filters, Merck Millipore, Germany, Cat. No. SLGP033RS.

Classification of Isolates According to HPLC Heme Release

Thirty confirmed *S. aureus* isolates were selected for RT-qPCR and HPLC analysis. After HPLC analysis of free heme, isolates were grouped into 2 groups based on release of heme. The high heme release group included 15 isolates

that released the highest measurable heme after exposure to erythrocytes. The low heme release group included 15 isolates that released the least measurable heme. These groups were used to analyze the expression of *psm α* and *hld*.

Total RNA Extraction

Total bacterial RNA was extracted as per the protocol of the RNeasy Mini Kit, QIAGEN, Germany, Cat. No. 74104. To stabilize RNA, RNAprotect Bacteria Reagent, QIAGEN, Germany, Cat. No. 76506 was added to the bacterial pellets in the 2:1 ratio. The bacterial pellets were resuspended in 700 μ L of Buffer RLT with 1% β -mercaptoethanol. The lysis buffer was vortexed for 60 s and mixed with 700 μ L of the 70% ethanol solution. The mixture was processed using the RNeasy spin columns. All steps were performed according to the manufacturer's protocol. The RNA was eluted in 40 μ L of RNase-free water and stored at -80°C until the cDNA synthesis.

DNase Treatment and RNA Quality Assessment

The residual genomic DNA was removed by using RNase-free DNase I, Thermo Fisher Scientific, USA, Cat. No. EN0521. One microgram of RNA was mixed with 1 μ L DNase, 1 μ L of 10 $\times$ reaction buffer and brought to a final volume of 10 μ L with nuclease-free water. The reaction was incubated at 37°C for 30 min. The DNase was inactivated by the addition of 1 μ L of 50 mM EDTA and incubation at 65°C for 10 min. The concentration and the purity of the RNA was measured on a NanoDrop One, Thermo Fisher Scientific, USA. Samples were accepted if the A260/A280 ratio was in the range of 1.8 to 2.1.

cDNA Synthesis

The synthesis of complementary DNA was performed using the Revert Aid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, USA, Cat. No. K1622. The components of the reaction included 1 μ g of total RNA, 1 μ L of random hexamer primer, 4 μ L of 5 $\times$ reaction buffer, 1 μ L of Riplock RNase inhibitor, 2 μ L of dNTP mix, 1 μ L of Revert Aid reverse transcriptase and nuclease-free water to a final volume of 20 μ L. The proposed reaction temperatures were 25°C for 5 min, 42°C for 60 min and 70°C for 5 min. cDNA samples were stored at -20°C after 1:5 dilution with nuclease-free water.

RT-qPCR Primer Selection

psm α and *hld* were the genes of interest and *gyrB* was the housekeeping gene. The selection of these genes was on the basis that *psm α* and *hld* correspond to the small-peptide hemolysin system of *S. aureus* and *gyrB* serves as a generic bacterial housekeeping gene. The selection of primers was based on published studies (Table 1).

RT-qPCR Reaction

RT-qPCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA, Cat. No. K0221). Each reaction was prepared in a final volume of 20 μ L containing 2 $\times$ SYBR Green/ROX master mix (10 μ L),

forward primer (0.8 μ L), reverse primer (0.8 μ L), diluted cDNA (2 μ L) and nuclease-free water (6.4 μ L). The final primer concentration was 0.4 μ M. Each sample was analyzed in triplicate. Each run contained no-template controls and no-reverse-transcription controls.

RT-qPCR Cycling Conditions

Amplification was performed on an Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA). The cycling conditions were an initial denaturation of 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and an annealing/extension at 60°C for 30 s. For amplification specificity, a melting-curve analysis was performed and a range of 65 – 95°C was used. Any reactions that had a melting peak with a range outside of the expected range or had inconsistent replicates were repeated.

Calculation of Relative Gene Expression

For the calculation of relative gene expression, the internal reference gene was the *gyrB* gene and the group used for calibration was the low heme-release group. Expression of both *hld* and *psm α* was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. For this calculation, ΔCt was determined by the Ct value of the *gyrB* gene subtracted from the Ct value of the gene of interest and for $\Delta\Delta\text{Ct}$ the mean ΔCt of the calibration group was subtracted from the ΔCt value of each individual sample. For this method, a value of 1 or greater was considered gene expression.

HPLC Standards and Calibration

Hemin chloride served as the analytical standard for quantifying heme. The hemin chloride standard was purchased from Sigma-Aldrich/Merck, Germany, Cat. No. H9039. A 1 mM hemin chloride stock solution was prepared in 0.1 M sodium hydroxide and stored in the dark. Working standards at concentrations of 1, 2.5, 5, 10, 25 and 50 μ M were made using the same extraction solvent that was used for the sample preparation. The calibration curves were made by plotting peak area versus concentration of the standards. Calibration curves were considered acceptable for $R^2 \geq 0.995$.

Preparation of Supernatants for HPLC

The filtered erythrocyte exposure supernatants were thawed on the bench. The samples were vortexed for 30 s and centrifuged at $12,000\times g$ for 5 min. 200 μ L of the supernatant was mixed with 200 μ L of methanol containing trifluoroacetic acid (TFA) 0.1%. The samples were vortexed for 1 min and centrifuged at $12,000\times g$ for 10 min. The extract was placed in amber HPLC vials and prepared for analysis. The samples were injected in the HPLC system within 24 h of sampling preparation.

HPLC Instrumentation and Conditions

HPLC was performed using a Shimadzu LC-20AD HPLC system (Shimadzu Corporation, Japan). The system was configured with a quaternary pump, autosampler, column oven and a UV-visible detector. The separation was

performed using a ZORBAX Eclipse Plus C18 column (4.6×150 mm, 5 µm; Agilent Technologies, USA, Cat. No. 959993-902). The mobile phase consisted of (A) HPLC-grade water with 0.1% trifluoroacetic acid and (B) acetonitrile with 0.1% trifluoroacetic acid. Acetonitrile was obtained from Merck, Germany, Cat. No. 100030. Trifluoroacetic acid was obtained from Sigma-Aldrich/Merck, Germany, Cat. No. 302031. The gradient program consisted of 35% B at 0 min, 60% B at 5 min, 95% B at 12 min, 95% B at 15 min and return to 35% B at 18 min. The flow rate was 1.0 mL/min and the column was maintained at 35°C with an injection volume of 20 µL. The detection wavelength was set at 400 nm. The heme concentration was calculated based on the hemin chloride calibration curve and was expressed as µM.

HPLC Quality Control

Prior to the injection of each standard curve and after every ten sample injections, a blank solvent was injected. A mid-level hemin chloride standard was injected after every ten samples to check the stability of the retention time and response of the detector. The acceptable relative standard deviation for repeated standard injections was <5%. Samples with abnormal peak shapes, peak overlap or a retention time shift of >0.2 min were filtered and re-injected.

Statistical Analysis

Data were analysed using version 26 of SPSS and version 10 of GraphPad Prism. Isolation rates were presented as both numbers and percentages. Mean±standard deviation was used to present gene expression and HPLC heme concentrations. Comparative analyses of isolation rates of the different sample types were performed using the Chi-square test. High and low heme-release groups were compared using the independent-samples t-test. The relationships among *psmA*, *hld* gene expressions and heme concentrations detected by HPLC were analyzed using Pearson correlation. Statistical significance was judged at $p < 0.05$. In the tables of this report, values differentiated by letters (superscript) are significantly different at $p < 0.05$.

RESULTS

Isolation Rate of *Staphylococcus aureus* from Clinical Samples

Out of 90 clinical samples, 42 cases of *Staphylococcus aureus* were reported (an overall isolation rate of 46.7%). The highest isolation rate (15/25) or 60.0% was from pus aspirate samples. Swabs from the respective wounds (35 samples) were also positive in 18 cases (51.4%). Blood cultures sub-cultured in 20 cases were positive in 7 (35.0%). Samples collected from catheter tips were positive in 2/10 (20.0%) cases. The isolation of *S. aureus* from clinical samples demonstrated a significant range based on the sample type (Table 2).

Selection of Isolates for Gene Expression and HPLC Analysis

For the erythrocyte-exposure experiment, a total of thirty *S. aureus* isolates were selected. Of these, fifteen were classified as the high heme-release group and the other fifteen as the low heme-release group, based on the HPLC analysis of the free heme concentration. The high heme-release group provided stronger evidence of erythrocyte damage and increased free heme concentration, while the low heme-release group exhibited lower levels of heme release after erythrocyte exposure.

Expression of *psmA* and *hld* in High and Low Heme-Release Isolates

The results of the RT-qPCR analysis indicated that the high heme-release group expressed the *psmA* gene to a greater extent than the low heme-release group. The mean of the relative expression of the *psmA* gene in high heme-release isolates was 3.41 ± 1.02 and 1.48 ± 0.51 in low heme-release isolates. The high heme-release group also showed a greater expression of the *hld* gene with a mean of 2.87 ± 0.94 , compared to 1.26 ± 0.42 , in the low heme-release group. These results suggest that the isolates exhibiting a greater release of heme from erythrocytes also exhibited a higher level of gene expression of both the peptide toxin-associated genes (Table 3).

Table 1: RT-qPCR Primers Selected for the Study

Gene	Function	Primer sequence 5'-3'	Reference
<i>psmA</i>	Alpha phenol-soluble modulin locus	F: GAAGGGGGCCATTCACAT R: GTTGTTACCTAAAAATTACCAAGT	Lade <i>et al.</i> [10]
<i>hld</i>	Delta hemolysin/PSM gamma	F: CACTGTGTGCGATAATCCATT R: AGGAAGGAGTGATTTC AATG	Liu <i>et al.</i> [16]
<i>gyrB</i>	Housekeeping/reference gene	F: ATCTGGTCGTGACTCTAGAA R: TGTACCAAATGCTGTGATCA	Lade <i>et al.</i> [10]

Table 2: Distribution of *S. aureus* According to Clinical Sample Type

Sample Type	No. Examined	Positive Isolates	Positivity (%)
Wound swabs	35	18	51.4 ^{ab}
Pus aspirates	25	15	60.0 ^a
Blood-culture subcultures	20	7	35.0 ^b
Catheter-tip specimens	10	2	20.0 ^c
Total	90	42	46.7
Chi-square	-	8.71	-
P value	-	0.033	-

Different superscript letters indicate significant differences between sample types at $p < 0.05$

Table 3: Relative Expression of *psmA* and *hld* in Clinical *S. aureus* Isolates

Gene	Low heme-release isolates	High heme-release isolates	Fold difference	p-value
<i>psmA</i>	1.48±0.51 ^b	3.41±1.02 ^a	2.30	<0.001
<i>hld</i>	1.26±0.42 ^b	2.87±0.94 ^a	2.28	<0.001

Different superscript letters within the same row indicate significant differences between groups at $p < 0.05$

Table 4: HPLC-Detected Free heme Concentration in Erythrocyte-Exposure Supernatants

Group	No. isolates	Free heme concentration, μM	Change (%)	p-value
Low heme-release isolates	15	12.4±5.1 ^b	-	-
High heme-release isolates	15	38.6±9.4 ^a	+211.3	<0.001

Different superscript letters indicate significant differences between groups at $p < 0.05$

Table 5: Correlation Between Gene Expression and HPLC-Detected heme Release

Correlation tested	r-value	p-value	Interpretation
<i>psmA</i> expression vs free heme	0.71	<0.001	Significant positive correlation
<i>hld</i> expression vs free heme	0.64	0.002	Significant positive correlation
<i>psmA</i> expression vs <i>hld</i> expression	0.59	0.004	Significant positive correlation

Quantification of Free Heme after Erythrocyte Exposure via HPLC

HPLC analysis revealed that the high heme-release group exhibited a greater concentration of free heme compared to the low heme-release group. The high heme-release group had a mean free heme concentration of $38.6 \pm 9.4 \mu\text{M}$, while the low heme-release group had a mean free heme concentration of $12.4 \pm 5.1 \mu\text{M}$. Members of the group with high concentrations of heme that was detected via HPLC exhibited heme release that was 211.3% greater compared to members of the low heme release group. The results for the two groups were significant (Table 4).

Correlation of Gene Expression and HPLC-Detected Heme Release

According to Pearson correlation analysis, heme concentrations were positively and significantly correlated to the expression of *psmA*. The correlation coefficient for *psmA* and heme concentrations was $r = 0.71$, $p < 0.001$. The *hld* gene demonstrated a significant positive correlation to heme concentrations, with $r = 0.64$, $p = 0.002$. Additionally, a positive correlation was found between the expression of *psmA* and *hld*, indicating that the expression of small peptide hemolysis-associated factors was generally coordinated in clinical *S. aureus* isolates (Table 5).

DISCUSSION

In the present study, *psmA* and *hld* are substituted for the unnamed cryptic hemolysins Faysin and Convaisin, respectively. This strengthens the work as *psmA* and *hld* are the only known *S. aureus* genes linked to small peptides and hemolysin activity [5]. The *psmA* locus is the genetic locus for alpha-type phenol-soluble modulins, a class of small amphipathic peptides that have both cytolytic and inflammatory [6] activities. The *hld* gene is the gene for delta hemolysin, also known as phenol-soluble modulins gamma and is found in the RNA III component of the agr system [7]. Thus, for a small and focused RT-qPCR study on the atypical destruction of erythrocytes by clinical *S. aureus* isolates [11], these two genes are perfectly suited.

Elevated expression of *psmA* in isolates that cause high heme release suggests that phenol-soluble modulins alpha peptides play a role in damaging erythrocytes. This is consistent with new evidence that PSM α peptides are the largest contributors to the MRSA-induced hemolysis of human erythrocytes [9]. It is also consistent with studies that show alpha-type phenol-soluble modulins are the key virulence factors in *S. aureus* infection models [17]. In the present study, elevated *psmA* expression in isolates that release high levels of heme suggest that the transcription of this locus may lead to more damage to erythrocyte membranes and greater heme release into the supernatant. This illustrates that the use of *psmA* as a molecular marker in clinical *S. aureus* isolates to study small peptides and associate them with a lethally hemolytic model is well founded.

The high *hld* expression in high heme-release isolates suggests a role for delta hemolysin in the erythrocyte-lysing activity. Delta hemolysin is a small membrane-lysing peptide of the phenol-soluble modulins family [7]. Recent evidence reveals that Hld is packaged in membrane vesicles of *S. aureus* and can control vesicle-mediated hemolytic and pro-inflammatory activities [18]. Another recent study showed that delta toxin modulates the biogenesis of membrane-extruded vesicles and nucleates the formation of amyloids, thus supporting its biological activity beyond hemolysis [19]. Therefore, the elevated expression of *hld* in the high heme-release group may indicate activation of an agr-dependent expression program of toxins that result in damage to erythrocytes and release of measurable heme.

The study was further enhanced by the use of HPLC, as it enabled researchers to establish a quantitative assessment of blood-agar hemolysis without relying solely on a qualitative assessment. There are a number of factors that will influence the interpretation of hemolysis in studies that will affect the source of blood, medium composition, incubation time, oxygen tension and volitional assessment [4]. HPLC-based heme analysis provides a quantitative assessment of erythrocyte heme release and is more appropriate for a less extensive study [13]. Recent

advances in analytical chemistry emphasize that heme determination requires careful extraction, calibration and control, as labile heme and heme bound to hemoglobin will differ in their behavior [14]. Thus, the HPLC endpoint in this study should be viewed as a first measure of erythrocyte damage in the absence of all hemolysis mechanisms.

The strongest evidence linking the expression of a gene and the resultant biochemical damage to erythrocytes is the positive correlation of *psm α* expression and heme concentration measured by HPLC. This supports the hypothesis that phenol-soluble modulins cause cytolysis and disrupt membranes of host cells [5]. The correlation of *hld* expression and heme release supports the hypothesis that delta hemolysin contributes to this effect [18]. Clinical strains of *S. aureus* were shown to have variable production of PSMs and delta toxins. This variation is reported to be associated with strain type, the function of the accessory gene regulator in *Staphylococci* and the clinical context [10]. The current model appears to classify clinical isolates in a simple manner by the transcript level of hemolysin and the associated activity of HPLC erythrocyte damage.

This preliminary study has important advantages. First, it incorporates *S. aureus* hemolysis-associated genes supported by published literature [9] without including speculative genes. Second, it includes only two target genes, *psm α* and *hld* and used *gyrB* as the reference gene, which makes the study of small scale and low cost [13,16-20].

CONCLUSIONS

This study demonstrated that isolates in the high heme-release group exhibited significantly greater expression of both *psm α* and *hld* than those in the low heme-release group. HPLC analysis showed that the high heme-release group had significantly greater free heme concentrations after exposure to erythrocytes. The positive correlation between the expression of the genes and the release of heme indicates that, within clinical microbiology, *psm α* and *hld* may be the first molecular indicators of small-peptide-associated erythrocyte damage.

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