

Quantitative RT-PCR Analysis of *spa* and *hla* Virulence Gene Expression in Nasal Isolates of *Staphylococcus aureus*: Association Between Virulence Transcription and Clinical Symptoms

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Abstract

Background: Nasal colonization by *Staphylococcus aureus* is common in humans and represents a major reservoir for subsequent infection. However, not all colonizing strains cause disease, suggesting that differences in virulence gene expression may contribute to the transition from asymptomatic carriage to symptomatic infection. **Aim:** The present study investigated the transcriptional profile of two key virulence genes, *spa* (protein A) and *hla* (alpha-hemolysin), in nasal swab isolates from healthy carriers and symptomatic patients using quantitative reverse transcription PCR (RT-qPCR). **Patients and Methods:** A total of 100 nasal swab samples were collected from two groups: 50 healthy carriers and 50 symptomatic individuals presenting with nasal or respiratory infection. Bacterial RNA was extracted from confirmed *S. aureus* isolates and converted to cDNA. Quantitative RT-PCR was performed to measure transcription levels of *spa* and *hla*, with *gyrB* serving as the internal reference gene. Gene expression levels were calculated using the $\Delta\Delta C_t$ method. Statistical comparisons between groups were performed using independent two-tailed Student's *t*-tests. Effect sizes and statistical power were also evaluated. **Results:** Both virulence genes demonstrated significantly lower *Ct* values in symptomatic patients. The mean *Ct* value of *spa* was 28.5 ± 1.4 in healthy carriers compared with 25.9 ± 1.2 in symptomatic patients ($P < 0.0001$). Similarly, *hla* showed *Ct* values of 30.1 ± 1.7 in healthy carriers and 26.8 ± 1.3 in symptomatic individuals ($P < 0.0001$). Normalized expression analysis revealed substantial differences between groups. The mean ΔC_t value for *spa* was 7.4 ± 1.1 in healthy carriers and 4.6 ± 1.0 in symptomatic patients, producing a $\Delta\Delta C_t$ value of -2.8 and corresponding to a 6.96-fold increase in expression. For the *hla* gene, the mean ΔC_t values were 9.0 ± 1.3 in healthy carriers and 5.5 ± 1.2 in symptomatic patients, generating a $\Delta\Delta C_t$ of -3.5 and an 11.31-fold increase in expression among symptomatic individuals. Statistical analysis confirmed highly significant differences between groups, with *t*-values of 13.2 for *spa* and 14.8 for *hla* (both $P < 0.0001$). Effect size analysis indicated very large differences in expression, with Cohen's *d* values of 2.6 for *spa* and 2.9 for *hla*. **Conclusions:** The findings demonstrate that nasal isolates from symptomatic individuals exhibit markedly increased transcription of key virulence determinants compared with isolates from healthy carriers. The observed 6.96-fold increase in *spa* expression and 11.31-fold increase in *hla* expression suggest that activation of virulence gene transcription plays an important role in the transition from asymptomatic colonization to clinical infection. Quantitative profiling of virulence gene expression using RT-qPCR may therefore represent a valuable molecular approach for identifying potentially pathogenic nasal strains and improving early detection of infection-associated bacterial phenotypes.

Keywords: alpha-hemolysin, gene expression, nasal carriage, quantitative RT-PCR, *spa* gene,

Staphylococcus aureus, virulence factors.

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Introduction

Staphylococcus aureus nasal colonization is a phenomenon recognized globally and is a significant source for future endogenous infections. The anterior nares have an ecological niche for the persistent colonization due to the presence of nutrients and epithelial receptors and local immune interference. Many epidemiological studies have illustrated a significant number of healthy individuals with asymptomatic *S. aureus* colonization and a mere minority developing disease in relation to the same colonizing strain (Wolde et al. 2023; Congdon et al. 2023). The colonization-infection continuum demonstrates the *S. aureus* pathogenicity is not attributable to the presence or absence of the bacterium, but to the host where there is a modulation of the components of pathogenicity.

Several studies have shown that the prevalence of nasal carriage varies within populations due to the influence of lifestyle, occupation, immune response, and other environmental factors. For instance, studies of healthcare personnel and members of the community have documented high carriage rates and noted the role of nasal colonization in the spread of pathogens and the associated infection risk (Wolde et al., 2023; Liu et al., 2025a). In the same way, the study of the collegiate cohort showed that lifestyle and environmental exposure might determine the colonization and antimicrobial resistance patterns of nasal *S. aureus* isolates (Congdon et al., 2023). This underlines the importance of the healthcare system monitoring the extent of nasal colonization as colonized individuals are both a transmission source and a potential infection case.

Aside from the epidemiological aspect, the molecular features of nasal isolates have been the focus of extensive studies. Several studies have demonstrated that colonizing isolates often contain genes that code for virulence factors that enable the bacterial isolate to adhere to the host, avoid the immune response, and cause damage to host tissues (Inagawa et al., 2024). In the clinical setting, the molecular epidemiology studies have also shown the circulation of virulent clones, where the clones can colonize a patient upon admission and then cause infection. Such studies help reinforce the notion that nasal carriage should not be viewed as neutral, but rather, that it may be part of a continuum of the pathogenesis of staphylococcal disease (Inagawa et al., 2024; Ibrahim et al., 2024).

Of the many virulence determinants of *S. aureus*, the *spa* gene and the *hla* gene are of particular interest. Protein A contributes to immune evasion of the host by binding the Fc region of immunoglobulins and thereby preventing opsonization and phagocytosis. In contrast, alpha-hemolysin is a pore-forming cytotoxin that can injure epithelial and immune cells, and as such, promotes the bacterial invasion and destruction of tissue. The expression of such virulence genes has been shown to increase or decrease depending on the particular environment and upon interaction with the host, indicating that some form of transcriptional control is activated to facilitate the change from asymptomatic colonization to active infection (Li et al., 2024).

The most recent studies on transcription have revealed how controlling virulence genes helps to understand the pathogenic potential of the virulence genes of the colonizing strains. Competitive

transcriptional adaptation studies showed that the *S. aureus* isolates adapt to the pressures of the immune system by altering their transcriptional profiles to adapt to the varying pressures of the immune system and tissue of the host during colonization and infection of the host (Li et al., 2024). Also, studies using molecular surveillance have shown that in viral isolates from the nose, persistent exposure to strains with virulence and antimicrobial resistance from the patients and medical caretakers is persistent (Ibrahim et al., 2024; Liu et al., 2025b). These studies waste the potential to understand the pathogenicity of the bacteria and susceptibility to disease.

The knowledge on nasal colonization has been rapidly growing, but the studies examining transcriptional differences from nasal isolates that asymptomatically carry the bacteria in comparison to those strains that symptomatically infect the patients remain scarce. The ability of *S. aureus* to adapt by flexibly considering the variation in pressure may in itself be a major determining factor in the adverse effects of the disease. The most direct means to address such evaluation is through quantitative reverse transcription PCR (RT-qPCR) which is a more sensitive measure of bacterial gene expression and enables a direct comparison of transcription rates of the different clinical isolates.

The current study sought to describe the transcriptional profiles of the two virulence genes, *spa* and *hla*, in nasal isolates of *Staphylococcus aureus* from healthy carriers and symptomatic patients. Gene expression levels were measured by RT-qPCR and normalized to the bacterial housekeeping gene *gyrB*. This study attempts to explain the degree of virulence gene expression in relation to the symptomatic infection and to elucidate the molecular mechanisms associated with the phenomenon of nasal carriage and clinical infection, by describing the differences in the levels of transcriptional activity in the two states of infection.

Materials and Methods

Study Design and Sample Collection

A cross-sectional clinical study was designed to assess the transcriptional profiling of virulence genes in *Staphylococcus aureus* nasal isolates. One hundred nasal swab samples from outpatient clinic attendees were collected. The study sample was comprised of two groups: 50 healthy carriers with no respiratory symptoms and 50 patients with symptoms of nasal or respiratory infections.

Nasal swabs (Copan Diagnostics, Brescia, Italy; Catalog No. 155C) were inserted into the anterior nares and rotated for 5 seconds to adequately sample the nasal mucosa. The swabs were then placed in sterile tubes containing 1 mL of RNeasy Protect Bacteria Reagent (Qiagen, Germany; Cat. No. 76506) to preserve the integrity of the bacterial RNA. The samples were then transported to the microbiology laboratory.

Isolation and Identification of Bacterial Isolates

Nasal swabs were inoculated on to Oxoid Mannitol Salt Agar (MSA) (UK, Cat. No. CM0085) and incubated for 24 hours at 37°C in aerobic conditions. Selective yellow fermenters were further confirmed. Each presumptive isolate was examined using Gram staining and biochemical techniques. Catalase was measured using 3% hydrogen peroxide, and coagulase was confirmed using Staphylase latex agglutination (Oxoid, UK; Cat. No. DR0595A). Identified isolates were kept in a Tryptic Soy Broth with 20% glycerol (Oxoid, UK; Cat. No. CM0129) and were kept at -80°C for future analysis.

Isolation of RNA

Total RNA from bacteria was obtained using the RNeasy Mini Kit (Qiagen, Germany, Cat. No. 74104) as per the manufacturer's instructions with slight modifications for Gram-positive bacteria. In short, a bacterial pellet was obtained by centrifugation at 10,000 g for 5 minutes. This pellet was resuspended in 100 µL of a TE buffer with 20 mg/mL of lysozyme (Sigma-Aldrich, USA; Cat. No. L6876) to promote lysis of cells.

The suspension was held at 37°C for 30 minutes and then Buffer RLT with beta-mercaptoethanol was added. The RNA was purified by membrane spin column method and was eluted in 30 µL of RNase free

water. The concentration and purity of RNA was determined using a NanoDrop 2000 (Thermo Fisher Scientific, USA). Only samples with an A260/A280 ratio of observed to be between 1.8 and 2.1 were taken for subsequent analysis.

To eliminate residual genomic DNA contamination, RNA samples were treated with RNase-free DNase I (Thermo Fisher Scientific, USA, Cat. No. EN0521) as per manufacturer's instructions.

cDNA synthesis

Complementary DNA (cDNA) was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA; Cat. No. K1622). Each reverse transcription reaction was prepared with 1 µg of total RNA, random hexamer primers, dNTP mix, reverse transcriptase enzyme, and reaction buffer for a total volume of 20 µL.

The reverse transcription reaction was performed in a thermal cycler under the following conditions: 25°C for 5 minutes, 42°C for 60 minutes, and 70°C for 5 minutes to terminate the reaction. The synthesized cDNA was kept at -20°C until analyzed by quantitative PCR.

Quantitative RT-PCR analysis

Quantitative PCR was conducted with PowerUp SYBR Green Master Mix (Applied Biosystems, USA, Cat. No. A25742) in a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). The components that made up each PCR reaction included: 10 µL of SYBR Green Master Mix, 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol), 2 µL of cDNA template, and 6 µL of nuclease-free water. This gives each reaction a total volume of 20 µL.

The conditions of the thermal cycling were as follows: Initial denaturation: 95°C for 2 minutes, Then, 40 amplification cycles: 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds

Lastly, a melting curve analysis from 60°C to 95°C was conducted to confirm amplification product specificity. Levels of gene expression were normalized to the bacterial housekeeping gene *gyrB*, and relative expression was analyzed with the method $\Delta\Delta Ct$. Table 1 shows the primers used in the present study.

Table 1. RT-PCR primer sequences used for virulence gene expression analysis

Gene	Primer sequence (5'–3')	Amplicon size (bp)	Reference
spa	F: TATGCCTAACTTAAATGCTG	119	Ngba Essebe et al. (2017)
	R: TTGGAGCTTGAGAGTCATTA		Ngba Essebe et al. (2017)
hla	F: TCCAGTGCAATTGGTAGTCA	132	Ngba Essebe et al. (2017)
	R: GGCTCTATGAAAGCAGCAGA		Ngba Essebe et al. (2017)
gyrB	F: CGACTTTGATCTAGCGAAAG	120	Lin et al. (2025)
	R: ATAGCCTGCTTCAATTAACG		Lin et al. (2025)

Ethical approval

The Institutional Research Ethics Committee in the University of Al-Qadisiyah, Iraq, reviewed and approved the study protocol under ethical approval number EC-CVM-2026-041. All participants provided written informed consent prior to sample collection, and all protocols adhere to the guidelines of the Helsinki Declaration on ethics of human subject research.

Statistical analysis

Statistical analysis was performed using version 26.0 of the SPSS (IBM Corp., USA) and version 9.0 of the GraphPad Prism (GraphPad Software, USA) software. The Ct values for the RT-qPCR were first normalized to the housekeeping gene (*gyrB*) to determine the ΔCt values. Relative gene expression levels

of the healthy carriers and the symptomatic patients were analyzed by the $2^{-\Delta\Delta C_t}$ formula.

The normal distribution of the study variables was assessed using the Shapiro–Wilk test. The independent two-tailed Student's t-tests were used to assess the differences in gene expression between the study groups. Data were presented as Mean $\pm$ Standard Deviation (SD).

Cohen's d was used to estimate the effect sizes and G Power version 3.1 was used to estimate the statistical power. A P value of less than 0.05 was considered to be statistically significant.

Results

Stability of the expression of the housekeeping gene gyrB

To check the reliability of the normalization method used for the quantitative RT-PCR analysis, the housekeeping gene gyrB was evaluated first. The Ct values for gyrB did not show much variation for the two study groups. The mean Ct value for healthy carriers was 21.1 ± 0.9 , while the mean Ct value for symptomatic patients was 21.3 ± 1.0 . The independent Student's t-test showed no significant difference for the two groups ($P = 0.281$). The absence of significant variation implies that there was stability in the expression of gyrB in all tested isolates, and therefore, it was a suitable internal reference gene for the normalization of the target gene expression. The housekeeping gene's stability showed that the changes in virulence gene transcription were not due to changes in the quantity of bacterial RNA or efficiency of PCR amplification, but rather true biological differences in gene expression in the isolates.

The high reproducibility of the RT-PCR assay, along with the consistent RNA quality across samples, is also evidenced by the relatively narrow standard deviation of the gyrB Ct values within both groups. This confirmed that the experimental conditions were appropriate and that the use of the ΔC_t and $\Delta\Delta C_t$ analysis were valid for the intended quantitative analysis of the transcriptional vrc of the virulence genes. The consistent amplification of gyrB across all the tested isolates, also provided a strong foundation for the analysis of the comparative gene expression (Table 1).

Analysis of Genes of Virulence, (spa and hla)

Analysis of RT-PCR with quantitative purposes, and comparative analysis of Ct values, revealed a clear difference in the virulence genes spa and hla of the isolates from asymptomatic patients as opposed to the ones from symptomatic patients. The spa gene, from asymptomatic patients, had a mean Ct value of 28.5 ± 1.4 , while the samples from symptomatic patients had a mean Ct value of 25.9 ± 1.2 , being significantly lower. The lower Ct values represent a higher transcriptional gene activity, and therefore, this evidenced a higher expression of the spa gene in the samples with clinical indications. The statistical analysis confirmed that difference in the two sample groups is hugely significant ($P < 0.0001$).

A similar trend was noted for the hla gene that encodes for the alpha-hemolysin toxin. Isolates from healthy carriers had an average mean Ct value of hla = 30.1 ± 1.7 , while symptomatic patients had an average mean Ct value of hla = 26.8 ± 1.3 , which is a significant difference. Statistical analyses once more showed a highly significant difference for these two groups ($P < 0.0001$). The size of the difference indicates that there is a strong relationship between the transcription (ang. 'transcription', which is likely a typo) of the gene for the cytotoxic alpha-hemolysin and the presence of clinical symptoms.

The results indicate that symptomatic individuals have more nasal isolates than asymptomatic carriers. The Ct value difference (ang. 'Comparison of the Ct value difference') is the basis for the Next Highest (Table 2).

Table 2: Ct values of housekeeping and virulence genes obtained by RT-qPCR from nasal isolates

Gene	Healthy carriers (n = 50) Mean Ct $\pm$ SD	Symptomatic patients (n = 50) Mean Ct $\pm$ SD	t value	P value
gyrB (housekeeping)	21.1 ± 0.9	21.3 ± 1.0	1.08	0.281

gene)				
spa (Protein A gene)	28.5 ± 1.4	25.9 ± 1.2	9.97	<0.0001
hla (Alpha-hemolysin gene)	30.1 ± 1.7	26.8 ± 1.3	10.95	<0.0001

Ct values

The first value obtained was the levels of exponential growth in the Ct value, while the second was the value obtained from the average spread of the exponent in the Ct value. This value is the average spread of the gene expression level.

The mean Δ Ct value for healthy carriers of the spa gene was 7.4 ± 1.1 , while it was significantly lower for symptomatic patients at 4.6 ± 1.0 . This means that symptomatic patients had much higher levels of spa transcription, as it was expressed much more in the lesion samples. A statistical analysis showed that there was a significant difference between the 2 populations where $t = 13.2$ and $P < 0.0001$.

The analysis of the hla gene also showed similar results, with mean Δ Ct value for hla in healthy carriers at 9.0 ± 1.3 while symptomatic patients had a mean Δ Ct value of 5.5 ± 1.2 . Here the difference between the 2 populations was also statistically highly significant ($t = 14.8$ and $P < 0.0001$). This means that both of the virulence genes are transcriptionally upregulated in lesion samples.

The large differences seen for both genes Δ Ct value show that the changes seen from asymptomatic colonization to symptomatic infection involves transcriptional upregulation of virulence factors (Table 3).

Table 3: Normalized gene expression based on Δ Ct values

Gene	Healthy carriers Mean Δ Ct $\pm$ SD	Symptomatic patients Mean Δ Ct $\pm$ SD	t value	df	P value
spa	7.4 ± 1.1	4.6 ± 1.0	13.2	98	<0.0001
hla	9.0 ± 1.3	5.5 ± 1.2	14.8	98	<0.0001

Relative Gene Expression Analysis ($\Delta\Delta$ Ct and Fold Change)

Utilizing the $2^{-\Delta\Delta$ Ct method, relative gene expression levels were ascertained with the ability to compare levels of transcription between the study groups. Considering the symptomatic and asymptomatic study groups, the $\Delta\Delta$ Ct value was determined by subtracting the average Δ Ct of asymptomatic study participants (aCtHC) from those of symptomatic study participants (aCtSC). For the spa gene, the calculated $\Delta\Delta$ Ct value was -2.8, which corresponds to a 6.96 increase in gene expression of symptomatic isolates as compared to healthy carriers. This reveals that transcription of the protein A gene was nearly 7 times higher for isolates with clinical manifestations. The hla gene showed an even greater increase in transcription. The calculated $\Delta\Delta$ Ct value was -3.5 which corresponds to an 11.31 increase in transcription of symptomatic isolates as compared to healthy carriers. This enormous increase in transcription supports that the production of the alpha-hemolysin may be of considerable importance in the pathogenic potential of nasal isolates. The hypothesis stating that transcriptional activation of virulence determinants leads to a more advanced state of infection, is supported by the increase in symptomatic isolates of the virulence genes that were examined in this study (Table 4).

Table 4: Relative gene expression calculated using the $\Delta\Delta$ Ct method

Gene	Mean Δ Ct Healthy	Mean Δ Ct Symptomatic	$\Delta\Delta$ Ct	Fold change ($2^{-\Delta\Delta$ Ct)	P value
spa	7.4	4.6	-2.8	6.96	<0.0001
hla	9.0	5.5	-3.5	11.31	<0.0001

Effect size and power calculations

In the scope of the study, we carried out an effect size analysis in order to quantify the scope of the deviations in expression of the genes that were of interest to the study in the two cohorts we analyzed. For the spa gene, the calculated Cohen's d value was 2.6, which indicates that this value is large. For the other gene, hla, the calculated Cohen's d value was 2.9, which indicates that this value is extremely large. Both of these show that the variations that were present in the cohorts analyzed were in fact significant and there was an obligation from a biological point of view.

Statistical Power analysis was conducted with sample size being 50 participants in every group, and an alpha of 0.05. For both genes, the calculated statistical power was >0.99, demonstrating that more than enough power was present to detect true differences in the gene expression levels in healthy carriers and patients.

The effect size and power calculated indicates a great likelihood of the validity of the results and that the transcriptional regulation of spa and hla is closely bound to symptomatic infection (Table 5).

Table 5: Effect size and statistical power analysis of gene expression differences

Gene	Cohen's d	Interpretation	Statistical power
spa	2.6	Very large effect	>0.99
hla	2.9	Extremely large effect	>0.99

Discussion

Our study revealed that nasal isolates of *Staphylococcus aureus* from symptomatic patients demonstrated significantly higher levels of transcription of the virulence factors spa and hla when compared to isolates from asymptomatic carriers. The 6.96-fold increase of spa and 11.31-fold increase of hla indicate that more virulence factors may help cause the shift from mere colonization to infection. Most studies have found nasal colonization to be the primary means by which a present and future clinical illness occurs. More than a few epidemiological studies documented the nasal carrier state as esp. important when determining infection risk. For example, the clinical importance of nasal *S. aureus* colonization and its role as a reservoir for disease was demonstrated in a large, multicenter study of blood donors (Dinh et al., 2025). Likewise, in studies of the nasal colonization of *S. aureus* in some immunocompromised patients, a number of host-related factors were identified to contribute to the persistent presence of the organism in the nasal cavity (Ogunleye et al., 2025). These studies were consistent with the hypothesis that colonizing strains of pathogenic organisms have specific traits that set them apart from non-pathogenic strains.

Increased transcriptional levels of spa in this study appears to be notable in light of immune evasion. It is understood that Protein A binds to the Fc region of immunoglobulins which inhibits the process of opsonization, thus preventing phagocytosis of the pathogen by the host immune system. Therefore, upregulation of this particular gene could aid in the survival of the pathogen within the host during the course of the infection. Furthermore, the significant levels of expression of hla noted in symptomatic isolates, indicates that tissue inflammation and damage may be due to the presence of cytotoxic activity. The alpha-hemolysin toxin which is known to create pores in the membranes of host cells, has been associated with the damage of epithelial cells, and the alteration of immunity during the course of an infection. Prior to this, molecular studies have shown that virulent clinical isolates usually have factors responsible for toxin production, biofilm formation, and immune evasion (Jarzynka et al., 2025). Also, among clinical patients, genomic studies have shown that the colonizing strains have the ability to promote infection if the conditions are appropriate (de Oliveira et al., 2025). Therefore, the activation of transcription of spa and hla in this study mirrors those studies that have been done pertaining to the presumed pathogenicity.

A recent study highlights influences on the nasal microbiome that may change virulent *S. aureus* strains. Zani et al. (2025) shows that *S. aureus* colonization could interact with certain / other bacteria or host(s)

that could affect risk of acquisition and/or infection. Also, nasal specimens cultured from COVID-19 infected patients revealed isolates with genetically diverse backgrounds, some of which may not even possess the so-called high-risk clonotypes, which may suggest that the pathogenic potential of an isolate may be affected by regulation of certain genes and not by the clonotype (Piechowicz et al., 2026). Furthermore, some clinical studies have demonstrated that nasal *S. aureus* colonization may affect the host immune status and even modify the degree and/or changes of inflammation in an allergic airway disease (Linton et al., 2025). These studies have suggested that virulence gene regulation could be the reason why some bacterial strains do not become pathogenic. The subsequent increase in virulence genes (*spa*, *hla*) was significant and supports the conclusion of gene regulation as a primary trigger of clinical infection in this study.

Conclusion

This study showed that nasal *Staphylococcus aureus* isolates from symptomatic cases had much higher expression rates of the virulence genes *spa* and *hla* than those from healthy carriers. Quantitative RT-PCR showed these genes had increased transcription. This activation of virulence determinants possibly explains the shift from asymptomatic colonization to symptomatic infection. This study demonstrates that the expression of virulence genes at the molecular level may be useful in identifying pathogenic nasal strains and understanding the role of bacteria in the pathogenesis of disease.

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