

# Analysis of Hyaluronidase Expression by qPCR in Egyptian Clinical Isolates of *Staphylococcus aureus* and Its Correlation with Phenotypic Plate Assay

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## Abstract

Hyaluronidase enzyme (HysA) is an extracellular enzyme that is considered to be an important virulence factor for Staphylococcus aureus. We screened the production of HysA enzyme in the spent media of Egyptian clinical isolates (32 isolates) via phenotypic plate assay. We found that 75% of the isolates (24 isolates) were able to produce HysA enzyme. We designed primers for qPCR analysis of hysA mRNA expression that was derived from the alignment of hysA gene sequences of 41 strains of S. aureus. The designed primers could be used for the amplification of hysA in 79.2% of the isolates (19 isolates) that were positive for HysA production as demonstrated by phenotypic plate assay. A significant positive correlation, as indicated by Pearson correlation analysis (r = 0.84 at P < 0.001), was found between phenotypic plate assay and qPCR of mRNA expression of hysA in the investigated isolates of S. aureus. In conclusion, we analyzed for the first time hysA mRNA expression via qPCR in S. aureus. Additionally, our work showed a good agreement between the phenotypic assay of HysA production via plate assay and hysA expression in S. aureus. The qPCR analysis of this study could be used as a more reliable quantitative method for hysA expression analysis particularly in infected animal models of S. aureus.

## **Keywords**

Hyaluronidase, qPCR, Staphylococcus aureus, Virulence Factor

## **1. Introduction**

Staphylococcus aureus is a Gram positive bacterium that causes a diverse range

of both community-associated and hospital-acquired infections [1] [2] [3]. *S. aureus* contains various virulence factors that consist of both surface-associated proteins and secreted proteins including hemolysins, immunomodulators and a number of exoenzymes [4] [5] [6].

Bacterial hyaluronidases (known as hyaluronate lyases) are a class of enzymes that degrade hyaluronic acid (HA) and to lower extent chondroitin and chondroitin sulfate [7] [8]. HA is a high molecular-weight polymer composed of alternating units of D-glucuronic acid and N-acetylglucosamine [9]. This polymer is a major component of the extracellular matrix of human tissues and it is abundant in the skin, skeletal tissue, umbilical cord, lungs, heart valves, brain, blood, liver and a number of other tissues [10]. Moreover, it plays important roles for inflammation, water homeostasis, in addition to being an immune regulator [11] [12]. Hyaluronidases have been found to be virulence factors that are involved in the invasion, and penetration of tissues in many of Gram positive bacteria [9].

*S. aureus* is able to infect many tissues which contain high HA concentrations using HysA as a virulence factor [13] [14]. The expression of HysA by *S. aureus* can be assayed phenotypically by various methods e.g. plate assay [15], turbide-metry [16], viscometry [17], colorimetry [18] and zymography [19]. The disad-vantages of these classical methods are that they often lack sensitivity, and selectivity, and require large amounts of samples [10]. Despite the vital role of hyaluronidase for *S. aureus* as a virulence factor, no study has analyzed the *hysA* mRNA expression via qPCR which would be a more reliable and accurate method for quantitative analysis of *hysA* expression particularly in infected animal models of *S. aureus*. Therefore, our main aim in this study was to analyze the expression of *hysA* in *S. aureus* clinical isolates via qPCR and to correlate these results with the phenotypic plate assay. The qPCR analysis of *hysA* expression will be a more reliable and accurate method for quantitative analysis of the enzyme expression particularly in infected animal models of *S. aureus*.

## 2. Materials and Methods

#### 2.1. Collection, Purification and Identification of S. aureus Isolates

32 isolates of *S. aureus* (S1-S32) were collected from different sources (wounds, burns, and throat) obtained from Mansoura university hospitals, Egypt. This work was done after approval of the administrative authorities (Research Ethics Committee) in the Faculty of Pharmacy, Mansoura University, Egypt. The isolates were purified and confirmed as *S. aureus* by Gram staining and biochemical reactions (Mannitol salt agar, catalase, and coagulase) and using *S. aureus* Newman as a reference strain [20] that was kindly provided by Dr. Mohammed Youssef Ali (Associate professor of microbiology and immunology, faculty of pharmacy, Mansoura university). All bacterial strains were stored in brain heart infusion (BHI) broth medium containing 20% glycerol at  $-80^{\circ}$ C, until further analyses were performed.

#### 2.2. Spent Media Preparation

Overnight cultures of different isolates in BHI broth were incubated at  $37^{\circ}$ C with shaking at 200 rpm. Later, subculturing into fresh BHI broth medium (starting  $OD_{630 \text{ nm}}$  equivalent to  $1.7 \times 10^7$  CFU/ml) was performed with shaking at 200 rpm, and  $37^{\circ}$ C. Samples (1 ml) were taken at the required time point, and centrifuged at 3200 x g for 5 min. Spent media (supernatant containing the produced HysA enzyme) were filter sterilized and stored at  $-80^{\circ}$ C till further analyses.

## 2.3. Screening and Quantitative Assay of the Hyaluronidase-Producing *S. aureus* Isolates Using Plate Assay Method

The procedure of [8] was followed with minor modifications. Sterile glass plates (12 cm diameter) containing 1% agarose, 1% bovine serum albumin (BSA), and 0.4 mg/ml of HA (Sigma, H-1504) in 0.3 M sodium phosphate buffer (pH 5.3) were prepared. After solidification of the agarose medium, wells (7 mm diameter) were made aseptically, and 100  $\mu$ l of spent media were added into each well.

Plates were incubated for 24 h at 37°C before flooding with 2 M acetic acid. Clear zones were observed against a background of opaque precipitated BSA conjugated to the undigested HA and their diameters were measured in millimeters. Hyaluronidase (Sanofi, 1500 IU) was used as a standard positive control and for construction of standard curve after dissolving in 500  $\mu$ l BHI broth to obtain a final concentration of 3000 IU/ml, where two fold serial dilutions from the concentration 3000 IU/ml till 5.8 IU/ml were prepared in BHI broth. Later, 100  $\mu$ l from each dilution was examined for its hyaluronidase as indicated above. The standard curve was constructed from plotting of the logarithmic concentration of standard hyaluronidase (3000 - 5.8 IU/ml) and the clear zones of HA hydrolysis subtracted from the cup diameter (**Figure 1**). The hyaluronidase activity present in the spent medium of each isolate was expressed as IU/ml according to the standard hyaluronidase (**Figure 1**).

## 2.4. Primer Design via Multiple Sequence Alignment for Detection of Hyaluronidase Expression by Quantitative Real-Time-PCR (qPCR)

Hyaluronidase DNA sequences of 41 *S. aureus* strains were obtained from the GenBank database and aligned by using the multisequence alignment tool (Clustal omega) that is provided by EMBL-EBI website (**Figure S1**). For qPCR analysis for hyaluronidase expression, forward (Hyalq-F) and reverse (Hyalq-R) primers (**Table 1**) were precisely designed from the conserved regions of the aligned sequences and with the consideration of the optimum GC% (40% - 60%).

#### 2.5. RNA Isolation, cDNA Synthesis, and qPCR

For qPCR analysis of *hysA* expression, the optimum time for RNA extraction was investigated by measurement of HysA activity in the spent media and the OD630nm at different time points (3, 5, 9, 24 h). For such optimization, two



**Figure 1.** Plate assay of different concentrations (IU/ml) of the HysA (a) and standard curve for enzyme activity (b).

Table 1	<b>Primers</b>	used in	this	study	y.
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Primer	Sequence (5'-3')	Relevance	Amplicon size (bp)	Reference
Hyalq-F	GGTGCTTATGGCGTTGTACT	qPCR of <i>hysA</i>	163	This study
Hyalq-R	CGTGATAAATCCATCATTTCACC	expression	105	This study
GyrB-F	CCAGGTAAATTAGCCGATTGC	qPCR of gyrB	121	[21]
GyrB-R	AAATCGCCTGCGTTCTAGAG	expression	121	[21]

F: Foward primer, R: Reverse primer.

strains were selected as representative samples; Newman, and S10.

Cell pellets were harvested from aliquots of 1.5 ml at each specified time for RNA isolation and centrifuged at 3200 x g and 4°C for 5 min. Glass beads (150 mg, 0.25 - 0.5 mm diameter) [Roth, Carlsruhe, Germany] and 300  $\mu$ l of triazole reagent (Zymo research) were added to the cell pellet. The mixture was subjected to alternating intervals of vortexing (30 sec) and immersion in ice (30 sec) for total of 10 min. Later, freezing at  $-80^{\circ}$ C (3 min) and thawing at 37°C (3 min) was performed 3 times. The disrupted cells were centrifuged at 3200 x g and 4°C for 5 min. RNA was precipitated according to the protocol of the triazole reagent company for isolation of RNA by traizole method. The precipitated RNA was dried, dissolved in 20  $\mu$ l RNAase free water, and stored at  $-80^{\circ}$ C till further analysis. The quality of RNA was checked by agarose gel electrophoresis and RNA concentration was determined using nanodrop.

The synthesis of cDNA from the purified RNA of different isolates was performed using the Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Real-time PCR was performed in 20  $\mu$ l reaction volumes using the Rotor-Gene Q (Qiagen) and 5x HOT FIREPol EvaGreen HRM Mix according to the manufacturers' instructions. Each reaction volume contained 4  $\mu$ l 5x HOT FIREPol EvaGreen HRM, 0.5  $\mu$ l forward (250 nM final), 0.5  $\mu$ l reverse primer (250 nM final), 13  $\mu$ l sterile water (PCR grade), and 2  $\mu$ l of template cDNA. The gene encoding GyrB was used as a reference gene and amplified using the GyrB-F and GyrB-R primers (**Table 1**). Negative control reactions without cDNA template were included in each PCR run. Additionally, strain S32 was included in each run as a control for the strains that were negative for HysA production by phenotypic assay. The PCR was performed using the following conditions: initial activation at 95°C for 12 min, followed by 40 cycles of denaturation (95°C for 12 sec), annealing (59°C for 20 sec), and extension (72°C for 20 sec).

Melting curves were analyzed to check PCR specificity, and the absence of primer dimers. The PCR efficiency (E) of each primer pair was determined by making dilution series (undiluted, 1/5, 1/10, 1/50, and 1/100) using cDNA of *S. aureus* strain Newman as a template. The cDNA sample of *S. aureus* strain Newman was used as a calibrator sample in all qPCR runs to minimize run-to-run variations. The threshold cycle (CT) values were calculated by the Rotor-Gene Q software, and exported to Excel for relative quantification analysis. To ensure the accuracy of amplification, all generated amplicons were separated by agarose gel electrophoresis in a 2% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

#### 2.6. Statistical Analysis

Results were statistically analyzed using Excel program. The values for either concentration/OD630nm (Phenotypic assay) or the fold of change in RNA expression (Genotypic assay) were calculated as the average mean  $\pm$  SD of 3 independent experiments. The correlation between the phenotypic production of HysA, as indicated by plate assay, and the qPCR analysis of *hysA* expression, was performed using the Pearson correlation analysis test.

#### **3. Results**

## 3.1. Phenotypic Screening of HysA Production by the Clinical Isolates of *S. aureus* via Plate Assay

Twenty four strains (S1-S24) out of 32 strains were able to produce HysA as indicated by the clear zones obtained from the plate assay. The concentration of hyaluronidase in the spent media was obtained from the standard curve of hyaluronidase enzyme (**Figure 1**), which was then normalized to the  $OD_{630 \text{ nm}}$  of each individual culture (Concentration/ $OD_{630 \text{ nm}}$ ). The highest HysA producing strain was S10, followed by Newman as indicated in **Table 2**.

## 3.2. qPCR Analysis of *hysA* mRNA Expression in the HysA Producing Clinical Isolates of *S. aureus*

As indicated in **Figure 2**, the highest production of HysA was detected after 3 h cultivation of subcultures of the strains; Newman and S10. Therefore, for qPCR analysis of *hysA* mRNA expression, the RNA was isolated from the subcultures of the HysA producing isolates of *S. aureus* after 3 h cultivation.

The efficiency of amplification of *hysA* and *gyrB* genes were 1.7 and 1.87 respectively, as determined by the software of Rotor gene Q. To correct for the

difference between amplification efficiencies of both genes, the Paffl method [22] was used in this study to determine the relative expression of *hysA* gene in different isolates using Newman as a calibrator strain. The fold of change (FC) in expression of *hysA* in different clinical isolates relative to *hysA* expression by Newman is shown as mean  $\pm$  SD of three independent experiments (**Table 2**).

**Table 2.** Phenotypic assay of HysA production and qPCR analysis of expression of *hysA* from different isolates of *S. aureus*. The values for either Concentration/OD<sub>630 nm</sub> or the fold of change are the average mean  $\pm$  SD of 3 independent experiments.

Strains producing HysA and their <i>hysA</i> expression could be detected by qPCR				
		Phenotypic assay	Genotypic assay	
Strain	Rank	Concentration/OD <sub>630 nm</sub> (Mean ± SD)	Fold of change (Mean ± SD)	Rank
S10	1	938.38 ± 94.22	127.86 ± 3.315	1
Newman	2	$397.00 \pm 4.24$	1	3
S5	3	$322.29 \pm 52.03$	$0.631\pm0.023$	4
S8	4	$295.50 \pm 21.92$	$0.579\pm0.028$	5
S3	5	$273.65 \pm 75.87$	$0.025\pm0.042$	17
S9	6	$270.16 \pm 64.83$	$0.326\pm0.027$	8
S7	7	$236.37 \pm 19.28$	$0.363\pm0.041$	7
S6	8	$219.50 \pm 14.85$	$2.243\pm0.604$	2
S2	9	$215.62 \pm 23.46$	$0.264\pm0.113$	9
S20	10	$212.16 \pm 17.20$	$0.175\pm0.004$	12
S4	11	201.30 ± 15.99	$0.413 \pm 0.424$	6
S13	12	$180.49\pm9.34$	$0.190\pm0.083$	11
S1	13	$145.31 \pm 7.51$	$0.145\pm0.050$	13
S12	14	$135.98 \pm 43.94$	$0.226\pm0.134$	10
S23	15	$107.50 \pm 17.68$	$0.005 \pm 0.0006$	19
S21	16	$107.30 \pm 10.32$	$0.036 \pm 0.0078$	16
S11	17	99.15 ± 12.93	$0.001 \pm 0.0003$	20
S22	18	$74.87 \pm 10.08$	$0.038\pm0.001$	15
S19	19	72.65 ± 3.33	$0.013\pm0.002$	18
S18	20	$52.64 \pm 10.42$	$0.137\pm0.049$	14

#### Strains producing HysA and their hysA expression could not be detected by qPCR

Strain	$Concentration/OD_{630 nm} (Mean \pm SD)$
S14	66.8 ± 15.77
S15	$30.1 \pm 6.52$
S16	$20.2 \pm 5.43$
S17	9.1 ± 2.22
S24	$63 \pm 14.34$



**Figure 2.** HysA production and growth curve of Newman and S10 strains at different time points of subculture cultivation.

#### 4. Discussion

Few reports have focused on the study of hyaluronidase production by *S. aureus*, despite the importance of hyaluronidase enzyme as a virulence factor for Gram positive cocci [9]. Such reports were interested in either phenotypic screening of the enzyme production by different species of Staphylococcus [23] [24] [25], or investigation of its role in the virulence of *S. aureus* [8] [14].

In this work, we performed phenotypic analysis for HysA production by different clinical isolates using a plate assay. The plate assay showed that 75% of the isolates (24 out of 32 isolates) were able to produce hyaluronidase. In previous studies such percent was either 91.2% [23], 93% [25] or 99.5% [24].

The previous studies on genotypic analysis of *hysA* in *S. aureus* were restricted on either the detection of the gene by Southern blot [25], DNA microarray [26] or semiquantitative analysis via Northern blot [8]. None of the previous studies tried to perform genotypic analysis of *hysA* expression via qPCR. Therefore, we were interested in quantitative detection of *hysA* mRNA expression via qPCR.

We determined the concentration/OD<sub>630 nm</sub> for HysA production at different time points for S10 and Newman strains (the 2 highest strains producing HysA as indicated from our preliminary investigations). As shown in **Figure 2**, the maximum productivity of the cells in both strains was found to be after 3 h of cultivation of the subcultures. Consequently, the optimum time for RNA extraction in this study was considered to be after 3 h cultivation in the early exponential phase of growth. We designed forward and reverse primers in the conserved regions of *hysA* after alignment of the gene sequences of 41 strains of *S. aureus* from the Genbank (**Figure S1**). The primers were used for the qPCR analysis of the mRNA expression of *hysA* in the isolates that were found to produce the enzyme after plate assay (24 out of 32). The ability of the designed primers to amplify *hysA* was investigated through both the analysis of the expected amplicons via agarose gel electrophoresis and the amplification plots after performing qPCR in the Rotor-Gene Q. Our results showed that the designed primers successfully amplified the *hysA* gene in 79.2% of the HysA producing isolates (19 out of 24 isolates) as demonstrated in **Table 2**. The last 19 isolates (target isolates) together with the Newman strain were used to investigate the correlation between the phenotypic assay via plate assay and the quantitative genotypic assay using qPCR. The reason that 5 isolates were associated with HysA production in the plate assay and could not be amplified by the designed primers, might be the presence of mutations in the conserved regions at which the primers bind in the *hysA* gene.

Previous studies correlated the measured phenotypic character for different genes and their genetic expression in different organisms e.g. antimicrobial resistance genes [27] and virulence genes [28] in *Pseudomonas aeruginosa*, genes regulating metabolite concentrations in the yeast *Saccharomyces cerevisiae* [29], and scoC gene in *Bacillus subtilis* [30]. Similarly, we studied the correlation between phenotypic plate assay and qPCR analysis for the *hysA* gene in the 19 target isolates using Pearson's correlation coefficient. A Pearson correlation coefficient (r) of 0.841 was calculated between the replicates of concentration/OD<sub>630 nm</sub> for HysA production and the fold of change in *hysA* mRNA expression for each isolate. The calculated r indicates a significant positive agreement between plate assay method and the quantitative assay of mRNA expression using qPCR at either P < 0.05 (Critical r value = 0.444) or P < 0.001 (Critical r value = 0.679).

### **5.** Conclusion

In conclusion, we established for the first time a protocol and primers for the qPCR analysis of the *hysA* mRNA expression in *S. aureus*. Moreover, our work showed the agreement between the phenotypic assay of HysA production via plate assay and *hysA* expression in *S. aureus*. Future work will be done to correlate the virulence of *S. aureus* in infected animal models and the *hysA* expression analysis using the qPCR protocol of this study.

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## **Conflict of Interest**

The authors declare that they have no conflict of interest.

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## **Supplementary Materials Description**

**Figure S1**. Multisequence alignment of hyaluronidase DNA sequences of 41 strains *S. aureus* obtained from the Genbank using Clustal omega tool. The forward primer (Hyalq-F) and reverse primer (Hyalq-R) were precisely designed from the conserved regions of the aligned sequences. The sequence of *S. aureus* strain Newman is indicated by lowercase letters of nucleotides.