مجله دنیای میکروب‌ها
سال دوازدهم شماره اول (پیاپی ۳۷) بهار ۱۳۹۸
صفحات ۸۳-۷۳

Original Article

اثر افزودن سرم گوساله بر بیان ژن‌های ویرولانس *استافیلوکوکوس اورئوس*

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چکیده

سابقه و هدف: *استافیلوکوکوس اورئوس* یک پاتوژن فرصت‌طلب و یکی از مهم‌ترین عوامل تهدید کننده سلامت در سطح جهان است. بیان عوامل بیماری زایی باکتری ها در شرایط محیط‌های کشت آزمایشگاهی و شرایط *in vivo* یکسان نیست. هدف از مطالعه حاضر ارزیابی اثرات افزودن ۵٪ سرم گوساله بر بیان ژن‌های ویرولانس *استافیلوکوکوس اورئوس* است.

مواد و روش ها: میزان بیان ژن‌های *agrA*، *RNAIII*، *hla*، *spa* و *mecA* در مدت رشد جدایه های‌های *استافیلوکوکوس اورئوس* در محیط کشت BHI براث و BHI براث غنی شده با ۵ درصد سرم گوساله و در فازهای مختلف رشد تعیین شد. به این منظور منحنی رشد ۵ جدایه از باکتری *استافیلوکوکوس اورئوس* در دو شرایط محیطی مختلف رسم شد. سپس RNA از فازهای مختلف رشد استخراج و میزان بیان ژن‌های یادشده با real-time PCR مورد آنالیز قرار گرفت.

یافته ها: به طور میانگین در انتقال از فاز لگاریتمی به سکون میزان بیان ژن‌های *agrA* و *RNAIII* در محیط سرم‌دار به ترتیب ۳/٤ و ۹/۵ برابر افزایش داشت، اما سیستم *agr* نتوانست نقش تنظیمی خود را بر بیان ژن‌های ویرولانس به خوبی بازی کند. به این صورت که میزان بیان ژن *hla* با کاهش ۰/۸۱ برابری نشان داد و میزان بیان ژن‌های *spa* و *mecA* به‌ترتیب تنها افزایش ۱/۲۵ و۱/۰۳ برابری را داشتند.

نتیجه گیری: صرف نظر از فاز رشد بیان همه ژن‌ها در محیط BHI براث حاوی سرم گوساله نسبت به محیط BHI براث افزایش نشان داد. از این رو به نظر می رسد که *استافیلوکوکوس اورئوس* در حضور فاکتورهای موجود در سرم گوساله توانایی القای بیان ژن‌های ویرولانس را مشابه با شرایط *in vivo* داشته باشد.

واژگان کلیدی: *استافیلوکوکوس اورئوس*، بیان ژن، سرم گوساله، ژن‌های بیماریزایی.

دریافت مقاله: شهریور ماه ۹۷ پذیرش برای چاپ: مهر ماه ۹۷

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Journal of Microbial World
Volume 12, No. 1, April 2019

Original Article

The effect of adding calf serum on the virulence genes expression of *Staphylococcus aureus*

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Abstract

Background & Objectives: *Staphylococcus aureus* is an opportunistic pathogen and one of the most important health-threatening agents worldwide. Expression of bacterial virulence factors is not similar in laboratory medium conditions and in vivo. The aim of the present study was to evaluate the effects of adding 5% calf serum on the virulence genes expression of *S. aureus*.

Materials & Methods: The expression levels of *agrA*, *RNAIII*, *hla*, *spa*, and *mecA* genes were determined during the growth of *S. aureus* isolates in BHI broth and BHI broth enriching with calf serum during different growth phases. Therefore, the growth curve of the five isolates of *S. aureus* in two different culture conditions was plotted. Subsequently, RNA was extracted from different phases of growth and the genes expression were analyzed by Real-time PCR.

Results: In average, the expression levels of *agrA* and *RNAIII* from stationary phase to the exponential phase in BHI broth containing calf serum was increased 3.4 and 9.5-fold, respectively, while the agr system could not appropriately play its regulatory role in the expression of virulence genes. As a result, the expression of *hla* gene was decreased 0.81-fold and the expression levels of spa and *mecA* genes was only increased 1.25 and 1.03-fold, respectively.

Conclusion: Regardless of the growth phase, the expression of the whole genes in BHI broth containing calf serum were increased in comparison to BHI broth. Consequently, it appears that *S. aureus* is capable to induce virulence genes expression in the presence of calf serum factors similar to conditions available in vivo.

Keywords: *Staphylococcus aureus*, Gene expression, Calf serum, Virulence genes.
Revised: September 2018 Accepted: October 2018

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Journal of Microbial World 2019, 12(1): 73-83.

Introduction

Staphylococcus aureus is an opportunistic pathogen and one of the most important health problems in the world. *S. aureus* is a part of the microbial flora of approximately 30% of the adult population. A wide range of human diseases, including benign skin infections to life-threatening systemic disease like osteomyelitis, endocarditis, pneumonia, bacteremia, sepsis, and toxic shock syndrome is associated to this bacteria. The increasing emergence of methicillin-resistant strains (MRSA) and CA-MRSA, which can cause problems such as nosocomial infections, has increased the importance of this bacteria (1).

The ability of this bacteria to create a wide range of diseases is related to the expression of a large number of virulence factors. Some of these factors are surface proteins such as Protein A, which interfere with bacterial binding and protecting against host immune system. Also this bacteria expresses a wide range of secretion virulence factors such as exogenous, hemolysin, leukocyidins and enterotoxins, which are very important in pathogenesis (2).

The most important regulatory system for the expression of virulence factors in *S. aureus* is known as Accessory Gene Regulator (*agr*) system that is the first regulatory system known for this bacterium. Agr system is the mediator of regulation of more than 70 genes, 23 of which were identified as virulence factors (3).

Agr system is a quorom sensing system in *Staphylococcus aureus* that activates following increased cellular concentration in the stationary growth phase and acts as an up-regulator of genes involved in producing secretory toxins such as gene encoding alpha hemolysin (*hla*) and down-regulator of genes involved in bacterial binding to host cells and evading the immunity, such as genes encoding protein A (*spa*) (4).

The pattern of expression of virulence factors in laboratory culture environments is completely different compared to *in vivo* conditions (5).

The aim of this study was to evaluate, the expression levels of *agrA* (as representative of *agr* system), *RNAIII* (the effecter molecule of the *agr* system), *hla* (encoding alpha-toxin), *spa* (encoding protein A) and *mecA* (encoding resistance of methicillin) genes of *S. aureus* isolates in BHI broth and BHI broth containing 5% calf serum during different growth phases. The purpose of adding the serum to BHI broth was to create conditions similar to *in vivo* conditions.

Materials and methods

Bacterial strains

Five bacterial strains of *Staphylococcus aureus* were used in this study. Of these, two strains were isolated from the nasal cavity of healthy volunteers, and two clinical strains were isolated from the patients. In addition to these, the standard COL strain was also used. Isolates

Table 1. Strains used in this study.

Strain	Characteristics
1C	Healthy carrier, Methicillin susceptible (MSSA)
2C	Healthy carrier, Methicillin resistant (MRSA)
1P	Clinical strain, Methicillin susceptible (MSSA)
2P	Clinical strain, Methicillin resistant (MRSA)
COL	Standard strain

from healthy carriers and patients were selected to include a methicillin-resistant *Staphylococcus aureus* (MRSA) strain and a methicillin-susceptible *Staphylococcus aureus* (MSSA) strains. Specifications of the strains used in this study are listed in Table 1.

The isolates were recovered then were identified with conventional laboratory techniques to identification of *S. aureus* including Gram stain, catalase test, hemolysis on blood agar and coagulase test (18).

Plotting of growth curve of isolates in studied culture media

The growth curves of five *S. aureus* isolates in BHI broth (BHI) and BHI broth (Merck, Germany) containing 5% calf serum (BHIS) were plotted during time of inoculation (0-time) through 24-hour incubation period using the method as described by Koohsari *et al* (6). In summary, suspension of containing 10^3CFU/ml of each of the isolates was prepared in BHI broth and BHI broth containing 5% calf serum and this concentration was considered as 0-time.

These cultures incubated at 37°C for 24-hours and were sampled at hourly intervals from the time of inoculation of the culture, 0-time, through 24-hours incubation period. Each sample were inoculated on the Muller Hinton agar (Merck, Germany) and incubated at 37°C for 24-hours. By counting the number of colonies, the number of bacteria per ml (CFU/ml) was obtained.

To plotting of the growth curve, the number of bacteria (log) per ml in the Y axis and the time in the X axis was placed. The generation time of each *S. aureus* isolates was determined by choosing two points on the exponential phase of the growth curve, using the following formula:

$$g = 0.301 \times t / \log Nt - \log No$$

g; Generation time, No; the initial population number (The first point), Nt; population at second point, t; the time interval (in hours) between the 2 points (6).

Also part of the samples were stored for RNA extraction and real-time PCR immediately frozen at -70°C until the tests.

Extraction and Purification of RNA

After plotting of the growth curve of *S. aureus* isolates and determining the different growth phases, four points were used to extraction of RNA and performing Real-time PCR. Consequently, 3 and 7 hours were selected as representatives of the logarithmic phase of growth and hours 11 and 15 as the representatives of the growth phase.

In this study,to extraction of RNA, RNeasy Mini Kit (Qiagene Company, Germany) was used. The base of this kit is guanidine thiocyanate buffer which by deactivation of Rnase enzymes provide the conditions for the extraction of high-quality RNA. For very sensitive applications such as RT-PCR, the RNA product should be treated with DNase to remove small amounts of residual DNA. For this purpose, was used the RNase-Free DNase set (Qiagene Company, Germany) which contains 1500 units of DNase I enzyme.

In order to determine the concentration of RNA extracted from Eppendorf biophotometer at the wavelength of 260 nm (A260) was used and the purity of the extracted RNA was identified

Table 2. Primers used in this study.

Name	Sequence (5′→3′)	Amp. (bp)	Ref.
agrA (F)	TGATAATCCTTATGAGGTGCTT	163	(7)
agrA (R)	CACTGTGACTCGTAACGAAAA		
***RNAIII* (F)**	CGATGTTGTTTACGATAGCTT	146	(5)
***RNAIII* (R)**	CCATCCCAACTTAATAACCA		
hla (F)	GGGGACCATATGATAGAGATT	154	(5)
hla (R)	TGTAGCGAAGTCTGGTGAAA		
spa (F)	GATGGTAACGGAGTACATGTCGTT	163	(8)
spa (R)	TTGCTGGTTGCTTCTTATCAACA		
mecA (F)	ACTGCTATCCACCCTCAAAC	163	(9)
mecA (R)	CTGGTGAAGTTGTAATCTGG		
gyrB (F)	CGCAGGCGATTTTACCATTA	141	(10)
gyrB (R)	GCTTTCGCTAGATCAAAGTCG		

from about 260 nm to 280 nm (A260/A280). Integrity and quality of RNA were tested by agarose gel electrophoresis and ethidium bromide staining tests so that two bands of 1.5 (16S) and 2.9 (23S) kb were observed.

After RNA extraction, extracted RNA samples were stored at -70 ° C until the operation of real-time PCR.

Real Time PCR and analysis of gene expression

For analysis of transcript levels in the extracted RNA, after the selection of appropriate primers for the genes, real-time PCR was performed using SYBR Green through the One-Step RT-PCR method and with QuantiFast SYBR Green RT -PCR kit (Qiagene Company, Germany). Real Time PCR was performed with an ABI Prism 7900 thermocycler (Applied Biosystems) and the target gene expression levels in RNA extracted were normalized based on *gyrB* gene as an internal control. Each test was repeated three times.

Primers used in this study are listed in Table 2 that were confirmed in NCBI website using BLAST pairing place and length of PCR product.

In this study, the relative quantitaion ($2^{-\Delta\Delta C}{}_T$ method) was used to analysis the gene expression (11). With operation of Real-time PCR, the device software reports the results as C_T. The increase or decrease in gene expression was compared with a reference gene as an internal control (ΔC_T). In this study the amount of target gene in the sample is normalized with a housekeeping gene of *gyrB* as internal control. To calculate the relative expression levels of a gene relative to a calibrator (BHI broth containing 5% calf serum relative to BHI broth or stationary phase relative to exponential phase), was operated based on Δ (ΔC_T) method.

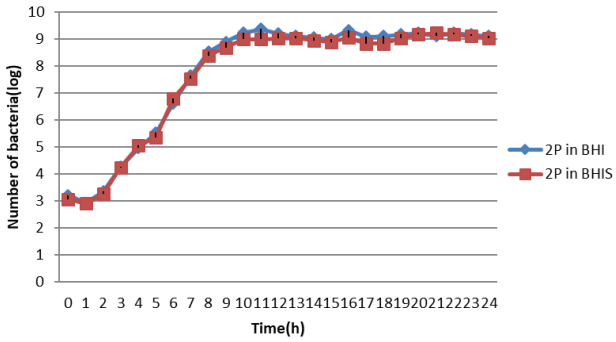

Figure 1. Growth curve Clinical isolate MRSA (2P) in BHI broth (BHI) and BHI broth containing 5% Calf serum (BHIS) at 24 h.

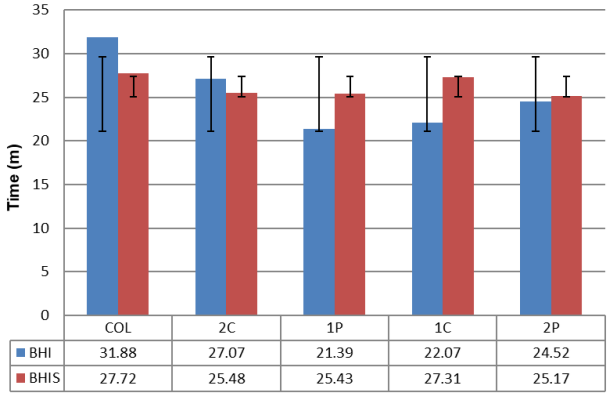

Figure 2. Generation time of *S. aureus* isolates in BHI and BHIS.

The amount of change in the gene expression (Fold Change) is calculated given by $2^{-\Delta\Delta C_T}$ (11).

(1): $\Delta C_T = C_{T,target} - C_{T,reference}$

(2): $\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (calibrator)

Statistical analysis

The tests were repeated three times and the results were analyzed by *SPSS* (version 15) software through analysis of variance (*ANOVA*) and Duncan test at the significant level of 0.05.

Results

Results of the growth curves of isolates

Fig 1 shows growth curve of Clinical isolate methicillin-resistant *S. aureus* (2P) in BHI broth (BHI) and BHI broth containing 5% calf serum (BHIS) at 24 h (Fig 1). The growth curves of other isolates were similar to 2P, in which the corresponding data were not shown.

The mean of generation time of five bacteria in BHI broth and BHI broth containing 5% calf serum was 25.4 and 26.2 min respectively which were not statistically significant (P>0.05). The mean generation time of MRSA was higher than MSSA isolates, which was 25.5 min and 24 min for MRSA and MSSA respectively. The mean generation time of clinical and healthy carrier isolates were 24.1 min and 25.5 min respectively (Fig 2).

Results of evaluation of genes expression

The results of plotting the growth curve showed that in all isolates and in both the medium of BHI and BHIS, the beginning of the exponential phase and middle exponential phase were at hours 3 and 7, respectively also beginning of the stationary phase and the middle stationary phase were at hours 11 and 15, respectively. Therefore, the average of C_T of hours 3 and 7 were considered as the exponential phase and the mean of C_T of hours 11 and 15 were considered as stationary phase.

One of the aims of this study is to study the pattern of gene expression in exponential and stationary phases. Fig 3 shows the mean of the expressions of the studied genes in five isolates in the stationary phase compared to the exponential phase in two different culture conditions.

In the BHI broth containing 5% calf serum the expression levels of regulatory genes increased, so that the expression of *agrA* and *RNAIII* genes in the stationary phase relative to exponential phase was increased 3.4 and 9.5

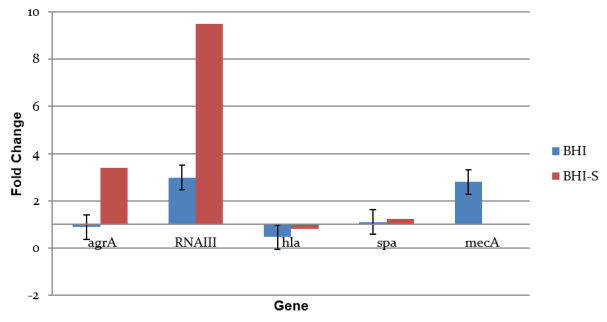

Figure 3. The mean of the gene expression of five isolates in the stationary phase compared to the exponential phase in BHI and BHIS.

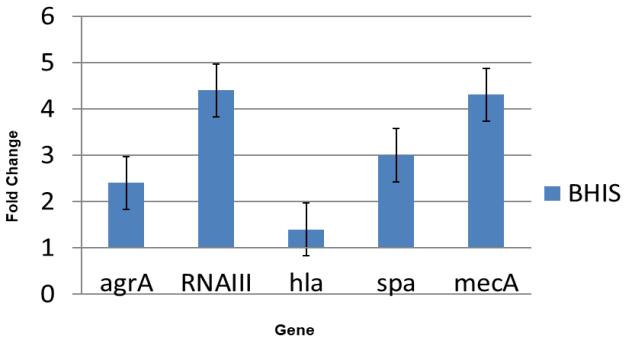

Figure 4. The mean of the gene expression of five isolates in BHIS relative to BHI (Regardless the growth phase).

fold, respectively. but this *agr* system could not play its regulatory role well in the expression of virulence genes so that the expression of *hla* gene was decreased 0.81 fold and the expression levels of *spa* and *mecA* genes just was increased 1.25 and 1.03 fold, respectively (Fig 3).

In the BHI broth the expression levels of regulatory gene of *agrA* in the stationary phase relative to exponential phase was decreased 0.89-fold, but 2.99-fold increase in gene expression of *RNAIII* gene could not apply regulatory effects. The reason for this is a 0.47 fold decrease of gene expression in stationary phase relative to exponential phase. Of course, in this condition, expression of the methicillin resistance gene, *mecA*, showed a 2.8 fold increase in stationary phase. The change in the expression of the *spa* gene was also not significant (Fig 3).

The other objectives of the this study are to compare the expression of genes in different culture media conditions, and to answer the question which of the culture media (BHI or BHIS) are more suitable for the expression of the studied genes? In this regard, the expression levels of each of the genes in the BHI broth containing 5% calf serum was compared with BHI broth.

Regardless the growth phase, expression of all genes in BHI broth containing 5% calf serum showed an increase compared to BHI broth. As seen in Fig 4, the genes expression levels of *agrA* (as representative of *agr* system), *RNAIII* (the effecter molecule of the *agr* system), *hla* (encoding alpha-toxin), *spa* (encoding protein A) and *mecA* (encoding resistance of methicillin) in BHI broth containing 5% calf serum relative to BHI broth was increased 2.4, 4.4, 1.4, 3 and 4.3-fold, respectively (Fig 4).

Comparison of the genes expression levels between MRSA and MSSA isolates and between clinical isolates and healthy carriers was one of the other goals of this study.

In Fig 5, the mean of ΔCT of each of the genes in the MRSA and MSSA isolates, regardless of the growth phase and the type of culture media, has been shown. As regards, less ΔCT indicates a higher expression of gene, as a result, all genes, except *spa*, are more expressed in MSSA isolates (Fig 5).

In Fig 6, the mean of ΔC_T of each of the genes in the clinical and healthy carriers isolates, regardless of the growth phase and the type of culture media, has been shown. As regards, less ΔC_T indicates a higher expression of gene, In

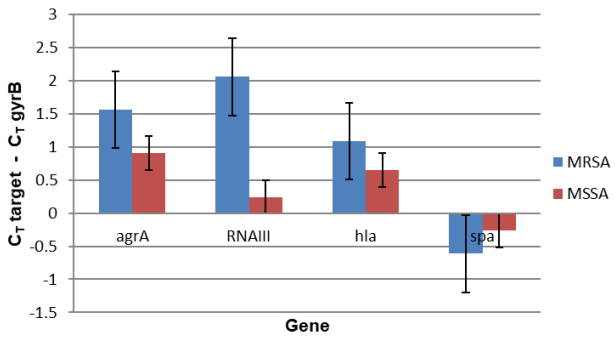

Figure 5. The mean of ΔCT of genes in the MRSA and MSSA isolates regardless of the growth phase and the type of culture media.

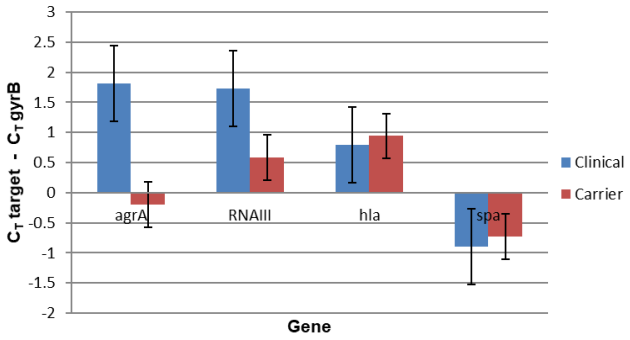

Figure 6. The mean of ΔCT of genes in the clinical and healthy carrier isolates regardless of the growth phase and the type of culture media.

general, this can be achieved that regulatory genes of *agrA* and *RNAIII* are more expressed in clinical isolates and in the case of the *hla* and *spa* genes, there is no significant difference between the clinical and healthy carrier isolates (Fig 6).

Discussion

The results of the growth curves plotting and generation time of isolates showed that addition of 5% calf serum in BHI culture media was not able to increase the growth rate of *S.aureus*. These findings are correspond with the results of others studies (5). BHI broth is an laboratory culture medium very nutritious and ideal for growth *S.aureus* and addition of calf serum is not able to alter nutrition conditions.

As mentioned *agr* system in *Staphylococcus aureus* as a quorom sensing system following increased cellular concentration in the stationary growth phase increases the transcription of secretory toxins genes such as alpha hemolysin (*hla*) and decreases th transcription of bacterial binding genes such as protein A (*spa*) (4).

But the results of this research the effectiveness of addition of 5% serum to BHI broth for activating *agr* and quorom sensing systems did not show in spite of the increased expression of *agrA* and *RNAIII* regulatory genes, however, this *agr* system could not play its regulatory role in expressing the virulence genes, and the expression of the *hla* gene decreased, and the expression levels of *spa* and *mecA* genes did not show significant changes. This findings confirm the results of the study by Abu Othman *et al* and Oogai *et al* that showed that agr system has is not effective to expression of virulence genes in the presence of serum (5,12). Are there factors in serum that prevents the *agr* system activity? The results of the study by Regassa *et al* showed the regulatory role of *agr* expression is not same in all strains and isolates. On the other hand, it is shown that the expression of this system is affected by various environmental factors and this system responds to environmental stimuli, for instance, there is evidence that some conditions like the glucose level and pH changes are effective in *agr* expression and toxin production (13). In addition, the diversity and polymorphism in the *agr* locus is associated with differences in activity level of strains (14).

Regardless of the growth phase, the expression of all genes in BHI broth containing serum was increased compared to the BHI medium. *Staphylococcus aureus* in the presence of calf serum factors may induce similar conditions to host and the expression profile of regulatory and virulence genes was similar to those seen *in vivo* conditions. This study suggest that addition of serum to bacteria culture media indicates that likely to the have a a host environment that produced more invasive factors than laboratory culture medium of BHI broth. this can confirm the fact that *agr* gene expression is influenced by environmental factors (13).

The regulatory genes expression of *agr* system in the BHI medium shows a relative decrease in the stationary phase, which means that despite the increase in the number quorom sensing system is not active. This finding are consistent with the results of Oogai et al. The researchers showed that in the laboratory culture medium

of Tryptic soy broth (TSB), the expression of *RNAIII* regulatory gene was increased in stationary phase, but this increase was associated with a decrease in the expression of some virulence genes, including *aur* (aerolysine encoding gene) and pantone-valentine leukocidin (*PVL*) (5).

The probable reason for this is that the laboratory culture medium of BHI broth, which is a very nutritious culture medium, and the bacteria do not need to invasion, and despite the increased cellular concentration, it does not activate the *agr* system to regulate virolence genes.

The impact of the culture medium type on the expression of virulence genes has been reviewed and approved in several studies; Malachowa et al demonstrated that expression of gamma-hemolysin (*hlg*) gene and Pantone-Valentine leukocidin (*PVL*) gene in three culture medium of BHI, TSB and CCY is different: PVL gene was expressed better in CCY medium than the other two culture media and *hlg* gene shows better expression in BHI broth. It is interesting that PVL gene expression in CCY medium is much more in the stationary phase than the exponential phase, while this gene in TSB medium is more expressed in exponential phase and in BHI broth expression of this gene in stationary and exponential phase is almost the same (15).

Comparison of the genes expression pattern in clinical and healthy carriers isolates showed that regulatory genes of *agrA* and *RNAIII* are more expressed in healthy carrier isolates, but the expression of virulence genes in the two groups there was no significant difference (Fig 6). Other studies have mentioned to the similarity of the frequency of virulence genes in isolated *S. aureus* from healthy carriers and patients (16). Abu Othman *et al* also concluded that the isolate origin cannot be without regard to of other genetic factors relating to the isolate effective in its pathogenicity (12).

The pattern of gene expression gene is variable depending on the growth conditions of the isolate. That's mean if clinical isolates cultured in laboratory conditions are in vivo conditions and in the host body, due to the their aggressiveness show a more realistic expression profile (8).

The results of this study indicate that all genes are more expressed in MSSA isolates. The reduction of expression of virulence factors due to the acquisition of antibiotic resistance in other studies has also been proven (17,18). Also decreased expression of virulence genes associated with type and size cassette *SCCmec* . As MRSA strains carrying the larger cassette *SCCmec,* there showed higher energy demand and reduction in virulence factor secretion. Reduction in the expression of virulence factors in MRSA isolates is related to size of the *SCCmec* cassette, since The expression of virulence factors in the MRSA isolates having *SCCmec II* cassettes is lower than the those having *SCCmec IV* cassettes because they have a larger cassette size (19).

Another reason may be that MRSA isolates, especially those with larger chromosomal cassettes, require more energy to grow and reproduction and expression of the *SCCmec* components, which reduces the expression of virulence genes, but MSSA isolates do not require this energy and expressed virulence factors (19,20). Lower generation time in

MSSA isolates as compared to MRSA isolates, which was also observed in this study, can be a proof to the validity of this theory.

Conclusion

In general, in the culture medium of BHI broth, which is a very nutritious culture medium, inactivity of the *agr* system and expression of virulence factors was observed that implies bacteria do not need to invasion, and despite the increased cellular concentration, it does not activate the *agr* system to regulate virulence genes. Regardless from growth phase, the expression levels all of genes in BHI broth containing 5% calf serum relative to BHI broth were increased. As a result *Staphylococcus aureus* in the presence of calf serum factors induce expression of virulence genes was similar to those seen *in vivo* conditions.

Acknowledgment

The authors thank all staff of the Microbiology laboratory of Golestan University of Medical Sciences.

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