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Research Article

VIRULENCE GENES EXPRESSION AMONG METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUSISOLATED FORM CANCER AND NON-CANCER PATIENTS

Alreshidi M. A[1]., Neela, V[2]., Alsharari A. S[3]., Hamat R. A[2]., Alsalamah A. A[4]., Alzoghaibi O[5] and Shamsudin M. N[2]

[1]Department of Medicla Laboratory Sciences, Faculty of Applied Medical Sciencese, Alrajhi colleges, Albukairiyah 5194, Saudi Arabia
[2]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
[3]Department of Basic Health Sciences, Faculty of Pharmacy, Northern Border University, Arar, Saudi Arabia
[4]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia
[5]Ministry of Health, Albkairiyah General Hospital, Saudi Arabia

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ABSTRACT

Relative quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) assay was designed and applied in order to study the expression levels of selected genes encoding the adherence and toxins virulent factors. Relative quantification qPCR showed a significant higher expression level of common genes tested among strains isolated from cancer patients not only within the clone but also among different lineages. This study demonstrated that although all MRSA strains studied from cancer and non-cancer patients possessed several virulence determinantsthe expression rather than presence of virulence determinants may mediate higher pathogenicity potential. These data will aid in developing more effective infection control strategy to improve the management of MRSA infection in cancer patients.

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INTRODUCTION

Although the extracellular and cell-bound virulence genes are under the coordinated expression by accessory gene regulator system (*agr*), the transcription profiles of virulence genes vary depending on the dynamic interaction between the host environment and the pathogen. With the lack of a definitive correlation between invasive MRSA strains and the carriage of virulence genes (Abu Othman *et al.*, 2011), the assessment of virulence genes expression has become crucial to understand the pathogenesis of staphylococcal infections. Since the host environment may be differentially altered in cancer compared to non-cancer patients, the expression of MRSA virulence genes may also vary.

Quantitative RT-PCR is now a common method for measuring gene expression, offering a highly sensitive, efficient and fast quantification of transcriptional levels of the gene of interest even with a single copy of a specific transcript (Wong & Medrano, 2005).

Assessment of virulence genes expression has become useful genetic tool to investigate the expression level of interested genes. Reverse transcription (RT) followed by polymerasechain reaction (RT-PCR) represents a powerful tool and it is the most suitable method for gene expression analysis (Chini *et al.*, 2007). With the lack of adefinitive correlation between invasive MRSA strains and the carriage of virulencegenes (Abu Othman *et al.*, 2011), the current study hypothesizes that, the genetic background of MRSA strains from cancer and non-cancer patients are similar in each country but the virulence genes

Corresponding author:* **Alreshidi M. A
Department of Medicla Laboratory Sciences, Faculty of Applied Medical Sciencese, Alrajhi colleges, Albukairiyah 5194, Saudi Arabia

expression level may vary, hence, the expression, rather than carriage of virulence determinants may mediate pathogenicity. The aim of this study was to develop and test a real-time qPCR assay for expression analysis of MRSA transcripts in cancer and non-cancer patients. Target genes chosen for analysis included several characterized MRSA virulence factors as well as putative antiseptic resistance virulence factors *qacA/B*. Consequently, comparing the expression of various MRSA virulence genes can provide a more effective evaluation of the pathogenic and toxic hazards of MRSA from different clinical sources.

MATERIALS AND METHODS

The relative expression of several virulence genes including *fnbA, fnbB, clfA, clfB, fib, eno, cna*, *sea* and *qacA/B* which were positive at least in three isolates each from cancer (sample group) and non-cancer (control group) patients in both countries was investigated using RT-PCR. A collection of 13 representative MRSA strains from different clinical specimens from different patients were selected based on the presence of gene interesting and their genetic profile according to the *agr*, SCC*mec*, MLST and *spa* types (Table 1).

Table 1 Clinical sources, genotypes and virulence genes content of MRSA strains used for gene expression study

agr*/SCC*mec*/ MLST / *spa	**Strain**	***fnbA***	***fnbB***	***clfA***	***clfB***	***fib***	***eno***	***cna***	***sea***	***qacA/B***
1/III/239/037	NCS 1	+	+	+	+	+	+	+	-	+
1/III/239/037	NCS 20	+	+	+	+	+	+	+	-	+
1/III/239/037	NCM 1	+	+	+	+	+	+	+	+	+
1/III/239/037	NCM 20	+	+	+	+	+	+	+	+	+
1/III/22/7604	NCS 3	+	-	+	+	-	+	+	+	-
1/III/08/304	CS 19	+	-	+	+	+	+	+	+	-
1/III/239/037	CS 44	+	+	+	+	+	+	+	+	+
1/III/239/030	CS 40	+	+	+	+	+	+	+	-	+
1/III/239/037	CS 23	+	+	+	+	+	+	-	-	+
1/III/239/037	NCM 10	+	-	+	+	+	+	+	+	+
1/III/239/037	CM 4	+	+	+	+	+	+	+	+	+
1/III/239/037	CM 1	+	+	+	+	+	+	+	+	+
1/III/239/032	CM 5	+	-	+	+	+	+	+	+	+
1/III/188/189	CM 6	+	+	+	+	-	+	+	+	-
1/III/239/037	NCM 3	+	+	+	+	+	+	+	+	+
2/III/71/02	NCS 9	+	-	+	+	+	+	+	+	-
3/III/30/019	NCS 22	+	-	+	+	+	+	+	+	-
1/III/241/363	NCS 54	+	-	+	+	+	+	+	+	-
1/III/239/037	NCS 57	+	+	+	+	+	+	-	-	+

RNA extraction

Total RNA was extracted from the culture using RiboPure™ Bacteria kit (Ambion).The kit also included Ambion's DNA*free*™ reagents for the post-elution removal of contaminating genomic DNA. Chemicals and reagents used were of RNase – free grade and standard precautions against including RNases were taken. Extracted RNA was stored at -80 °C until further use.

Measurement of RNA concentration purity and integrity

The RNA extracted was subjected to determine its concentration and purity. The RNA was diluted to 50 folds by adding 1µl of extracted RNA to 49 µl of distilled water. The ratio of A_{260}/A_{280} indicated the concentration and the purity of the sample were measured using the BioPhotometer plus (Eppendorf, Germany). The quality of RNA is considered pure when the A_{260}/A_{280}ratio is within the range of 1.9 and 2.1.

In order to assay the quality of the RNA samples and to check the DNA contamination, the samples were separated through electrophoresis using 1 % agarose gel in 1X TBE buffer at 65 V for 90 min. A ready-to-use lambda hind III ladder (Vivantis) was used as the standard marker.

Genomic DNA contamination was tested by PCR with the specific primers and extracted RNA as template using the appropriate reaction mix composition and thermal conditions for the gene of interest. In case of a positive result, sample was discarded and RNA extraction was repeated for this sample.

cDNA synthesis

A thin walled 0.2 ml PCR tube containing 25 µl of 2X SYBR® RT Buffer, 2.5 µl of 20X RT enzyme mix and 12.5 µl of nuclease-free water mixed with 10µl containing 3 µg of total RNA was incubated in thermal cycler at 37°C for 60 min, followed by 95°C for 5 min to inactivate the RT enzyme. A synthesized cDNA was stored at -20°C until further use. Minus-RT controls which contained all the RT reaction components except the 20X RT Enzyme Mix (substituted with water) was included. Minus-RT controls were used to demonstrate that the template for the PCR was cDNA, not genomic DNA.

Primers were designed from GenBank sequences (http://www.ncbi.nlm.nih.gov) with the aid of BeaconDesigner 7.91 software (http://www.premierbiosoft.com) and PerlPrimer v1.1.20 (http://perlprimer.sourceforge.net). Gene specificity of all primers was confirmed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Melting curve and electrophoresis analysis were performed to confirm the presence of a single product and absence of primer-dimers. Representative of synthesized primer reports and parameter used in this study are shown in appendix E.

PCR efficiency

The PCR efficiency (E) for each set of primers was determined by generating cDNA dilution curves. Known amounts of serially diluted cDNA were subjected to qRT-PCR analysis. The threshold cycle (CT) for each cDNA amount was plotted against the log of the cDNA concentration. The slope of this curve was determined and the PCR efficiency was calculated according to Realplex software. Quantification of DNA for

standard curve generation was done by UV spectrophotometry.

Quantitative real-time Polymerase Chain Reaction (qPCR)

Real-time (RT)-PCR was carried out to assess the transcriptional level of the selected virulence genes with newly synthesized primers (Table 2). The PCR reactions were carried out in 25-µL volumes containing 10 µl Fast SYBR Green (Ambion, Austin, TX), 1µl of each primer (300 nM final) 5 µl of a 1/100 template and 3 µl of nuclease-free water. The real-time PCR analysis was performed using a LightCycler 2.01 Instrument system Mastercycler ep Realplex (Eppendrof) with the cycling conditions which consisted of an initial step at 95°C for 20 seconds followed by an amplification program for 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C with fluorescence acquisition at the end of each extension. The amplification program was followed immediately by a melt program consisting of 1 minute at 95°C, 1 minute at 65°C, and a gradual increase to 90°C at a temperature transition rate of 0.2°C per second with fluorescence acquisition at each temperature transition. Two controls were included with every run: no-template and no-amplification controls. No-template controls (NTC) contain all the PCR components except the cell lysate (substituted with water). If the no-template control yields a fluorescent signal, the RT or PCR reagents may be contaminated with DNA.

As fluorescent contaminants can cause false-positive results in real-time PCR using SYBR Green Dye, a no-amplification control (NAC) that contained sample and all the PCR components except the Fast SYBR Green PCR Master Mix was included. If the fluorescence of the NAC is greater than that of the NTC after PCR, fluorescent contaminants may be present in the sample or in the heat block of the thermal cycler. The *16s* gene was used as an internal control housekeeping gene to normalize the levels of expression between samples. The real-time RT-PCR data were analyzed by the (ΔΔCt) method using the Realplex and Relative Expression Software Tool (REST) V.2.0.13 (Qiagen, Gmbh).

RESULTS

RNA concentration, purity and integrity

The extracted RNA was analyzed for concentration, purity and integrity. Based on absorbance readings at 260 nm and 280 nm,

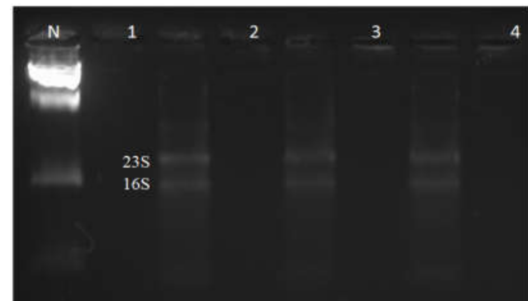

Figure 1 Representative gel pictures shows the quality of total RNA

Lanes 1, 2, 3: Isolates NCM 10, CM 4 and NCS
Negative control (N), M: Hind III marker.

the concentration and A_{260}/A_{280} ratio of each RNA preparation were determined. The concentrations of extracted RNA ranged from 43 to 550 µg/ml and the averages of A_{260}/A_{280} ratio were in the range of 1.7 to 2.1, indicating protein-free RNA samples. Agarose gel electrophoresis of the RNA preparations showed defined and intact RNA bands (Figure 1).

Validation of reaction specificity and efficiency

The specificity of each primer pair was analyzed by qRT-PCR using the melting curve analysis feature of the Realplex software. The melting curve analysis revealed a single, product-specific signal for each primer pair indicating that no primer dimers interfered with the fluorescence measurement (Figure 2).

PCR efficiency of each primer pair was determined by qRT-PCR. For each assay, duplicate reactions were conducted on each dilution of five-fold cDNA dilution series.

Table 2 The sequences of the primers utilized in gene expression study

Gene	Primer sequence	Genbank accession	Product size (bp)	Reference
FnbA	F: 5- CAATCATATAACGCAACAG-3 R: 5- AGAAGAGGTAGTTAAGGAA-3	AM749014	141	This study
FnbB	F: 5- GGTGGAGTTAGAGATAATTGG-3 R: 5- AACCTAACAACCAGTCGT-3	AM076084	112	This study
ClfA	F: 5-CTCAGATAGCGACTCAGAT-3 R: 5-AGATTCCGACAGTGACTC-3	Z18852	87	This study
Fib	F: 5-CGGCAATAGGTATTACTA-3 R: 5-AATCACAATATCGTAGAGT-3	X72014	108	This study
Eno	F: 5- TGTATCTATCGCAGTAGCA-3 R:5- CGTTAATGGTGGTTCTCA-3	AF065394	126	This study
Cna	F: 5- GGAGATATGCTACCAGAAGATACG-3 R: 5- TTCAAGGTGGACAGCAGTTAG-3	M81736	121	This study
qacA/B	F: 5- GTAGCAGTACTTGGTAGCC-3 R: 5- AACGAGGCTGTAACATCAT-3	X56628	145	This study
Sea	F: 5- TTGGAAACGGTTAAAACGAA-3 R: 5- TGTTTTTGATGGGAAGGTTC-3	M18970	121	This study
16s	F: 5- CCGCCTGGGGAGTACG-3 R: 5- AAGGGTTGCGCTCGTTGC-3			(Lee, Moon, Park, Chang, & Kim, 2007

Statistical Analysis

Data were analyzed using statistical software packages SPSSversion 18.0 (SPSS Inc., Chicago, IL, USA). A *p* value less than 0.05 was considered statistically significant.

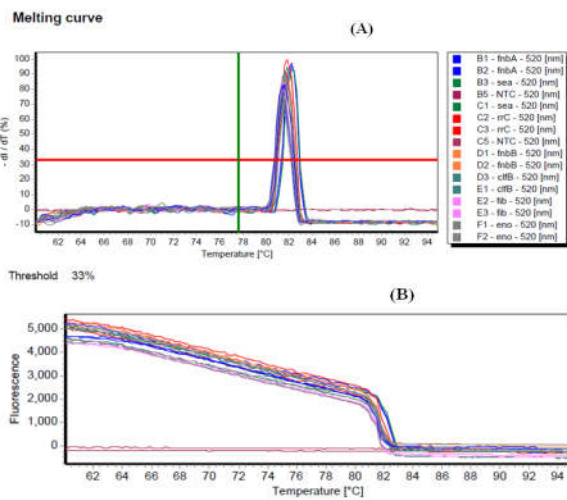

Figure 2 Specificity of synthesized primers for amplification of MRSA genes *fnbA*, *sea*, *rrC*, *fnbB*, *clfB*, *fib*, and *eno*, as determined by melting peak analysis consists of a single peak, indicating a highly specific PCR reaction (A).
Raw data shows a dramatic drop in fluorescence when DNA is melted (denatured); the fact that the drop is seen in several samples at the same time indicates the presence of a single product, i.e., a highly specific reaction (B)

Standard curves were generated by plotting the relative dilution of cDNA versus the cycle number required to elevate the fluorescence signal above the threshold (Figure 3).

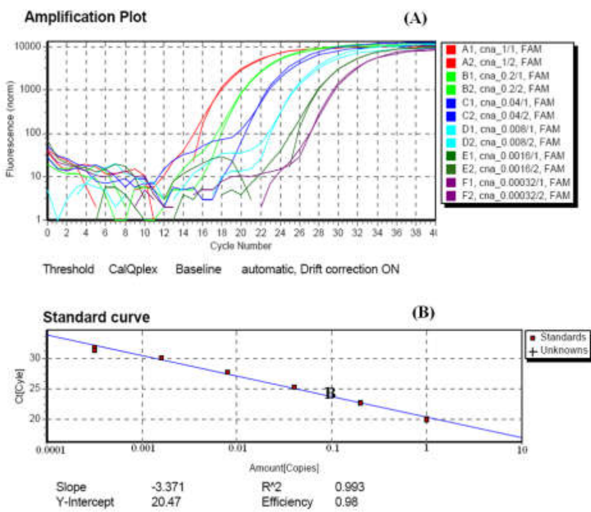

Figure 3 Representative picture of a real-time PCR output for efficiency determination. A two-fold dilution series wwere amplified (A) to create a standard curve (B). The results were generated by Realplex software.

The efficiencies ranged from 0.98 for the *clfA* primer pair to 1.03 for f*nbA* (Table 3).

Table 3 The slope, R2 and efficiency values for each of the genes, calculated from the standard curves

Genes	slope	efficiency	R_2	*Y-intercept Ct*
fnbA	-3.319	1	0.980	18.67
fnbB	-3.372	0.98	0.993	20.47
clfA	-3.369	0.98	0.996	19.54
clfB	-3.357	0.99	0.996	20.57
fib	-3.319	1	0.990	19.47
cna	-3.371	0.98	0.993	20.47
sea	-3.319	1	0.990	19.47
qacA/B	-3.316	1	0.991	8.93
16s	-3.256	1.03	0.980	14.87

The high PCR efficiencies for each primer pairs, as well as primer specificity shown by melting curve are indicative of an optimal qRT-PCR setup.

Relative expression of MRSA virulence genes by qRT-PCR

All genes were up-regulated in MRSA strains isolated from cancer patients compared to non-cancer patients except for *fnbB*, *clfB*, *fib* and *eno* which were down regulated (Table 4).

Table 4 Relative gene expression values in cancer compared to non-cancer patients

Gene	Type	Reaction efficiency	Expression	95% C.I.	Result
16s	REF	1.0	1.000		
fnbA	TRG	1.0	4.272	2.634 - 6.190	UP
fnbB	TRG	1.0	0.085	0.033 - 0.203	DOWN
clfA	TRG	0.98	62.136	18.304 - 153.745	UP
clfB	TRG	0.99	0.148	0.106 - 0.261	DOWN
fib	TRG	1.0	0.129	0.086 - 0.167	DOWN
eno	TRG	1.0	0.129	0.086 - 0.167	DOWN
cna	TRG	0.98	7.825	4.214 - 15.276	UP
sea	TRG	1.0	23.835	9.824 - 62.099	UP
qacA/B	TRG	1.0	33.282	15.402 - 80.759	UP

REF: reference, TRG: target

The fold changes ranged from 1.77 for *fnbA* gene to 182 folds for *clfA* gene (Figure 4).

Gene expression profiles were analyzed using the relative expression software REST© and the detailed report is shown in Figure 4.

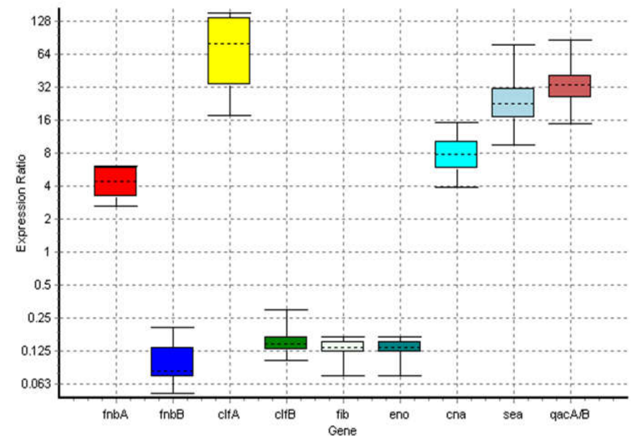

Figure 4 Graph view showing the relative gene expression folds. Boxes represent the interquartile range, or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

DISCUSSION

The success of *S. aureus* especially MRSA as a pathogen is primarily due to its ability to produce a large number of virulence factors. However, the role of different virulence factors in the development of staphylococcal infections remains incompletely understood (Gordon & Lowy, 2008). It appears that carriage of virulence genes by *S. aureus* does not influence the infection prognosis in general. Several authors have compared the frequency of presence of certain adhesion genes between isolates from diseased and healthy individuals. Donnell *et al*., (2008) indicated that, no significant correlation between the presence of 17 virulence genes including adhesion

and enterotoxin genes and invasiveness of MRSA isolates (O'Donnell, Humphreys, & Hughes, 2008). Another study by Lindsay *et al*., (2006) also demonstrated this non-association of specific genes or combinations of genes with invasive isolates and there were no consistent differences in gene content that could be used to distinguish between invasive and carriage isolates (Lindsay *et al*., 2006).

Although it is clear that the carriage of certain toxin genes is associated with toxin-mediated diseases such as toxic shock syndrome and scalded skin syndrome. However, Sharma *et al*., (2000) reported that toxin genes were detected in toxin-non-producing strains of *S. aureus*, due to low-level production of enterotoxin, mutations in the coding region or affected by the growth conditions used (Sharma, Rees, & Dodd, 2000). Another recent study demonstrated that enterotoxin production varies greatly among clinical strains grown to the same density under identical conditions (Varshney *et al*., 2009). Hence, it is important to recognize that the presence of the enterotoxin genes in *S. aureus* isolates does not necessarily indicate the ability of these isolates to produce intact and biologically active toxin or to produce sufficient toxin to induce disease (Naffa, Bdour, Migdadi, & Shehabi, 2006). As highlighted by the above mentioned studies and our findings in this study, the inability to find a definitive correlation between infectivity *S. aureus* strains and the carriage of virulence genes, suggests that either it is the expression of virulence determinants rather than the presence of the genes themselves that mediates pathogenicity or that host immune factors may play a significant role in the disease outcome. Hence, the elucidation of these virulence genes expression levels in cancer patients versus patients with MRSA infections will explain complexities of MRSA pathogenesis and contribute to our understanding of the association between gene expression and high infectivity of MRSA in cancer patients.

The aim of this study was to develop and test a real-time qPCR assay for expression analysis of MRSA transcripts in cancer and non-cancer patients. Target genes chosen for analysis included several characterized MRSA virulence factors as well as putative antiseptic resistance virulence factors *qacA/B*. Consequently, comparing the expression of various MRSA virulence genes can provide a more effective evaluation of the pathogenic and toxic hazards of MRSA from different clinical sources.

Real-time qPCR is both a thermodynamic and an enzymatic process. Successful real-time PCR requires amplification and detection under optimal conditions and each reaction component can affect the result. The important steps in the design and evaluation of theexperiment are: (*i*) select an internal control gene, (*ii*) validate the internal control to determine that it is not affected by experimental treatment, and (*iii*) PCR perform on dilutions of cDNA for both the targetand internal control genes to ensure that the efficiencies are similar (Livak & Schmittgen, 2001).

Prerequisites for successful real-time qPCR include design of optimal primer pairs for each reaction. The use of primers that have been used in traditional PCR assays is not recommended as the criteria of successful design for real-time PCR may not have been used in the design of primers used in other applications. For efficient amplification in real-time qPCR, primers should be designed so that the size of the amplicon is ideally <150 bp, enabling comparison of amplification reactions for different targets. The Amplification efficiency and sensitivity of the reaction drop significantly with increasing amplicon size. Hence, all primer set used in this study were newly designed with product size ranging from 87 to 145bp.

It is particularly important to minimize nonspecific primer annealing so that high yields of specific PCR product are obtained. The annealing step in PCR is affected by both primer design and primer concentration. When primers anneal to the template with high specificity, this leads to high yields of specific PCR products and increases the sensitivity of the amplification reaction. However, due to the high primer concentration in the reaction, primers will also hybridize to non-complementary sequences with mismatches. If the primers anneal to the template sequence with low specificity, amplification of nonspecific PCR products and primer–dimers may occur. Competition in the amplification reaction between these artifacts and the desired PCR product may reduce the yield of the specific product, thereby reducing the sensitivity and linear range of the real-time reaction.

Relative quantitation study requires optimally designed procedure. When all assays are designed under the same guidelines, all assays can be run at the same time with the same conditions and requires little, if any optimization. By this universality of design, same annealing and extension temperature was used during this experiment to prevent non-specific bindings and increases the sensitivity of the amplification reaction.

Real-time PCR specificity can also be increased by using a hot start, where an inactive DNA polymerase is activated at the start of PCR by incubation at a high temperature. A hot start increases PCR specificity because it prevents the formation of primer–dimers and nonspecific products during reaction setup and the initial heating step. After a hot start, these PCR artifacts are also absent in every PCR cycle. In this study temperature at 95°C was used as hot start to increases PCR specificity and prevents the formation of primer-dimers.

The SYBR Green dye was used in this study, which is a fluorescent dye that binds to the minor groove of the DNA double helix. It is an easy and cost-effective approach to real-time, as it does not require the design of sequence-specific probes (Chini, Foka, Dimitracopoulos, & Spiliopoulou, 2007). However, low PCR specificity can significantly affect quantitative PCR particularly when using SYBR Green for detection. As SYBR Green binds to any double-stranded DNA sequence, primer-dimers and other nonspecific PCR products will generate a fluorescent signal. This reduces the overall sensitivity of the assay and also leads to inaccurate quantification of the transcript of interest. This issue can be eliminated with well primer designed and appropriate reaction conditions.

One of the most important steps in relative quantitation experimental design is the selection of an appropriate endogenous control. Choosing the “correct” normalization approach therefore is both essential and critical for obtaining reliable expression data. Normalization to an endogenous control (often referred to as a housekeeping gene or reference gene) allow to correct results that can be varied by differing

amounts of input nucleic acid template. Any gene shown to be expressed at the same level in all study samples can potentially be used as reference gene.

Because the reference gene is used to normalize differences in the amount of cDNA that is loaded into PCR reaction wells, reference gene expression levels must the same in all samples in the study. So, it is critical to determine if the study treatment or intervention is affecting the expression level of candidate reference gene genes. Reference gene expression levels should vary only slightly.

In this study, the expression stability of four potential reference genes among MRSA isolated from cancer and non-cancer patients were investigated. The candidate reference genes were chosen from three different pathways to minimize chances that their expression is co-regulated and therefore affected by the same experimental conditions.

All our analyses on the stability of the references genes using the different algorithms showed consistent results with only slight differences in the ranking order. The ribosomal gene *16s* was shown to be stably expressed in the isolates from cancer and non-cancer patients used in this study.

The quantification procedures differ depending on whether the target and the endogenous reference gene are amplified with comparable or different efficiencies (Duquenne *et al*., 2010). The ΔΔCT method does not take into account the efficiency correction and should only be chosen if the PCR efficiency of the target gene and endogenous reference gene are similar (Chini *et al*., 2007). Although in biological samples the reaction efficiency is rarely 100% (Axtner & Sommer, 2009). The differences in efficiency between the target and the endogenous reference gene can be corrected by using efficiency-corrected calculation programs, such as the Relative Expression Software Tool (REST©). All genes investigated in this study were with comparable efficiencies with reference gene and within acceptable range from 0.97 to 1.03.

After identified the optimal real-time qPCR setup, the genes expression were confirmed by a real-time qPCR where the *16s* was used as the reference gene and the virulence genes as the target genes among MRSA isolates from cancer and non-cancer patients. The expressed internal reference gene is usually quantified at the same time, and the number of copies of the target gene normalized against the number of copies of the reference gene.

We first compared the levels of target gene transcripts in each MRSA strain from cancer and non-cancer patients. The profiles of gene expression for MRSA isolates from cancer patients were characterized by relative up-regulation of genes encoding components of several virulence genes (*fnbA, clfA,* and *cna*) and a relative decrease in the expression of *fnbB*, *clfB*, *eno* and *fib*.

Down regulation of many adhesion genes in cancer patients may due to the low immunity defense in these patients, hence, the pathogen need only to express a few adhesion factors such as *clfA* gene which has been shown to have a critical virulence factor in several experimental models of infection (Hall *et al*., 2003) to saving some of the metabolic energy that is lost during modulation of unnecessary gene expression.

In contrast in non-cancer patients as multiple virulence factors may perform the same function (Gordon & Lowy, 2008), the pathogen needs to express multiple adhesion factors to overcome the host defense and establish infection. Other virulence factors including *sea* and *qacA/B* genes were always up-regulated in cancer patients to produce much harmful effect due to low immunity level of these patients. In order to investigate the effect of clonality on gene expression, the selected samples from different clonal background were separately investigated in each cancer and non-cancer patient groups. The individual different genotypes strains displayed similar trends in differential gene expression regardless the clonal types.

The qRT-PCR established in this study showed that although all studied MRSA strains possessed several virulence determinants, the expression, rather than carriage of virulence determinants may mediate high infectivity of MRSA in cancer patients.

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