Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Absolute and relative real-time PCR in the quantification of *tst* gene expression among methicillin-resistant *Staphylococcus aureus*: evaluation by two mathematical models

V. Chini, A. Foka, G. Dimitracopoulos and I. Spiliopoulou

Department of Microbiology, School of Medicine, University of Patras, Patras, Greece

Keywords
Absolute and relative real-time RT-PCR, MRSA, quantification, SYBR® Green I, *tst* expression.

Correspondence
Iris Spiliopoulou, Dept. of Microbiology, School of Medicine, University of Patras Patras 26500, Greece
E-mail: spiliopl@med.upatras.gr

2006/1426: received 10 October 2006, revised 7 March 2007 and accepted 11 June 2007

doi:10.1111/j.1472-765X.2007.02208.x

Abstract

Aim: Absolute and relative quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) by the use of two mathematical models were applied in order to study the expression of *tst* gene encoding the toxic shock syndrome toxin-1 (TSST-1), among methicillin-resistant *Staphylococcus aureus* (MRSA).

Methods and Results: Thirteen epidemic MRSA belonging to different clones and carrying a variety of toxin genes were selected. *tst* gene expression was achieved by using absolute and relative quantitative real-time RT-PCR and the SYBR® Green I. Absolute RT-PCR showed a statistically significant higher level of *tst* expression among strains isolated from soft tissue infections. Relative quantification was performed in relation to 23S rRNA expression by the application of two mathematical models, the $2^{-\Delta\Delta Ct}$ and the Pfaffl analysis methods.

Conclusions: *tst* gene expression was best calculated by the relative real-time RT-PCR analysis applying the Pfaffl analysis method, taking into account the reactions' efficiencies. Level of *tst* expression was related to patients' infection and did not depend on the MRSA genetic profile.

Significance and Impact of the Study: The results indicate that the application of the Pfaffl analysis method in the evaluation of relative real-time RT-PCR is more adequate.

Introduction

Staphylococcus aureus is one of the most significant bacterial pathogens causing a wide spectrum of serious infections. It produces a variety of proteins and toxins that contribute to its ability to colonize and cause disease (Dinges *et al.* 2000). The emergence and spread of methicillin-resistant *S. aureus* (MRSA) as nosocomial and recently as community pathogen has posed a clinical threat worldwide (Vandenesch *et al.* 2003).

Assessment of toxin genes expression has become crucial to understand the pathogenesis of staphylococcal infections. Reverse transcription (RT) followed by polymerase chain reaction (RT-PCR) represents a powerful tool and it is the most suitable method for gene expression quantification through the detection and quantification of mRNA. Real-time RT-PCR is increasingly used because of high sensitivity, good reproducibility and wide dynamic quantification range (Pfaffl 2004). Quantification is realized by two approaches: absolute and relative. The absolute RT-PCR relates the PCR signal to input copy numbers using a calibration curve, and neither comparisons nor references are needed (Pfaffl 2004). The relative RT-PCR determines the expression level in comparison with a reference sample. It is based on the expression levels of a target gene *vs* a housekeeping gene (Pfaffl 2004).

Toxic shock syndrome (TSS) is an acute and potentially fatal situation, associated with staphylococcal and streptococcal infections, due to the production of the toxic shock syndrome toxin-1 (TSST-1) by *S. aureus*. This

toxin belongs to the pyrogenic toxin superantigens' group (PTSAgs), which is characterized by the extensive T-cell activation and cytokine release (Dinges *et al.* 2000). TSST-1 is encoded by the *tst* gene, which is present on the bacterial chromosome within a 15·2-kb mobile genetic element called staphylococcal pathogenicity island and its expression is regulated by the accessory gene regulator system (*agr*) (Novick *et al.* 1998). According to the polymorphism in the sequence of autoinducing peptide and its receptor within *agr*, clinical strains can be classified into four groups (*agr* 1–4) (Jarraud *et al.* 2002; Lina *et al.* 2003). In a previous study performed at the Department of Microbiology, University of Patras, the majority of the *tst*-carrying MRSA belonged to the pulsed-field gel electrophoresis (PFGE) type A (ST30) and *agr* group 3, while the remaining three *tst*-carrying MRSA isolates belonged to clone B (ST239) and *agr* 1 (Chini *et al.* 2006).

In the present study, we have quantified the *tst* gene expression with absolute and relative real-time RT-PCR in 13 representatives of MRSA isolated from variable infection sites. For the relative quantification we have used the 23S rRNA as reference gene and we have applied two mathematical models, in order to define the most reliable. The real-time RT-PCR was carried out using the SYBR® Green I.

Materials and methods

Bacterial strains

A collection of 13 representative *tst*-positive MRSA from clinical specimens of different patients, admitted at the University Hospital of Patras during 2001–2003, was studied. The strains were selected on the basis of their genetic profile according to the presence of *mecA*, *tst*, the enterotoxin gene cluster (*egc*) and the PVL (Panton–Valentine leukocidin) genes, tested by PCR (Jarraud *et al.* 2001; Chini *et al.* 2006). According to patients' data, infections caused by the selected isolates were categorized as invasive and as soft skin tissue infections (SSTI) (Table 1). Absence of PCR inhibitors in the DNA extracts was tested by amplifying a 420-bp part of the domain V region on the 23S rRNA (Rahim *et al.* 2003). All MRSA were characterized by PFGE of *Sma*I DNA digests, SCC*mec* and *agr* groups according to published protocols (de Lencastre *et al.* 1994; Tenover *et al.* 1995; Oliveira and de Lencastre 2002; Lina *et al.* 2003). Fri913 and Fri137 were used as the *tst*-positive and -negative reference strains, respectively (Chini *et al.* 2006).

RNA extraction

Total RNA was isolated with the Trizol (Invitrogen, Carlsbad, CA, USA) method from the postexponential growth phase. Isolates were grown in 5 ml of tryptic soy broth (TSB) after 16 h of incubation with agitation at 37°C, up to an optical density of 3 at 540 nm, corresponding to the postexponential growth phase during which TSST-1 is primarily transcribed (Dinges *et al.* 2000). Colony-forming units (CFU) were measured by inoculating serial dilutions from the bacterial suspensions onto blood agar plates. A volume of 2 ml of the bacterial suspension (2–2·5 McFarland density) was centrifuged and washed with 1·5 ml of DEPC-treated water (0·1%)

Table 1 Clinical sources and type of infections caused by MRSA isolates, genotypes and expression of *tst* by RT-PCR

agr/PFGE/ SCC*mec*	Strain	Sample	Infection	Genotype	Absolute RT-PCR (copies μl^{-1})	Relative RT-PCR $2^{-\Delta\Delta Ct}$	Relative RT-PCR 'Pfaffl method'
	Fri913	Positive control		*tst*-positive	3594·77	1	1
	Fri137	Negative control		*tst*-negative	0·01	0·045	0·002
1/B/IIIA	1118	Abscess	Invasive	*tst/seo/sei/seu/sen/seg*	44·05	1408·55	34·65
	1176	Wound	Invasive	*tst/seo/sei/seu/sen/seg*	44·26	3·972	0·46
	261	Blood	Invasive	*tst/seg*	71·78	25·28	2·29
3/A/IVvar	1194	Tissue	Invasive	*tst/egc2*	66·29	0·74	0·156
	1200	Wound	Invasive	*tst/egc2*	56·37	1·21	0·26
	1213	Wound	SSTI*	*tst/egc2*	8666·65	15·1	2·78
	1267	Wound	Invasive	*tst/egc2*	4072·13	1·31	0·153
	1273	Wound	Invasive	*tst/egc2*	3264·4	49·52	3·68
	1351	Wound	SSTI	*tst/egc2*	7746·68	1·87	0·51
	232	Urine	Invasive	*tst/egc2*	28·17	2·25	0·39
	246	Skin lesion	SSTI	*tst/egc2*	148·6	51·62	4·49
	267	Wound	SSTI	*tst/egc2*	86·63	3·03	0·63
	279	Wound	Invasive	*tst/egc2*	31·98	6401595·1	9386·6

*SSTI, skin and soft tissue infections; RT-PCR, reverse transcriptase polymerase chain reaction; MRSA, methicillin-resistant *Staphylococcus aureus*.

(DEPC: diethyl pyrocarbonate, Sigma, St Louis, MO). The pellet was resuspended in 100 μl of TE (10 mM Tris-HCL/1 mM EDTA, pH 8) containing 2 mg lysozyme (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and RNA extraction was carried out as described (Petinaki *et al.* 2006). RNA was dissolved in 50 μl of DEPC water and treated with 3 IU of RNase-free DNase (Promega, Madison, WI, USA) at 37°C for 40 min. RNA integrity was tested with electrophoresis on 1% agarose gel. RNA quantification was performed measuring the absorbance at 260 nm. Nucleic acid purity was assessed measuring $A_{260\ nm}/A_{280\ nm}$ ratio (acceptable when the ratio was >1·6).

Genomic DNA contamination was tested by PCR with the specific primers, using the appropriate reaction mix composition and thermal conditions for the *tst* and 23S rRNA genes (Jarraud *et al.* 2001; Rahim *et al.* 2003). In case of a positive result, samples were discarded.

cDNA synthesis

Reverse transcription was performed in a final volume of 20 μl, using 0·5 μg of total RNA, 50 ng of random hexamer primers and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's instructions for the first-strand cDNA synthesis.

Real-time PCR thermal conditions

The real-time PCR was carried out with the RotorGene device (RG-3000; Corbett Research, Sydney, Australia). The same primers for *tst* and 23S rRNA used for the conventional PCR, were applied in the RT-PCR. Reaction efficiency was established for each set of primers, after quantification of six different dilutions of the DNA pool with different primer concentrations. Reactions were set up in a total volume of 25 μl using 5 μl of cDNA (diluted 1 : 4), 2× SYBR® Green I master mix (Invitrogen) and 0·4 μmol of each gene-specific primer. Reaction mixture's composition was the same for *tst* and 23S rRNA genes. For the *tst* gene the cycling conditions were: 95°C for 4 min; 45 cycles of 95°C for 20 s, 58°C for 20 s and 72°C for 20 s; a final elongation step at 72°C for 4 min. For the 23S rRNA gene, thermal conditions were the same, with a modified amplification step at 72°C for 30 s and a final elongation step for 5 min. Specificity of the PCR products was confirmed by analysis of the dissociation curve. The one-cycle melting curve program, that was common for both genes, consisted of heating at 90°C and cooling to 50°C every 0·2°C per step, with 2 s hold per step. Each run included the *tst*-negative and a nontemplate control. Fluorescence acquisition was carried out at 74°C. Results were evaluated using the Rotor-Gene Analysis Software 6.0. The amplicons' expected size and the absence of non-specific products were confirmed by conventional PCR with the same primers. Efficiency of reactions was calculated from the slope according to the equation $E = 10^{-1}/\text{slope}$ (Pfaffl 2004).

Real-time absolute RT-PCR

For the development and analytical evaluation of the *tst* real-time RT-PCR, a *tst* gene-specific PCR amplicon of the reference strain was prepared as a standard. PCR products were analysed by 1% agarose gel electrophoresis and purified (Wizard PCR Preps DNA Purification System; Promega). Concentration of the purified nucleic acid was calculated by measuring the absorbance at 260 nm and its corresponding concentration was converted into copies per microlitre by using the Avogadro constant ($6{\cdot}023 \times 10^{23}$) and its molecular weight (number of bases of the PCR product multiplied by the average molecular weight of a pair of nucleic acids, which is 660 Da) (Sambrook *et al.* 1989). Tenfold serial dilutions of the quantified *tst* stock amplicon solution were kept in aliquots at −20°C and used throughout the study as external standards of known concentration for the *tst* real-time PCR reaction (range of the standards: 10–10^5 *tst* copies μl^{-1}).

The calibration curve was created by plotting the threshold cycle (Ct) corresponding to each standard, *vs* the value of their corresponding log number of *tst* concentration (expressed as copies μl^{-1}). Ct is the number of required cycles to reach the defined threshold level.

Real-time relative RT-PCR

The relative quantification measures the levels of a target gene expressing it relatively to the levels of an internal control, usually a housekeeping gene. Therefore, relative quantification does not require standards with known concentrations and all samples are expressed as a ratio towards the housekeeping gene. Comparison between the ratios is performed.

Normalization of target gene expression is useful in order to compensate sample-to-sample and run-to-run variations and to ensure the experimental reliability. For this purpose, the domain V region of the 23S rRNA gene was used as the reference gene (Rahim *et al.* 2003), because it is a stable marker and has been applied for *S. aureus* identification (Straub *et al.* 1999). Both rRNA (16S and 23S) are characterized by high expression rate and stability (Scheu *et al.* 1998) and the 420-bp segment of domain V was already used in our laboratory as a control in all nucleic acid preparations (Rahim *et al.* 2003).

The number of cells expressing the *tst* gene and the housekeeping gene was determined by comparing the Ct of each sample with a corresponding standard curve. A standard curve for real-time PCR was established with serial dilutions of cDNA of the reference strain Fri913, inoculated in TSB and then on blood agar plates for CFU measurement. Finally, two standard curves, for *tst* and for 23S rRNA genes were obtained, by plotting the Ct values *vs* the concentration expressed as CFU ml^{-1} (range: $7{\cdot}5 \times 10^9$–3×10^7 CFU ml^{-1}). The 23S rRNA gene's length was 420 bp, at the upper limit of the range of SYBR® Green I, while the *tst* gene was within the optimal range (180 bp). As target and internal standard genes had different sequences and amplicon lengths, they showed different PCR efficiencies. PCR efficiencies were first established for each couple of primers. Results were analysed using the $2^{-\Delta\Delta Ct}$ method and the Pfaffl mathematical model (Pfaffl 2004).

Statistical analysis

Statistical analysis was conducted using the SPSS v.12·0 software package for Windows (SPSS Inc.), applying the Mann-Whitney *U*-test and the ANOVA test.

Results

Development of the real-time PCR assay

Optimization of real-time PCR is critical for reliable and reproducible results. In order to develop a trustworthy real-time RT-PCR methodology, primer and sample concentrations, as well as temperatures and times, were optimized. Best results were obtained with a final primer concentration of 0·4 μmol l^{-1} for each reverse and forward primer, for both pairs of primers, while the appropriate dilution for the samples' cDNA was determined to be 1 : 4 ($7{\cdot}5 \times 10^9$ CFU ml^{-1} for the reference strain). Annealing temperature at 58°C was sufficient for both genes while for the 23S rRNA gene the extension times were longer. In each run, the corresponding standard curve was used to determine the amplification efficiency (E), expressed as $E = 10^{-1/\text{slope}}$.

Real-time absolute quantification of *tst* gene

Absolute quantification refers to the analysis of unknown samples compared with a standard curve. Efficiency was 2·04 and the concentrations of the expressed gene (*tst*), in copies per microlitre, ranged between 28·17 and 8666·65 (Table 1). Normalization was achieved by using the same amount of total RNA (0·5 μg) for reverse transcription in all samples.

Real-time relative quantification of *tst* gene

Relative quantification is based on the expression levels of a target gene *vs* a reference gene, thus standards with known concentrations are not required. To determine the relative expression ratio two published mathematical models were used: (i) without efficiency correction, using the $2^{-\Delta\Delta Ct}$ method (Livak 1997 and 2001; Livak and Schmittgen 2001) and (ii) with kinetic PCR efficiency correction, the 'Pfaffl analysis method' (Pfaffl 2001; Pfaffl *et al.* 2002).

The efficiency of the target gene PCR (*tst*) was 1·95 whereas that of the housekeeping gene (23S rRNA) PCR was 1·6. The lower efficiency of the 23S rRNA PCR was because of the length of the amplified part that was 420 bp, at the limits of SYBR® Green I chemistry. Therefore, results were additionally expressed by applying the second method, taking into account the efficiencies that were not similar (Table 1). Ratios resulted from the $2^{-\Delta\Delta Ct}$ method ranged from 0·74 to 6401595·1 and were calculated with the equation, $R = 2^{-\Delta(\text{Ct sample}-\Delta\text{Ct control})}$, while ratios resulting from the 'Pfaffl analysis method' had a range between 0·153 and 9386·6 and were derived from the equation,

$$R = E_{\text{target}}^{\Delta\text{Ct target(control}-\text{sample)}} / E_{\text{ref}}^{\Delta\text{Ct ref(control}-\text{sample)}}.$$

The results obtained by both analysis methods of samples with higher expression ratios did not differ (Table 1, strains 1118, 1273, 246, 279). Among the remaining strains with lower *tst* expression, differences on the expression rate were calculated by the two methods. For example, with the $2^{-\Delta\Delta Ct}$ method, strain 232 showed higher *tst* expression (2·25) than strain 1351 (1·87). On the contrary, with the 'Pfaffl analysis method' strain 232 had lower gene expression (0·39), than strain 1351 (0·51) (Table 1).

tst gene expression, genotypes and infections caused by MRSA strains

A statistically significant difference was detected for *tst* gene expression calculated by the absolute RT-PCR among the MRSA isolated from SSTI compared with those recovered from invasive infections ($P = 0{\cdot}0308$). The differences were not statistically significant comparing the results of the relative RT-PCR calculated by both methods with patients' infections ($P > 0{\cdot}05$).

Ten out of 13 MRSA belonged to clone A, SCC*mec* type IVvar, *agr* type 3 carrying the *tst* and *egc2* genes. Among the strains of clone B, SCC*mec* type IIIA and *agr* type 1, two harboured the *tst, seo, sei, seu, sen, seg* genes and one the *tst* and *seg* genes. Comparison of *tst* gene expression by the absolute and relative RT-PCR among

the different clones and genotypes did not reveal statistically significant differences ($P > 0{\cdot}05$).

Discussion

A real-time PCR assay was developed for the quantification of *tst* gene in *S. aureus*. This assay generated results faster, than the conventional PCR methods and it was specific and highly reproducible, targeting solely *tst* gene. We used the SYBR® Green I detection, 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 and new primers (Pfaffl 2004).

By the developed method of absolute quantification the results were reproducible and constant, meaning that the assay can be applied in the routine laboratory. The statistically significant difference of *tst* gene expression among strains associated with SSTI, suggests that such strains may be the cause of TSS among patients.

Relative quantification was based on the expression of the *tst* gene towards the 23S rRNA housekeeping gene giving a ratio of the gene of interest and not actual copy numbers in a defined concentration of mRNA (Pfaffl 2004). A high ratio may not necessarily mean a high expression of the gene of interest, as this ratio is sensitive to the expression level of the normalizing gene and it depends on the whole productivity of the cell (Pfaffl 2004). Consequently, results derived from absolute and relative quantification are not exactly comparable and it is normal to detect variations, as they refer to different variables and depend on different factors.

The $2^{-\Delta\Delta Ct}$ method does not take into account the efficiency correction and it is usually used for reactions with similar efficiencies (Livak 1997 and 2001; Livak and Schmittgen 2001). Using as reference the 420-bp part of the 23S rRNA we expected to get different efficiencies from the reactions. Thus, we could further differentiate and compare the results by additionally calculating the ratios with the 'Pfaffl analysis method', which is a mathematical model with kinetic PCR efficiency correction (Pfaffl 2001; Pfaffl *et al.* 2002).

Comparing the ratios calculated by both analysis methods, different results for the same strains were obtained for the relative quantification of *tst* gene expression (Table 1). The detected variations of the expression levels mainly among strains with lower and similar expression values can be explained by the fact that at very low copy numbers the random variation due to sampling error (Poisson's error law) becomes significant (Peccoud and Jacob 1996; Pfaffl 2004). Besides, the 'Pfaffl analysis method' takes into account the different efficiencies, namely not only the *tst* mRNA production, but also the entire mRNA productivity of the cell. Results deriving from this method are closer to the true cell situation. Therefore, we suggest the use of this mathematical model, even in reactions with similar efficiencies, as the more precise and reliable method.

tst-carrying MRSA may be crucial for the clinical outcome of patients, as *tst* gene is responsible for the production of TSST-1, the main causative agent of the TSS. We have selected to study gene expression through mRNA quantification in order to approach the molecular basis of the TSST-1 production. In the present study we have developed a real-time RT-PCR for the rapid detection and quantification of *tst* gene in *S. aureus*, applying the SYBR® Green I. A statistically significant higher expression rate was detected among strains isolated from SSTI by the absolute RT-PCR. The implementation of the 'Pfaffl analysis method' allows accurate quantification of the gene's expression especially when a housekeeping gene of different sizes is used as reference.

Acknowledgements

These results were partially presented as a poster at the 12th International Symposium on Staphylococci and Staphylococcal Infections, 3–6 September 2006, Maastricht, The Netherlands. The authors wish to thank Prof Jerome Etienne for kindly providing the reference *S. aureus* strains, Dr Efthimia Petinaki for active support and critically reading the manuscript and Dr Markos Michalatos for his technical assistance. This work was supported by a grant 'K. Karatheodori' no. B116 of the University of Patras awarded to Dr I. Spiliopoulou.

References

Chini, V., Dimitracopoulos, G. and Spiliopoulou, I. (2006) Occurrence of the enterotoxin gene cluster and the toxic shock syndrome toxin-1 gene among clinical isolates of methicillin-resistant *Staphylococcus aureus* is related to clonal type and *agr* group. *J Clin Microbiol* **44**, 1881–1883.

Dinges, M.M., Orwin, P.M. and Schlievert, P.M. (2000) Exotoxins of *Staphylococcus aureus*. *Clin Microbiol Reviews* **13**, 16–34.

Jarraud, S., Peyrat, M.A., Lim, A., Tristan, A., Bes, M., Mougel, C., Etienne, J., Vandenesch, F., *et al.* (2001) *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol* **166**, 669–677.

Jarraud, S., Mougel, C., Thioulouse, J., Lina, G., Meugnier, H., Forey, F., Nesme, X., Etienne, J., *et al.* (2002) Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles) and human disease. *Infect Immun* **70**, 631–641.

de Lencastre, H., Couto, I., Santos, I., Melo-Cristino, J., Torres-Pereira, A. and Tomasz, A. (1994) Methicillin-resistant *Staphylococcus aureus* disease in a Portuguese hospital: characterization of clonal types by a combination of DNA typing methods. *Eur J Clin Microbiol Infect Dis* **13**, 64–73.

Lina, G., Boutite, F., Tristan, A., Bes, M., Etienne, J. and Vandenesch, F. (2003) Bacterial competition for human nasal cavity colonization: role of staphylococcal *agr* alleles. *Appl Environ Microbiol* **69**, 18–23.

Livak, K.J. (1997 and 2001) *ABI Prism 7700 Sequence detection System User Bulletin #2 Relative quantification of gene expression;* http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf (accession 2006).

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^[delta-delta C(T)] method. *Methods* **25**, 402–408.

Novick, R., Lindsay, J., Ruzin, A. and Ross, H. (1998) Molecular genetics of the *tst* element, a mobile pathogenicity island in *Staphylococcus aureus*. In *European Conference on Toxic Shock Syndrome. International Congress and Symposium Series 229* ed. Arbuthnott, J. and Furman, B. pp. 109–112 New York: Royal Society of Medicine Press Ltd.

Oliveira, D.C. and de Lencastre, H. (2002) Multiplex PCR strategy for rapid identification of structural types and variants of the *mec* element in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* **46**, 2155–2161.

Peccoud, J. and Jacob, C. (1996) Theoretical uncertainty of measurements using quantitative polymerase chain reaction. *Biophys J* **71**, 101–108.

Petinaki, E., Spiliopoulou, I., Liakos, P., Kontos, F. and Maniatis, A.N. (2006) Emergence of *Enterococcus faecalis* susceptible to quinupristin/dalfopristin in Greece. *Int J Antimicrob Agents* **28**, 155–158.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, 2002–2007.

Pfaffl, M.W. (2004) Quantification strategies in real-time PCR. In *A–Z of Quantitative PCR* ed. Bustin, S.A. pp. 89–113 La Jolla: International University Line.

Pfaffl, M.W., Horgan, G.W. and Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* **30**, 1–10.

Rahim, S., Pillai, S.K., Gold, H.S., Venkataraman, L., Inglima, K. and Press, R.A. (2003) Linezolid-resistant, vancomycin-resistant *Enterococcus faecium* infection in patients without prior exposure to linezolid. *Clin Infect Dis* **36**, 146–148.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Concentration of DNA solution. In *Molecular Cloning: A Laboratory Manual* ed. Nolan, C. pp. C1 New York: Cold Spring Harbor Laboratory Press.

Scheu, P.M., Berghof, K. and Stahl, U. (1998) Detection of pathogenic spoilage micro-organisms in food with the polymerase chain reaction. *Food Microbiol* **15**, 13–31.

Straub, J.A., Hertel, C. and Hammes, W.P. (1999) A 23S rRNA targeted polymerase chain reaction-based system for detection of *Staphylococcus aureus* in meat starter cultures and dairy products. *J Food Prot* **62**, 1150–1156.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H. and Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.

Vandenesch, F., Naimi, T., Enright, M.C., Lina, G., Nimmo, G.R., Heffernan, H., Liassine, N., Bes, M., *et al.* (2003) Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis* **9**, 978–984.