Supplementary Data 2

Minimum Information for Publication of Quantitative Real-Time PCR Experiments

Minimum Information for Publication of Quantitative Real-Time PCR Experiments

All information designed according to the MIQE guidelines as essential (E) is presented. Desirable information (D) is attached if it is available.

Experimental Design

Definition of experimental and control groups (E)

Staphylococcus aureus strains were grown in Brain Heart Infusion broth (BHI, DIFCO) at 37°C under shaking (180 rpm).

All cultures were performed by inoculating 50 ml-BHI flask with 50 µl of an overnight preculture (an 18-hr-old culture incubated in 10 ml BHI, 37°C, 180 rpm) to reach exponential phase (optical density $OD_{620\,nm} = 0.5–0.7$).

Microorganisms: Heterogeneous vancomycin-intermediate *S. aureus* (hVISA) recovered from a single patient suffering bone and joint infection, before and after 36 days of vancomycin treatment (D1 and D2, respectively), and two D2 laboratory-derived vancomycin intermediate *S. aureus* (VISA) mutants (D23C9 and D2P11) that were selected by independent serial passages in the presence of increasing concentrations of vancomycin, selected from D2. Control group was incubated in BHI broth, and treated group was incubated in BHI broth with vancomycin ¼ MIC.

Number within each group (E)
Three cultures of each *S. aureus* strains (designed D1, D2, D3C9, and D2P11).

Assay carried out by core laboratory or investigator's laboratory? (D)
Investigator's laboratory.

Sample

Description (E)
Samples were taken at exponential phase during the cell growth, all cultures were performed by inoculating 50 ml-BHI flask with 50 µl of an overnight preculture (an 18-hr-old culture incubated in 10 ml BHI, 37°C, 180 rpm) to reach the exponential phase (optical density $OD_{620nm} = 0.5–0.7$).

Volume/mass of sample processed (D)
Volume of sample processed was 2 ml, in exponential phase.

Microdissection or macrodissection (E)
Not applied.

Processing procedure (E)
Cells were collected by centrifugation for 3 min at 13,000 rpm, supernatant was discarded.

If frozen—how and how quickly? (E)
Not applied.

If fixed, with what and how quickly? (E)
Not applied.

Sample storage conditions and duration (especially for formalin-fixed, paraffin-embedded samples) (E)
RNA extraction was performed immediately after the collection of the samples.

Nucleic Acid Extraction

Procedure and/or instrumentation (E)
Bacterial pellets were resuspended in 100 µl of Lizozyme 15 mg/ml (Sigma-Aldrich), incubated 1 hr at 37°C, and centrifuged 1 min at 13,000 rpm, supernatant was discarded.

RNA was extracted using TRIZOL® Reagent (Invitrogen) with the Pure Link® RNA Mini Kit (AMBION) according to manufacturer's recommendations.

Name of kit and details of any modifications (E)
TRIZOL Reagent (Invitrogen) with the Pure Link RNA Mini Kit (AMBION).

Details of DNase or RNase treatment (E)
DNase I used (RQ1 RNase free DNase; Promega) according to manufacturer's protocol.

Samples were treated by 3 U DNase I/1 µg RNA/37°C/60 min.

Contamination assessment (DNA or RNA) (E)
DNA contamination was assessed using qPCR with treated RNA samples and matching cDNA samples.

RNA samples were diluted the same way as cDNA samples and resulted Cq values were compared.

Only samples with a nondetected signal in RNA samples or with a higher difference than 10 Cq between RNA and cDNA samples were assessed to be free of DNA contamination.

Nucleic acid quantification (E)
RNA concentration was quantified by measuring absorbance at 230, 260, and 280 nm (A_{230}, A_{260}, A_{280}) using NanoDrop™ 1000 EspectroPhotometer (Thermo Scientific).

Instrument and method (E)
NanoDrop 1000 EspectroPhotometer (Thermo Scientific) used according to manufacturer's protocol.

Purity (A_{260}/A_{280}, A_{260}/A_{230}) (D)
Ratio A_{260}/A_{280} was from 1.8 to 2.1 indicating good purity.

Ratio A_{260}/A_{230} was >1 indicating good purity.

Yield (D)
Yield of RNA extraction was from 8g to 20 µg.

RNA integrity method/instrument (E)
RNA integrity was electrophoretically verified by ethidium bromide staining.

RIN/RQI or Cq of 3′ and 5′ transcripts (E)
Not applied.

Inhibition testing (Cq dilutions, spike or other) (E)
Not applied.

Reverse Transcription

Complete reaction conditions (E)
M-MLV™ Reverse Transcriptase (Invitrogen) was used according to manufacturer's recommendations.

One microliter 50 µM Random Hexamers (Invitrogen), 1 µl 10 mM dNTP Mix (Invitrogen), 4 µl 5× First-Strand Buffer (Invitrogen), 2 µl 0.1 M DTT (Invitrogen), 1 µl (40 U) RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen), 1 µl (200 U) of M-MLV Reverse Transcriptase (Invitrogen), and 10 µl RNA (0.5 µg).

RT temperature profile: 65°C for 5 min (mix of primers, dNTPs and RNA), quick chill on ice, 37°C for 2 min, 25°C for 10 min, 37°C for 50 min, and 70°C for 15 min.

Amount of RNA and reaction volume (E)
Ten microliters RNA (0.5 µg RNA), reaction volume 20 µl.

Priming oligonucleotide (if using gene-specific primer) and concentration (E)
Fifty micromolar Random Hexamers (Invitrogen).

Reverse transcriptase and concentration (E)
M-MLV Reverse Transcriptase (Invitrogen), 200 U.

Temperature and time (E)
65°C for 5 min (primer's mix, dNTPs and RNA), quick chill on ice, 37°C for 2 min, 25°C for 10 min, 37°C for 50 min, and 70°C for 15 min.

Cqs with and without reverse transcription (D)
Cq with reverse transcription <40; Cq without reverse transcription—undetected.

Storage conditions of cDNA (D)
In a freezer (−20°C).

qPCR Target Information

Gene symbol (E)
gyrB, pta, pbp2, pbp4.

Sequence accession number (E)
gyrB YP_498613.1, *pta* YP_499142.1, *pbp2* ABD30552.1, *pbp4* ABD29781.1.

Amplicon length (E)
gyrB 121 bp, *pta* 121 bp, *pbp2* 85 bp, *pbp4* 140 bp.

***In silico* specificity screen (BLAST, etc.)** (E)
Not applied.

Location of each primer by exon or intron (if applicable) (E)
Not applied, prokaryote.

What splice variants are targeted? (E)
Not applied, prokaryote.

qPCR Oligonucleotides

Primer sequences (E)

Location and identity of any modifications (E)
Not applied.

qPCR Protocol

Complete reaction conditions (E)
Reaction mixture consisted of 10 µl of 2×SYBR® Select Master Mix (Applied Biosystems), 0.3 µl of each 10 µM primer (150 nM final), 4.7 µl of nuclease free milliQ water, and 5 µl cDNA (1:100 dilution)—final volume = 20 µl.

Thermocycling program consisted of one hold at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Melting curve analysis was performed after each run.

Reaction volume and amount of cDNA/DNA (E)
Total reaction volume was 20 µl, volume of cDNA was 5 µl (100-times diluted cDNA after reverse transcription polymerase chain reaction [RT-PCR]).

Primer, (probe), Mg^{2+}, and dNTP concentrations (E)
One hundred fifty nanomolar of each primer, Mg^{2+} and dNTPs were components of commercial SYBR Select Master Mix (Applied Biosystems), concentration unknown.

Polymerase identity and concentration (E)
AmpliTaq® DNA Polymerase—a component of commercial SYBR Select Master Mix (Applied Biosystems), concentration unknown.

Buffer/kit identity and manufacturer (E)
SYBR Select Master Mix (Applied Biosystems).

Additives (SYBR Green I, DMSO, etc.) (E)
Not applied.

Manufacturer of plates/tubes and catalog number (D)
MicroAmp® Optical 96-Well Reaction Plate (catalog number 4306737; Applied Biosystems).

MicroAmp Optical 96-384-Well Optical Adhesive films (catalog number 4360954; Applied Biosystems).

Complete thermocycling parameters (E)
Thermocycling program consisted of one hold at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Melting curve 60°C–95°C.

Reaction setup (manual/robotic) (D)
Manual.

Manufacturer of qPCR instrument (E)
7500 Real-Time PCR System (Applied Biosystems).

qPCR Validation

Specificity (gel, sequence, melt, or digest) (E)
Melting curve analysis performed.

For SYBR Green I, Cq of the no template control (E)
No signal in NTCs or Cq >37.

Name	*Sequence*	*Reference*
gyrB F *gyrB* R	CCAGGTAAATTAGCCGATTGC AAATCGCCTGCGTTCTAGAG	[1]
pta F *pta* R	AAAGCGCCAGGTGCTAAATTAC CTGGACCAACTGCATCATATCC	[1]
pbp2 F *pbp2* R	CGGTGCTGAAACTTATTCACAATA AGTGTATTGAGGTGTAAAGCCGTTAA	[2]
pbp4 F *pbp4* R	GCACCTTCCAATGAAAAGTTG GTAACGACTGCTAGAGACTATGCC	This study

Calibration curves with slope and *y*-intercept (E)
gyrB (slope = −3.292; *y*-intercept = 33.016).
pta (slope = −3.373; *y*-intercept = 31.944).
pbp4 (slope = −3.179; *y*-intercept = 34.455).
pbp2 (slope = −3.509; *y*-intercept = 32.344).

PCR efficiency calculated from slope (E)
gyrB (E = 101.277).
pta (E = 97.916).
pbp4 (E = 106.35).
pbp2 (E = 92.737).

r^2 of calibration curve (E)
gyrB ($R^2 = 0.996$).
pta ($R^2 = 0.996$).
pbp4 ($R^2 = 0.998$).
pbp2 ($R^2 = 0.993$).

Linear dynamic range (E)
Five points of a 10-fold serial dilution tested (designed 10^5–10^1, the real copy number unknown).
Reactions were linear in the following tested ranges: 10^0–10^{-4} for *pta* and *gyr*B genes, and 10^{-1}–10^{-4} for *pbp*2 and *pbp*4 genes.
Cq values achieved during experiments were in these ranges.
All reactions have a potential to cover wider range of Cq values.

Cq variation at limit of detection (E)
Not determined.

Evidence for limit of detection (E)
Not determined.

If multiplex, efficiency and LOD of each assay (E)
Not applied.

Data Analysis

qPCR analysis program (source, version) (E)
7500 Software (version 2.0.6; Applied Biosystems)

Method of Cq determination (E)
Second max. derivate.

Outlier identification and disposition (E)
Not determined.

Results of NTCs (E)
No signal in NTCs or Cq >37.

Justification of number and choice of reference genes (E)
Identification of reference genes: reference genes (*gyr*B and *pta*) previously described for *S. aureus* were used.[1]
The combination of two reference genes was selected as most stable following the M parameter and the CV%. The reference genes had to meet the M value less than 0.5 and having less than 25% CV when they were compared to each other.[3]

Description of normalization method (E)
The relative quantities (RQ) of each gene and the reference gene were calculated, considering the reaction efficiency of qPCR calculated from the standard curve. Geometric averaging of multiple internal reference genes method was performed. With these data, subsequently, the normalized RQ (NRQ) for each gene of interest was calculated.[3,4].

Number and stage (RT or qPCR) of technical replicates (E)
Distribution of technical replicates: RT-PCR—1; qPCR—2.

Repeatability (intra-assay variation) (E)
Standard deviation of duplicates were <0.5.

Statistical methods for results significance (E)
Two-way analysis of variance.

Software (source, version) (E)
Infostat.[5]

References

1. Valihrach, L., and K. Demnerova. 2012. Impact of normalization method on experimental outcome using RT-qPCR in *Staphylococcus aureus*. J. Microbiol. Methods 90: 214–216.
2. Goldstein, F., J. Perutka, A. Cuirolo, K. Plata, D. Faccone, J. Morris, A. Sournia, M.D. Kitzis, A. Ly, G. Archer, and A.E. Rosato. 2007. Identification and phenotypic characterization of a beta-lactam-dependent, methicillin-resistant *Staphylococcus aureus* strain. Antimicrob. Agents Chemother. 51: 2514–2522.
3. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman . 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.
4. Hellemans, J., G. Mortier, A. De Paepe, F. Speleman, and J. Vandesompele. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8:R19.
5. Di Rienzo, J.A., F. Casanoves, M.G. Balzarini, L. Gonzalez, M. Tablada, and C.W. Robledo. 2013. InfoStat versión 2013. Available at www.infostat.com.ar (accessed July 23, 2013).