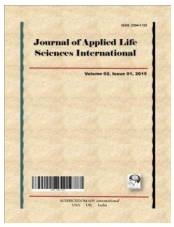

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Optimization and Evaluation of Triplex Real-time PCR Assay for Detection of Genes Encoding Staphylococcal Virulence and Methicillin Resistance Using Two Different Multi-channel Emission Instruments

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Authors' contributions

This work was carried out in collaboration between both authors. Author CEO designed the study, wrote the protocol, performed the wet experiments and wrote the first draft of the manuscript. Author RJ had oversight of the group and managed the funds. Both authors performed statistical analysis, read and approved the final manuscript.

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ABSTRACT

Aim of Study: To optimize a triplex real-time PCR assay developed elsewhere and to evaluate the performance characteristics of two different multi-channel real-time PCR systems on the newly optimized assay.
Methodology: A triplex real-time PCR assay developed for three key genes encoding virulence and antibiotic resistance in *Staphylococcus aureus*, namely, *lukSF-PV*, *mecA*, and *spa* was optimized and evaluated using two different real-time PCR instruments (7500SDS and LightCycler 480). Bacterial strains (N=230), including staphylococcal and non-staphylococcal isolates, were

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used for the study.
Results: Following optimization, cycling and data analysis completed within one hour compared with the former three hours. Assay specificity became 100% on both instruments. The negative predictive value (NPV) and the positive predictive value (PPV) rose to 100%.
Conclusion: The optimized triplex real-time PCR assay is highly reproducible across the two systems without loss of speed, sensitivity and specificity. The results also suggested that user-familiarization with each machine operational system would allow assay performance across various real-time PCR platforms currently being used.

Keywords: Molecular diagnostics; instrumentation; real-time PCR; MRSA; PVL.

1. INTRODUCTION

Public health challenges from *Staphylococcus aureus* are associated with molecules which cause therapy refractory conditions. The molecules include staphylococcal proteinA (Spa) encoded by *spa* gene, penicillin-binding protein 2a encoded by *mecA* gene, and Panton–Valentine leucocidin (PVL) encoded by phage-borne bi-component *lukSF*-PV operon [1]. Spa, coagulase and PVL have been incriminated in diverse *S. aureus* infections including abscesses, skin and soft tissue infections and musculo-skeletal damage [2-4], while *mecA*-driven methicillin resistant *S. aureus* (MRSA) is of massive diagnostic, therapeutic and infection control importance. Whereas MRSAs first emerged in UK hospital patients [5,6], victims of PVL-associated disease are often young and otherwise healthy persons and outcome is usually independent of hospitalization and staphylococcal methicillin resistance status [7]. The convergence of virulence and antibiotic-resistance signals high-level therapy refractory infections and more significant threat than the two factors separately [8].

Bacteriological culture-based tests for staphylococcal methicillin resistance take ≥24hrs. One of the diagnostic challenges from the convergence of rapidly mortifying PVL-associated toxicity and methicillin resistance is that there is currently no reliable bacteriological assay for PVL. It has been suggested that application of multiplex PCR to detect multiple genetic markers within the same sample will enhance molecular diagnostics [9]. In line with this, several multiplexed assays have been developed in conventional and real-time PCR platforms, some of which have been reported to be ineffectual following evaluation studies. A recent evaluation revealed discrepancies between a triplex real-time PCR assay for *nuc*, *pvl*, and *mecA* [10] and phenotypic antimicrobial susceptibility testing (AST) which led to modification of the same triplex assay into a quadruplex assay [11]. To enable application across instruments and to keep performance at par with recent PVL-MRSA assays, we optimized a similar triplex real-time PCR assay for detection of *spa, lukSF-PV* and *mecA* [12]. Here we report the performance evaluation outcomes using two different multi-channel real-time PCR instruments.

2. MATERIALS AND METHODS

To enable application across instruments and to keep performance at par with recent assays for MRSA and PVL, the cycling conditions and reaction factors of a triplex real-time PCR assay originally described elsewhere [12] were optimized. Two real-time PCR instruments (ABI 7500SDS: Applied Biosystems, CA, USA and LightCycler™ 480: Roche, Germany) were used for the study. Staphylococcal type culture strains (n=50) used for this study are listed (Table 1). The assay was further evaluated using randomly selected local clinical staphylococcal strains (n = 121) isolated in the Nottingham area during the period August 2003 to December 2004 from blood, sputum, pleural effusion, cerebrospinal fluid (CSF), breast fluid, spinal fluid, wound swab, vaginal swab, abscess, urine and other specimens analysed by the Queen's Medical Centre (QMC) microbiology laboratory. The above 171 strains were collected and stored (-80°C) by Dr. Richard Spence who used them for validation of microarrays [13]. Additional *S. Aureus* strains (n=17) and other staphylococci (n=20) were also used for the evaluation including *S. capitis, S. sciuri, S. hemolyticus, S. saprophyticus, S. lugdunensis* and *S. epidermidis.* Non-staphylococcal bacterial strains/DNA (n=22) including *Escherichia coli*, Group A Streptococcus, *Pseudomonas* spp., *Proteus* spp., and *Klebsiella* spp. were also used for performance evaluation. Thus, a total of 230 DNA samples from bacteria were used for the evaluation. Extraction of DNA from the bacterial

isolates was performed by boiling and centrifugation as described elsewhere [14].

All primers were supplied by Sigma (Sigma Genosys, Cambridge, UK).Probes used on the AB7500 were exactly the same as published by Nakagawa and were purchased from Applied Biosystems (Applied Biosystems, Warrington, UK). The LCR670 probe used for the detection of *mecA*on the LC480 (Table 2) was purchased from TIB MOLBIOL (TIB MOLBIOL, Berlin, Germany). Manufacturer-specific master-mixes for the two instruments were used. To evaluate the specificity of the optimized assay, sequencing followed by standard nucleotide BLAST were used to confirm the identity of the PCR amplification products. Antibiotic susceptibility test (AST) was performed for staphylococcal oxacillin susceptibility. Tube coagulase (TC) test was used to confirm *S. aureus*. Ten-fold serial dilutions were performed on 0.5MacFarland standardized inoculum to determine the limit of detection (LoD) of the optimized assay. Bacterial DNA was extracted from each dilution and used for real-time PCR assay.

2.1 Statistical Analysis

The R^2 values were calculated from the mean of five most linear points, an approach which increases the accuracy of the DNA quantification by allowing the real-time PCR software to perform a five-point standard curve [15]. Sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of the triplex real-time PCR assay were analysed according to the Clinical and Laboratory Standards Institute (CLSI) Guidelines for molecular diagnostics [16].

3. RESULTS

The optimized PCR cycling conditions which increased assay speed on both instruments and without spectral cross-talk consists of an initial single cycle for 5 minutes at 95ºC (to activate the polymerase system) and 40 cycles of two-temperature cycling consisting of 15s at 95ºC (for denaturation) and 5s at 60ºC (for polymerization). The optimized triplex real-time PCR assay completed one diagnostic cycle including data analysis within one hour. This is a major speed advantage over the original assay by Nakagawa et al. [12]. In addition to the optimized PCR cycling conditions, the oligonucleotide primer–probe sets driving the triplex real-time PCR assay are listed (Table 3). Other optimized PCR factors which necessarily removed spectral cross-talk are also presented (Table 4). When the crossing point (Cp) values were plotted against the $\log_{10}$ of the bacterial DNA load, a good linearity was observed over the range from 1.0-2.0 $x10^8$ colony forming units/ml (abbreviated: CFU/ml), which was confirmed phenotypically by serial dilution methods. This sensitivity result applied to both instruments.

Using MRSA strains from NARSA to study assay specificity in monoplexes and multiplexes including NRS194, NRS192, and *S. aureus*USA400 (MW2) whose genomes harbour all the three key genes, the assay reliably identified *spa*, *lukSF-PV* and *mecA* on both instruments. PVL-negative MSSA strains generated only the *spa* signal, PVL-positive MSSA strains yielded positive signals for *spa* and *lukSF-PV*, while producing negative signal for *mecA*. Methicillin-resistant coagulase-negative staphylococci (MRCoNS) including *S. epidermidis* strain NRS8, *S. hemolyticus* NRS9 and *S. epidermidis* NRS69 all gave positive signals for *mecA* and and not for *spa* and *lukSF-PV*. Also the 22 bacterial strains of non-staphylococcal background generated no amplification signal with the triplex PCR assay.

When we evaluated the triplex real-time PCR assay on direct bacterial cultures from frozen stocks, agar plates, and from 0.5McFarland broth used for phenotypic identification and AST, they all yielded concordant gene detection results. Despite differences in user interface and display of emission signals (Fig. 1), discordant performance characteristics were not found between the two instruments.

4. DISCUSSION

Optimization of the cycling conditions which led to completion of cycling and data analysis under one hour. This compares favourably with a recent real-time PCR assay reported by Pichon et al. [11]. Interestingly, the Pichon assay was developed following evaluation of a similar 2005 triplex real-time PCR assay [10] which the Pichon group found erroneous that they modified and upgraded it to a quadruplex assay. The reduction in assay cycling time from 3 hours to less than one hour will empower microbiology laboratories to provide speedier diagnostic service. This will help reduce the rapid victim deterioration associated with therapy-refractory *S. aureus* PVL syndrome.

Table 1. Reference staphylococcal strains (n = 50) and their sources

S/N	Isolate identity[a]	Nomenclature (species level)	Source
1	NRS1	*S. aureus*	NARSA[b]
2	NRS102	*S. aureus*	NARSA
3	NRS103	*S. aureus*	NARSA
4	NRS110	*S. aureus*	NARSA
5	NRS111	*S. aureus*	NARSA
6	NRS112	*S. aureus*	NARSA
7	NRS113	*S. aureus*	NARSA
8	NRS114	*S. aureus*	NARSA
9	NRS123	*S. aureus*	NARSA
10	NRS13	*S. aureus*	NARSA
11	NRS147	*S. aureus*	NARSA
12	NRS149	*S. aureus*	NARSA
13	NRS153	*S. aureus*	NARSA
14	NRS157	*S. aureus*	NARSA
15	NRS158	*S. aureus*	NARSA
16	NRS162	*S. aureus*	NARSA
17	NRS164	*S. aureus*	NARSA
18	NRS165	*S. aureus*	NARSA
19	NRS167	*S. aureus*	NARSA
20	NRS170	*S. aureus*	NARSA
21	NRS171	*S. aureus*	NARSA
22	NRS172	*S. aureus*	NARSA
23	NRS176	*S. aureus*	NARSA
24	NRS179	*S. aureus*	NARSA
25	NRS182	*S. aureus*	NARSA
26	NRS185	*S. aureus*	NARSA
27	NRS188	*S. aureus*	NARSA
28	NRS191	*S. aureus*	NARSA
29	NRS192	*S. aureus*	NARSA
30	NRS194	*S. aureus*	NARSA
31	NRS227	*S. aureus*	NARSA
32	NRS229	*S. aureus*	NARSA
33	NRS231	*S. aureus*	NARSA
34	NRS233	*S. aureus*	NARSA
35	NRS244	*S. aureus*	NARSA
36	NRS248	*S. aureus*	NARSA
37	NRS249	*S. aureus*	NARSA
38	NRS255	*S. aureus*	NARSA
39	NRS260	*S. aureus*	NARSA
40	NRS265	*S. aureus*	NARSA
41	NRS70	*S. aureus*	NARSA
42	NRS71	*S. aureus*	NARSA
43	NRS72	*S. aureus*	NARSA
44	NRS8	*S. epidermidis*	NARSA
45	NRS9	*S. haemolyticus*	NARSA
46	NRS69	*S. haemolyticus*	NARSA
47	NCTC12217	*S. lugdunensis*	NCTC[c]
48	NCTC11042	*S. haemolyticus*	NCTC
49	NCIMB9993	*S. epidermidis*	NCIMB[d]
50	NCIMB700787	*S. capitis*	NCIMB

a Identity of isolate at source, b NARSA, Network for Antimicrobial resistance in Staphylococcus aureus
c NCTC, National Collection of Type Cultures, d NCIMB, National Collection of Industrial and Marine Bacteria

Table 2. Emission wavelengths of DNA probe fluorophores and channels used for detection of bacterial DNA on ABI 7500 and LC480 instruments

DNA probe fluorophore	Emission channel	Wavelength(nm) on ABI 7500	Wavelength(nm) on LC480
6FAM	FAM	500 (FAM)	533 (FAM)
VIC	VIC/HEX	560 (VIC)	568 (HEX)
TET/LCR670	TET/Cy5	530 (TET)	670 (LCR670)

Table 3. Primer-probe sets used for evaluation of performance on both instruments[a]

Target sequence	Amplicon size (bp)	Primer and probe identity[b]	Primer and probe[c] sequence 5' → 3'
mecA	155	mecA-F	TGGTATGTGGAAGTTAGATTGGGAT
		mecA-P	[c]F-TTCCAGGAATGCAGAAAGACCAAAGCA-BBQ[d]
		mecA-R	CTAATCTCATATGTGTTCCTGTATTGGC
lukSF-PV	118	lukSF-PV-F	TTACACAGTTAAATATGAAGTGAACTGGA
		lukSF-PV-P	FAM-AAGTGAAAGGACATAATTG-MGBNFQ[e]
		lukSF-PV-R	AGCAAAAGCAATGCAATTGATG
spa	101	spa-F	CAGCAAACCATGCAGATGCTA
		spa-P	VIC-TCAAGCATTACCAGAAAC-MGBNFQ
		spa-R	CGCTAATGATAATCCACCAAATACA

a Primer and probe oligonucleotide sequences are all copied from Nakagawa et al. [12].
b F = forward primer; P = Probe; R = Reverse primer. c the 5'-end of the probe was labelled with TET for use with ABI 7500, while the 5'-end of the probe was labelled with LightCycler®Red 670 (LCR670) for use with LC480.d the 3'-end was labelled with a blackberry quencher. e the 3'-end of the probe was labelled with a minor groove binder molecule and non-fluorescent quencher (MGB-NFQ)

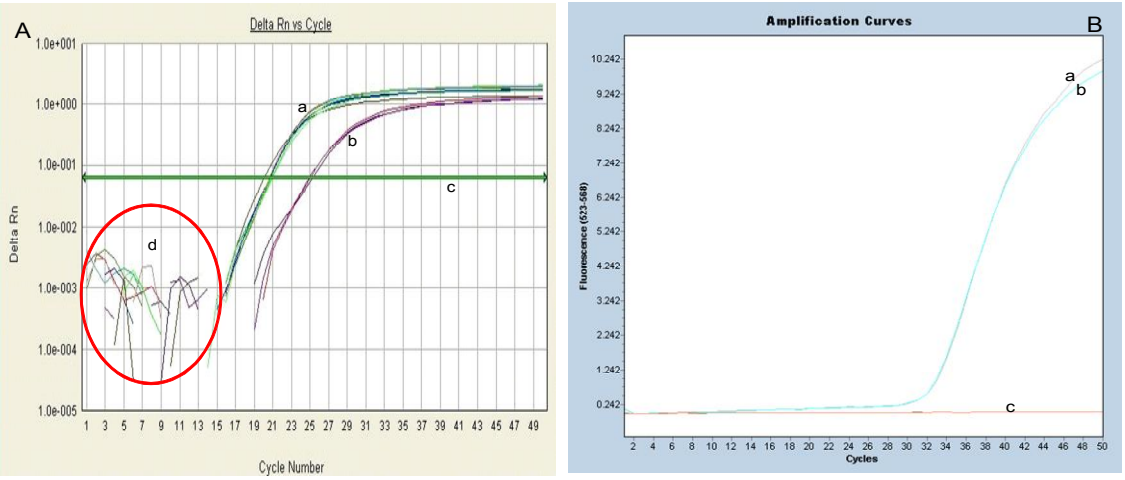

Fig. 1. User interface output display of fluorescence signals for 50 cycles by ABI 7500SDS (A) and LC480 (B)

Sensitivity and good linearity over the range 1.0-2.0x10^8 CFU/ml suggests the optimized triplex real-time PCR assay is of comparable sensitivity as the original assay developed by Nakagawa using a different machine (AB7900HT). Therefore the gain in speed associated with the newly optimized assay was without loss of sensitivity. PCR was commended by early experimenters for its sensitivity [17]. Also, the fact that no amplification signal was detected from the 22 non-staphylococcal bacteria examined with the optimized triplex real-time PCR assay shows the assay did not lose specificity (precision and accuracy) following optimization.

The newly optimized triplex real-time PCR assay identified all *mecA*-positive oxacillin resistant strains, including high-level (MIC oxacilin ≥32 g/L) and very low-level (0.5 g/L) expressors. The assay also detected *mecA* gene from all the *SCCmec* types collected by Dr Spence, including those local to Nottingham as well as the National (NCTC and NCIMB) and international (NARSA) type culture strains. Thus, the capacity of the optimized real-time PCR assay to detect the expected gene sequences as confirmed by sequencing and BLAST was 100%. This represents a 100% positive predictive value (PPV=100%). Evaluation of a similar assay developed in 2005 [10] showed it was unable to detect some *SCCmec* types which encoded phenotypically confirmed oxacillin resistance [11]. Also, the 22 eubacteria of non-staphylococcal background yielded no amplification signal, representing a negative predictive value (NPV) of 100%. Other than obvious differences in the user interface and signal output style (Fig. 1), there was no difference in speed between the two instruments.

Table 4. PCR factors of the optimized triplex real-time PCR assay

Factor	Quantity
Probe mastermix	25.0 µL
spa and *lukSF-PV* primers	0.75 µM
mecA primers	1.0 µM
spa probe	0.04 µM
lukSF-PV probe	0.08 µM
mecA probe	0.1 µM
PCR grade water (Roche)	Make up to 50.0 µL

The difference between R^2 values reported by Nakagawa who used ABI 7900HT and the R^2 values we obtained in this evaluation using two different instruments were not significant (<0.01, Student's t test: p=0.99). Thus the analytical quantitative essence of the triplex real-time PCR assay is highly reproducible on both instruments.

Quantification of input DNA is very important in real-time PCR and constitutes a major advantage of real-time PCR over end-point PCR. This is especially important when quantifying input template DNA in clinical samples.

5. CONCLUSION

NPV and PPV values of 100% suggest that the triplex real-time PCR assay is capable of confirming or ruling out the presence of the three key genes in a given bacterial isolate. Thus, the primer–probe sets for *lukSF-PV*, *mecA* and *spa* yielded 100% gene detection specificity on both instruments. The sensitivity, speed, specificity and reproducibility generated from using the triplex real-time PCR assay on both instruments show the assay is readily adaptable for use in a routine diagnostic microbiology laboratory across different instruments. The sensitivity and specificity of the assay on direct detection from broths and colony PCR suggests the applicability of the assay for direct detection of the three key genes from biological samples. Completion of cycling and data analysis under an hour means the optimized assay is speedy enough to support timely infection control and clinical therapeutic interventions than would be possible by conventional microbiological culture-based assays.

Despite difference in signal detection emission channels, the triplex real-time PCR assay developed using one machine had been packaged and operated on another real-time PCR proprietary instrument(s) from a completely different manufacturer. What is required is sound understanding of the operational style of each unique instrument, including the wavelengths for the different emission channels as well as the output signal and the user-interface data presentation style.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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