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Comparison of a multiplexed MassARRAY system with real-time allele-specific PCR technology for genotyping of methicillin-resistant *Staphylococcus aureus*

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Abstract

The Sequenom MassARRAY iPLEX single-nucleotide polymorphism (SNP) typing platform uses matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) coupled with single-base extension PCR for high-throughput multiplex SNP detection. In this study, we investigated the use of iPLEX MassARRAY technology for methicillin-resistant *Staphylococcus aureus* (MRSA) genotyping. A 16-plex MassARRAY iPLEX GOLD assay (MRSA-iPLEX) was developed that targets a set of informative SNPs and binary genes for MRSA characterization. The method was evaluated with 147 MRSA isolates, and the results were compared with those of an established SYBR Green-based real-time PCR system utilizing the same SNP–binary markers. A total of 2352 markers belonging to 44 SNP–binary profiles were analysed by both real-time PCR and MRSA-iPLEX. With real-time PCR as the reference standard, MRSA-iPLEX correctly assigned 2298 of the 2352 (97.7%) markers. Sequence variation in the MRSA-iPLEX primer targets accounted for the majority of MRSA-iPLEX erroneous results, highlighting the importance of primer target selection. MRSA-iPLEX provided optimal throughput for MRSA genotyping, and was, on a reagent basis, more cost-effective than the real-time PCR methods. The 16-plex MRSA-iPLEX is a suitable alternative to SYBR Green-based real-time PCR typing of major sequence types and clonal complexes of MRSA.

Keywords: MassARRAY, MRSA, PCR, SNP

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Introduction

Infections caused by community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) and healthcare-associated methicillin-resistant *S. aureus* (HA-MRSA) pose a significant problem to healthcare facilities and within the community, because of their association with significant morbidity and mortality (1–4). CA-MRSA is often linked to production of the Panton–Valentine leukocidin (PVL) toxin, which is associated with tissue necrosis and fatal necrotizing pneumonia (5). The increased prevalence of CA-MRSA and HA-MRSA worldwide has highlighted the need for accurate epidemiological surveillance systems (6,7), with methicillin-resistant *S. aureus* (MRSA) genotyping now being an integral part of clinical and health facility management (8,9). Traditional tools for MRSA epidemiological surveillance include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), staphylococcal cassette chromosome *mec* (SCC*mec*) element typing, and staphylococcal protein A (*spa*) sequence typing (10,11). PFGE is highly discriminatory and is

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typically employed for short-term surveillance, whereas MLST/SCC*mec* and *spa* sequencing are used for long-term surveillance of genetic relatedness (11). Although the results obtained with these tools are highly reproducible, their limitations include high technical complexity, expense, and prolonged result turn-around times (10,11).

More recently, MRSA genotyping based on a set of informative single-nucleotide polymorphisms (SNPs) and binary markers has been employed. These methods are effectively mini-MLSTs, and have proved to have a similar performance to full MLST data for resolving clonal complexes and certain sequence types (STs) (10,12), with the added advantage of using real-time PCR technology for rapid and cost-effective SNP discrimination with simple data analysis software (13–15). The MRSA typing method used by our laboratory was originally described by Huygens *et al.* (13), and consists of 17 separate SYBR Green-based real-time PCRs, including two assays to confirm MRSA (*mecA* and *nuc*), eight assays to discriminate seven informative SNPs (*arcC*210, *tpi*241/243, *arcC*162, *gmk*318, *pta*294, *tpi*36*T, *tpi*36*C, and *pta*383), five assays targeting binary genes (*pvl*, *cna*, *sdrE*, pUB110, and pT181), one additional SNP assay (*aroE*252*G*) for confirmation of the Queensland CA-MRSA ST93, and one internal control reaction (16S). The method is robust, is able to discern major Australian and certain international MRSA STs and clonal complexes (CCs), including the South West Pacific (SWP) (ST30) and Queensland CA-MRSA (ST93) clones, as well as the HA-MRSA ST239 clone (colloquially known as Aus-2 and Aus-3 EMRSA), and has since been employed in numerous studies (16–18). The limitation of this method is that throughput can be severely impeded by the number of individual reactions that need to be performed (17 per isolate), particularly in a routine laboratory, where the number of MRSA isolates is high and valuable space on real-time PCR instruments may be quickly sequestered by other testing.

The Sequenom (Brisbane, Australia) MassARRAY iPLEX SNP typing platform uses matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) coupled with single-base extension PCR for high-throughput multiplex SNP detection, which is capable of multiplexing up to 40 SNPs per single reaction (19–21). In this study, we adapted the above MRSA SNP–binary typing method to the iPLEX system (MRSA-iPLEX). MRSA-iPLEX was validated with 147 clinical MRSA isolates, and the results were compared with those of the real-time PCR methods.

Materials and Methods

Bacterial isolates

A total of 147 clinical MRSA isolates were retrospectively tested with SYBR Green real-time PCR and MRSA-iPLEX. These included 110 clinical MRSA isolates from previous studies (15,22), 25 local outbreak isolates (Princess Alexandria Hospital in 2006, *n* = 17, and Royal Brisbane Hospital in 2007, *n* = 8), and 12 MRSA control isolates used in our laboratory. Of the 147 isolates, 103 were major STs or CCs (summarized in Table 1), and the remaining 44 were either minor CCs or otherwise not assigned. A subset of isolates (*n* = 68) were characterized by PFGE, MLST and SCC*mec* typing, as previously described (23), and are summarized in Table S1.

Nucleic acid extraction

Isolates were grown on horse blood agar at 37°C overnight. For each isolate, a 1-μL loop of a 24-h colony was suspended in 250 μL of HPLC-grade water and treated with lysostaphin (40 mg/μL). Nucleic acids were purified with the Corbett CAS-1820 X-tractor Gene, following the manufacturer's instructions (Qiagen, Doncaster, Australia). The DNA concentration was determined with the NanoDrop 1000 (Thermo Fisher Scientific, Scoresby, Australia), and this was followed by dilution to a final concentration of 5 ng/μL. Diluted extracts were used immediately or stored at −20°C until further analysis.

MRSA detection, typing and analysis with SYBR Green real-time PCR

The SYBR Green real-time PCR assays were performed and analysed as previously described (13). For confirmatory and binary assays, a positive or negative result was coded as one or zero. SNP assay results (*arcC*210, *tpi*241/3, *arcC*162,

TABLE 1. Major sequence types (STs) or clonal complex (CC) profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates

CC or ST complex	SNP profile[a]	No. of isolates	Included PFGE types
1	CGATAACT/TGATTACT	10	WA-MRSA-10, WA-MRSA-38, WA-MRSA-60
5	CGATTACA	22	WA-MRSA-3, WA-MRSA-11, Mu50
8	TGATACCA	18	USA300, Irish EMRSA-1
30	TGGATCCA	8	PAH58, WSPP MRSA
93	TGGTTCTA	8	QLD, RBH98
239	TGAAACCA	37	AUS EMRSA-2, AUS EMRSA-3

PFGE, pulsed-field gel electrophoresis; SNP, single-nucleotide polymorphism.
[a]SNP results in the order *arcC*210, *tpi*241 + 243, *arcC*162, *gmk*318, *pta*294, *tpi*36, and *pta*383.

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*gmk*318, *pta*294, *tpi*36, and *pta*383) were coded as G, C A, or T. For the *aroE*252 assay, the confirmatory assay for the Queensland clone ST93, positive results were coded as G.

MRSA detection and typing with MassARRAY MALDI-TOF MS (MRSA-iPLEX)

MRSA-iPLEX design. Sequence data available on the NCBI/GenBank/UCSC Gene Browser and *S. aureus* MLST database (http://www.mlst.net) were utilized. Optimal amplification primers and extension primers for use in a multiplex format were designed with Assay Designer v3.1 (Sequenom). Primers were supplied by Sigma Genosys (Castle, Hill, Australia), and contained the 5′ 10mer extension (ACGTTGGATG) to remove them from the observed mass window and provide stability in a multiplex PCR reaction. PCR and extension primers for multiplex MRSA typing are listed in Tables 2 and 3. PCR primers were mixed equimolar at 1.0 μM, and the extension primer mix was prepared by dividing primers into two groups on the basis of mass, and diluting the high-mass primers to 14.0 μM and the low-mass primers to 9.0 μM. The extension primer adjustment was fine tuned with MALDI-TOF MS and Typer 4 to ensure that all primers were present in the mix and that peak intensities were equalized.

Primary PCR reaction and shrimp alkaline phosphatase (SAP) treatment. The PCR multiplex assay was performed on the GeneAMP PCR system 9700 (Applied Biosystems, Mulgrave, Australia), with the Sequenom PCR Reagent Set. Each reaction contained 1 × PCR Buffer, each primer at 0.1 μM (Table 2), 4 mM $MgCl_2$, 500 μM dNTP mix, 0.5 units of PCR enzyme, and 10 ng of DNA template. The final reaction volume was adjusted to 5.0 μL with HPLC-grade water. Samples and reaction mix were loaded into a 384-well microplate (Thermo Fisher Scientific). PCR parameters included an initial denaturation at 95°C for 2 min, 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 5 min. Each run included negative controls (water and human DNA), and all samples were tested in duplicate. Non-incorporated dNTPs were dephosphorylated by adding 2.0 μL of SAP cocktail (Sequenom), consisting of 0.5 units of SAP enzyme, to the PCR reaction mix, and incubated on the GeneAMP PCR system 9700 at 37°C for 40 min, and then at by 85°C for 5 min.

TABLE 2. Primers for primary PCR reaction

Gene target	Forward primer[a]	Reverse primer[a]	Amplicon size (bp)
16S	10mer-GAGATATGGAGGAACACCAG	10mer-TGTTTGATCCCCACGCTTTC	102
nuc	10mer-AGAGTTGTGGATGGTGATAC	10mer-TTCGGTTTCACCGTTTCTGG	118
mecA	10mer-GCTTTGGTCTTTCTGCATTC	10mer-AAGAAGATGGTATGTGGAAG	91
*arcC*210	10mer-ATAGTGATAGAACTGTAGGC	10mer-CTTTCGTATAAAAAGGACCA	125
*tpi*241/3	10mer-GCAATCGGAACTGGTAAATC	10mer-CGAGTTGCTTCTGATACTTC	130
*gmk*318	10mer-GAAGGTGCAAAGCAAGTTAG	10mer-CCTCTACCTACTAATCGCTC	121
*pta*294	10mer-GTTAGTGGTGCAGCACATTC	10mer-GGATTGATTGCACAATCACC	169
*tpi*36	10mer-CACGAAACAGATGAAGAAA	10mer-ACGCTCTTCGTCTGTTTCAC	116
pvl	10mer-GGCTATACAAAGCCAAATCC	10mer-AATGTTGTACTTAGAACCCC	89
cn	10mer-TGATGTTTCGGGATTTGCAG	10mer-TTCCCAGATGTTATCGTAGC	116
sdr	10mer-TACGAAGATGTAACAAGTC	10mer-AGTTCGCCATCTGATGTAGG	157
pUB110	10mer-CGTGATGGAAAACTGCAAGG	10mer-ACCACGCTCCAACTCAAAAC	131
pT181	10mer-TGAGCTGTCTTGGTTCATTG	10mer-AATGCAGCAGATCCTACTCC	117
*aroE*252	10mer-GTCTGGATAAACGCTGTGCAA	10mer-GTGGATAGGGTATAATACAG	83

[a]10mer = 10-mer tag ACGTTGGATG placed at 5′-end of each primer.

TABLE 3. Primers and masses for ASPE reaction

Target	UEP	EP1[a] (Da)	EP2[a] (Da)	EP3 (Da)
16S	gctAAGGCGACTTTCTGGTCTG	6757.4	A, 7028.6	T, 7084.5
nuc	gAAAGAAATTAAAGTTAGGCTTATAG	8090.3	G, 8377.5	T, 8417.4
mecA	TGGAAGTTAGATTGGGAT	5633.7	G, 5880.9	C, 5920.9
*arcC*210	gggagAAAAAGGACCAATTGGTTT	7489.9	T, 7761.1	C, 7777.1
*tpi*241/3	ccCATCAACATCTGAAGATGC	6359.2	GA, 6630.4	GG, 6646.4
*arcC*162	ACTGTAGGCACAATCGT	5194.4	A, 5465.6	T, 5521.5
*gmk*318	ATCGCTCTCTCAAGTG	4832.2	T, 5103.4	A, 5159.3
*pta*294	cccCAGCACATTCAACAGG	5726.7	C, 5973.9	A, 5998
*tpi*36	gggtAAATTAACAAAAAAGCGCA	7122.7	C, 7369.9	T, 7449.8
*pta*383	ATCTTCTTTATGATTAAAGGTGATG	7701	A, 7972.2	T, 8028.1
pvl	cccGTACTTAGAACCCCAATAAAAT	7563	T, 7834.2	A, 7890.1
can	TAGCTACTTTGTCATCTGA	5768.8	T, 6040	A, 6095.9
sdrE	gggcATATCACCAAAATCCAATGT	7329.8	T, 7601	A, 7656.9
pUB110	CGTCAAGAACTGTTATGGCT	6132	A, 6403.2	T, 6459.1
pT181	AACCTACCAAAAATCAAAATAAAAAA	7934.3	G, 8181.4	C, 8221.5
*aroE*252	gACAGATGGTATCGGTTATGT	6516.2	A, 6787.5	G, 6803.5
T, 6843.3				

[a]EP1 denotes a positive result and EP2 a negative result for binary and confirmatory genes.

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iPLEX reaction and final cleanup. The single-base extension reaction was performed on the GeneAMP PCR system 9700 with the iPLEX Gold Reaction Kit, following the manufacturer's instructions (Sequenom). Briefly, 2.0 μL of the iPLEX extension primer cocktail, consisting of 0.2 μL of iPLEX Buffer, 0.25 μL of iPLEX Terminator Mix, 0.05 μL of iPLEX Enzyme, and 0.94 μL of the adjusted iPLEX extension primer mix (Table 3), was added to each reaction well. PCR conditions included an initial incubation at 94°C for 30 s, followed by 40 cycles of one step at 94°C for 5 s with five subcycles of 52°C for 5 s and 80°C for 5s, and a final extension at 7°C for 3 min. Each extension product was conditioned with 6 mg of CLEAN Resin (Sequenom) and 16 μL of HPLC-grade water. The microplate was rotated for 15 min, and then centrifuged at 500 *g* for 5 min with a standard plate centrifuge. Approximately 10 nL of product was dispensed onto a 384-format SpectroCHIP (Sequenom), with the MassARRAY Nanodispenser RS 1000 (Sequenom).

MassARRAY analysis and data quality control. MALDI-TOF MS analysis was performed on a MassARRAY Compact Analyzer (Sequenom). Data acquisition was automatically performed by the use of SpectroAcquire, with ten laser shots per raster position and a threshold of five good spectra per sample pad. The mass window of analyte peak observation was set at 4500–9000 Da. The data were analysed with Sequenom's MassARRAY Typer software version 4.0.3. Genotyping calls were viewed in call cluster plots, and peak intensities were reviewed in each respective sample spectrum. Genotyping calls were considered to be final if results between duplicates were concordant. SNP and binary markers were coded as per the real-time PCR assays, except that results of the *aroE*252 assay, the confirmatory assay for ST93, were further distinguished into either a T or an A base.

Sequence analysis of discrepant results

DNA sequencing was used to investigate representative markers providing discrepant results between SYBR Green real-time PCR and MRSA-iPLEX. Primers for DNA sequencing were developed to encompass both real-time PCR and MRSA-iPLEX primer target sites (data not shown). PCR products were submitted to the Australian Genome Research Facility, St Lucia, Queensland, for DNA sequencing.

Results

A total of 2352 markers belonging to 44 SNP–binary profiles were analysed by both real-time PCR and MRSA-iPLEX, and are summarized in Table 4 (all 147 isolates) and Table S1 (the subset of 68 isolates). With the real-time PCR as the reference standard, MRSA-iPLEX correctly assigned 2298 of the 2352 (97.7%) markers, providing an overall error rate of 2.3% (54/2352). Among the 147 isolates examined, MRSA-iPLEX correctly identified all 16 markers for 124 isolates.

There were 23 isolates that accounted for the 54 incorrect/failed MRSA-iPLEX results. Of these, 16 were represented by only three SNP–binary profiles, including CC9 (n = 3), ST93 (n = 7), and an unassigned group (n = 6), with the latter six isolates accounting for 36 of 54 incorrect/failed results.

TABLE 4. SYBR Green real-time PCR and MRSA-iPLEX results. Erroneous MRSA-iPLEX profiles are denoted by an 'X' and discrepant results are in bold italics

No. of isolates	CC/ST	SYBR real-time PCR[a]	MRSA-iPLEX[a]
1	15	111CGATAACA01100	111CGATAACA01100T
1	15	111CGATAACA11100	111CGATAACA11100T
4	1	111CGATAACT01100	111CGATAACT01100T
5	1	111CGATAACT11100	111CGATAACT11100T
1	NA[b]	111CGATACCA00101	111CGATACCA00***0***01T X
1	NA[b]	111CGATACCA00000	111CGATACCA00000T
14	5	111CGATTACA00100	111CGATTACA00100T
4	5	111CGATTACA00110	111CGATTACA00110T
2	5	111CGATTACA00101	111CGATTACA00101T
2	5	111CGATTACA11100	111CGATTACA11100T
1	72	111CGATTCCA10100	111CGATTCCA10100T
1	45	111CGGATCCA01100	111CGGATCCA01100A
1	45	111CGGATCCA01100	111CGGATCCA01***0***00A X
2	80	111CGGTACCA10101	111CGGTACCA10101T
1	80	111CGGTACCA10100	111CGGTACCA10100T
2	22	111CGGTTACA01100	111CGGTTACA01100T
1	509	111CGGTTCCA01000	111CGGTTCCA01000T
1	509	111CGGTTCCA01100	111CGGTTCCA01100T
20	239	111TGAAACCA01100	111TGAAACCA01100T
12	239	111TGAAACCA01110	111TGAAACCA01110T
2	239	111TGAAACCA01110	1***0***1TGAAACCA01110T X
1	239	111TGAAACCA01101	111TGAAACCA01101T
1	239	111TGAAACCA01010	111TGAAACCA01010T
1	239	111TGAAACCA11110	111TGAAACCA11110T
9	8	111TGATACCA00100	111TGATACCA00100T
1	8	111TGATACCA00100	111TGATACA00100T X
3	8	111TGATACCA00101	111TGATACCA00101T
1	8	111TGATACCA00010	111TGATACCA00010T
2	8	111TGATACCA10101	111TGATACCA10101T
3	8	111TGATACCA10100	111TGATACCA10100T
4	78	111TGATTACA00100	111TGATTACA00100T
1	7	111TGATTACA01100	111TGATTACA01100T
1	1	111TGATTACT01100	111TGATTACT01100T
1	30	111TGGATCCA01000	111TATCCA01000T X
1	30	111TGGATCCA01010	111TGGATCCA01010T
5	30	111TGGATCCA11000	111TGGATCCA11000T
1	30	111TGGATCCA11100	111TGGATCCA11100T
1	NA[b]	111TGGTAACA11100	111TGGTAACA11100T
1	97	111TGGTAACT00100	111TGGTAACT00100T
1	OCC	111TGGTACCA01100	111TGGTACCA01100T
6	NA[b]	111TGGTTCCA00100	1***0***1GG–TA00***0***00T X
7	59, 121, 133	111TGGTTCTA00100	111TGGTTCTA00100T
1	59, 121, 133	111TGGTTCTA01100	111TGGTTCTA01***0***00A X
3	59, 121, 133	111TGGTTCTA10101	111TGGTTCTA10101T
2	59, 121, 133	111TGGTTCTA10100	111TGGTTCTA10100T
7	93	111TGGTTCTA10100G	1***0***1TGGTTCTA10100G X
1	93	111TGGTTCTA11100G	111TGGTTCTA11100G
3	9	111TGATAACT00100	111TGATAACT00***0***00T X

CC, clonal complex; MRSA, methicillin-resistant *Staphylococcus aureus*; NA, not assigned; OCC, other clonal complex profile; ST, sequence type.
[a]Results are in the order 16S, *nuc*, *mecA*, *arcC*210, *tpi*241 + 243, *arcC*162, *gmk*318, *pta*294, *tpi*36, *pta*383, *pvl*, *cna*, *sdrE*, pUB110, pT181, and *aroE*252.
[b]Not assigned to a particular CC or ST.

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TABLE 5. Sequence analysis of erroneous MRSA-iPLEX results (shown in bold italics)

SYBR Green real-time PCR profile	MRSA-iPLEX profile	No. of isolates	Sequence analysis
111CGATACCA00101	111CGATACCA00***0***01T	1	Deletion in *sdrE* primer target (primary PCR)
111CGGATCCA01100	111CGGATCCA01***0***00A	1	ND
111TGAAACCA01110	1***0***1TGAAACCA01110T	2	ND
111TGATAACT00100	111TGATAACT00***0***00T	3	Deletion in *sdrE* primer target (primary PCR)
111TGATACCA00100	111TGATACA00100T	1	Single base mismatch in 3′-end of *tpi*36 extension primer
111TGGATCCA01000	111TATCCA01000T	1	Single base mismatch in 3′-end of *tpi*241/3 extension primer
111TGGTTCCA00100	1***0***1GGTA00***0***00T	6	Variations in *arcC*210, *arcC*162, t*pi*36, *nuc* and *sdrE* primer targets (primary PCR); single base mismatch in 3′-end of *pta*294 extension primer
111TGGTTCTA01100	111TGGTTCTA01***0***00A	1	Deletion in *sdrE* primer target (primary PCR)
111TGGTTCTA10100G	1***0***1TGGTTCTA10100G	7	Variations in *nuc* primer targets (primary PCR)

MRSA, methicillin-resistant *Staphylococcus aureus*; ND, not detected—variations not observed in respective primer targets.

The 54 incorrect/failed results included failure to detect *nuc* (15 instances), *sdrE* (12 instances), and various SNPs (27 instances). The basis for these discrepancies was further investigated by DNA sequencing, and the results are summarized in Table 5. Briefly, a failure to amplify or extend because of sequence variation or deletion in the MRSA-iPLEX primer targets accounted for the majority of incorrect/failed results.

Discussion

Epidemiological surveillance of MRSA, which is one of the most important nosocomial pathogens worldwide, is typically performed with techniques that have high discriminatory power but are limited by expense and prolonged result turnaround times (6,9). Ideal bacterial genotyping systems should be highly accurate, specific, fast, reproducible, inexpensive, and simple, and have a high level of throughput and multiplexing capabilities (11,12,20). Recently, Huygens *et al.* (12) created a simple SYBR Green-based real-time PCR system for MRSA typing. Although not as discriminatory as MLST and PFGE, the system could readily be used to differentiate important Australian and certain international STs and CCs, including the Queensland clone ST93, and was easier and cheaper to perform. However, one limitation of this system was that it utilized 17 monoplex PCR reactions per isolate, and was therefore laborious to perform. This may not present a problem for laboratories running small batches of isolates, but is particularly cumbersome for large-scale investigations. The development of post-genomic PCR technology such as MALDI-TOF MS combined with iPLEX GOLD chemistry has facilitated higher-throughput analysis of SNPs (20,21). This technology provides similar simplicity in design as real-time PCR systems, but enables multiplexing of up to 40 SNPs in a single reaction (21). In this study, we successfully multiplexed the original 16 MRSA targets into the MRSA-iPLEX system, and found that the system provided optimal throughput for MRSA genotyping for a diverse range of local and international isolates, and was, on a reagent basis, considerably less expensive than use of the monoplex SYBR Green real-time PCR reactions. The MRSA-iPLEX result turn-around time for complete profiles for all 16 markers for all 147 isolates, including duplicates, was <12 h, at a consumable cost of $AUD5.26 per sample. Results were obtained in three working days with SYBR Green real-time PCR, at a cost of $AUD18.00 per sample. The cost benefits of using MALDI-TOF MS technology for multiplex SNP detection will probably improve in the foreseeable future as the costs of the MALDI-TOF MS instrumentation decrease, making the technology more accessible to the routine clinical laboratory.

The error rate of MRSA-iPLEX was determined to be 2.3%, and seems to pose a technical limitation. However, the majority of these errors were limited to particular SNP–binary groups, suggesting that these issues are probably related to the sequence composition of certain MRSA types. DNA sequencing confirmed that the majority of these problems were caused by sequence variation in the MRSA-iPLEX targets. In the design of MRSA-iPLEX, many of the MRSA-iPLEX primer targets were no longer shared with those of the original real-time PCR assays, being moved either upstream, downstream, or to the opposite DNA strand. Changing the primer targets may have been a problem for certain genes, particularly *nuc* and *sdrE*, resulting in primers being moved away from well-conserved targets. However, the observed errors are not considered to be a major limitation affecting the utility of MRSA-iPLEX, given that: (i) 36 of the observed 54 incorrect calls were limited to only six isolates, and these isolates did not belong to clinically significant CCs or STs; and (ii) 15 errors related to *nuc*, which is merely used for confirmation of *S. aureus* DNA and does not affect the genotype. Redesigning MRSA-iPLEX to accommodate sequence variants, new emerging clones or use in

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different epidemiological contexts is possible by changing the affected primer set. Such a change could potentially, but not necessarily, impact on other primers used in the multiplex, so the resulting multiplex would need to go through a brief revalidation procedure on a control sample cohort to establish the performance of all assays in the multiplex. Redesign of the monoplex SYBR Green real-time PCR assays would involve a similar validation procedure, with an advantage being that only the individual assay in question needs to be considered.

In conclusion, the iPLEX GOLD is a highly useful technology as an alternative for SYBR Green-based real-time PCR typing of SNPs and binary markers of major Australian and certain international MRSA clones. This assay would be suitable for large-scale investigations in research or clinical fields. Notwithstanding the results of this study, MRSA-iPLEX would require evaluation against established MRSA typing schemes prior to use in different epidemiological contexts.

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Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Subset of 68 isolates further characterized by PFGE, MLST and SCC*mec* typing.

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