A Novel 3base™ Multiplex PCR Assay for the Rapid Detection and Stratification of MRSA

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important global nosocomial pathogens. Intensive care patients colonised with MRSA are at a greater risk of developing a more serious infection, compared to those patients carrying methicillin-susceptible *S. aureus* (MSSA). Rapid molecular detection of MRSA will aid in patient management and care. Recently a *mecA* homologue was discovered (*mecALGA251*) and it has been suggested that MRSA strains harbouring only the *mecALGA251* homologue may be wrongly identified as MSSA using molecular methods which only target the *mecA* gene. Furthermore, *nuc* deficient MRSA strains have also been identified, again leading to the potential of false negative results in molecular assays that only target the *nuc* gene (2). We aimed to produce a rapid real time PCR assay that could detect the presence of MRSA from clinical samples in less than 3 hours and that also addresses the current limitations of molecular methods.

Materials & Methods

Previously characterised MRSA isolates or clinical specimens either in saline solution or transport media were tested with the *EasyScreen*™ MRSA Detection Kit. Briefly, a total of 150 µL of each specimen was added to 400 µL of *EasyScreen*™ buffer, heated for 15 minutes at 95°C and the nucleic acids were extracted via the Kingfisher Flex (Thermofisher, Waltham) according to the protocols supplied (Genetic Signatures, Sydney, Australia). The purified nucleic acids were resuspended in 50 µL of *EasyScreen*™ Reagent 7 and 2 µL of the eluate was added directly to the PCR mix. The nucleic acid purification and the PCR set up (into 384 wells) can also be performed using the dedicated GS1 instrument, which reduces user hands on time to around 1 minute per sample. The real time PCR detection is compatible with common hardware such as the LC480 (Roche, Pleasanton, USA), 7500 fast (Applied Biosystems, Foster City, USA), Rotorgene-Q (Qiagen, Hilden, Germany), the Smartcycler II (Cepheid, Sunnyvale, USA) and the CFX96/384 (Bio-Rad, Hercules, USA).

Sensitivity & Specificity

The Genetic Signatures' *EasyScreen*™ sample processing method converts the native nucleic acids from the traditional 4-bases to a 3base™ form (3, 4). The conversion process simplifies the design of multiplex PCR reactions by eliminating the large Tm differences that can be present when targeting multiple pathogens (see Figure 1). On testing, all assay components demonstrated a sensitivity of between 5-20 copies and no cross reactivity was observed against a wide range of non-target organisms at an input of 10^4/copies per PCR.

Conventional Sequence		Tm
Primer 1	GTACACACCGCCCGTCGCTCCTACC	77°C
Primer 2	GAAGGAGAAGTCGTAACAAG	56°C
Probe 1	TGAATAAAGAGGTGAAATTCTAGG	59°C
Probe 2	GAAGGGCCGCGAGCCCCCGCGC	87°C

3base™ Sequence		Tm
Primer 1	GTATATATTGTTTGTTGTTTTTATT	52°C
Primer 2	GAAGGAGAAGTTGTAATAAG	50°C
Probe 1	TGAATAAAGAGGTGAAATTTTAGG	59°C
Probe 2	GAAGGGTTGTGAGTTTTTGTGT	62°C

Figure 1: The sequence of 2 primers and probes directed to regular sequence are shown (top) and compared to the equivalent 3base™ sequence (bottom). As seen, the 3base™ designs have Tm's better suited to real time PCR.

Study Design

The *EasyScreen*™ MRSA Detection Kit contains a common primer and probe located in the orfX gene and over 10 specific primers to detect all commonly encountered SCCmec subtypes. To detect *nuc* deficient and *mec*A negative MRSA strains the assays also include additional primer and probes directed to *fem*A and the *mecALGA251* homologue (Table 1). The assay was shown to detect between 5-20 copies and no cross reactivity was observed with a wide range of non-target bacteria (Figure 2 and Table 2). Typical results of the multiplex assay is shown in Figure 3. All MRSA samples provided as part of the Qnostics validation panel were detected with the exception of one sample which only contained 2 cfu of MRSA, which is below the LOD of the assay (Table 3). All 18 reference strains examined were correctly identified in the assay (Table 4). In addition, 39/39 provided isolates covering SCCmec subtypes I-V were correctly identified. All (36/36) previously characterised clinical specimens were positive and 1 negative clinical sample was also reported as positive. Further random screening of 183 clinical specimens showed a prevalence rate of 11.5% and we are awaiting confirmation of these results.

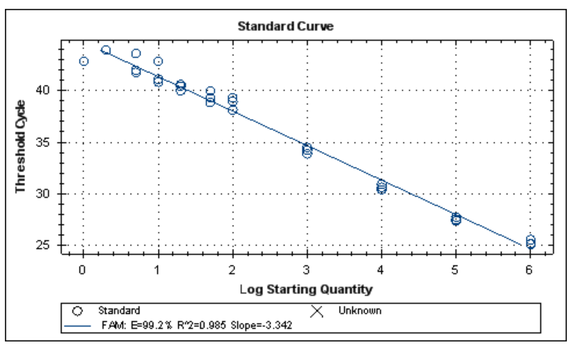
Preclinical Studies

Table 1: Components of the *EasyScreen*™ MRSA Detection Kit

Target	Gene Region
MRSA	orfX/SCCmec
Extraction control	16s rDNA
Methicillin resistance	mecA/mecALGA251
Staphylococcus	nuc/femA

To assess the sensitivity and specificity of the panel 110bp oligonucleotides and synthetic plasmids (Mr. Gene GmbH) were synthesised containing the identical target region to each of SCCmec types and all other components targeted in the reaction. The oligonucleotides/plasmids were quantified by Nanodrop™, then diluted to a final concentration of 10^6 copies/µL. The samples were added to the *EasyScreen*™ lysis buffer, heated for 15 minutes at 95°C, then purified by isopropanol precipitation and resuspended in 50µL of reagent 7. Serial dilutions were performed down to 1 copy and amplified in the multiplex reaction (see figure 2). Cross reactivity was assessed by adding 10^4 copies of each of the organisms listed in Table 2 to the PCR reaction. The results obtained using the multiplex assay on the Qnostics MRSA proficiency panel are shown in Table 3.

Results



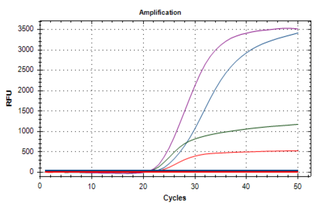
SCCmec type	Limit of detection (triplicate reactions)
Mec 5C1	5 copies
Mec IVe	5 copies
Mec V	20 copies
Mec III	20 copies
Mec IV	5 copies

Figure 2: Linearity (top) and sensitivity (bottom) for the detection of various SCCmec subtypes

Table 2: Organisms tested to assess cross reactivity of the *EasyScreen*™ MRSA Detection Kit.

Organism	Organism	Organism	Organism
S. aureus	*C parapsilosis*	*M. arginine*	*S schleiferi*
St. sanguinis	*St salivarius*	*M arthritiditis*	*St mitis*
S epidermidis	*St. agalactiae*	*M genitalium*	*C albicans*
N. flava	*S enterica*	*M hyorhinis*	*M fermentas*
N. perflava	*C jejuni*	*M orale*	*St pneumoniae*
S. saprophyticus	*St thermophilus*	*M pirum*	*C difficile*
Mycobacterium spp.	*S. flexineri*	*M salivarium*	*C sordelli*
C. glabrata	*B cereus*	*St mutans*	
S. lugdunensis	*B pertussis*	*B subtilis*	
C. kefyr	*E coli*	*C perfringens*	
C tropicalis	*H pylori*	*S hominis*	

Clinical Studies



Colour	Target	Ct value
Blue	orfX/SCCmec	22.76
Green	Extraction control	21.25
Red	Methicillin resistance	21.60
Purple	Staphylococcus specific	21.42

Figure 3: Typical real time traces obtained using the EasyScreen™ MRSA Detection Kit.

Table 3: Results obtained when testing the Qnostics MRSA proficiency panel

Qnostics MRSA proficiency panel				
Sample#	Content	cfu	orfX/SCC	nuc
1	MSSA	$2x10^1$	Negative	Positive
2	MSSA	20	Negative	Negative
3	MRCoNS	$2x10^1$	Negative	Negative
4	MRCoNS+MSSA	20+100	Negative	Positive
5	E. coli	$2x10^1$	Negative	Negative
6	Negative	0	Negative	Negative
7	MSSA	TBD	Negative	Positive
8	MRCoNS	TBD	Negative	Negative
9	MRCoNS	TBD	Negative	Negative
10	MRSA	$2x10^1$	Positive	Positive
11	MRSA	$2x10^4$	Positive	Positive
12	MRSA	20	Positive	Positive
13	MRSA	20	Positive	Positive
14	MRSA	2	Negative	Negative

Table 4: Results obtained when testing reference strains and clinical samples

Sample type tested	Results	Comments
MRSA Isolates (n=39)	39/39 PCR positive	
MRSA Reference Strains (n=18)	18/18 PCR positive	
MRSA positive patient samples (n=36)	36/36 PCR positive	
MRSA negative patient samples (n=36)	35/36 PCR negative	1 discrepant result
Screening samples (n=183)	21/183 PCR positive	11.5% positivity rate, awaiting conformation

Discussion

Although no *nuc*/*mec*A deficient samples were detected in this small pilot study the inclusion of both *fem*A/*mecALGA251* will safeguard against possible false negative reactions. A number of SCCmec/*nuc* positive, *mec*A negative samples, were detected in the routine screening assay demonstrating the importance of the inclusion of the *mec*A/*mecALGA251* markers for the stratification of MRSA samples. The assay can be fully automated using the GS1 platform reducing hands on time to minutes. Alternatively the assay can be run on common laboratory equipment e.g KingFisher Flex (Thermo), MagNA™ Pure (Roche), EasyMag (bioMerieux) Qiasymphony and EZ1 workstations (Qiagen).

References

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