Real-time PCR detection of MRSA

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The Spartan DX™ can be used for on-demand, non-batched testing of methicillin-resistant Staphylococcus aureus.

Introduction

MRSA strains are resistant to a wide range of antibiotics. The mortality rate for patients with MRSA infection is 2-2.5 times higher than for methicillin-susceptible *S. aureus* (MSSA) (Ref 1). In terms of economic costs, patients with MRSA had a 1.5-fold longer length of stay (19.1 vs. 14.2 days) and a 2-fold increased cost of hospitalization ($21,577 vs. $11,668) (Ref 2).

Infection control relies on rapid and sensitive tests for the detection of MRSA carriers; however, standard culture methods are labor-intensive and require 48 h, to produce a result. DNA-based tests can faster quick results by detecting an *S. aureus*-specific gene and the *mecA* gene, which encodes methicillin resistance (Ref 1). Manufacturers have developed DNA-based tests for batched systems such as the 16-well SmartCycler® (Cepheid) and the 32- or 384-well LightCycler® (Roche).

The 4-well Spartan DX™ is designed for non-batched applications.

The purpose of this study was to develop a real-time PCR assay for MRSA on the Spartan DX.

Materials and Methods

Characterization of clinical isolates

All isolates tested were recovered from blood cultures. *S. aureus* was identified based on positive slide agglutination (Pastorex®, Bio-Rad) and tube agglutination (Rabbit coagulase plasma, VWR). Methicillin resistance was identified by growth on oxacillin salt agar screen plate (NCCLS M100-S17, M7), and PBP2 testing. Coagulase-negative *Staphylococci* were identified based on negative slide agglutination test and oxacillin sensitivity using the Vitek II system from bioMerieux.

In this way, clinical isolates of *Staphylococci*, from 52 different patients, were characterized into four phenotypes: (1) MRSA, (2) MSSA, (3) Methicillin-resistant Coagulase-negative *Staphylococci* (MR-CNS), and (4) Methicillin-sensitive CNS (MS-CNS).

DNA extraction

Crude DNA was extracted from the 52 *Staphylococci* isolates by resuspending 4 medium-sized colonies in 100 µl of lysis buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, pH 8) with 2 µl of 1 mg/ml lysostaphin (Sigma-Aldrich, Cat No. l7386). The samples were incubated at 37°C for 15 min. Following this incubation, 5 µl of 20 mg/ml Proteinase K (Sigma-Aldrich, Cat. No. p2308) were added to the mixture and the sample was shaken at 50°C for 15 min. The samples were then boiled for 10 min to inactivate the Proteinase K. Extracted DNA was stored at 4°C for up to 2 weeks.

Real-time PCR

Oligonucleotide primers were designed against the *mecA* gene which codes for methicillin and oxacillin resistance, the *nuc* gene which codes for a thermostable nuclease

Primer (Ref)	Forward (5'-3')	Reverse (5'-3')	Amplicon size (bp)
mecA	tccaggaatg-cagaaagaccaa	ggccaattcca-cattgtttcg	92
nuc	tcgtcaaggcttg-gctaaagt	cagcgttgtcttc-gctccaac	125
16S	cgaaagcgt-ggggatcaaac	cccaggcggagt-gcttaatg	125

Table 1. Primer sequences and amplicon sizes.

Reaction Component	Final Amount
10X PCR Reaction Buffer (No $MgCl_2$) (Invitrogen)	1X
$MgCl_2$ (Invitrogen)	2.5 µM
dNTP mix (Invitrogen)	0.125 mM
Taq DNA polymerase (Biotools)	1 U
SYBR Green I (Invitrogen)	0.5 X
PCR primers (Sigma-Aldrich)	1 µM each
Template DNA	2 µl
Sterile water	
Total reaction volume	**20 µl**

Table 2. Components of PCR amplification mixture.

Step	Temperature	Time	Cycles
Initial denaturation	97.6°C	30 s	1
Denaturation	97.6°C	30 s	30
Annealing/extension	56.9°C	25 s	30

Table 3. Cycling parameters.

specific to *S. aureus*, and the *16S rRNA* gene which codes for 16S ribosomal RNA and is highly conserved across bacterial species. The primers were designed with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were optimized to increase their melting temperatures using online software (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer). The presence or absence of these genes enables determination of genotype, such that MRSA should be positive for *mecA*, *nuc* and *16S,* MSSA should be positive for *nuc* and *16S*, MS-CNS should be positive for *16S,* and MR-CNS should be positive for *mecA* and *16S.*

Genotype	# Analyzed	# Concordant
MRSA	17	17
MSSA	15	14
MR-CNS	14	14
MS-CNS	6	6
Total	**52**	**51**

Table 4. Concordance of phenotypes and results.

Table 1 shows primer sequences and expected amplicon sizes. Components of the PCR amplification mixture are listed in Table 2 and cycling parameters are listed in Table 3. Note that a two-temperature cycling program was performed by combining the annealing and extension steps. Reactions were performed using the Spartan DX and 0.2 ml thin-wall flat cap PCR tubes (Axygen, Cat. No. PCR 02C), and topped with 15 µl of mineral oil (Biotools, Cat. No. 20.032)

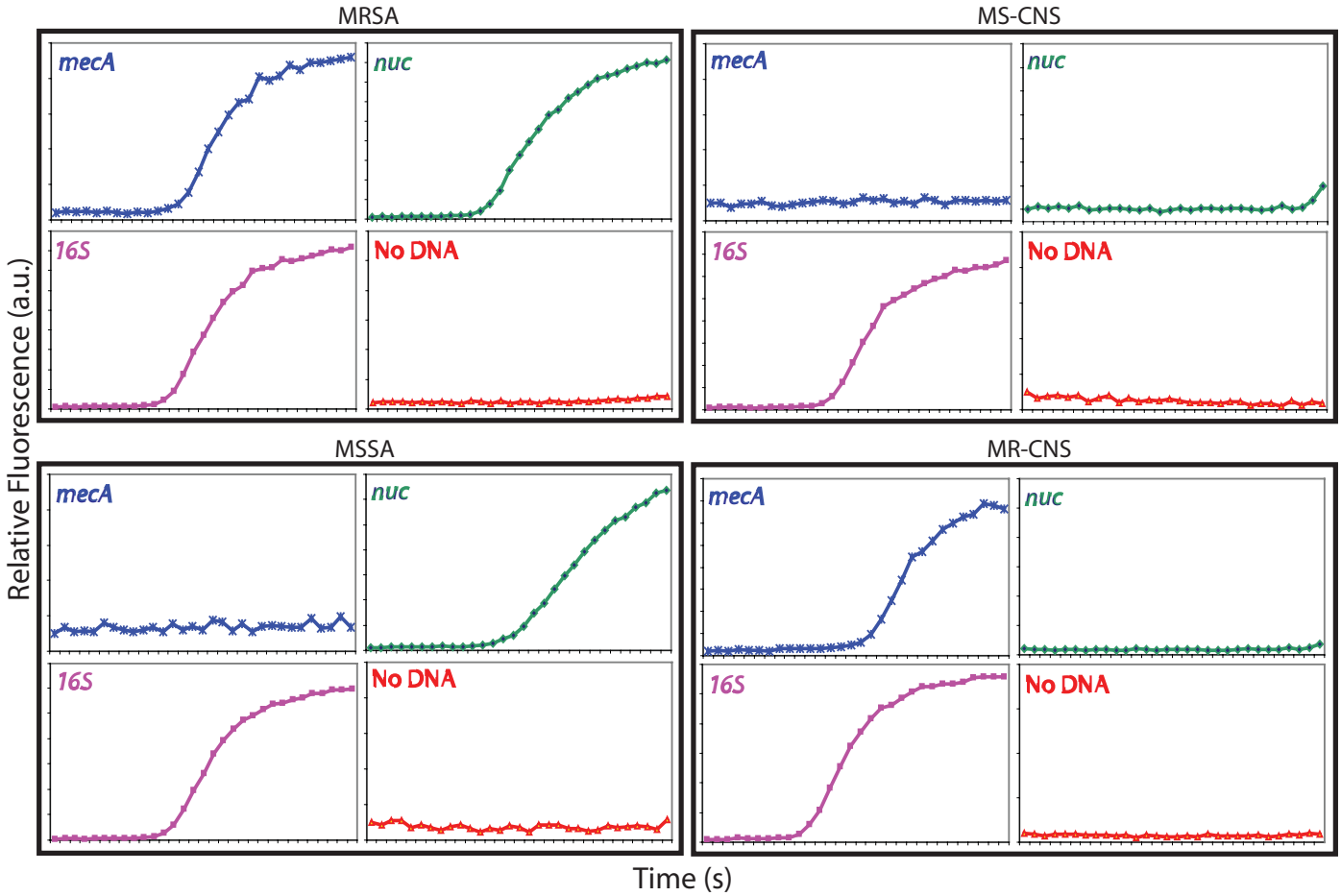

Figure 1. Example of real-time PCR output for 4 different genotypes: Methicillin resistant *Staphyloccus aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA), methicillin-resistant coagulase-negative *Staphylococci* (MR-CNS) and methicillin sensitive-coagulase negative *Staphylococci* (MS-CNS). SYBR Green I fluorescence was used to visualize formation of the amplicons.

	mecA	nuc	16S
Genotype	Average Ct	Average Ct	Average Ct
MRSA	13 ± 1	12 ± 2	10 ± 2
MSSA	32 ± 2	14 ± 3	11 ± 3
MR-CNS	14 ± 2	28 ±1	12 ± 2
MS-CNS	32 ± 4	27	12

Table 5. Threshold cycle (Ct) values

to prevent evaporation. SYBR Green® I dye (Invitrogen, Cat. No. S-7563) was used for fluorescent detection. Negative controls consisted of reactions mix components with the exception of DNA template.

DNA analysis

Fluorescence data was downloaded from the Spartan DX to a computer and graphed using Microsoft Excel®. Real-time results were confirmed by gel electrophoresis.

Results

Negative controls with *mecA, nuc,* and *16S* primers had average threshold cycle (Ct) values of 35, 34 and 27 respectively. Table 4 compares the real-time PCR results with the original phenotypic characterizations.

Figure 1 shows representative real-time PCR graphs and Table 5 shows average Ct values for the MRSA, MSSA, MR-CNS, and MS-CNS genotypes.

Discussion and Conclusions

For the real-time PCR results, it was determined that positive samples had Ct values that were within three standard deviations of the average Ct value. Therefore, a positive result was defined as a threshold Ct value that was ≤19 for *mecA*, ≤19 for *nuc*, and ≤17 for *16S*. Using these threshold values, genotypic results were concordant with phenotypic characterizations for 51 out of 52 samples.

The single discordant sample was characterized as an MSSA phenotype but was negative for *nuc* by real-time PCR. This sample tested positive for nuc when DNA was re-purified from the original bacterial colonies, which suggested that the problem was purification-related human error.

For the *16S* controls, the average Ct value was lower than for the *mecA* and *nuc* primers. The reason may have been the presence of *16S rRNA* gene fragments in the recombinant Taq DNA polymerase because it was cloned and purified from *E. coli*. This phenomenon did not affect interpretation of the results.

In conclusion, a real-time PCR assay for MRSA was successfully developed for the Spartan DX. This assay may be particularly useful for situations where on-demand, non-batched results are desired.

References

1. Huletsky A et al. (2005). Identification of methicillin-resistant *Staphylococcus aureus* carriage in less than 1 hour during a hospital surveillance program. *Clin Infect Dis.* 40:976-981.

2. Lodise TP, McKinnon PS. (2005). Clinical and economic impact of methicillin resistance in patients with *Staphylococcus aureus* bacteremia. *Diag Micro Infect Dis.* 52:113-122.

3. Kloos WE, Bannerman TL. (1999). "*Staphylococcus* and *micrococcus*". In PR Murray et al. (Eds.). *Manual of Clinical Microbiology, 7th ed.* (pp 264–282). Washington, DC: ASM Press.