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DETECTION OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) AND REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

BY

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ABSTRACT

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Title: Detection of Methicillin-Resistant Staphylococcus aureus (MRSA) Using Loop-Mediated Isothermal Amplification (LAMP)

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Staphylococcus aureus is one of the most common pathogens that cause a wide range of infections ranging from skin and soft tissue infections to invasive, life-threatening infections. The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) substantially increased healthcare burdens associated with *Staphylococcal* infections because of high morbidity and mortality and increasing the need for efficient and cost-effective screening methods, for high-risk patients. The objective of this study is to develop two molecular methods, real-time PCR and loop-mediated isothermal amplification (LAMP), and validate them following Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologists (CAP) standards. The real-time PCR assay was developed targeting *mecA*, *mecC*, *nuc*, and *coa* to detect *S. aureus* and methicillin-resistance. The assay had high precision, a linear range of 10^4-10^8 CFU/ml, and 95% accuracy. The assay detects MRSA, MSSA, MR-CoNS, and MS-CoNS. The LAMP assay was developed targeting the same genes; however, its lower limit of detection was 10^6 CFU/ml, which was much higher than that of the real-time PCR assay. Additional studies are required to optimize the performance characteristics of the LAMP assay further. Nevertheless, the real-time PCR assay developed in this study will be useful for the detection of MRSA in a cost-effective manner.

DEDICATION

This work is dedicated to my mother, who showed me the value of hard work and education.

Without her, I would not be who I am.

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Chapter 1: Introduction

The Clinical Significance of *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive bacterial species that is a common part of the normal human flora. It is a commensal colonizer of the axillae, nares, pharynx, vagina, rectum, perineum and damaged skin [1, 2]. Depending on bacterial virulence and host factors, this colonization may turn into an infection and cause various complications, including skin and soft tissue infections (SSTIs), pneumonia, surgical site and medical implant infections, and bacteremia. *S. aureus* is the most commonly isolated pathogen in SSTIs, accounting for up to 50% of isolates [3]. It is increasingly problematic in neonatal intensive care units (NICUs) worldwide, due to the vulnerability of preterm and critically ill neonates and the ability of *S. aureus* to survive on environmental surfaces for long periods [4]. Another particularly vulnerable subset of patients is those with cystic fibrosis (CF). *S. aureus* is the third most prevalent bacterial isolate in CF patients in the United States of America (USA) and is increasing in other countries [5]. The emergence of antimicrobial resistance (AMR), particularly methicillin-resistant *S. aureus* (MRSA), has magnified the health care burden associated with *S. aureus* infections. MRSA was found to result in substantially higher morbidity and mortality than methicillin-sensitive *S. aureus* (MSSA) and increased health care costs [6-8].

Methicillin-Resistant *S. aureus* (MRSA)

MRSA was first isolated shortly after the introduction of the antibiotic methicillin in 1961 in the United Kingdom (UK) [9-12]. A few years later, more isolates were identified in Canada and followed by an outbreak a little more than a decade after their initial emergence [12]. Up until the mid-1980s, MRSA was a healthcare-acquired pathogen, meaning that infections occur in a hospital setting only. Eventually, however,

cases of young patients, with no history of hospitalization, with MRSA infections started appearing in indigenous communities in Canada and Australia [11]. Therefore, MRSA isolates have been classified as either hospital-acquired (HA-MRSA) or community-acquired (CA-MRSA). The two types can differ in their AMR patterns and genotypes. For example, methicillin resistance in HA-MRSA and CA-MRSA develops through different mobile genetic elements [13-15]. However, as MRSA strains have become more widespread in the community, the distinction between HA-MRSA and CA-MRSA has become less useful.

Nasal and skin MRSA colonization are a significant risk factor for SSTIs both in the hospitals and in the community [3]. The health care burden of MRSA has been progressively increasing in the past years, leading to increases in morbidity, mortality, and health care costs [3]. By 2005, mortality from MRSA in the US exceeded that of *Salmonella*, tuberculosis, influenza, and HIV combined [16, 17]. Proper identification and control measures for MRSA must be implemented to prevent increases in carriage and infection rates, as is the case with northern Saskatchewan, Canada, where the incidence of CA-MRSA increased from 8.2 cases per 10,000 in 2001 to 168.1 per 10,000 in 2006 [18]. In 2015, MRSA accounted for 20-30% of SSTIs in Europe and Latin America and up to 35% in North America [3]. Significant efforts have been made in the reduction of mortality due to MRSA, with much of this effort driven by the early identification of carriers of MRSA to prevent its spread in health care settings and rapidly identify *S. aureus* isolates that are MRSA.

MRSA Surveillance

Successful efforts in the prevention of MRSA and subsequent reduction in mortality focused on active surveillance programs (ASP) [19]. Most MRSA carriers are asymptomatic and thus would not typically be subjected to bacterial cultures. It is

estimated that, in the absence of ASP, routine cultures only identify 18% of MRSA carriers [20]. These individuals, while asymptomatic, can develop infections later or spread MRSA to others. It is estimated that 25% of carriers in intensive care units (ICUs) develop MRSA infections [20]. Thus, ASPs are necessary to prevent and control MRSA spread. ASPs may include screening all patients for MRSA during general admission at the hospital or screening specific risk groups. Patient risk groups include pre-operative, immunosuppressed, ICU and NICU patients [3]. Contact precautions are used for health care providers and visitors to patients who may carry MRSA to prevent the spread of the organism in hospital settings. In some situations, the successful decolonization of patients with MRSA may reduce the chance of MRSA infections. A clinical trial investigating the impact of *S. aureus* surveillance and pre-operative decolonization with joint replacement surgeries found that decolonization of pre-operative patients reduced surgical site infections by 50% [21].

Furthermore, a prospective, controlled, interventional cohort-study of 33 surgical wards in 10 hospitals across Europe and Israel demonstrated that active surveillance, contact precautions, and decolonization are significantly associated with reduced risk of MRSA infections [22]. However, decolonization of patients remains a controversial issue. Despite stringent decolonization measures, patients can remain positive or become re-colonized upon readmission to the hospital [23]. In other cases, poor sample collection techniques can produce false-negative results. Successful ASPs depend on the methods used to screen for MRSA. These range from various culture methods to molecular methods. When choosing a screening method, the laboratory must consider sensitivity, specificity, turn-around time, labor and cost.

Screening for MRSA is usually performed on swabs from nares or a combination of swabs from nares, throat, axilla, rectal, and perirectal areas [24, 25].

When testing one site only, the nares swabs are typically used as they are the most common site of colonization. However, testing nares swabs alone can miss up to 35% of patients colonized with MRSA [19]. In the past, the swabs were cultured on non-selective media (blood agar and chocolate agar) or selective media (mannitol salt agar). More recently, chromogenic agar and molecular testing have become more widely available [24, 25]. Most laboratories choose between chromogenic media and real-time polymerase chain reaction (PCR), as both can produce results within 24 hours [26]. Chromogenic media has a sensitivity of about 80% at 25 hours, and it must be incubated longer, up to 48 hours, for the sensitivity to approach 100% [26]. As for molecular assays, the first commercial assay for *S. aureus* was the "Gen-Probe Accuprobe *Staphylococcus aureus* Culture Identification Test" for positive blood cultures in the 1990s [27]. The assay uses a chemiluminescent DNA probe for the *S. aureus*-specific 16S rRNA gene and provides results in less than an hour, but the assay is designed for use on clinical isolates and not on clinical specimen [27, 28]. Later, molecular testing moved towards commercial and in-house developed PCR assays targeting genes unique to *S. aureus,* such as *femA, nuc* or *spa* [29]. The further differentiation of MSSA from MRSA is possible by incorporating primers and probes for the *mecA* gene, which codes for the methicillin resistance [30]. Some coagulase-negative staphylococcal (CoNS) species also carry the *mecA* gene, so *mecA* PCR must be performed only on confirmed *S. aureus* isolates or in conjunction with PCR targeting *S. aureus*-specific genes.

Methicillin-Resistance and its Detection

The *mecA* gene codes for a penicillin-binding protein (PBP2a) homolog that has less affinity to β-lactam antibiotics [5, 31]. β-lactams work by binding the penicillin-binding proteins and inhibiting peptidoglycan cross-linking, resulting in cell lysis. The PBP2a encoded by the *mecA* gene confers β-lactam resistance to MRSA by decreasing

its affinity to β-lactam antibiotics [32]. MRSA evolved from MSSA after it acquired the SCC*mec* element, a 21-67kbp mobile staphylococcal genetic element that carries the *mecA* gene, site-specific recombinase genes (*ccr* complexes) and a joining region [31, 33]. SCC*mec* is located near the origin of replication of the staphylococcal chromosome (*orfX*) [31]. The position of SCC*mec* relative to *orfX* provides a specific target site for PCR amplification for MRSA. Eleven types of SCC*mec* have been identified in staphylococcal species, differing in the *mec* gene and *ccr* complexes [31] The SCC types I, II and III give methicillin resistance in HA-MRSA, while CA-MRSA gains its resistance through SCC*mec* types IV and V [34]. The SCC*mec* types in HA-MRSA are generally larger than CA-MRSA and confer resistance to more antibiotics. HA-MRSA, for instance, is resistant to clindamycin, while CA-MRSA is susceptible [34]. The varying resistance profiles can be attributed to the *ccr* region, which may harbor multiple antibiotic resistance genes [31]. In 2007, a new variant of *S. aureus* was found in the UK, which was phenotypically determined to be MRSA, but confirmatory tests were negative for *mecA* [32]. Sequencing found that the gene conferring the resistance had only ~69% nucleotide identity to *mecA,* and the encoded PBP (PBP2c) was only ~63% identical at the amino acid level [32]. This new gene was termed *mecC,* and a new type of SCC*mec* was identified. Due to the low homology between *mecA* and *mecC,* molecular assays targeting *mecA* can miss many *mecC* MRSA.

The SCC*mec* element is not unique to MRSA and can be found in coagulase-negative staphylococcal species. Molecular screening requires a robust method to specifically detect MRSA rather than detecting methicillin-resistance in other staphylococcal species. Also, patients can be colonized with multiple species of organisms. While the gold standard for MRSA identification is by culture, molecular methods offer more rapid results. Real-time PCR is the most common method used,

and multiple commercial assays are available. A literature search of real-time PCR for MRSA also reveals a plethora of in-house developed assays. What these, as well as commercial assays, have in common is the algorithm used in interpreting the results of a multiplex PCR. The assays target a *S. aureus*-specific gene, and the *mecA* gene or a portion of SCC*mec*. Some newer assays also incorporate the *mecC* gene. Hirvonen (2014) reviewed multiple molecular methods for the detection and identification of MRSA. The review included a comparison of multiple nucleic acid-based tests, both in-house and commercial, and non-nucleic acid-based tests [35]. Hirvonen compared the assays in terms of sensitivity, specificity, and turn-around time and run-time. Turn-around time is the time taken from specimen receipt to result reporting, while run-time is the time taken from starting the test to completion. Chromogenic agar had a turn-around time of 16-48 hours, sensitivity ranging from 44.1-100%, and specificity of 97.2-100%.

Other non-nucleic acid-based assays included latex agglutination, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), and biochemical identification and susceptibility testing by Vitek 2. The run-time for these assays ranged between 3 minutes to 12 hours. However, all of them require bacteriologic culture and organism isolation first, adding at least 16 hours to the total turn-around time [35]. For nucleic acid-based assays, Hirvonen compared multiple in-house and commercial real-time PCR assays, including GeneOhm MRSA (Becton Dickson), Xpert MRSA and Xpert MRSA/SA (Cepheid Diagnostics), BD MAX MRSA (Becton Dickson), Light Cycler MRSA (Roche Diagnostics), in-house developed assays and a LAMP assay. The sensitivity from clinical specimens ranged from 69.2-100% and specificity from 64.5-100%. The turn-around time ranged from 1 hour to 3 hours [35]. Even when including nucleic acid extraction before the assay, the turn-around time is significantly shorter

than non-nucleic acid-based assays. Particularly interesting is the LAMP assay (loop-mediated isothermal amplification), which showed the shortest run-time (1 hour), high specificity (100%) and sensitivity ranging from 91.3-98.4%. When considering molecular assays, the biggest hurdle tends to be the cost. Real-time PCR instruments are expensive due to the complex components required for thermal cycling and fluorescence detection. LAMP, however, is isothermal, thus removing the need for such sophisticated machinery, requiring only the maintenance of constant temperature. LAMP was first described by Notomi *et al*. (2000) as a novel method for nucleic acid amplification. The method is capable of amplifying DNA from a few copies to 10^9 in less than an hour [36]. Unlike PCR, LAMP is performed under isothermal conditions and without requiring a thermal cycler. Furthermore, LAMP is more specific than other isothermal nucleic acid amplification methods, such as nucleic acid sequence-based amplification (NASBA) and self-sustained sequence replication (3SR), and does not require the use of expensive modified nucleotides as in strand displacement amplification (SDA). Furthermore, LAMP is not significantly affected by the presence of non-target DNA [36].

Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) was developed by Kary Mullis in 1983 and marked a significant milestone in the field of molecular biology [37]. PCR is a powerful technique that has become one of the most widely used techniques in molecular biology [38, 39].

The technique is based on the cellular DNA replication mechanism. In a PCR reaction, a DNA molecule is replicated, and each product is replicated again in another cycle, resulting in exponential amplification of the DNA molecule [39]. A typical PCR reaction includes a DNA polymerase, deoxyribonucleotides (dNTPs, adenine, thymine,

guanine, and cytosine), and primers [39].

The reaction is dependent on thermal cycling, in which each cycle consists of three steps, denaturation, annealing, and extension, at varying temperatures. The double-stranded DNA is denatured at temperatures around 90-97°C separating it into single strands, each acting as a template for the following steps [39]. Then, the primers anneal to strands to prime extension at a lower temperature (50-60°C) to allow the primers to anneal. Finally, the temperature is increased to the optimum temperature for the polymerase for DNA synthesis to occur (Figure 1). These three steps constitute a cycle. The reaction can be performed for multiple cycles to increase the amount of product DNA [39]. The final products of the reaction can be analyzed by agarose gel electrophoresis, where the amplicons can be visualized as distinct bands of the expected size (Figure 2).

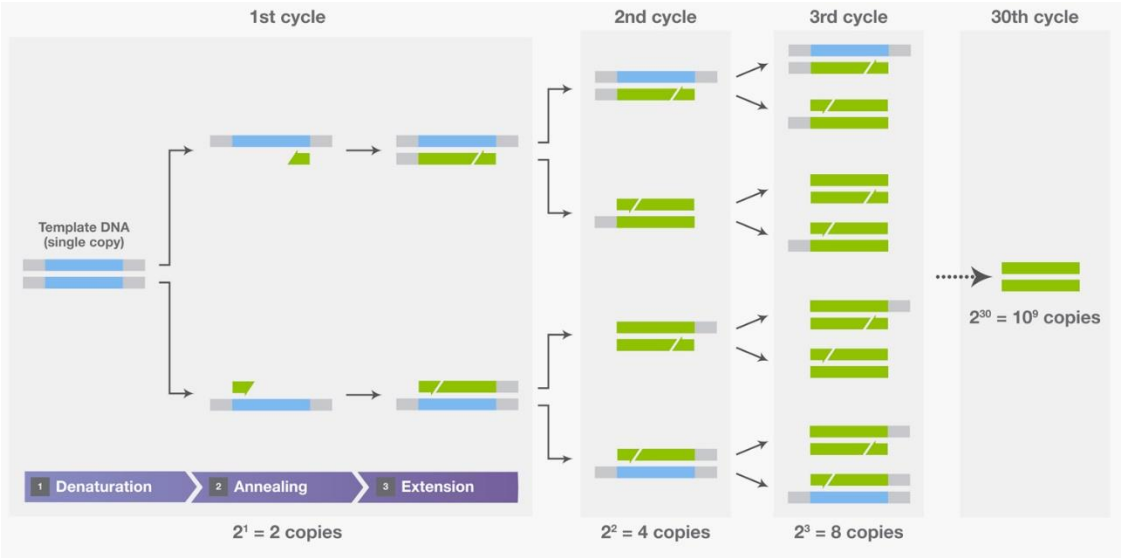


Figure 1. PCR reaction schematic

(*Source: Thermo Fisher Scientific Website*).

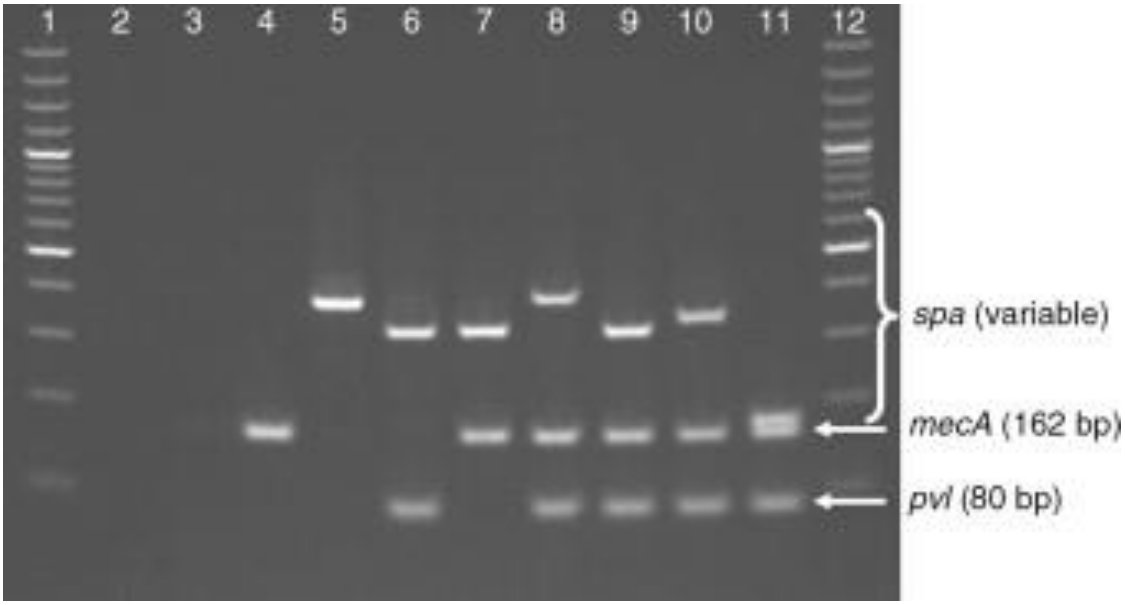


Figure 2. PCR Product Gel Electrophoresis

(*Source: Larsen et al. 2008 [40]*).

During the extension step, the DNA polymerase binds to the annealed primer and synthesizes complementary DNA using the available dNTPs. The polymerase most commonly used in PCR reactions is Taq polymerase or one of its variants [39]. It is a thermostable DNA polymerase, isolated from *Thermus aquaticus,* that can withstand the high temperatures used in the PCR reaction [41]. Other polymerases have also been developed and vary in their properties to suit their intended applications [41]. When choosing a polymerase, some considerations are its specificity, thermostability, fidelity, and processivity. As with the type of polymerase used, there are other reagent considerations to be taken into account when performing a PCR reaction. Primers are designed to be specific to the region intended to be amplified, and thus their sequence must be complementary to the region but not to others.

Additionally, two primers must be designed, each complementary to one strand of the DNA. However, with the varying sequences of primers needed for different targets, there are important properties to be noted to ensure a successful reaction,

including the primer melting temperatures, GC-content, secondary structure formation, and dimerization. The tools and software to perform all these analyses *in silico* are widely available with both free-to-use and commercial options [42].

Almost a decade after PCR was first described, the concept of monitoring amplification in real-time was introduced [43]. Real-time PCR follows the same principle as traditional PCR with the addition of fluorescent molecules to monitor the amplification reaction. In real-time PCR, detection can be done with two chemistries, DNA binding dyes or fluorophore-labeled oligonucleotides [44]. DNA binding dyes bind to double-stranded DNA, resulting in a complex that absorbs light and subsequently fluoresces [44]. The most commonly used DNA binding dye is SYBR® Green I [44]. Fluorophore-labeled oligonucleotides are segments of DNA bound to a fluorescent molecule. They can be divided into multiple groups based on their structures and mechanism of fluorescence; these are hairpin primer-probes, hydrolysis probes, and hybridization probes [44]. One such oligonucleotide is the so-called Taqman probe. Taqman probes are segments of DNA complementary to a region in the target DNA between the two primer binding sites and has a fluorophore and quencher on each end [45]. In solution, the intact probe does not fluoresce because the quencher absorbs the energy released from the fluorophore due to proximity. However, during the extension step, the 5′-3′ exonuclease activity of the DNA polymerase excises the probe and releases the quencher and fluorophore. The excision enables the fluorophore to emit a signal that can be detected (Figure 3). With both DNA binding dyes and fluorophore-labeled oligonucleotides, the amount of fluorescence is proportional to the amount of DNA, which enables both relative and absolute DNA quantification.

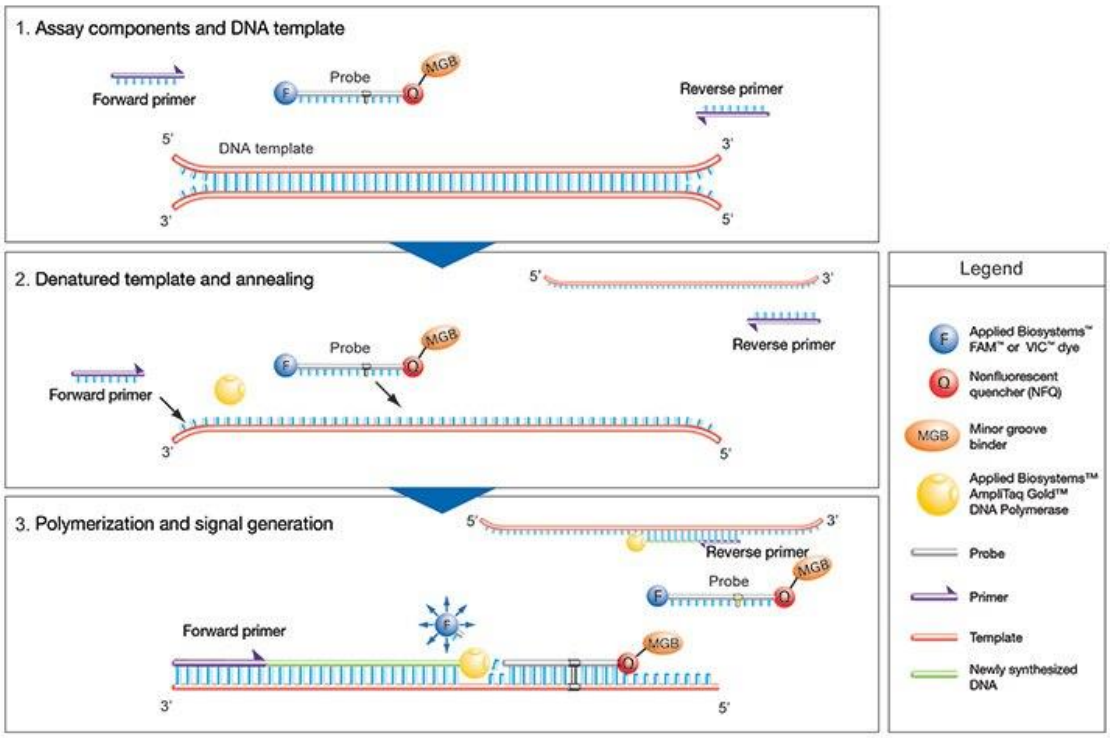


Figure 3. TaqMan Probe

(*Source: Thermofischer Scientific Website*).

Loop-Mediated Isothermal Amplification (LAMP)

The LAMP method relies on auto-cycling strand displacement DNA synthesis. The reaction is performed using a DNA polymerase with high strand displacement activity aided by sets of two inner and two outer primers. *Bst* DNA polymerase, derived from *Bacillus stearomophilus*, is frequently used in LAMP reactions. The enzyme has optimum activity at 60-65°C and high concentrations of magnesium. It can be inactivated by incubating for 15 minutes at 80°C [41, 46]. LAMP primers are designed based on six regions within the target. Two 23-34 nucleotide long DNA sequences inside both ends of the target, designated F2c and B2; two 40 nucleotide long DNA sequences from the inner ends of F2c and B2, designated F1c and B1; and two sequences outside the ends of F2c and B2, designated F3c and B3 (Figure 4) [36]. The sequences of the primers termed Forward Inner Primer (FIP) and Backwards Inner

Primer (BIP) for the inner primers and F3 and B3 for the outer primers, are based on the designated sequences on the target. FIP contains the F1c sequence (complementary to F1), a polyT spacer, and the F2 sequence (complementary to F2c). Similarly, BIP contains B1, a polyT spacer, and B2c. The two outer primers contain the sequences F3 and B3 for the forward and backward primers, respectively. A mixture of the target and four primers are heat denatured at 65°C and rapidly cooled on ice, then mixed with the DNA polymerase, and the reaction proceeds for 1 hour at 65°C [36].

Figure 4. LAMP target DNA structure

(*Source: Eiken Chemical Co. Ltd. website*).

The LAMP reaction mechanism proceeds in three stages: 1) production of a stem-loop structure, 2) cycling amplification, 3) elongation, and recycling [36]. The reaction starts when FIP displaces the DNA strands, and the F2 sequence anneals to its complementary sequence. A DNA polymerase with high strand displacement activity then extends the primer while displacing the strands, starting with the 3′ end of the F2 segment. The result is a strand of DNA complementary to the target and linked to FIP. Then, the outer primer (F3) anneals to its complementary region, upstream of F2. Extension of this primer displaces the FIP-linked strand. The displaced FIP-linked strand forms a stem-loop structure due to the reverse complementarity of the F1c region and a region within the target. This stem-loop structure serves as a template for BIP and B3 elongation in a similar reaction as outlined for FIP. The product is a DNA segment

with a stem-loop at each end, formed by F1 and F1c on one side, and B1 and B1c on the other. This dumbbell-shaped structure serves as the base for the subsequent cycling amplification. The structure contains multiple sites for primer annealing and DNA synthesis. Amplification occurs through auto-cycling, resulting in continuous strand displacements and exponential amplification of the target (Figure 5) [36, 47].

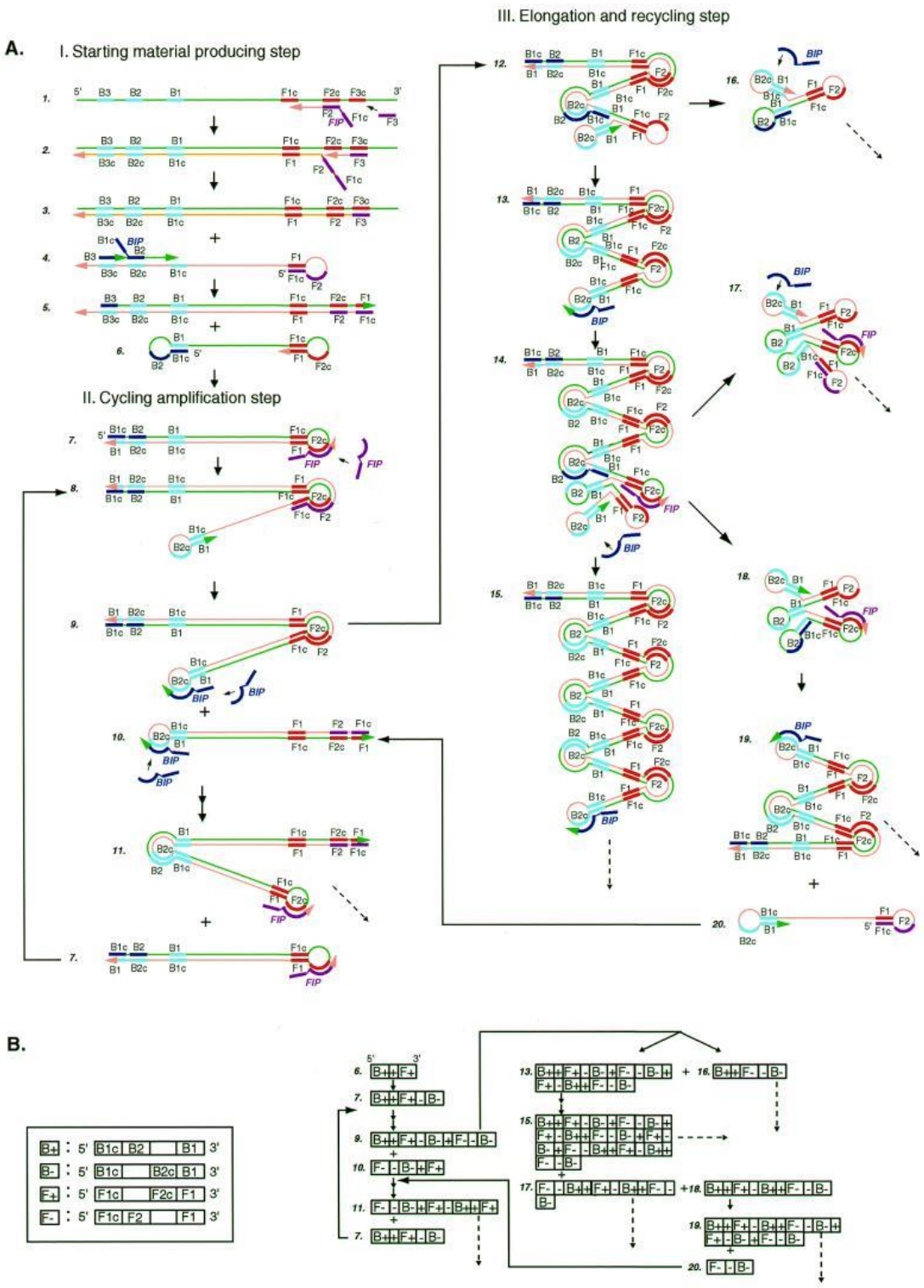


Figure 5. LAMP mechanism outline

(*Source: Notomi et al. 2000*).

In addition to F3, B3, FIP, and BIP, specific primers targeting the loop regions of the amplified products (Termed Loop Primers, LF, and LB) can result in a reduction of the time to amplification by half or two-thirds [48]. These loop primers correspond to two sequences within the target. The forward loop primer (LF) corresponds to the region between F1 and F2, and the backward loop primer (LB) corresponds to the region between B1 and B2.

LAMP Primer Design

When designing LAMP primers, several factors need to be considered. First, the meting-temperature (Tm) of the F2 and B2 sequences in the FIP and BIP primers have to be within the optimal range of the polymerase [36]. The Tm for F1c and B1c (the other sequences of FIP and BIP) need to be slightly higher, however, so that the loop structure can form immediately after the denaturation of the target DNA. Finally, the Tm for F3 and B3 (the outer primers) needs to be lower than F2 and B2, to ensure that the inner primers (FIP and BIP) anneal and synthesis from them starts first [36].

Another factor in LAMP primer design is the length of the FIP and BIP sequences as it affects the size of the stem-loop structure, the formation of which is essential for the amplification reaction. Notomi *et al*. (2000) experimented with multiple sizes and found a length of 40 bases or longer gave the best amplification efficiency [36]. Additionally, the distance between the F2 and B2 and between F2 and F3 should be 120-180bp and 0-20bp respectively. Furthermore, the stability of binding at the 3′ end of the primer is essential for the reaction to proceed, as it is the starting point DNA synthesis. LAMP is an isothermal reaction that depends on auto-cycling. Thus, annealing of the primers must generate a negative change in free energy (ΔG) to promote the auto-cycling, specifically at the 3′ end, where DNA synthesis proceeds

[49]. Similarly, the GC content of the primer should be between 40-65% to ensure the stability of primer binding, and secondary structures should be minimized.

Objectives

The overarching aim of this study is to develop a cost-effective method for the detection of MRSA from clinical samples for use in the Molecular Infectious Diseases Laboratory at Sidra Medicine. To that end, our goal is to develop and validate in-house real-time PCR and LAMP assays for the detection of MRSA from patient specimens with high sensitivity and specificity, short turn-around time, and low cost. The in-house developed LAMP assay will be compared to both culture-based testing and the in-house developed real-time PCR assay targeting the same genes. The sensitivity, specificity, accuracy, precision, robustness, specimen preparation time, assay run-time, and cost per specimen will be compared using clinical specimens sent to the microbiology laboratory for MRSA screening by culture (N= 200). The accuracy of the MRSA PCR results will be assessed based on the agreement with microbiology culture results. Successful completion of the project will result in two in-house developed, cost-effective molecular assays for the rapid screening of MRSA. Ultimately, the better assay will be adopted as an alternative method for MRSA detection at the Molecular Infectious Diseases Laboratory of Sidra Medicine. The goal of the study will be achieved through the following specific objectives:

- **Specific objective 1:** Develop an in-house real-time PCR assay for MRSA detection.
    - *Sub-objective 1.1: design primers and probes targeting S. aureus and methicillin-resistance.*
    - *Sub-objective 1.2: Design plasmids for custom synthesis (commercial) to serve as a) positive controls for different PCR assays b) to optimize*

assay conditions and c) to determine analytical performance characteristics of different PCR assays.

- *Sub-objective 1.3: optimize assay conditions (annealing temperature and reagent concentration).*
- *Sub-objective 1.4: determine the performance characteristics of the assay (precision, analytical sensitivity, analytical specificity, and linearity).*

- **Specific objective 2:** Develop an in-house LAMP assay for MRSA detection.
  - *Sub-objective 2.1: design primers for S. aureus and methicillin-resistance.*
  - *Sub-objective 2.2: optimize assay conditions (reaction temperature, reaction time, and reagent concentrations).*
  - *Sub-objective 2.3: evaluate and compare the analytical performance characteristics of in-house real-time PCR assay and in-house LAMP assay.*
  - *Sub-objective 2.4: determine the performance characteristics of the assay (precision, analytical sensitivity, analytical specificity, and linearity).*

- **Specific objective 3:** Clinically validate both the in-house developed MRSA molecular assays.
  - *Sub-objective 3.1: DNA extraction from retrospective, residual clinical samples.*

- *Sub-objective 3.2: test the PCR assay on the patient specimens to assess the clinical sensitivity and specificity.*
- *Sub-objective 3.3: test the LAMP assay on the patient specimens to assess the clinical sensitivity and specificity.*

Chapter 2: Literature Review

MRSA Detection by PCR

The literature on the detection of MRSA by real-time PCR is extensive. Many studies explored gene targets, chemistries, and platforms. The utility of PCR assay is substantial, especially in critical cases where rapid detection has a significant impact on treatment and prognosis. For example, Chan *et al*. (2015) describe a workflow combining MALDI-TOF MS and a home-brew real-time PCR with melt-curve analysis for the rapid detection of MRSA and vancomycin-resistant enterococci (VRE) from colonies and, most importantly, blood culture bottles [50]. For the detection of MRSA in blood culture, they used a duplex real-time PCR targeting *mecA* and *nuc* for resistance and identification of *S. aureus* respectively [50]. The results of the workflow were 100% concordant with culture and antibiotic susceptibility testing and took only 2.5 hours [50].

Multiple publications are available assessing home-brew real-time PCR assay. The studies assessed various targets and conditions and their effects on the assay performance. Paule *et al*. (2004) designed a real-time PCR assay to detect *S. aureus* from neonatal nasal swabs by targeting the *femA* gene, a gene containing a conserved region in *S. aureus* [51]. They found that the assay sensitivity was higher with PCR (98%) than culture (90%). Additionally, the measured turn-around time of the assay was 2 hours.

The sensitivities and specificities of the in-house developed MRSA real-time PCR assays vary but are generally more sensitive and faster than culture. However, there are cases where culture was found to perform better. For example, Kali *et al*. (2014) compared home-brew PCR assay for the detection of *mecA* with commercial culture methods, including mannitol salt agar, MeReSA (HiMedia, India), and oxacillin screening agar. Interestingly, they found that the sensitivity was higher for MeReSA

and oxacillin screening agar compared to PCR; however, PCR had the superior specificity [52]. The lower sensitivity of the PCR was attributed to primer design and assay optimization. The point raises important issues when designing real-time PCR assays. The primer design process has to be stringent enough to increase specificity, but not too stringent as to reduce sensitivity. The stringency can be improved by exploring different amplification targets. The resistance marker *mecA* is widely used in in-house developed assays. However, these assays may have resulted in some false-negative results because of their inability to detect the newly emergent *mecC* gene. More recently developed assays, however, have incorporated the gene into the detection repertoire [53]. As for *S. aureus* specific genes, multiple targets have been proposed and used, including *femA*, 16S rRNA, *nuc*, and *spa* [50, 52, 54-58].

With further development, more commercial assay kits became available in the market, and many researchers investigated their performance and compared them to each other. Examples of these kits are the FluroType® MRSA assay (Hain Lifescience), Xpert MRSA Gen 3 (Cepheid), BD-Max MRSA XT, BD StaphSR, BD GeneOhm [59-62]. For MRSA detection, instead of multiple targets, many of the commercial kits target *SCCmec*, using primers directed towards the junction at which *SCCmec* is inserted into the *S. aureus* chromosome [53, 59-63]. The applications of PCR in the detection of MRSA are widely present and continuously developing. PCR offers potentially higher sensitivity and specificity, as well as faster turn-around times. It does come at a higher cost, however. Culture methods tend to be much cheaper than real-time PCR due to the requirement for fluorescent probes, reaction reagents, and sophisticated instruments. An assessment of the cost of commercial assay could not be made from the literature, as the prices are seldom mentioned and they vary between countries and regions. Nevertheless, when comparing real-time PCR assays to each

other, an in-house developed assay tends to be much cheaper than commercial assays. However, it should be noted that the cost-effectiveness of an in-house developed PCR assay is highly dependent on how well it is designed and optimized.

MRSA Detection by LAMP

Since Notomi *et al.* 's publication of LAMP in the year 2000, the technique has seen many applications, ranging from environmental surveys to diagnostics. Currently, 2206 publications are available on LAMP in the PubMed database, many of which applied the technique for the detection of microorganisms. It has been applied for the detection of various human pathogens, including *E. coli* O157, *L. monocytogenes*, *P. aeruginosa*, *Salmonella*, *V. parahaemolyticus,* and *Y. pseudotuberculosis* [64]. A comprehensive search of publications in the databases: PubMed, SCOPUS and Web of Science using the terms "loop-mediated isothermal amplification" and "MRSA," limited to publications related to human infection and those published in English, yielded 17 publications form the year 2000 (when the technique was developed and reported) to 2018 (Table 1).

The first report of using LAMP for the detection of MRSA was in 2007 by Misawa *et al.* [65]. LAMP was applied to detect MRSA directly from positive blood cultures. The LAMP primers were designed to target the *spa* gene (protein A specific to *S. aureus*) and the *mecA* gene for methicillin-resistance [65]. The assay was compared to a duplex real-time PCR targeting the *nuc* and *mecA* genes. The limit of detection (LOD) of the assay was determined using serial 10-fold dilutions of an American Type Culture Collection (ATCC) MRSA strain and detection by a turbidity meter. By LAMP assay, the LOD of detecting *spa* and *mec*A genes were 10^3 and 10^2 copies/reaction, respectively, compared to 10 copies/reaction for both the *nuc* and *mecA* genes by real-time PCR assay. As for specificity, they tested the assay against several

common infectious pathogens, including MRSA, MSSA, methicillin-resistance coagulase-negative staphylococci (MRCoNS), methicillin-sensitive coagulase-negative *Staphylococci* (MSCoNS), several enteric pathogens, *Streptococcus pneumoniae,* and many others. The LAMP assay was positive for *spa* in MRSA and MSSA only and positive for *mecA* in MRSA and MRCoNS only, giving the assay 100% specificity. When tested on blood cultures, the LAMP assay was found to have sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 92.3%, 100%, 100%, and 96.6%, respectively. PCR on the other hand, had 96.2% sensitivity, 100% specificity, 100% PPV, and 98.4% NPV [65]. Thus, it can be concluded that the LAMP assay developed by Misawa *et al.* can detect MRSA from blood cultures with superior sensitivity and specificity, but the PCR assay was analytically more sensitive. However, the LAMP assay offers the advantage of being more practical and cheaper. One limitation of this comparison between PCR and LAMP was that each assay targeted different genes for *S. aureus,* and thus the differences may be due to the characteristics of the targets rather than the detection method.

All the studies set out to develop a molecular assay that is more sensitive and rapid than culture methods. The studies investigated various amplification targets and specimen types. The most common targets used for *S. aureus* detection are *spa* and *femA*, with some studies using *femB*, and one study each using *nuc* and *orfX*. Most of the studies compared in-house developed PCR and LAMP assays. The LODs between the studies are not directly comparable because of the different units of measurements. Nevertheless, the majority of reports concluded that LAMP is at least ten times more sensitive than PCR. The amplification and detection platforms used in these studies are also different. One study is exceptional in that the results were evaluated based on detection by the naked eye using a reaction with SYBR Green I, which was visualized

under UV [66]. However, the LOD using this method was found to be ten times higher than conventional PCR, possibly because their extraction method was unable to remove LAMP inhibitors [66]. Other studies employed more sophisticated platforms such as microfluidics or surface plasmon resonance imaging (SPR) [67-70]. While these methods have been shown to work, they increase the cost of the assay with the requirement of expensive instruments and consumables.

All the studies had the common goal of developing a cost-effective, rapid, and accurate assay. The majority of the studies evaluated LAMP on sterile fluids or clinical isolates, both of which typically contain fewer non-target organisms as opposed to the swab specimens used for screening. Also, all the in-house developed LAMP assays targeted only the *mecA* gene for antibiotic resistance. As detailed in the introduction, another resistance gene has emerged recently, the *mecC* gene. The exclusion of the *mecC* gene can lead to higher rates of false negatives. Because of that, there is a need to develop an assay that is sensitive, specific, and cost-effective, and targets both the *mecA* and *mecC* genes. Although there are commercial kits available that include the *mecC* gene [71, 72], they are still more expensive than an in-house developed assay.

Table 1. LAMP Literature Review Summary

Year	Target genes	Specimen	LOD [a]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
2007	*spa*, *mecA*	Blood Culture	10^3 and 10^2 copy/reaction	92.3	100	100	96.6	Misawa *et al*. [65]
2010	*spa*, *mecA*	plaque sputum	<400 and <4000 CFU/ml	100 52.9% and 69.2	100 100	100 100	100 92	Koide *et al*. [66]

Year	Target genes	Specimen	LOD [a]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
2011	NM	Swab	17 copy/ reaction	NM	NM	NM	NM	Bearinger *et al.* [73]
2011	*spa*, *mecA*	Sputum and serum	1-10 fg/µl	NM	100%	NM	NM	Wang *et al.* [67]
2011	*femB, mecA*	Culture	NM	NM	NM	92.5	NM	Hanaki *et al.* [74]
2012	*femA, mecA*	Culture	800 and 8000 CFU/ml	98.5 and 94.3	100	100	98.1 and 92.3	Xu *et al.* [75]
2014	*femA, mecA*	Blood Culture	NM	91.7%	100	100	100	Metwally *et al.* [76]
2014	*orfX*	Culture	400 CFU/ml	NM	100	100	92.7	Su *et al.* [64]
2015	*spa*, *mecA*	Culture	10^3 CFU/ml	NM	100	NM	NM	Guo *et al.* [68]
2015	*femA, mecA*	Culture	10 copies/µl	NM	NM	NM	NM	Nawattanapaiboon *et al.* [70]
2015	*nuc, mecA*	Abscess Fluid and Blood Culture	NM [c]	NM [c]	NM [c]	NM [c]	NM [c]	Sudhaharan *et al.* [77]

Year	Target genes	Specimen	LOD [a]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
2015	*nuc, mecA*	Blood Culture	<1.47 and <14.7 pg/µl	NM [c]	100	NM [c]	NM [c]	Wang *et al.* [78]
2016	*femB, mecA*	Blood Culture, Sputum	100 CFU/ml	100 and 93.3	NM	NM	100 and 88.9	Nawattanapaiboon *et al.* [79]
2017	16S rRNA, *femA, mecA, orfX*	Respiratory Specimen	10^4 CFU/ml for 16S rRNA, *femA orfX* 10^5 CFU/ml for *mecA*	97.2	NM	NM	NM	Lin *et al.* [80]
2017	*nuc, femB, mecA*	Culture	NM [c]	NM	NM	NM	NM	Chen *et al.* [81]
2017	Easyplex MRSA® (Amplex)	Pleural and Synovial Fluids	$6.4x10^3$ CFU/ml	83.3	97.8	NM	NM	Henares *et al.* [71]
2017	Easyplex MRSA® (Amplex)	Blood Culture	NM	100	92.3	NM	NM	Rodel *et al.* [72]

a. The LOD units could not be standardized, as many publications did not disclose the amplicon length.
b. NM: not mentioned
c. The specific values were not mentioned, but the results are reported to be identical to the PCR assay developed by the authors.

Chapter 3: Methods

Real-time PCR primers and probes

Primer and probe design

Novel primers and probes for real-time PCR were designed targeting the *mecA* and *mecC* genes to detect methicillin resistance, the staphylococcal coagulase gene, *coa*, to detect coagulase-positive *Staphylococcus* species, and the thermonuclease gene, *nuc*, specific to *S. aureus*. The primers and probes were designed using the Primer Express v3.0.1 software (Applied Biosystems™) using reference gene sequences obtained from the National Center for Biotechnology Information Reference Sequence database (NCBI RefSeq; Appendix A).

For each gene, the sequences were imported into the software primer/probe sets were automatically generated using the "Find primers/probe" feature under the "TaqMan Quantification" type and default parameters. The software generated a list of candidate primer/probe sets arranged by a penalty score. The software calculates the score based on various parameters, including length, GC-content, secondary structures, dimerization, etc. The sets with the lowest penalty score were assessed for hairpin structures, self-dimers, and cross-dimers using the software's built-in features. One set for each of *mecC*, *mecA*, *coa*, and *nuc* were designed, and one set for each of *mecA*, *coa*, and 16S rRNA genes were retrieved from previously published literature by Hasan *et al.* (2013) [82]. To differentiate the two *coa* and *mecA* sets, they were numbered as CoA1 and mecA1 for the sets designed by Hasan *et al*. and CoA2 and mecA2 for the novel sets.

Primer and probe assessment

The primer and probe specificities were assessed *in silico* using the NCBI Nucleotide Basic Local Alignment Search Tool (BLAST) against the non-redundant

nucleotide collection (nr/nt) database [83]. The BLAST search was conducted with the "somewhat similar sequences" algorithm to ensure comprehensiveness. The algorithm allows for more lenient sequence alignment and enables the comparison to less homologous sequences. The primer/probe sets meeting the conditions: 1) complementary to the target gene, 2) not complementary to other similar genes, were selected for further *in silico* analysis The assessment showed alignment to the target genes in *S. aureus* over the primer and probe regions for *mecA*, *mecC*, and *coa*. (Appendices C-F). The *nuc* forward primer and probe had single-nucleotide mismatches with some *S. aureus* strains. The mismatches were resolved by introducing mixed base pairs in the sequences.

The amplicon sequences, including the primers and probe annealing sites for each primer/probe set were generated from the reference sequences and used for further specificity assessment. Another BLAST search was conducted with the amplicon sequences against a sample of randomly selected genome sequences for multiple bacterial species, including various strains of *S. aureus* and other *Staphylococcus* species (Appendix B). The results were analyzed for the complementarity of the primer and probe regions on their target sequence. In the case of *mecA* and *mecC*, complementarity was sought for any species carrying the genes, for *coa* complementarity was sought for the coagulase gene in coagulase-positive staphylococci, and for *nuc*, complementarity was sought only for the gene in *S. aureus*. The primer/probe sets showed complementarity to their respective targets and no significant complementarity to other genomic. The novel sets and those previously published by Hasan *et al.* [82] were synthesized by Integrated DNA Technologies (IDT) and purified under standard de-salting conditions (Table 2). Because the intended purpose is to create a multiplex reaction, the probes were synthesized with different

fluorescent dyes. *mecA* and *mecC* used FAM and *nuc*, and 16S rRNA used Cy5. As for *coa*, each probe was synthesized in duplicate, one with ROX and the other with JOE.

Table 2. Real-time PCR Primers and Probes

Source	Primer / Probe	Sequence (5′- 3′)	5’ Label	3’ Label
Hasan *et al.*	CoA1-F	TAGATTGGGCAATTACATTTTGGAG		
	CoA1-R	CATCTGCTTTGTTATCCCATGT	ROX	Iowa Black RQ
	CoA1-P	CGCTAGGCGCATTAGCAGTTGCATC		
Novel	CoA2-F	TCGTTCAAGGTCCCGATTTT		
	CoA2-R	CGGTGGGTTTGTATAATTATTGCTT	ROX	Iowa Black RQ
	CoA2-P	CAATGGAACAAAGCGGCCCATCA		
Hasan *et al.*	mecA1-F	GTAACATTGATCGCAACGTTC		
	mecA1-R	CTTTGGAACGATGCCTAATCTC		
	mecA1-P	TTCCAGGAATGCAGAAAGACCAAAG C	FAM	Iowa Black RQ
Novel	mecA2-F	TTAGATTGGGATCATAGCGTCATTAT		

Source	Primer / Probe	Sequence (5′- 3′)	5’ Label	3’ Label
	mecA2-R	AATTCCACATTGTTTCGGTCTAAAA		
	mecA2-P	CCAGGAATGCAGAAAGACCAAAGCATACA	FAM	Iowa Black RQ
	mecC-F	GCAAGCAATAGAATCATCAGACAAC		
	mecC-R	TCTTGCATACCTTGCTCAAATTTT		
Novel	mecC-P	CCGCATTGCATTAGCATTAGGAGCCA	FAM	Iowa Black RQ
	nuc-F	ATTTCGCTACTAGTTG**Y**TTAGTGTTAACTTTAG		
Novel	nuc-R	CACTATATACTGTTGGATCTTCAGAACCA		
	nuc-P	TCAGCAAATGCATCACAAACAGATAA**Y**GGC	Cy5	Iowa Black RQ
Hasan *et al.*	S16S-F	TCGTGAGATGTTGGGTTAA		
	S16S-R	ACTTAATGATGGCAACTAAGC		

Source	Primer / Probe	Sequence (5′- 3′)	5’ Label	3’ Label
	S16S-P	CCCGCAACGAGCGCAACCC	Cy5	Iowa Black RQ

*Y: mixed nucleotide with C and T.

Finally, the primers were tested *in-vivo* against American Type Culture Collection (ATCC) strains, ATCC BAA 976 and ATCC BAA 1026, first, then against randomly collected MRSA clinical isolates. Figure 6 below shows the workflow of the primer design and assessment process.

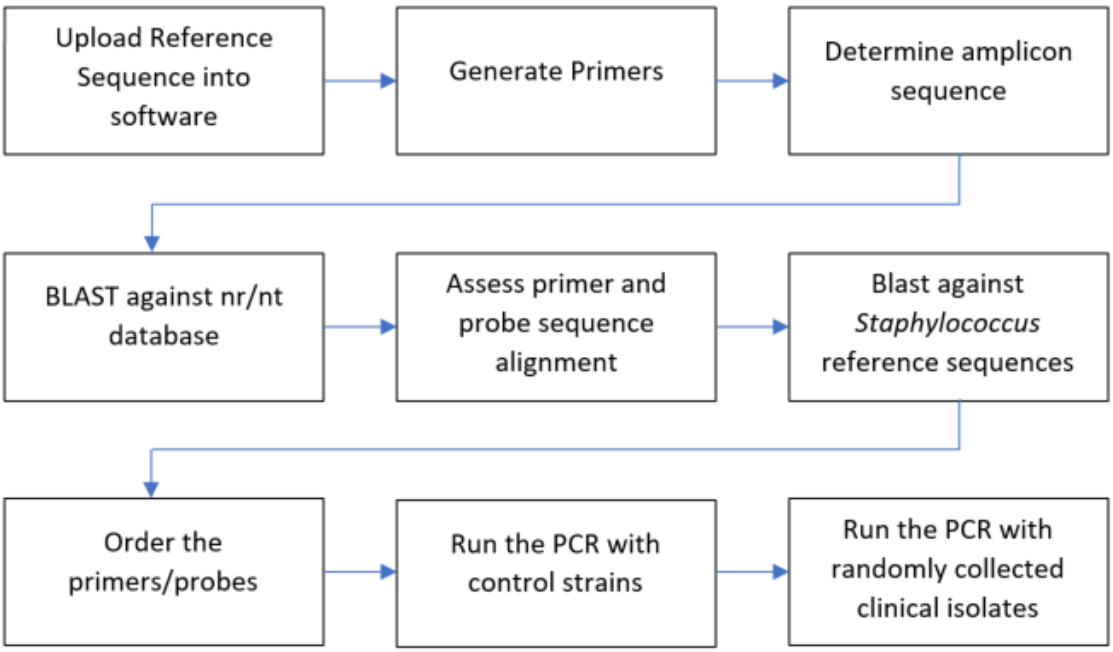


Figure 6. Primer design work-flow.

LAMP primers

Primer design

The LAMP primers were designed using the open-source software Primer Explorer V5 (Eiken Chemical Co. Ltd.) targeting the same genes as the real-time PCR assay. The same reference sequences were used to design the primers, except for the coagulase gene, as initial testing of the PCR primers showed no amplification. The new reference sequence used for the coagulase gene is AJ306908.1 (Appendix A). The Primer Explorer software runs an algorithm that generates sets of primers with specific parameters for melting temperature (Tm), GC-content, and ΔG.

The primers for this study were designed using the default software parameters. The default parameters for Tm are 64-66°C for F1c and B1c, 59-61°C for F2 B2, and 64-66°C for the loop primers and the default parameter for the GC content is 40-65% [49]. The reference sequences were uploaded to the software, and five candidate primer sets were generated for each gene under the default parameters. The software lists the primer sets along with the 3′ and 5′ stability in the form of ΔG. As per the software's guidelines, primer set with the ΔG of the 3′ ends of the F2 and B2 primers and the 5′ ends of the F1c and B1c be ≤ -4.0 kcal/mol were chosen. Then, primer data files were downloaded for each set and then re-uploaded into the software to generate compatible loop primers. The generated loop primers were evaluated based on the 3′ ΔG and the loop primer sets with negative ΔG were selected and compared for the 5′ ΔG. The loop primer sets with the lowest 3′ and 5′ ΔG for each set of primers were selected. Also, sets of primers for *mecA* and *nuc* were retrieved from the literature [78].

Primer set assessment

The generated sets, including their loop primers, were assessed for hairpins, self-dimers, and cross-dimers using the AutoDimer V1 software (National Institute of Standards and Technology). Then each set was evaluated with BLAST. All primer sets were also synthesized by IDT for further in vitro assessment. All primers were purified by standard desalting. The processes of assessment with BLAST and in vitro testing were conducted similarly to the real-time PCR primers. Regardless of the result of the results of the specificity assessment, all primers were synthesized by IDT with standard desalting purification. Table 3 below contains the sequences of all the primers.

Table 3. LAMP Primers

Source	Primer	Sequence (5′→3′)
Novel	mecA1-F3	TGATGCTAAAGTTCAAAAGAGT
	mecA1-B3	GTAATCTGGAACTTGTTGAGC
	mecA1-FIP	TGAAGGTGTGCTTACAAGTGCTAATTTTTCAACAT GAAAAATGATTATGGCTC
	mecA1-BIP	TGACGTCTATCCATTTATGTATGGCTTTTAGGTTCT TTTTTATCTTCGGTTA
	mecA1-LF	AATTCACCTGTTTGAGGGTGG
	mecA2-F3	GCGACTTCACATCTATTAGGT
	mecA2-B3	GCCATCTTTTTTCTTTTTCTCT
	mecA2-FIP	GTCCCTTTTTACCAATAACTGCATCTTTTTATGTTG GTCCCATTAACTCT

Source	Primer	Sequence (5′→3′)
Wang et al.	mecA2-BIP	AAGCTCCAACATGAAGATGGCTTTTCGATTGTATT GCTATTATCGTCAA
	mecA3-F3	AAAAAACGAGTAGATGCTCAA
	mecA3-B3	TGGCCAATTCCACATTGT
	mecA3-FIP	TCCCAATCTAACTTCCACATACCATTTTTAAAACAA ACTACGGTAACATTGA
	mecA3-BIP	TAGCGTCATTATTCCAGGAATGCATTTTCGGTCTAA AATTTTACCACGT
	mecAL-F3	TGATGCTAAAGTTCAAAAGAGT
	mecAL-B3	GTAATCTGGAACTTGTTGAGC
	mecAL-FIP	TGAAGGTGTGCTTACAAGTGCTAATTTTTCAACAT GAAAAATGATTATGGCTC
	mecAL-BIP	TGACGTCTATCCATTTATGTATGGCTTTTAGGTTCT TTTTTATCTTCGGTTA
	mecAL-LF	TCACCTGTTTGAGGGTGGA
Novel	mecC1-F3	AGATGCTAGAGTACAAGAAAGT
	mecC1-B3	GAACCTGGTGATGTAGTGAT
	mecC1-FIP	GATGGGGTACTTACCAAAGCTAAAATTTTAACATA TGAAAAATGACGATGGA

Source	Primer	Sequence (5′→3′)
	mecC1-BIP	TGGATTAAGCAATAATGACTACCGTTTTTTTGAAATTTGTTGAGCAAAGG
	mecC1-LF	CTCCAGTTTTTGGTTGTAATGCTGT
	mecC2-F3	AAGATGCATCATGGGGKAA
	mecC2-B3	GCTTTATAAAAGGGATAATCACTCG
	mecC2-FIP	TGTCTGATGATTCTATTGCTTGCTTTTTATCACAAGATTTAAAGTAGTAGACG
	mecC2-BIP	GCCCGCATTGCATTAGCATTTTTTATTTTCACCGATTCCCAAAT
	mecC3-F3	AATAAACACTATAAAAAGCCGTG
	mecC3-B3	TGTGTCTAAAGGTTTATTGTCAT
	mecC3-FIP	CGTCAGAATTAATTGGACCCACATTTTTTTATCCATTGAACGAAGCAAC
	mecC3-BIP	AGGCTTAGAACGCCTCTATGATTTTTCAATGGATACCTTAAAACCATCA
	nuc1-F3	CGATTGATGGTGATACGGTTA
	nuc1-B3	CAGTTCTTTGMCCTTTGTCA
Novel	nuc-1FIP	GCTTTGTTTCAGGTGTATCAACCAATTTTATTAATGTACAAAGGTCAACCAATG

Source	Primer	Sequence (5′→3′)
Wang et al.	nuc-1BIP	AAGGTGTAGAGAAATATGGTCCTGATTTTTCGACT TCAATTTTCTTTGCA
	nuc2-F3	GCATTTACGAAAAAAATGGTAGA
	nuc2-B3	TGTTCATGTGTATTGTTAGGTT
	nuc2-FIP	GCCACGTCCATATTTATCAGTTCTTTTTAAATGCAA AGAAAATTGAAGTCG
	nuc2-BIP	TATGCTGATGGAAAAATGGTAAACGTTTTTAAACA TAAGCAACTTTAGCCAAG
	nuc3-F3	AACAGTATATAGTGCAACTTCAA
	nuc3-B3	CTTTGTCAAACTCGACTTCAA
	nuc3-FIP	ATGTCATTGGTTGACCTTTGTACATTTTTAAATTAC ATAAAGAACCTGCGA
	nuc3-BIP	GTTGATACACCTGAAACAAAGCATCTTTTATTTTTT TCGTAAATGCACTTGC
	nucL-F3	AACAGTATATAGTGCAACTTCAA
	nucL-B3	CTTTGTCAAACTCGACTTCAA
	nucL-FIP	ATGTCATTGGTTGACCTTTGTACATTTTTAAATTAC ATAAAGAACCTGCGA
	nucL-BIP	GTTGATACACCTGAAACAAAGCATCTTTTATTTTTT TCGTAAATGCACTTGC

Source	Primer	Sequence (5′→3′)
	nucL-LF	GTATCACCATCAATCGCTTT
Novel	CoA1-F3	ACTACAGGATGCATTAAAGAGA
	CoA1-B3	CCAGTTTTGCTCGTAACTCT
	CoA1-FIP	TGCTGCATTAAAAGTTTTCAAGTCTTTTTGCACTGGATGATTTTCACA
	CoA1-BIP	AGGAAGTATACGATCTCGTATCTGATTTTCCCCATAATCCTTATCACCATA
Novel	CoA1-F3	GAAGAAAGTTGAAGAACCTCAA
	CoA1-B3	ATCTGGACCTTGAACGATT
	CoA1-FIP	TGGTTGTGTTGTTTCTTCAGCTTTATTTTCTAAAGTTGGAAACCAGCAA
	CoA1-BIP	AATTCCACAGGGCACAATTACATTTTCCTTGTAACGTTTTATTTTCCATAG
	CoA1-LB	GGTGAAATTGTAAAAGGTCCAGACT
Novel	CoA2-F3	AAGCGATAATTATACTCAACCG
	CoA2-B3	GATACCTGTACCAGCATCTC
	CoA2-FIP	CCTTTMAACGTTGATTCAGTACCTTTTTTTTTAGAAGGTCTTGAARGTAGC

Source	Primer	Sequence (5′→3′)
	CoA2-BIP	TGATATTGAMGTTAAACCTCAAGCATTTTGTGTTTTGTTAAATTGCGGTC
	CoA2-LB	AGAAGCATCACATTATCCAGCG
	CoA3-F3	ACTACAGMGATGCATTAAAGAGA
	CoA3-B3	CCAGTTTTGCTCGTAACTCT
	CoA3-FIP	TGCTGCATTAAAAGTTTTCAAGTCTTTTTGCACTGGATGATTTTCACA
	CoA3-BIP	GCAACTAAGGAAGTATACGATCTCGTTTTCCCCATAATCCTTATCACCATA
	CoA4-F3	CTTGGAAAAAGAAAACTGTCAA
	CoA4-B3	GGACCTTTTACAATTTCACCT
	CoA4-FIP	TTGCTGGTTTCCAACTTTAGGTAATTTTTGAGAAACTGAAACAAAATCGC
	CoA4-BIP	AAGCTGAAGAAACAACACAACCATTTTTAATTGTGCCCTGTGGAATT

Positive controls

Two positive controls were used in this study, extracted DNA from an American Type Culture Collection (ATCC) *S. aureus* strain BAA 976 for *mecA, coa,* and *nuc* and

a plasmid for the *mecC* gene. The ATCC strain was previously confirmed to be *S. aureus* by the Bruker MALDI-TOF Biotyper (Bruker, USA) and methicillin-resistant by BD Phoenix (Becton-Dickinson, USA). The isolate was cultured on sheep blood agar (BAP; International for Medical Equipment's and Supplies, Qatar) and incubated at 35°C in a 5% CO_2 incubator for 24 hours. Then, a 0.5 McFarland (McF) suspension was created in normal saline. The solution was then diluted into nine 10-fold serial dilutions. The fifth, sixth, and seventh dilutions were subsequently cultured on BAP in duplicate and incubated at 35°C in a 5% CO_2 incubator for 24 hours. The colonies were counted and used to quantify the bacteria in the solution in CFU/ml (Figure 7). Finally, DNA was extracted from each dilution using NucliSENSE EasyMAG (Biomerieux, USA) magnetic silica-based automated extraction platform (Table 4).

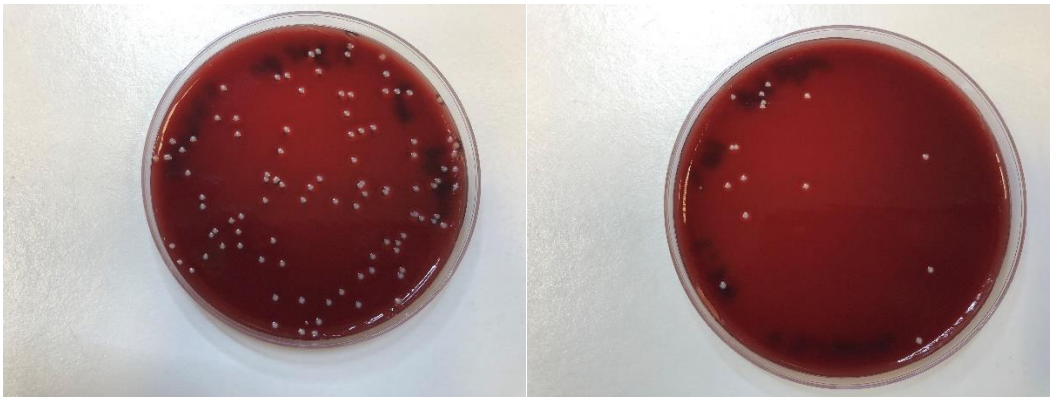


Figure 7. Positive control plates for *mecA*, *nuc*, and *coa*.

The image on the left shows the fifth dilution, and the image on the right shows the sixth dilution.

Based on the experiments, more dilutions were required to specify the limit of detection of the assay. Thus, the positive control was further diluted 2-fold from PC4 (Table 4)

Table 4. Positive Control Quantification for *mecA*, *nuc*, and *coa*

ID	Quantity (CFU/ml)
PC8	$9.95x10^{7}$
PC7	$9.95x10^{6}$
PC6	$9.95x10^{5}$
PC5	$9.95x10^{4}$
PC4	$9.95x10^{3}$
M1	$4.98x10^{3}$
M2	$2.49x10^{3}$
M3	$1.24x10^{3}$
PC3	$9.95x10^{2}$
M4	$6.22x10^{2}$
M5	$3.11x10^{2}$
M6	$1.55x10^{2}$
PC2	$9.95x10^{1}$
M7	78
M8	39
PC1	9.95

DNA from all the controls was extracted as described in the methods and used for testing. As for *mecC*, a plasmid containing the target sequence was synthesized by IDT. The plasmid was accompanied by a specification sheet that contained the mass of the plasmid. The concentration of the plasmid was calculated using the formula:

$$\frac{Copy}{ml} = \frac{(mass\ of\ DNA\ (g)\ \text{x Average number of base pairs per mole})}{length\ of\ DNA\ \text{x}\ average\ mass\ of\ one\ mole\ of\ base\ pairs}$$

The mass of the plasmid, as specified by IDT, was $1.98x10^{-6}$g, and the length of the plasmid was 3078 bp. The average number of base pairs per mole is $6.02x10^{23}$ g/mol, and the mass of one mole of base pairs is 650 g/mol. The concentration was

calculated to be $5.96x10^{12}$ copy/µl. The concentration was confirmed by NanoDrop (Thermo Fisher Scientific, USA). The plasmid was serially diluted 10-folds and into ten dilutions, then 2-folds to make eight intermediary dilutions similar (Table 5).

Table 5. *mecC* Plasmid Dilutions and Quantification

ID	Quantity (copy/ml)
mecC-1	$5.96x10^{10}$
mecC-2	$5.96x10^{9}$
mecC-3	$5.96x10^{8}$
mecC-4	$5.96x10^{7}$
mecC-5	$5.96x10^{6}$
mecC-6	$5.96x10^{5}$
D1	$2.98x10^{5}$
D2	$1.49x10^{5}$
D3	$7.45x10^{4}$
mecC-7	$5.96x10^{4}$
D4	$3.72x10^{4}$
D5	$1.86x10^{4}$
D6	$9.31x10^{3}$
mecC-8	$5.96x10^{3}$
M7	$4.66x10^{3}$
M8	$2.33x10^{3}$
mecC-9	$5.96x10^{2}$
mecC-10	$5.96x10^{1}$

In addition to the above, a new set of positive controls was created for the LAMP assay as the original controls were depleted. First, the DNA from 200µl of PC8 was extracted in quadruplicate and eluted in 60µl each then mixed. The quantity calculation was adjusted to account for the further extraction then serially diluted. The range of dilutions and quantities is shown in Table 6 below.

Table 6. LAMP Positive Control Quantification (*mecA*, *coa*, and *nuc*)

ID	Quantity (CFU/ml)
L1	$3.32x10^{8}$
L2	$3.32x10^{7}$
L3	$3.32x10^{6}$
L4	$3.32x10^{5}$
L5	$3.32x10^{4}$
L6	$1.66x10^{4}$
L7	$8.29x10^{4}$
L8	$4.15x10^{3}$
L9	$3.32x10^{3}$
L10	$2.07x10^{3}$
L11	$1.04x10^{3}$
L12	$5.18x10^{2}$
L13	$3.32x10^{2}$
L14	$2.59x10^{2}$
L15	$1.30x10^{2}$
L16	$3.32x10^{1}$

Real-time PCR assay optimization

Primer/probe initial verification

The initial verification of the real-time PCR reaction was performed using TaqMan™ Universal PCR Master Mix (Thermo Fisher Scientific, USA) in a 25µl reaction volume containing 1X TaqMan Universal PCR Master Mix, 0.3µM of the forward and reverse primers, 0.2 µM of the probe, and 5µl of the sample. The reaction was run in an Applied Biosystems® Fast Dx Real-Time PCR System (Thermo Fisher Scientific, USA) under the manufacturer's recommended conditions. The thermal cycling profile included an initial denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C denaturation for 15 seconds and 60°C annealing and extension for 1 minute. For *mec*C, the primer/probe set was verified against six dilutions of the plasmid

(10^{11} – 10^{1} with 100-fold dilutions) along with a nuclease-free water sample as a negative control. For *mecA, coa, nuc*, and the 16S rRNA genes, the sets were verified against 0.5 McF and 10-fold dilutions of two ATCC MRSA strains, BAA 976 and BAA 1026, and a nuclease-free water negative control. The sets with the best amplification were chosen.

Optimization

Two master mixes, TaqMan™ Universal PCR Master Mix (henceforth abbreviate as TaqMan) and TaqPath™ 1-Step RT-qPCR Master Mix (Thermo Fisher Scientific, USA*;* henceforth abbreviated as TaqPath), were compared using all the primer sets to optimize the reaction. First, singleplex reactions were compared between both. For TaqMan, the reactions were performed using the same conditions as above. As for TaqPath, the reaction volume was 20µl containing 1X TaqPath™ 1-Step Multiplex Master Mix, 0.3µM of the forward and reverse primers, 0.2µM of the probe, and 5µl of the sample. The reaction was run under the manufacturer's recommended thermal cycling conditions, which is an initial denaturation at 95°C for 2 minutes followed by 40 cycles of 95°C denaturation for 3 seconds and 60°C annealing and extension for 30 seconds. The samples used were 0.5McF for the two ATCC strains BAA 976 and BAA 1026 and 10^{5} copy/ml sample of the *mecC* plasmid. The best primer/probe sets were selected based on the previous two experiments and used to design a multiplex assay. The multiplex assays were run with both master mixes and using the same conditions as the singleplex assays. 0.3µM of each forward and reverse primers and 0.2µM of each probe were mixed into the reaction. The multiplex reactions were tested using the same samples.

Final verification

To further verify the primers and probes and assess their coverage of the local strain, the optimized multiplex real-time PCR reaction was performed with ten random clinical isolates collected from the Sidra Medicine Microbiology Laboratory. The isolates were confirmed MRSA positives by MRSA CHROM agar. A single colony was picked directly from the agar and re-suspended in Tris-EDTA (TE) buffer. The suspension was then diluted 10^5-fold and 10^6-fold. Both dilutions were used in the assay. The reaction was run using the multiplex assay with the TaqPath master mix under the manufacturer's recommended conditions.

LAMP assay optimization

Primer/probe initial verification

The primer sets for the LAMP assay were verified using the same process as for the real-time PCR. First, a 10X primer mix was created for each set by mixing 16µM of each of FIP and BIP, 2µM of F3 and B3, and 4µM of each of the loop primer. The reactions were performed using the WarmStart® LAMP KIT (New England BioLabs, USA; henceforth abbreviated as WarmStart) in a 25µl reaction volume containing 1X WarmStart LAMP Master Mix, 1X of the fluorescent dye supplied with the kit, 1X primer mix, and 1µl of the sample DNA. The reactions were performed on an Applied Biosystems® Fast Dx Real-Time PCR System. The program used consisted of 30 cycles at 65°C with detection at each cycle using the SYBR Green I channel. Melt curve analysis was performed following the reaction to confirm and compare the amplification results. The samples tested were extracted DNA from the two ATCC strains used for PCR, a plasmid containing *mecC*, and nuclease-free water as a negative control. The primer sets with the shortest time to result were chosen for optimization.

LAMP Optimization

Similar to PCR, two master mixes were tested, the WarmStart® LAMP Master Mix and LavaLAMP™ DNA Master Mix (Lucigen, USA; henceforth abbreviated as LavaLAMP). The WarmStart reactions were run using the same reaction mixture as above. As for LavaLAMP, a reaction mixture of 25µl containing 1X LavaLAMP master mix, 1µl of the supplied fluorescent dye, 1X primer mix, and 1µl of the sample was used. The same temperature and time were used for the reaction, with the addition of a 2-minute denaturation at 95°C. The master mix producing the best result was used for further reaction optimization in terms of primer mix concentration, and reaction temperature (Table 7).

Table 7. LAMP Optimization Matrix

Temperature (°C)	Primer Mix Reaction 1	Reaction 2	Reaction 3	Reaction 4
65	1X	1.25X	1.5X	2X
66	1X	1.25X	1.5X	2X
67	1X	1.25X	1.5X	2X
68	1X	1.25X	1.5X	2X
69	1X	1.25X	1.5X	2X

All reactions were performed on the Applied Biosystems® Fast Dx Real-Time PCR System (Thermo Fisher Scientific, USA) using the SYBR Green I channel for detection. The samples tested were the same as those used in the initial verification. The conditions showing the shortest time to result were chosen for the assay.

Clinical specimens

Patients in Sidra Medicine undergo risk-based screening for MRSA upon admittance to the hospital through the collection of swabs from the nares, throat, groin, and in newborns, the umbilical cord. MRSA screening specimens are cultured on MRSA CHROM agar and incubated at 35°C in a non-CO_2 incubator. The culture plates are read after 24 hours of incubation, and the growth of pink colonies is interpreted as the presence of MRSA. Then, Bruker MALDI-TOF Biotyper (Bruker, USA) is used to confirm that the colony is *S. aureus*. Confirmation of the resistance is performed for isolates from cardiology patients by BD Phoenix (Becton-Dickinson, USA). At present, molecular testing for MRSA is reserved for urgent cases and is performed by the Cepheid Xpert MRSA Gen 3 kit.

For the present study, retrospective, residual swab (E-swabs and dry swabs, VWR, USA) specimens were collected from the Microbiology Laboratory in Sidra Medicine. Two-hundred specimens were collected, consisting of 122 E-swabs and 78 dry swabs divided among nares (n=67), throat (n=71), groin (n=61), and umbilical cord (n=1).

DNA extraction

The collected clinical specimens were extracted using the NucliSENSE EasyMAG magnetic silica-based automated extraction platform. Dry swabs were immersed in 1mL of 1X PBS, pH 7.4 (gibco, Thermo Fisher Scientific; USA), and vortexed for 15 seconds to re-suspend the bacterial cells. As for E-swabs, the tube was vortexed for 15 seconds, and the solution was used for extraction. 600µl of the specimens were used for extraction under the generic protocol, following the manufacturer's instructions. The elution volume was 60µl.

Assay validation

Assay validation was performed following the recommendations of Burd's (2010) paper, "The Validation of Laboratory-Developed Molecular Assays for Infectious Diseases." The paper describes requirements and proposed experiments to validate assays following the Clinical Laboratory Improvement Amendments (CLIA) and the College of American Pathologists (CAP) [84]. The validation criteria are linearity and reportable range, analytical sensitivity (LOD), precision, analytical specificity, and accuracy. Linearity, analytical sensitivity, and precision were measured simultaneously using serial dilutions of quantified positive control tested in triplicates once a day for five days and recording the Ct for real-time PCR or the time-to-result (Tt) for LAMP. The experiment encompasses 15 data points for each dilution and 135 data points for the experiment as a whole. The analytical specificity was assessed by testing the assays against a panel of bacterial organisms commonly isolated in the lab setting (Table 8). The panel was created by collecting colonies directly from culture plates after identification by MALDI-TOF. Finally, the accuracy was measured by testing the clinical specimens and comparing the results with chromogenic MRSA culture results.

Table 8. List of Organisms in the Specificity Panel

Acinetobacter baumannii	*Campylobacter jejuni*	*Candida albicans*	*Citrobacter amalyticus*
Clostridium perifringens	*Enterobacter cloacae*	*Enterococcus faecalis*	*Enterococcus faecieum*
Escherichia coli	*Haemophilus influenza*	*Klebsiella pneumoniae*	*Morganella morganii*
Proteus hauseri	*Pseudomonas aeruginosa*	*Salmonella* Group B	*Staphylococcus aureus* (MRSA)
Staphylococcus capitis	*Staphylococcus epidermidis*	*Staphylococcus hominis*	*Stentrophomonas maltophilia*
Streptococcus aglactiae	*Streptococcus mitis*	*Streptococcus oralis*	*Streptococcus pneumoniae*
Streptococcus pyogenes			

Statistical analysis

The data collected from the study was saved on an Excel spreadsheet for the initial organization and analysis. The linearity of the assays was measured with linear regression using a plot of $\log_{10}$ quantity vs. Ct (or Tt) and overlaying a best-fit line. The r^2 value was used as the measure of linearity and was considered acceptable when $r^2 \geq 0.98$. The reportable range of the assay was determined as the range of concentrations over-which the assay result was linear.

Analytical sensitivity was measured as the LoD at 95% confidence by applying probit analysis [84]. In short, probit analysis is a specialized regression analysis for binomial variables. It is used in dose-response analysis to measure the lowest dose that will generate a response 95% of the time. As for the precision, the standard deviation (SD) between the replicates was measured for the same concentration of analytes.

Precision was measured for both intra-test (replicates tested together at the same

time) and inter-test (replicates over multiple different days). The precision of the assay was considered acceptable if 95% of the replicates fall within ±2 SD for all replicates or ±3 SD for replicates within 20% of the LoD. The specificity was calculated as the ratio of true positives to total positives, and the accuracy was calculated as the percentage of results in agreement with the chromogenic agar culture result.

Ethical compliance

Ethical approval was obtained from the Institutional Review Board (IRB) of Sidra Medicine before collecting the clinical samples and conducting the experiments. The study involves the secondary use of residual anonymous biological materials that were previously collected for diagnostic purposes by the Microbiology laboratory. The patients' identifiers and demographic information were not required. As such, each sample was given a serial number, and all patient identifiers were removed to ensure the specimen could not be linked back to the patients. The IRB at Sidra Medicine granted an exemption letter as the study was deemed not to involve human subjects.

Chapter 4: Results

Real-time PCR assay Verification and Optimization

The primer/probe sets designed for the real-time PCR assay were verified in vitro using the MRSA ATCC strains BAA 976 and BAA 1026 for the mecA, nuc, 16S rRNA, and CoA genes. While the pmecC plasmid was used to verify the mecC primer/probe set. Ideally, the mecC set would have been verified using a bacterial strain; however, no known mecC strains were available in the laboratory. The verification was conducted with both the TaqMan and TaqPath master mixes and using both the CoA sets labelled with ROX and the sets labelled with JOE.

mecC, mecA1, and mecA2 showed positive amplification with both master mixes, with mecA2 having consistently lower Cts than mecA1. As for the *S. aureus* specific targets, both nuc and 16S amplified in the samples. However, 16S also showed amplification in the negative control, suggesting non-specific amplification. Finally, only CoA1 showed amplification in both master mixes. CoA2 was negative for one of the control strains, ATCC BAA 1026, suggesting lack of coverage of *S. aureus* strains.

The comparison of the master mixes overall showed that TaqPath consistently performed better than TaqMan, owing to its optimization for multiplex reactions. Notably, the CoA1 set labelled with ROX performed worse than the one labelled with JOE in the TaqMan master mix, likely due to the presence of ROX as a passive dye.

Thus, the mecC, mecA2, CoA1, and nuc sets were chosen for the multiplex assay. From the results, the TaqPath master mix performed better and enables the addition of an extra internal control. Nevertheless, the multiplex reaction was tested using both master mixes, the results of which are shown in Table 9 below.

Table 9. Multiplex Real-Time PCR: TaqMan vs. TaqPath

Target	Sample	TaqMan		TaqPath	
		Ct1	Ct2	Ct1	Ct2
mec	ATCC BAA 976	25.13	27.53	23.99	23.97
	ATCC BAA 1026	25.69	25.97	25.09	25.35
	pmecC (10^5 copy/ml)	33.23	34.18	33.72	33.68
	H_2O	Negative	Negative	Negative	Negative
coa	ATCC BAA 976	25.14	27.41	23.67	23.80
	ATCC BAA 1026	24.45	25.13	24.92	25.52
	pmecC (10^5 copy/ml)	38.62	38.75	Negative	Negative
	H_2O	32.36	22.31	Negative	Negative
nuc	ATCC BAA 976	25.91	27.24	24.57	24.40
	ATCC BAA 1026	26.00	25.56	25.43	25.50
	pmecC (10^5 copy/ml)	Negative	Negative	Negative	Negative
	H_2O	Negative	Negative	Negative	Negative
IC	ATCC BAA 976	26.15	24.76	20.53	25.28
	ATCC BAA 1026	23.96	23.40	25.37	22.63
	pmecC (10^5 copy/ml)	18.72	23.90	24.48	22.62
	H_2O	22.62	21.37	27.23	19.38

Similar to the previous results, the TaqPath master mix also performed better. The *coa* target showed false-positive amplification in the negative control when using the TaqMan master mix, likely attributed to the presence of the ROX passive dye. As for the internal control, the criterion set by the laboratory was that it must be below a Ct of 33 to be considered acceptable. Based on this criterion, the internal control performed well with both the TaqMan and TaqPath master mixes. Then, to further verify the performance of the TaqPath master mix, and ensure no loss of sensitivity during the multiplex reaction, an experiment was performed to compare singleplex and multiplex reactions. No significant difference was found (Table 10).

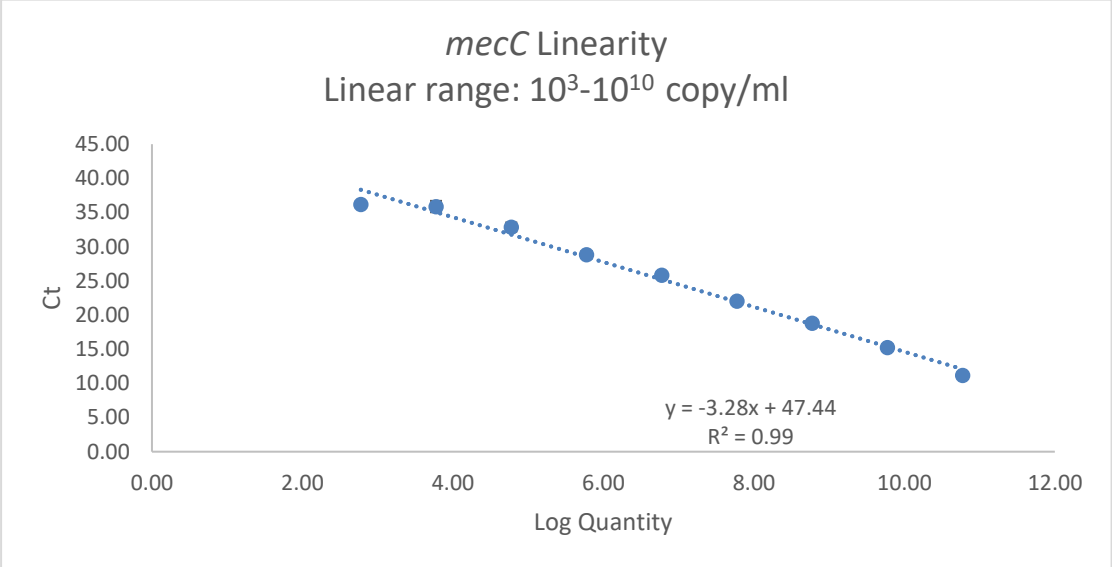
Table 10. Singleplex vs. Multiplex Real-Time PCR with TaqPath.

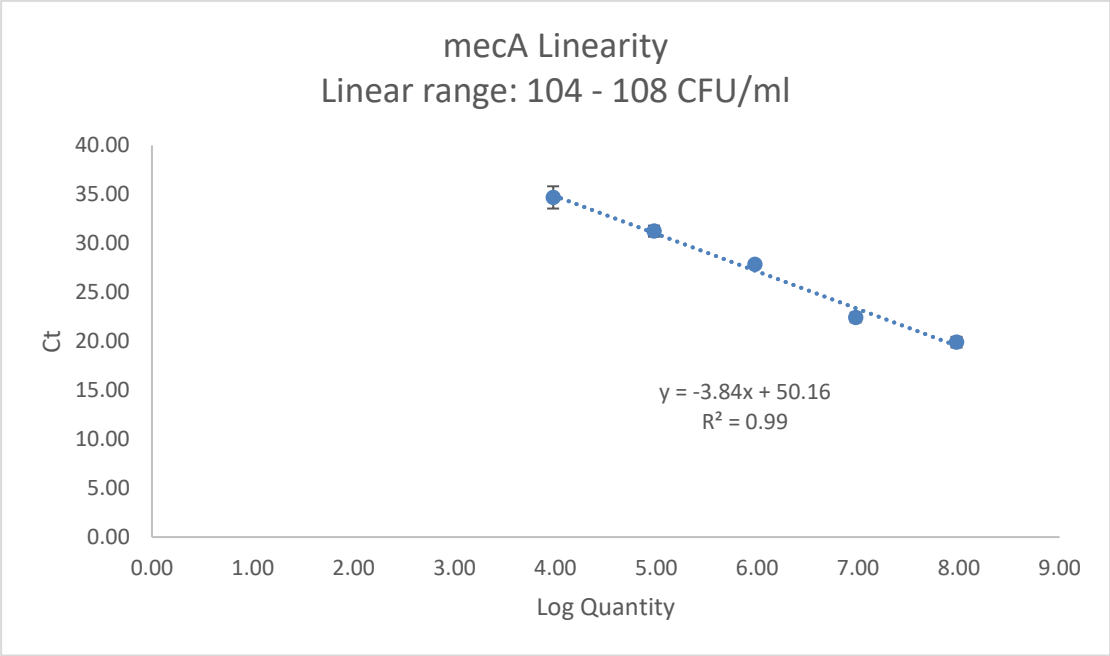
Target	Sample	Singleplex Average Ct	Multiplex Average Ct
mec	ATCC BAA 976	23.33	23.01
	ATCC BAA 1026	24.66	23.51
	pmecC (10^5 copy/ml)	30.99	31.52
	H_2O	Negative	Negative
coa	ATCC BAA 976	23.40	24.43
	ATCC BAA 1026	23.91	24.94
	pmecC (10^5 copy/ml)	Negative	Negative
	H_2O	Negative	Negative
nuc	ATCC BAA 976	25.48	25.72
	ATCC BAA 1026	26.81	26.10
	pmecC (10^5 copy/ml)	Negative	Negative
	H_2O	Negative	Negative
IC	ATCC BAA 976	Not tested	24.77
	ATCC BAA 1026		24.83
	pmecC (10^5 copy/ml)		22.95
	pmecC (10^5 copy/ml)		Negative

The results of the singleplex and multiplex assays are all within 2 Cts of each other, showing no substantial loss of sensitivity. Thus, the assay was deemed satisfactory, and further optimization was not necessary. The final step of the evaluation was to test the multiplex assay against bacteria isolated from patients. The multiplex assay was positive for all targets on all ten isolates. Also, the Cts between the targets were close to each other. Which is expected, as all the targets exist on the bacterial chromosome, of which there is one copy. Thus, it was decided to continue with the TaqPath master mix for validation.

Real-time PCR assay validation

The multiplex assay was tested with quantified controls to measure precision, sensitivity, linearity, and reportable range. The assay was found to be within the acceptance criteria in terms of precision (SD<2) and linearity (R^2>0.98). The limits of detection were found to be 10^3 copy/ml for *mecC*, 10^3 CFU/ml for *coa*, and 10^4 CFU/ml for *mecA* and *nuc*. The detailed results of the experiment are presented in appendices G and H, and the plots for the linearity are shown in Figure 8.





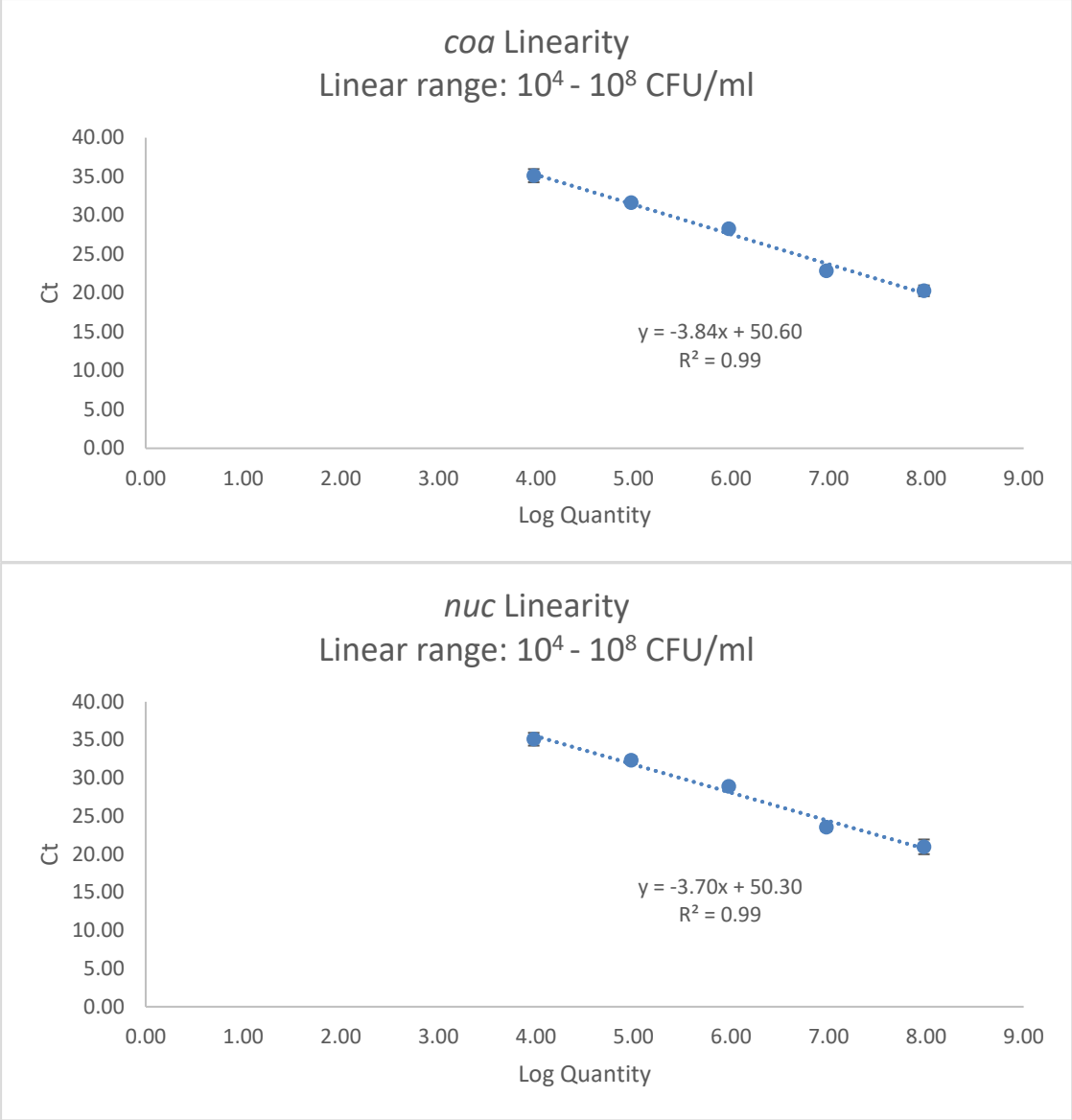


Figure 8. Real-time PCR linearity plots

The specificity of the assay was tested using a panel of microorganisms, including MRSA, MSSA, CoNS, and other organisms commonly isolated in the laboratory. Amplification was observed for all targets with MRSA and mec in *S. epidermidis* and *S. oralis*, while all other organisms were negative for all targets, resulting in an apparent 100% specificity. Finally, the clinical specimens were tested with the assay to assess its accuracy by comparison with chromogenic agar and was found to be 95% (Appendix I). The assay's performance characteristics are shown in

table 11.

Table 11. Real-Time PCR Assay Performance Characteristics

Target Gene	Intra-experiment mean Ct standard deviation	Inter-Experiment mean Ct standard deviation	Linearity Range (R^2)	LOD	Assay Specificity (%)	Accuracy
mecC	0.39	0.41	10^3-10^{10} copy/ml (0.99)	10^3 copy/ml	100%[a]	95%
mecA	0.45	0.61	10^4-10^8 CFU/ml (0.99)	10^4 CFU/ml		
coa	0.38	0.50	10^4-10^8 CFU/ml (0.99)	10^3 CFU/ml		
nuc	0.47	0.64	10^4-10^8 CFU/ml (0.99)	10^4 CFU/ml		

[a] positive result was obtained for mec when testing against *S. epidermidis* and *s. oralis*. The assay was negative for all other targets, so it was considered as 100% specific because it was not *S. aureus*.

LAMP Assay Verification and Optimization

The primer sets that met the design criteria were all tested to verify their function in vitro. In total, four sets for *nuc*, four sets for *mecA*, and four sets for *coa*. The *mecC* primer sets were not tested due to the unavailability of a positive control. The pmecC plasmid used for the real-time PCR assay was not compatible because of different target sequences. Of the tested primer sets, nuc3, nucL, and mecA1 showed positive amplification with the ATCC BAA 976 and BAA 1026 MRSA strains, the

remaining sets did not show any amplification. A melt curve analysis was performed to ensure that the amplification detected was from a single product (Figure 9).

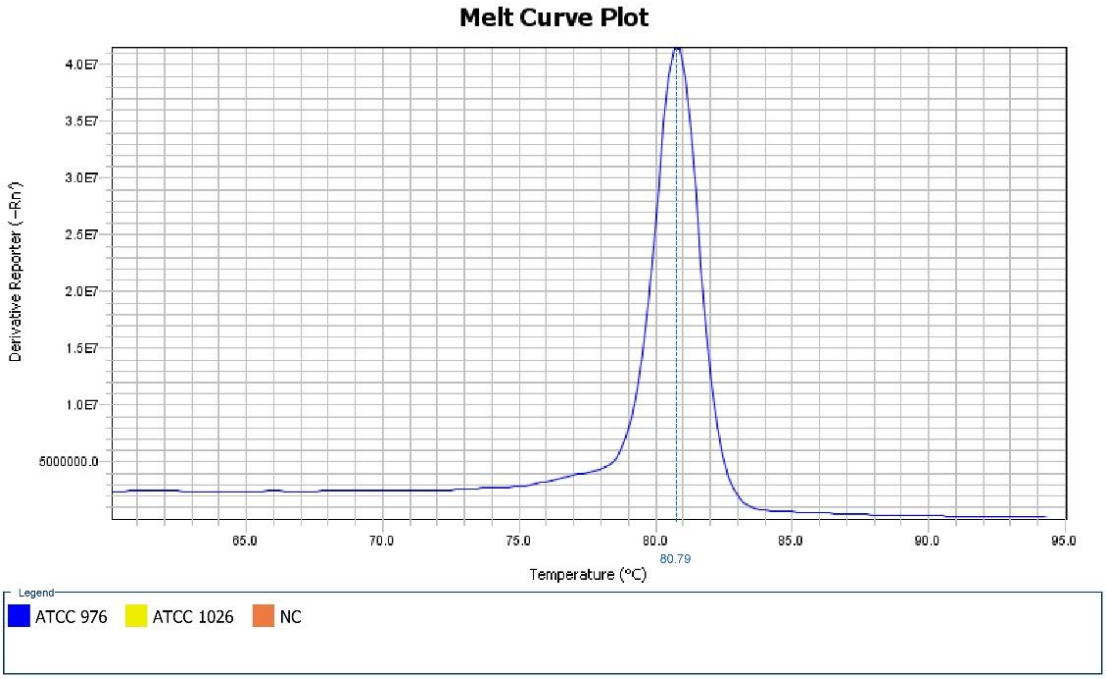


Figure 9. Melt curve plot for mecA1 LAMP primer set with ATCC 976

The three primer sets were then tested with the same ten clinical isolates used with the real-time PCR assay and showed positive amplification for all ten isolates. Nuc3 consistently showed a faster time to result than nucL. Thus, it was chosen for the assay. However, when the sets were tested with the positive control dilutions, positive amplification was observed down to L3 dilution ($3.32x10^6$ CFU/ml) for both targets. The assay was repeated using both the WarmStart and LavaLAMP master mixes with primer concentrations of 1X, 1.5X, and 2X and temperatures of 65, 68, and 70°C with no improvement to the results.

The sensitivity of the primers was assessed by performing a PCR reaction with the outer pair (F3 and B3) of each of nuc3 and mecA1 and visualizing with agarose gel

electrophoresis using the positive control with the highest concentration (L1) and a negative control (Figure 10). The gel showed no bands for both mecA1 and nuc3 with the positive control L1, which suggests that the primers had reduced sensitivity.

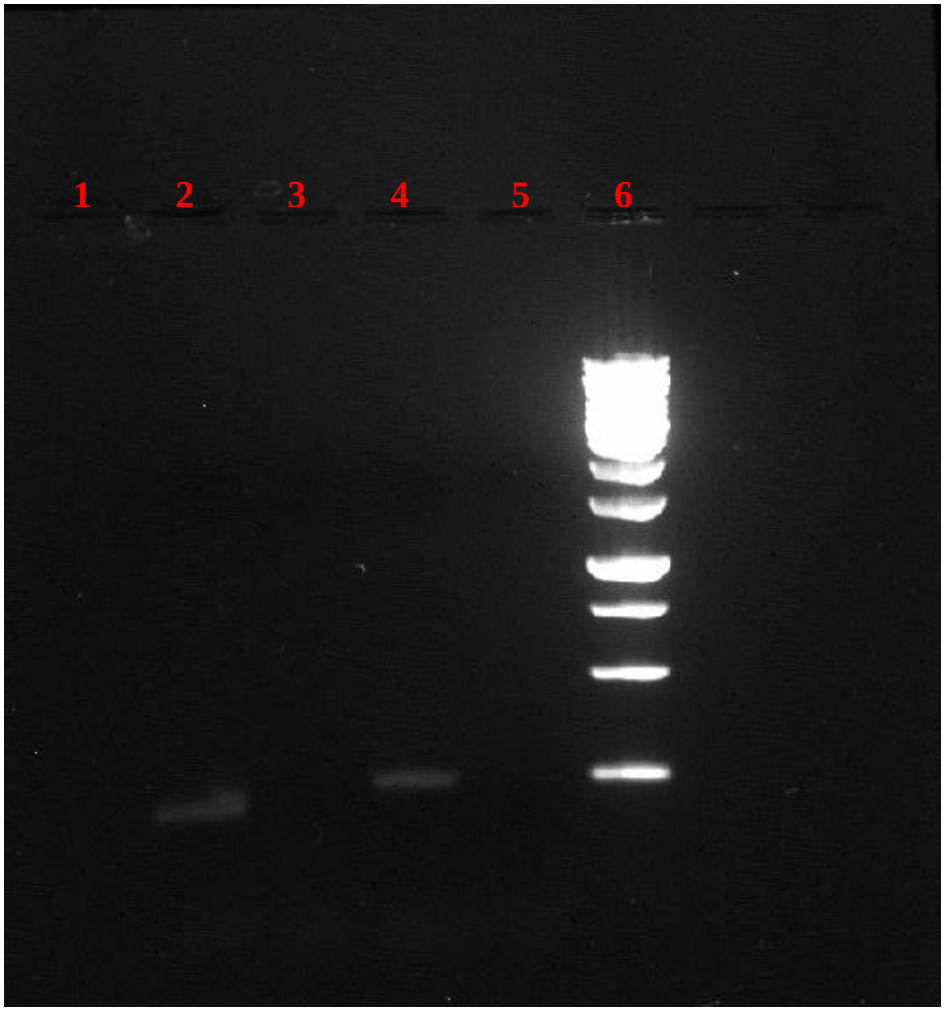


Figure 10. Gel picture of the mecA1 and nuc3 outer primers in a PCR reaction

From left to right: lanes 2 and 3 contained the reactions of mecA1 F3 and B3 with L1 and negative control, respectively. The expected size of the fragment is 210bp. Lanes 4 and 5 contained the reactions of nuc3 F3 and B3 with L1 and negative control, respectively. The expected fragment size is 248bp Lane 6 contains a DNA ladder.

Chapter 5: Discussion

Designing a Molecular Assay

The process of developing an in-house molecular assay to detect or identify a pathogenic microorganism - PCR, LAMP, or any other technique - starts with the question of what should be detected. The primary aim of the present study is to develop PCR and LAMP assays to detect MRSA in patient specimens. The assay must be sensitive and specific enough, so it does not produce false-negative and false-positive results. In order to design these assays, first, it was essential to determine which genetic regions to target.

A search of the literature showed various possible target genes. Many of the developed assays use *SCCmec* or *orfX* as targets for MRSA detection. However, these two genes show variability between MRSA strains and may fail to detect some MRSA cases [85-87]. To date, there are eleven recognized *SCCmec* types based on sequence variation, with two more proposed in the literature [88]. The variation in the *SCCmec* region extends to *orfX*, leading to many of the available assays producing false negatives. A study evaluating PCR assays for the detection of MRSA in Denmark showed that the tested assay, which targets *SCCmec*, gave 15.4% false negatives [89]. Moreover, there are cases where *SCCmec* is present without methicillin resistance. Such *SCCmec* elements do not carry a *mec* gene; rather they carry resistance markers for other substances [90]. Targeting those elements, in turn, would produce false-positive results. The variability of *SCCmec* introduces uncertainty in testing, which would significantly impact the workflow of a clinical laboratory and might increase the cost of testing with the need for follow up confirmatory tests, especially if there is a clinical indication of MRSA.

Thus, instead of targeting *SCCmec* or *orfX*, this assay targets the more conserved *mec* genes as methicillin resistance markers. The *mecA* gene is responsible

for the majority of resistance. However, the recently emergent *mecC* confers similar resistance. Thus, both of these were considered as targets for the assays. However, these genes are also harbored by species other than *S. aureus*. Therefore, it was integral to add a target that is specific to *S. aureus*. Existing literature includes many suggested targets, *spa*, *nuc*, *femA*, *femB*, among others for *S. aureus*. The target genes chosen for the assay were *nuc*, *coa*, *mecA*, and *mecC* so that PCR detects MRSA, as well as provide more information. Specifically, the targets were chosen such that the result can be interpreted as MRSA, MSSA, methicillin-resistant coagulase-negative staphylococci (MR-CoNS), methicillin-sensitive coagulase-negative staphylococci (MS-CoNS), methicillin-resistant or sensitive coagulase-positive staphylococci other than *S. aureus*, or a mixture of the above. While some of the results may not be useful to screening tests (i.e., CoNS), they could be significant when applied to sterile-site samples in the appropriate clinical context.

While designing the assay, one consideration was the creation of a multiplex reaction. An internal control (IC) was planned to be incorporated into the multiplex reaction to conform with regulatory requirements. Furthermore, to maintain consistency between the assays performed in the laboratory at Sidra Medicine and to prevent additional costs of synthesizing a new IC, it was decided to use the same IC used in the other assays. To that extent, the dyes on the five probes (*mecA*, *mecC*, *coa*, *nuc*, and IC) were chosen to minimize overlap between their spectra. The same color dye, FAM, was chosen for *mecA* and *mecC* because it was not necessary to differentiate between them. The *nuc* and 16S rRNA probes were synthesized with the Cy5 dye. As for CoA, it was synthesized in duplicate, one with the ROX dye and the other with the JOE dye. The reason is to assess the performance of the assay with both of the used master mixes. The TaqMan master mix contains ROX as a passive dye. Therefore the

coa probe with the JOE dye was used in its reaction, which prevented the use of the IC. TaqPath, on the other hand, lacks the passive reference dye, enabling the incorporation of the IC with the use of the ROX labeled *coa* probe.

Designing the LAMP assay was more challenging as the software does not offer sufficient resources or guidance on how to set and optimize the parameters. Also, due to the nature of the amplification reaction in LAMP and the vast array of structures that form, it is challenging to create a multiplex reaction. Also, regular practice is to use SYBR Green chemistry rather than probes for detection. Thus, no probes were designed for the assay. Furthermore, to account for the fact that LAMP is not as well established as PCR, all generated primer sets were synthesized for in vitro evaluation

Real-Time PCR

Clinical Validation

The assay validation was performed following Burd's (2010) paper. The performance characteristics passed the set criteria for precision and linearity of the assay. While the assay is qualitative, the establishment of the linear range allows quantification by using quantitation standards. After the performance criteria were deemed satisfactory, the assay was evaluated using 200 screening specimens collected from the Microbiology Laboratory. At a glance, the assay would have low accuracy (Appendix I), due to the nature of the specimen. Because the specimens were swabs of body sites that typically contain a mixture of bacteria, including *S. aureus* and other methicillin-resistant CoNS It would be expected that a large portion of the specimen would have a positive result for *mec* and thus, the results must be interpreted with this in mind.

First, all three targets, *mec*, *coa*, and *nuc*, must be positive in MRSA. The *coa* gene is used to differentiate between coagulase-positive and negative species. A

positive result excludes the presence of CoNS. The *nuc* gene then differentiates *S. aureus* from other coagulase-positive species, and, finally, the *mec* gene differentiates MRSA from MSSA. Thus, having all three genes increases the probability that the organism detected is MRSA. The probability would be very high in more invasive infections, where the specimen is less likely to have multiple species present. In the case of screening samples, however, there would likely be a mixture of species, which complicates the interpretation. For instance, how can the test differentiate if there was MRSA or a mixture of MSSA and MR-CoNS?

Further investigation of the results shows that there are differences in the Ct values in those cases. Therefore, a second criterion must be implemented in the interpretation. Because MRSA would possess a *mec* gene, *coa* gene, and *nuc* gene, and all three are in the chromosome, it is expected that all three would be present at similar levels to each other, assuming equal efficiency of amplification for each target. The cases that were positive for all three targets by the real-time PCR assay, but were MRSA negative by chromogenic culture consistently had a Ct difference greater than two between the average of *nuc* and *coa* and the Ct of *mec*. Accordingly, the criteria for a positive MRSA interpretation of the results are 1) all three targets are positive, and 2) the difference between the average Ct of *coa* and *nuc* and the Ct of *mec* is less than 2. Implementing these two criteria increases the accuracy from approximately 85% to 96.25%. The accuracy is after the adjusted interpretation is consistent with published MRSA assays and commercial kits. Furthermore, accuracy $\geq$ 95% is acceptable to diagnostic laboratories and CLIA and Cap requirements.

Nevertheless, the assay does suffer from limitations. While it is useful in screening for MRSA and differentiating other groups of species with high accuracy, it still falls short in some instances. For example, false-positive results can occur if both

MSSA and MR-CoNS are present at similar levels. In this case, differentiation is difficult, as it would meet the interpretation criteria. Similarly, a false negative result can occur when MR-CoNS are present in large quantities, which can cause the Ct for *mec* to be much lower than *nuc* and *coa* and thus not meeting the interpretation criteria. Six false positive and six false negatives were found in this study; however, it is unknown if the reason is the quantity of MR-CoNS present, a limitation of the assay, or differences in the sensitivities of the PCR and chromogenic agar.

By considering all the results, the assay is very useful in the detection of MRSA in invasive infections or from sterile body sites (e.g., blood). It is also useful for screening purposes; however, there are some limitations. The assay detects multiple organisms, MSSA, MRSA, MS-CoNS, and MR-CoNS. Giving it an advantage over other assays described in the literature, which detect MRSA or MSSA and MRSA [50-62]. The wider detection range is particularly helpful in cases of blood infection. Finally, being an in-house developed assay, it reduces the testing cost compared to commercial assays. Currently, in Sidra Medicine, MRSA detection is performed using the Cepheid Xpert MRSA Gen 3 kit, which costs an approximate $100 per specimen. The present assay costs approximately $3 per sample for the primers, probes, and PCR reagents. The extraction cost can vary; in the case of this study, the extraction cost was an approximate $15, bringing the total cost per specimen to $18. Thus, the present assay reduces the cost of molecular MRSA detection by up to 80%.

LAMP

While the primers designed for LAMP met the design criteria and showed promise when assessed *in silico*, they did not perform well in vitro. The primers resulted in amplification during the initial verification, which used ATCC MRSA strains and

clinical isolates. However, these samples contained large amounts of bacteria. When tested with dilutions of the positive control, the limit of detection was found to be $3.32x10^6$ CFU/ml, which is contradictory to the literature as LAMP reactions were found to be at least 10-times more sensitive than PCR. Further investigation was conducted by testing the outer primers with a PCR reaction. The lack of bands on the agarose gel further supported the fact that the primers were not sensitive. Attempts to increase the primer concentrations were also unsuccessful in improving the sensitivity of the assay.

The low sensitivity can be due to various reasons. A scan of the available literature revealed that the recommendation is that the primers be purified with HPLC. The primers used in this study were purified with standard desalting, which yields lower purity than HPLC. Another possible explanation could be inherent to the primers themselves. Redesigning the primers with criteria that are more stringent and different properties or a different target sequence in the gene might prove useful.

Chapter 6: Conclusion

The present study aimed to develop a sensitive and cost-effective molecular assay for the detection of MRSA. The study had three objectives, to develop a real-time PCR assay, to develop a LAMP assay, and to validate the assays for use in clinical laboratories. The real-time PCR assay had 95% accuracy, >95% specificity and high precision. The assay was validated following the recommendations of Burd (2010) for the validation of in-house developed molecular assays following CLIA. The assay has the advantage of lower cost than the available commercial methods and a wider range of detection (MRSA, MSSA, MR-CoNS, and MS-CoNS) compared to other laboratory-developed assays.

Nevertheless, it can be improved further to increase its utility. At present, it is suitable as a detection method in specimens where polymicrobial infections are uncommon. For screening, however, it suffers from the limitations outlined above. Multiple tweaks to the methodology can be investigated to alleviate some of these limitations and increase utility. First, the concentrations of the primer/probe sets in the multiplex reactions can be further optimized to increase sensitivity. For instance, the sensitivity of the *nuc* set was consistently slightly lower than the sensitivities of the other targets. Perhaps experimenting with increased proportions of the set can yield higher sensitivity. Similarly, experimenting with other proportions of the sets could fine-tune the results. Second, to reduce the probabilities of false results, more testing of clinical isolates and comparison with other methods should be conducted to tune the interpretation algorithm further. To increase the accuracy and specificity of the assay, one solution is to use *orfX* for the detection of methicillin-resistance and *S. aureus* simultaneously. Using *orfX* will also reduce the necessary targets, as *mecA*, *nuc*, and *coa* will not be needed. This comes, however, at the cost of reduces information from

the result. Finally, the PCR reaction with the TaqPath master mix takes approximately 40 minutes. The reaction includes two minutes of UNG incubation, 15 minutes of reverse transcription, and 22 minutes for the thermal cycling. The time can be shortened to 22 minutes by omitting the UNG incubation and reverse transcription; however, there will be a trade-off between time and sensitivity and specificity. Using the assay with other master mixes might provide a solution to minimize the trade-off, but will require investigation. Another major factor in the turn-around time (from specimen reception to result) is DNA extraction. This study used the NucliSENSE EasyMAG extraction platform; however, other extraction methodologies can be used, some of which might reduce the total time needed. As for cost, while made-to-order primers and probes are cheaper than commercial kits, the cost of the instrumentation can be a problem. Hence, it is up to the laboratory to choose between time and cost.

As for the developed LAMP, it had low sensitivity (limit of detection= 10^6 CFU/ml). The reasons for the low sensitivity range from the design of the primers to the conditions at which they were synthesized. In both assays, further investigations may improve the results. For the LAMP assay, a better understanding of the mechanism and the factors that affect it, such as inhibitory molecules, can improve the design workflow. As can be seen in the study, the LAMP assay design is not as well established as PCR assays. With PCR, there exists a well-defined workflow with various software dedicated to the design of the primers for many purposes. Additionally, the expertise in PCR is widespread, and resources are plentiful. LAMP is a relatively recently developed technique and as of yet, a relatively less applied technique in infectious disease diagnostics.

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APPENDIX A: Target Gene Reference Sequences

Gene	Accession	Sequence
mecA	NG_047945.1	ATGAAAAAGATAAAAATTGTTCCACTTATTTTAATAGTTGTAGTTGTCGGGTTTGGTATATATTTTTAT GCTTCAAAAGATAAAGAAATTAATAATACTATTGATGCAATTGAAGATAAAAATTTCAAACAAGTTT ATAAAGATAGCAGTTATATTTCTAAAAGCGATAATGGTGAAGTAGAAATGACTGAACGTCCGATAAA AATATATAATAGTTTAGGCGTTAAAGATATAAACATTCAGGATCGTAAAATAAAAAAAGTATCTAAA AATAAAAAACGAGTAGATGCTCAATATAAAATTAAAACAAACTACGGTAACATTGATCGCAACGTTC AATTTAATTTTGTTAAAGAAGATGGTATGTGGAAGTTAGATTGGGATCATAGCGTCATTATTCCAGGA ATGCAGAAAGACCAAAGCATACATATTGAAAATTTAAAATCAGAACGTGGTAAAATTTTAGACCGA AACAATGTGGAATTGGCCAATACAGGAACAGCATATGAGATAGGCATCGTTCCAAAGAATGTATCTA AAAAAGATTATAAAGCAATCGCTAAAGAACTAAGTATTTCTGAAGACTATATCAAACAACAAATGG ATCAAAATTGGGTACAAGATGATACCTTCGTTCCACTTAAAACCGTTAAAAAAATGGATGAATATTT AAGTGATTTCGCAAAAAAATTTCATCTTACAACTAATGAAACAAAAAGTCGTAACTATCCTCTAGAA AAAGCGACTTCACATCTATTAGGTTATGTTGGTCCCATTAACTCTGAAGAATTAAAACAAAAAGAAT ATAAAGGCTATAAAGATGATGCAGTTATTGGTAAAAAGGGACTCGAAAAACTTTACGATAAAAAGC TCCAACATGAAGATGGCTATCGTGTCACAATCGTTGACGATAATAGCAATACAATCGCACATACATT AATAGAGAAAAAGAAAAAAGATGGCAAAGATATTCAACTAACTATTGATGCTAAAGTTCAAAAGAG TATTTATAACAACATGAAAAATGATTATGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTAT TAGCACTTGTAAGCACACCTTCATATGACGTCTATCCATTTATGTATGGCATGAGTAACGAAGAATAT AATAAATTAACCGAAGATAAAAAAGAACCTCTGCTCAACAAGTTCCAGATTACAACTTCACCAGGTT CAACTCAAAAAATATTAACAGCAATGATTGGGTTAAATAACAAAACATTAGACGATAAAACAAGTT ATAAAATCGATGGTAAAGGTTGGCAAAAAGATAAATCTTGGGGTGGTTACAACGTTACAAGATATGA AGTGGTAAATGGTAATATCGACTTAAAACAAGCAATAGAATCATCAGATAACATTTTCTTTGCTAGA GTAGCACTCGAATTAGGCAGTAAGAAATTTGAAAAAGGCATGAAAAAACTAGGTGTTGGTGAAGAT ATACCAAGTGATTATCCATTTTATAATGCTCAAATTTCAAACAAAAATTTAGATAATGAAATATTATT AGCTGATTCAGGTTACGGACAAGGTGAAATACTGATTAACCCAGTACAGATCCTTTCAATCTATAGC GCATTAGAAAATAATGGCAATATTAACGCACCTCACTTATTAAAAGACACGAAAAACAAAGTTTGGA AGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCAACAAGTCGTAAATAA AACACATAAAGAAGATATTTATAGATCTTATGCAAACTTAATTGGCAAATCCGGTACTGCAGAACTC AAAATGAAACAAGGAGAAACTGGCAGACAAATTGGGTGGTTTATATCATATGATAAAGATAATCCA AACATGATGATGGCTATTAATGTTAAAGATGTACAAGATAAAGGAATGGCTAGCTACAATGCCAAAA TCTCAGGTAAAGTGTATGATGAGCTATATGAGAACGGTAATAAAAAATACGATATAGATGAATAACA AAACAGTGAAGCAATCCGTAACGATGGTTGCTTCACTGTTTTATTATGAATTATTAATAAGTGCTGTT ACTTCTCCCTTAAATACAATTTCTTCATTT

Gene	Accessio	Sequence
mecC	NG_047955.1	TGTTCACACCTCACTTCTTAACTATTATATCATTATTTTGACAAACAGACTACAAATGTAATATTATTG GATTACATTTGTAGTACAAAAGGAGGAAGAGATGAAAAAAATTTATATTAGTGTGCTAGTTCTTTTA CTAATTATGATTATAATAACTTGGTTATTCAAAGATGACGATATTGAGAAAACAATTAGTTCTATTGA AAAAGGAAACTATAACGAAGTATATAAAAATAGTTCAGAAAAATCTAAACTGGCATATGGAGAAGA AGAAATTGTAGATAGGAATAAAAAAATTTACAAAGATTTAAGTGTCAATAACTTAAAAATTACTAAT CATGAAATTAAAAAAACTGGAAAAGATAAAAAGCAAGTTGATGTTAAATATAACATATATACAAAA TATGGAACTATACGACGTAATACACAATTAAACTTTATTTATGAAGATAAGCATTGGAAATTAGATT GGAGACCAGACGTAATAGTACCTGGTTTGAAAAATGGACAGAAAATTAATATAGAAACATTAAAAT CAGAGCGAGGCAAAATAAAAGATAGAAATGGTATAGAATTAGCTAAAACTGGAAATACATATGAAA TCGGTATTGTCCCTAACAAAACACCCAAAGAAAAATATGATGATATTGCTCGTGACTTACAAATTGA TACAAAAGCTATAACCAATAAAGTTAATCAAAAATGGGTTCAGCCAGATTCATTTGTACCAATTAAA AAGATAAATAAACAAGATGAATATATAGACAAATTAATTAAATCATACAATTTACAAATAAACACTA TAAAAAGCCGTGTTTATCCATTGAACGAAGCAACAGTACACCTTTTAGGTTATGTGGGTCCAATTAAT TCTGACGAGTTAAAAAGTAAGCAATTTAGAAACTATAGCAAAAATACTGTTATTGGAAAAAAAGGCT TAGAACGCCTCTATGATAAACAATTGCAAAACACTGATGGTTTTAAGGTATCCATTGCAAATACTTAT GACAATAAACCTTTAGACACATTATTGGAGAAAAAGGCTGAAAACGGAAAAGATCTTCATTTAACTA TAGATGCTAGAGTACAAGAAAGTATTTATAAACATATGAAAAATGACGATGGATCTGGTACAGCATT ACAACCAAAAACTGGAGAAATTTTAGCTTTGGTAAGTACCCCATCGTACGATGTTTATCCATTCATGA ATGGATTAAGCAATAATGACTACCGTAAATTAACTAACAATAAAAAAGAGCCTTTGCTCAACAAATT TCAAATCACTACATCACCAGGTTCAACCCAAAAAATATTAACATCTATTATAGCCTTAAAAGAAAAT AAACTAGACAAAAATACTAATTTTGATATTTATGGTAAGGGTTGGCAAAAAGATGCATCATGGGGTA ATTATAATATCACAAGATTTAAAGTAGTAGACGGCAATATCGATTTAAAGCAAGCAATAGAATCATC AGACAACATATTTTTTGCCCGCATTGCATTAGCATTAGGAGCCAAAAAATTTGAGCAAGGTATGCAA GATTTGGGAATCGGTGAAAATATCCCGAGTGATTATCCCTTTTATAAAGCACAAATCTCAAATAGTA ATTTAAAAAATGAAATATTATTAGCAGATTCAGGATATGGCCAAGGCGAGATACTAGTAAACCCTAT ACAAATTTTATCAATATACAGTGCTTTAGAAAATAACGGAAATATACAAAATCCTCATGTTTTACGTA AAACAAAATCTCAAATATGGAAAAAAGATATTATACCTAAAAAAGACATAGATATATTAACTAATG GTATGGAACGTGTAGTTAATAAAACACATAGGGATGATATATACAAAAATTATGCCCGAATTATTGG TAAATCTGGCACAGCAGAATTAAAAATGAATCAAGGGGAAACTGGAAGACAAATAGGTTGGTTTGT TTCATATAATAAAAATAATCCTAATATGTTAATGGCGATTAATGTTAAAGACGTTCAAAATAAAGGG ATGGCCAGCTATAATGCTACTATATCTGGAAAAGTTTATGATGATTTGTATGATAATGGAAAAACTC AATTTGATATAGATCAGTAATTCGAATACTTCTTTTGACTTGGTATTAATTAAAAATAATAGTGAGAA GCGTTTCCACAAAGATTACATTTGTAATATATAGGAGGAATAAAATTGAAAA

Gene	Accessio	Sequence
nuc	NC_007795.1:800034-800720	ATGACAGAATACTTATTAAGTGCTGGCATATGTATGGCAATCGTTTCAATATTACTTATAGGGATGGC TATCAGTAATGTTTCGAAAGGGCAATACGCAAAGAGGTTTTTCTATTTCGCTACTAGTTGTTTAGTGT TAACTTTAGTTGTAGTTTCAAGTCTAAGTAGCTCAGCAAATGCATCACAAACAGATAATGGCGTAAA TAGAAGTGGTTCTGAAGATCCAACAGTATATAGTGCAACTTCAACTAAAAAATTACATAAAGAACCT GCGACATTAATTAAAGCGATTGATGGTGATACGGTTAAATTAATGTACAAAGGTCAACCAATGACAT TCAGACTATTATTGGTTGATACACCTGAAACAAAGCATCCTAAAAAAGGTGTAGAGAAATATGGTCC TGAAGCAAGTGCATTTACGAAAAAAATGGTAGAAAATGCAAAGAAAATTGAAGTCGAGTTTGACAA AGGTCAAAGAACTGATAAATATGGACGTGGCTTAGCGTATATTTATGCTGATGGAAAAATGGTAAAC GAAGCTTTAGTTCGTCAAGGCTTGGCTAAAGTTGCTTATGTTTATAAACCTAACAATACACATGAACA ACTTTTAAGAAAAAGTGAAGCACAAGCAAAAAAAGAGAAATTAAATATTTGGAGCGAAGACAACGC TGATTCAGGTCAATAA

Gene	Accession	Sequence
coa	AJ396908	GTTTATTCTAGTTAATATATAGTTAATGTCTTTTAATATTTTGTTTCTTTAATGTAGATTGGGCAATTA CATTTTGGAGGAATTAAAAAATTATGAAAAAGCAAATAATTTCGCTAGGCGCATTAGCAGTTGCATC TAGCTTATTTACATGGGATAACAAAGCAGATGCGATAGTAACAAAGGATTATAATGGGAAATCACAA GTTAATGCTGGGAGTAAAAATGGGACATTAATAGATAGCAGATATTTAAATTCAGCTCTATATTATTT GGAAGACTATATAATTTATGCTATAGGATTAACTAATAAATATGAATATGGAGATAATATTTATAAA GAAGCTAAAGATAGGTTGTTGGAAAAGGTATTAAGGGAAGATCAATATCTTTTGGAGAGAAAGAAA TCTCAATATGAAGATTATAAACAATGGTATGCAAATTATAAAAAAGAAAATCCTCGTACAGATTTAA AAATGGCTAATTTTCATAAATATAATTTAGAAGAACTTTCGATGAAAGAATACAATGAACTACAGGA TGCATTAAAGAGAGCACTGGATGATTTTCACAGAGAAGTTAAAGATATTAAGGATAAGAATTCAGAC TTGAAAACTTTTAATGCAGCAGAAGAAGATAAAGCAACTAAGGAAGTATACGATCTCGTATCTGAAA TTGATACATTAGTTGTATCATATTATGGTGATAAGGATTATGGGGAGCACGCGAAAGAGTTACGAGC AAAACTGGACTTAATCCTTGGAGATACAGACAATCCACATAAAATTACAAATGAACGTATTAAAAAA GAAATGATTGATGACTTAAATTCAATTATTGATGATTTCTTTATGGAAACTAAACAAAATAGACCGA AATCTATAACGAAATATAATCCTACCACACATAACTATAAAACAAATAGTGATAATAAACCTAATTT TGATAAATTAGTTGAAGAAACGAAAAAAGCAGTTAAAGAAGCAGATGATTCTTGGAAAAAGAAAAC TGTCAAAAAATACGGAGAAACTGAAACAAAATCGCCAGTAGTAAAAGAAGAGAAGAAAGTTGAAG AACCTCAATTACCTAAAGTTGGAAACCAGCAAGAGGATAAAACTACAGTTGATAAAGCTGAAGAAA CAACACAACCAGTGGCACAGCCATTAGTTAAAATTCCACAGGGCACAATTACAGGTGAAATTGTAAA AGGTCCAGACTATCCAACTATGGAAAATAAAACGTTACAAGGTGAAATCGTTCAAGGTCCAGATTTC CCAACAATGGAACAAAACAGACCATCTTTAAGCGATAATTATACTCAACCGACGACACCGAACCCTA TTTTAGAAGGTCTTGAAAGTAGCTCATCTAAACTTGAAATAAAACCACAAGGTACTGAATCAACGTT GAAAGGTATTCAAGGAGAATCAAGTGATATTGAGGTTAAACCTCAAGCATCTGAAACAACAGAAGC ATCACATTATCCAGCGAGACCGCAATTTAACAAAACACCTAAATATGTTAAATATAGAGATGCTGGT ACAGGTATCCGTGAATACAACGATGGAACATTTGGATATGAAGCGAGACCAAGATTCAATAAGCCAT CAGAAACAAACGCATACAACGTAACGACAAATCAAGATGGCACAGTAACATATGGCGCTCGTCCAA CACAAAACAAGCCAAGTAAAACGAACGCATACAACGTAACGACAAATCAAGATGGCACAGTAACAT ATGGCGCTCGTCCGACATACAAGAAGCCAAGCGAAACAAATGCATACAATGTAACAACACATGCAA ACGGTCAAGTATCATACGGCGCTCGTCCGACATACAACAAGCCAAGCAAAACAAATGCATATAACGT AACAACACATGCAAACGGTCAAGTATCATATGGCGCCCGCCCGACATACAACAAGCCAAGCAAAAC AAATGCATACAACGTAACAACACATGCAAACGGCCAAGTATCATATGGCGCTCGCCCGACACAAAA CAAGCCAAGCGAAACAAACGCATATAACGTAACAACACATGCAAATGGTCAAGTGTCATACGGGGC TCGCCCGACACAAAACAAGCCAAGTAAAACAAACGCATATAACGTAACAACACATGCAGATGGTAC TGCGACATATGGGCCTAGAGTAACAAAATAA

Gene	Accessio	Sequence
coa	AJ306908.1	GTTTATTCTAGTTAATATATAGTTAATGTCTTTTAATATTTTGTTTCTTTAATGTAGATTGGGCAATTA CATTTTGGAGGAATTAAAAAATTATGAAAAAGCAAATAATTTCGCTAGGCGCATTAGCAGTTGCATC TAGCTTATTTACATGGGATAACAAAGCAGATGCGATAGTAACAAAGGATTATAATGGGAAATCACAA GTTAATGCTGGGAGTAAAAATGGGACATTAATAGATAGCAGATATTTAAATTCAGCTCTATATTATTT GGAAGACTATATAATTTATGCTATAGGATTAACTAATAAATATGAATATGGAGATAATATTTATAAA GAAGCTAAAGATAGGTTGTTGGAAAAGGTATTAAGGGAAGATCAATATCTTTTGGAGAGAAAGAAA TCTCAATATGAAGATTATAAACAATGGTATGCAAATTATAAAAAAGAAAATCCTCGTACAGATTTAA AAATGGCTAATTTTCATAAATATAATTTAGAAGAACTTTCGATGAAAGAATACAATGAACTACAGGA TGCATTAAAGAGAGCACTGGATGATTTTCACAGAGAAGTTAAAGATATTAAGGATAAGAATTCAGAC TTGAAAACTTTTAATGCAGCAGAAGAAGATAAAGCAACTAAGGAAGTATACGATCTCGTATCTGAAA TTGATACATTAGTTGTATCATATTATGGTGATAAGGATTATGGGGAGCACGCGAAAGAGTTACGAGC AAAACTGGACTTAATCCTTGGAGATACAGACAATCCACATAAAATTACAAATGAACGTATTAAAAAA GAAATGATTGATGACTTAAATTCAATTATTGATGATTTCTTTATGGAAACTAAACAAAATAGACCGA AATCTATAACGAAATATAATCCTACCACACATAACTATAAAACAAATAGTGATAATAAACCTAATTT TGATAAATTAGTTGAAGAAACGAAAAAAGCAGTTAAAGAAGCAGATGATTCTTGGAAAAAGAAAAC TGTCAAAAAATACGGAGAAACTGAAACAAAATCGCCAGTAGTAAAAGAAGAGAAGAAAGTTGAAG AACCTCAATTACCTAAAGTTGGAAACCAGCAAGAGGATAAAACTACAGTTGATAAAGCTGAAGAAA CAACACAACCAGTGGCACAGCCATTAGTTAAAATTCCACAGGGCACAATTACAGGTGAAATTGTAAA AGGTCCAGACTATCCAACTATGGAAAATAAAACGTTACAAGGTGAAATCGTTCAAGGTCCAGATTTC CCAACAATGGAACAAAACAGACCATCTTTAAGCGATAATTATACTCAACCGACGACACCGAACCCTA TTTTAGAAGGTCTTGAAAGTAGCTCATCTAAACTTGAAATAAAACCACAAGGTACTGAATCAACGTT GAAAGGTATTCAAGGAGAATCAAGTGATATTGAGGTTAAACCTCAAGCATCTGAAACAACAGAAGC ATCACATTATCCAGCGAGACCGCAATTTAACAAAACACCTAAATATGTTAAATATAGAGATGCTGGT ACAGGTATCCGTGAATACAACGATGGAACATTTGGATATGAAGCGAGACCAAGATTCAATAAGCCAT CAGAAACAAACGCATACAACGTAACGACAAATCAAGATGGCACAGTAACATATGGCGCTCGTCCAA CACAAAACAAGCCAAGTAAAACGAACGCATACAACGTAACGACAAATCAAGATGGCACAGTAACAT ATGGCGCTCGTCCGACATACAAGAAGCCAAGCGAAACAAATGCATACAATGTAACAACACATGCAA ACGGTCAAGTATCATACGGCGCTCGTCCGACATACAACAAGCCAAGCAAAACAAATGCATATAACGT AACAACACATGCAAACGGTCAAGTATCATATGGCGCCCGCCCGACATACAACAAGCCAAGCAAAAC AAATGCATACAACGTAACAACACATGCAAACGGCCAAGTATCATATGGCGCTCGCCCGACACAAAA CAAGCCAAGCGAAACAAACGCATATAACGTAACAACACATGCAAATGGTCAAGTGTCATACGGGGC TCGCCCGACACAAAACAAGCCAAGTAAAACAAACGCATATAACGTAACAACACATGCAGATGGTAC TGCGACATATGGGCCTAGAGTAACAAAATAA

APPENDIX B: *In silico* Specificity Reference Sequences

NCBI Accession	Organism
NZ_CP009623.1	*Staphylococcus agentis* strain 908
NC_016941.1	*Staphylococcus argenteus* MSHR1132
NZ_CP025023.1	*Staphylococcus argenteus* strain XNO106
NZ_CP010296.1	*Staphylococcus aureus* strain 31b_MRSA
NZ_CP014791.1	*Staphylococcus aureus* Strain MCRF184
NZ_CP019117.1	*Staphylococcus aureus* strain SJTUF_J27
NC_017340.1	*Staphylococcus aureus* 04-02981
NZ_CP025395.1	*Staphylococcus aureus* O46
NZ_CP009361.1	*Staphylococcus aureus* strain ATCC 25923
NZ_CP010402.1	*Staphylococcus aureus* strain GR2
NZ_CP020020.1	*Staphylococcus aureus* strain ATCC 6538
NZ_CP022290.1	*Staphylococcus aureus* strain EDCC5458
NZ_CP022291.1	*Staphylococcus aureus* strain EDCC5464
NZ_CP029031.1	*Staphylococcus aureus* strain CTI
NC_022226.1	*Staphylococcus aureus* strain CN1
NC_002951.2	*Staphylococcus aureus* strain COL
NZ_CP011526.1	*Staphylococcus aureus* strain DSM 20231
NC_009632.1	*Staphylococcus aureus* strain JH1
NC_009487.1	*Staphylococcus aureus* strain JH9
NC_017338.1	*Staphylococcus aureus* strain JKD6519
NC_002758.2	*Staphylococcus aureus* strain Mu50
NC_007795.1	*Staphylococcus aureus* strain

NCBI Accession	Organism
NC_017341.1	*Staphylococcus aureus* strain JKD6008
NZ_CP012409.1	*Staphylococcus aureus* strain Tager 104
NZ_CP007601.1	*Staphylococcus capitis* strain AYP1020
NZ_CP016760.1	*Staphylococcus carnosus* strain LTH 3730
NZ_CP015114.1	*Staphylococcus condiment* strain DSM 11674
NZ_CP018776.1	*Staphylococcus condiment* strain StO 2014-01
NZ_CP009046.1	*Staphylococcus epidermidis* strain SEI
NZ_CP020463.1	*Staphylococcus epidermidis* strain 1457
NZ_CP022247.1	*Staphylococcus epidermidis* strain ATCC 12228
NZ_CP018842.1	*Staphylococcus epidermidis* strain 14.1.R1
NC_004461.1	*Staphylococcus epidermidis* strain ATCC 12228
NC_002976.3	*Staphylococcus epidermidis* strain RP62A
NZ_CP013114.1	*Staphylococcus equorum* strain KS1039
NZ_CP013980.1	*Staphylococcus equorum* strain KM1031
NZ_CP027770.1	*Staphylococcus felis* strain ATCC 49168
NZ_CP013911.1	*Staphylococcus haemolyticus* strain S167
NZ_CP025396.1	*Staphylococcus haemolyticus* strain 83131B
NZ_CP024809.1	*Staphylococcus haemolyticus* strain 83131A
NZ_CP025031.1	*Staphylococcus haemolyticus* strain SGAir0252
NZ_CP027846.1	*Staphylococcus kloosii* strain ATCC 43959
NZ_CP014022.1	*Staphylococcus lugdunensis* strain FDAARGOS_141
NC_013893.1	*Staphylococcus lugdunensis* strain HKU09-01
NC_017353.1	*Staphylococcus lugdunensis* strain N920143
NZ_CP027848.1	*Staphylococcus muscae* strain ATCC 49910

NCBI Accession	Organism
NZ_CP017460.1	*Staphylococcus nepalensis* strain JS1
NZ_CP017463.1	*Staphylococcus pasteuri* strain JS7
NC_022737.1	*Staphylococcus pasteuri* strain SP1
NZ_CP016072.1	*Staphylococcus pseudintermedius* strain NA45
NZ_CP016073.1	*Staphylococcus pseudintermedius* strain 081661
NZ_CP015626.1	*Staphylococcus pseudintermedius* strain 063228
NC_017568.1	*Staphylococcus pseudintermedius* strain ED99
NC_014925.1	*Staphylococcus pseudintermedius* strain HKU10-03
NC_007350.1	*Staphylococcus saprophyticus* strain ATCC 15305
NZ_CP010309.1	*Staphylococcus schleiferi* strain 2317-03
NZ_CP009762.1	*Staphylococcus schleiferi* strain 2142-05
NZ_CP009676.1	*Staphylococcus schleiferi* strain 5909-02
NZ_CP009470.1	*Staphylococcus schleiferi* strain 1360-13
NZ_AP014944.1	*Staphylococcus schleiferi* strain TSCC54
NZ_CP018199.1	*Staphylococcus succinus* strain 14BME20
NC_020164.1	*Staphylococcus warneri* strain SG1
NZ_CP007208.1	*Staphylococcus xylosus* strain HKUOPL8
NZ_CP008724.1	*Staphylococcus xylosus* strain SMQ-121
NZ_CP013922.1	*Staphylococcus xylosus* strain S170

APPENDIX C: Novel Real-Time PCR *coa* Primer/Probe Sequence Alignment for *In Silico* Specificity

Forward primer

Reverse Primer

Probe

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Query  1        TCGTTCAAGGTCCCGATTTTCTAACAATGGAACAAAGCGGCCCATCATTAAGCAATAATTATACAAACCCACCG  74

CP031661.1  212331   ....................................................................  212404
CP031670.1  232946   ....................................................................  233019
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CP022902.1  250353   ....................................................................  250426
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CP029678.1  266092   ....................................................................  266165
CP029667.1  2570180  ....................................................................  2570107
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CP029166.1  246416   ....................................................................  246489
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CP027101.1  2002719  ....................................................................  2002646
CP027476.1  267559   ....................................................................  267632
CP012119.2  2292025  ....................................................................  2292098
CP026962.1  354305   ....................................................................  354378
CP026961.1  1094306  ....................................................................  1094379
CP026960.1  2374652  ....................................................................  2374725
CP016858.2  1113219  ....................................................................  1113146
CP016855.2  1803936  ....................................................................  1803863
CP007539.3  2137715  ....................................................................  2137642
CP026080.1  1791865  ....................................................................  1791792
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CP026077.1 952476 952549

CP026076.1 1114607 1114680

CP026072.1 1399855 1399782

CP026070.1 823364 823291

CP026068.1 2410506 2410579

CP012120.2 1104379 1104306

CP017094.2 1809782 1809709

CP016861.2 1104378 1104305

CP025495.1 1104378 1104305

CP016863.2 2687172 2687099

CP022290.1 210693 210766

CP023500.1 2727892 2727965

CP023391.1 212868 212941

CP023390.1 212868 212941

AP014921.1 234758 234831

CP019590.1 268244 268317

CP020619.1 267787 267860

CP014444.1 267401 267474

CP014441.1 267399 267472

CP014438.1 267399 267472

CP014435.1 267397 267470

CP014432.1 267399 267472

CP014429.1 267397 267470

CP014426.1 267397 267470

CP014423.1 267397 267470

CP014420.1 267397 267470

CP014415.1 267401 267474

CP014412.1 267397 267470

CP014409.1 267397 267470

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CP014397.1 267321 267394

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CP014381.1 267397 267470

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CP014362.1 267278 267351

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LT671859.1 235438 235511

CP018205.1 212890 212963

AP017377.1 248942 249015

LT598688.1 212861 212934

CP013231.1 2172073 2172146

CP007676.1 233217 233290

CP007672.1 257132 257205

CP007657.1 236894 236967

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CP007674.1 240684 240757

CP007670.1 256996 257069

CP011526.1 212090 212163

CP010300.1 267564 267637

CP010299.1 267564 267637

CP010298.1 267563 267636

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CP010296.1 267564 267637

CP010295.1 267564 267637

CP007499.1 245816 245889

CP007690.1 267456 267529

CP007176.1 268276 268349

HF937103.1 264602 264675

CP003033.1 212890 212963

AB489885.1 1160 1233

AB489883.1 1160 1233

AB489874.1 1148 1221

AB489873.1 1160 1233

Note: All accession listed are *coa* genes

APPENDIX D: Novel Real-Time PCR *mecA* Primer/Probe Sequence Alignment for *In Silico* Specificity

Forward primer

Reverse Primer

Probe

Query 1 TTAGATTGGGATCATAGCGTCATTATTCCAGGAATGCAGAAAGACCAAAGCATACATATTGAAAATTTAAAATCAGAACGTGGTAAAATTTTAGACCGAAACAATGTGGAAT 112

CP031779.1 45152 45041

CP031537.1 1601507 1601396

CP022905.1 40763 40652

CP022908.1 40691 40580

CP022906.1 40691 40580

CP022904.1 40763 40652

CP022903.1 40763 40652

CP022902.1 40691 40580

CP022894.1 40763 40652

CP022893.1 40763 40652

CP031131.1 44502 44391

LT992477.1 1662989 1663100

LT992476.1 176025 176136

LT992475.1 2423162 2423273

LT992474.1 632787 632676

LT992473.1 1868717 1868828

LT992472.1 985333 985222

LT992471.1 91457 91346

LT992470.1 1772428 1772539

LT992469.1 2249342 2249453

LT992468.1 1364086 1363975

LT992467.1 1426438 1426327

LT992466.1 227147 227258

LT992465.1 1690005 1690116

LT992464.1 2638320 2638431

LT992463.1 232902 232791

LT992462.1 2614889 2614778

LT992461.1 1000036 999925

LT992460.1 923512 923623

LT992458.1 564210 564321

LT992456.1 1272784 1272895

CP030326.1 39812 39701

MH188482.1 17127 17016

MH188467.1 10841 10730

CP030323.1 453890 454001

MF774211.1 145 256

LS483319.1 40836 40725

LS483316.1 42876 42765

LS483309.1 87510 87399

LS483301.1 39799 39688

LS483484.1 46037 45926

CP029673.1 45158 45047

CP029664.1 2632451 2632340

CP029663.1 45148 45037

CP029657.1 45158 45047

CP021171.1 42529 42418

CP021141.1 41377 41266

CP029680.1 2499821 2499932

CP029681.1 2373863 2373974

CP029655.1 518324 518435

CP029658.1 93474 93585

CP029678.1 40224 40113

CP029649.1 1747793 1747904

CP029675.1 46116 46005

CP029667.1 2796949 2797060

CP029669.1 2205161 2205272

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CP015447.2 78441 78330

CP029172.1 48683 48572

CP029166.1 42052 41941

MF185206.1 4665 4554

CP029087.1 45158 45047

CP029086.1 1377533 1377422

CP029082.1 843862 843973

CP029080.1 505508 505397

CP029032.1 40761 40650

CP029030.1 40761 40650

CP029031.1 40761 40650

MF383340.1 196 307

CP020544.1 45797 45686

CP021105.1 42587 42476

CP020553.1 48473 48362

CP028190.1 904738 904627

CP028163.1 453460 453571

MG787423.1 7731 7620

CP027486.1 40969 40858

CP027101.1 2227741 2227852

CP027476.1 40761 40650

MG674089.1 24813 24924

MF278654.1 1673 1562

MF278653.1 1661 1550

CP012119.2 2065305 2065194

CP025031.1 879787 879898

CP026968.1 2723285 2723174

CP026958.1 1157965 1158076

CP026957.1 2093267 2093378

CP026953.1 1317804 1317693

CP016858.2 1339966 1340077

CP016855.2 2031446 2031557

CP007539.3 2343240 2343351

CP014119.1 1040946 1041057

CP014107.1 1760952 1760841

CP026074.1 1401227 1401116

CP026064.1 1417047 1417158

CP025396.1 70801 70912

CP024809.1 70801 70912

CP026079.1 826739 826850

CP026073.1 2081857 2081968

CP026072.1 1602957 1603068

Note: All accession listed are *mecA* genes

APPENDIX E: Novel Real-Time PCR *mecC* Primer/Probe Sequence Alignment for *In Silico* Specificity

Forward primer

Reverse Primer

Probe

```
Query       1  GCAAGCAATAGAATCATCAGACAACATATTTTTTGCCCGCATTGCATTAGCATTAGGAGCCAAAAAATTTGAGCAAGGTATGCAAGA  87
CP028165.1  1562939  ..........................................................................  1562853
NG_047955.1 1459  ..........................................................................  1545
KU867950.1  16862  ..........................................................................  16948
KR732654.1  2623  ..........................................................................  2537
KT192641.1  1359  ..........................................................................  1445
LK024544.1  5173  ..........................................................................  5087
HF569116.1  2380  ..........................................................................  2294
KC110686.1  1237  ..........................................................................  1323
JN794592.1  151  ..........................................................................  237
FR821779.1  36320  ..........................................................................  36234
FR823292.1*  2380  ..........................................................................  2294
KF955540.2  1794  ..................................A..T..........T.......T.....................  1880
HG515014.1  43635  ..................T..T..T.....................G..T.....................  43549
HG515014.1  12528  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  12445
HE993884.1  12672  ............G.....................A..T..........T.....C..T.....................  12586
MH155596.1  120181  ..................T.............A..T..........T.......T.............A.....A....  120095
MG334392.1  60  ............G.................A.....A..T.....  1
MG334391.1  60  ............G.................A.....A..T.....  1
NG_047950.1 1379  .....T..T..........T..T..C..C.....GA.AG.....C.T.A.....AG.....C..AG.....A..  1462
KF058902.1  1282  .....T..T..........T..T..C..C.....GA.AG.....C.T.A.....AG.....C..AG.....A..  1365
KF058901.1  1291  .....T..T..........T..T..C..C.....GA.AG.....C.T.A.....AG.....C..AG.....A..  1374
KF058900.1  1291  .....T..T..........T..T..C..C.....GA.AG.....C.T.A.....AG.....C..AG.....A..  1374
AY820253.1  1379  .....T..T..........T..T..C..C.....GA.AG.....C.T.A.....AG.....C..AG.....A..  1462
CP031779.1  44156  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  44073
MH607131.1  16  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  99
CP031537.1  1600511  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  1600428
LC414617.1  94  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  177
CP022905.1  39767  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  39684
CP022908.1  39695  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  39612
CP022906.1  39695  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  39612
CP022904.1  39767  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  39684
CP022903.1  39767  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  39684
CP022902.1  39695  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  39612
CP022894.1  39767  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  39684
CP022893.1  39767  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  39684
CP022582.1  40554  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  40637
CP031130.1  43507  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  43424
CP031131.1  43506  ..................T.....T..C.....TA.AG.A...C.C.A......CAGT..G.....AA....C...A..  43423
```

LT992477.1 1663985 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1664068

LT992476.1 177021 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 177104

LT992475.1 2424158 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2424241

LT992474.1 631791 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 631708

LT992473.1 1869713 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1869796

LT992472.1 984337 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 984254

LT992471.1 90461 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 90378

LT992470.1 1773424 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1773507

LT992469.1 2250338 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2250421

LT992468.1 1363090 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1363007

LT992467.1 1425442 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1425359

LT992466.1 228143 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 228226

LT992465.1 1691001 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1691084

LT992464.1 2639316 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2639399

LT992463.1 231906 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 231823

LT992462.1 2613893 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2613810

LT992461.1 999040 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 998957

LT992460.1 924508 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 924591

LT992458.1 565206 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 565289

LT992456.1 1273780 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1273863

CP030326.1 38816 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 38733

MH188482.1 16131 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 16048

MH188467.1 9845 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 9762

CP030323.1 454886 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 454969

CP029685.1 61204 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 61287

CP027788.1 77623 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 77540

LS483319.1 39840 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 39757

LS483316.1 41880 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 41797

LS483309.1 86514 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 86431

LS483301.1 38803 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 38720

LS483484.1 45041 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 44958

CP029673.1 44162 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 44079

CP029664.1 2631455 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2631372

CP029663.1 44152 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 44069

CP029657.1 44162 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 44079

CP021171.1 41533 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 41450

CP021141.1 40381 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 40298

CP029680.1 2500817 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2500900

CP029681.1 2374859 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2374942

CP029655.1 519320 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 519403

CP029658.1 94470 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 94553

CP029678.1 39228 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 39145

CP029649.1 1748789 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1748872

CP029675.1 45120 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 45037

CP029667.1 2797945 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2798028

CP029669.1 2206157 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 2206240

CP029653.1 47550 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 47467

CP029652.1 1638880 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1638963

CP015447.2 77445 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 77362

CP029172.1 47687 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 47604

CP029166.1 41056 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 40973

MF185206.1 3669 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 3586

CP029087.1 44162 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 44079

CP029086.1 1376537 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 1376454

CP029084.1 735457 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 735374

CP029082.1 844858 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 844941

CP029080.1 504512 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 504429

CP029032.1 39765 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 39682

CP029030.1 39765 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 39682

CP029031.1 39765 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 39682

CP020544.1 44801 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 44718

CP021105.1 41591 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 41508

CP020553.1 47454 .....T.....T..C.....TA.AG.A...C.C.A.....CAGT..G.....AA....C...A.. 47371

Note: All accession listed are *mecC* genes. All accessions after the underlined accession marked with * are non *S. aureus* species.

APPENDIX F: Novel Real-Time PCR *nuc* Primer/Probe Sequence Alignment for *In Silico* Specificity

Forward primer

Reverse Primer

Probe

Query 1

ATTTCGCTACTAGTTGTTTAGTGTTAACTTTAGTTGTAGTTTCAAGTCTAAGTAGCTCAGCAAATGCATCACAAACAGATAATGGCGTAAATAGAAGTGGTTCTGAAGATCCAACAGTATAT

AGTG 126

CP031661.1 813802 813927

CP031670.1 831670 831795

CP031664.1 813802 813927

CP031673.1 2089402 2089277

CP031667.1 831670 831795

CP031537.1 2435625 2435750

CP022910.1 813119 813244

CP022908.1 854898 855023

CP022906.1 854901 855026

CP022902.1 854897 855022

CP022892.1 817272 817397

CP029685.1 978832 978957

CP027788.1 938327 938452

LS483319.1 852640 852765

LS483316.1 837535 837660

LS483309.1 926632 926757

LS483301.1 874700 874825

LS483365.1 808776 808901

LS483350.1 834943 835068

CP029664.1 1819486 1819361

CP029678.1 884151 884276

CP029667.1 1952260 1952135

CP029669.1 1318637 1318512

CP015447.2 896144 896269

CP029166.1 919631 919756

CP029032.1 866327 866452

CP029030.1 866327 866452

CP029031.1 866327 866452

CP027101.1 1384945 1384820

CP027476.1 866322 866447

CP012119.2 2906380 2906505

CP026962.1 950714 950839

CP026961.1 1690714 1690839

CP026960.1 186985 187110

CP016858.2 500327 500202

CP016855.2 1189831 1189706

CP007539.3 1496696 1496571

CP026080.1 1195457 1195332

CP026077.1 1548751 1548876

CP026076.1 1719528 1719653

CP026072.1 755923 755798

CP026070.1 226957 226832

CP026068.1 309366 309491

CP018629.1 1606009 1605884

CP012120.2 486372 486247

CP017094.2 1191716 1191591

CP016861.2 486372 486247

CP025495.1 486372 486247

CP016863.2 2069105 2068980

CP022290.1 815291 815416

CP023560.1 591769 591894

CP023500.1 564559 564684

CP023391.1 856936 857061

CP023390.1 856936 857061

AP014921.1 873798 873923

CP019590.1 886187 886312

CP020619.1 881214 881339

CP014444.1 880279 880404

CP014441.1 875758 875883

CP014438.1 875758 875883

CP014435.1 880038 880163

CP014432.1 875758 875883

CP014429.1 880278 880403

CP014426.1 880278 880403

CP014423.1 880278 880403

CP014420.1 880278 880403

CP014415.1 880279 880404

CP014412.1 880278 880403

CP014409.1 880038 880163

CP014407.1 880280 880405

CP014402.1 880131 880256

CP014397.1 880131 880256

CP014392.1 880131 880256

CP014387.1 880131 880256

CP014384.1 880045 880170

CP014381.1 880038 880163

CP014371.1 880131 880256

CP014368.1 880038 880163

CP014365.1 868983 869108

CP014362.1 868983 869108

CP014376.1 880131 880256

CP009423.1 868803 868928

LT671859.1 839228 839353

CP018205.1 798946 799071

AP017377.1 857787 857912

LT598688.1 856932 857057

CP013959.1 938078 938203

CP013957.1 937408 937533

CP013231.1 2790083 2790208

CP012018.1 907785 907910

CP012015.1 908191 908316

CP012013.1 907519 907644

CP012012.1 909281 909406

CP012011.1 953277 953402

CP007676.1 850613 850738

CP007672.1 862033 862158

CP007657.1 862321 862446

CP007674.1 845630 845755

CP007670.1 875208 875333

CP011526.1 818097 818222

Query 1

ATTTCGCTACTAGTTGTTTAGTGTTAACTTTAGTTGTAGTTTCAAGTCTAAGTAGCTCAGCAAATGCATCACAAACAGATAATGGCGTAAATAGAAGTGGTTCTGAAGATCCAACAGTATAT

AGTG 126

NC_017341.1 882081 882206

NC_007795.1 800146 800271

NZ_CP011526.1 818097 818222

NC_002951.2 887822 887947

NZ_CP029031.1 866327 866452

NZ_CP022290.1 815291 815416

NZ_CP010296.1 866327 866452

NZ_CP020020.1 826880 .....C..... 827005

NZ_CP012409.1 736282 .....C.....C..... 736158

NC_002758.2 894377 .....C.....C..... 894501

NC_009487.1 897433 .....C.....C..... 897557

NC_009632.1 897308 .....C.....C..... 897432

NZ_CP009361.1 823306 .....C.....C..... 823430

NC_017340.1 853079 .....C.....C..... 853203

NZ_CP019117.1 866224 .....C.....C..... 866348

NZ_CP010402.1 841618 .....A.....C.....C..... 841743

NC_017338.1 842204 .....C.....T.....C..... 842328

NC_022226.1 828265 .....C.....C.....A..... 828389

NZ_CP025395.1 870953 .....C.....G.....C..... 871077

NZ_CP022291.1 800682 .....C.....C.....T.....T..... 800806

NZ_CP014791.1 803688 .....C.....C.....G...C.....A.....C.... 803812

Note: All accession listed are *nuc* genes.

APPENDIX G: Real-Time PCR LOD Results

mecC

Sample	Quantity (Copy/ml)	Log Quantity	Number of Replicates	number of positives	% positive	Probit value
D5	1.86E+04	4.27	12	12	100.00	
D6	9.31E+03	3.97	12	12	100.00	
mecC-8	5.96E+03	3.78	12	12	100.00	
D7	4.66E+03	3.67	12	11	91.67	6.34
D8	2.33E+03	3.37	12	7	58.33	5.2
mecC-9	5.96E+02	2.78	12	3	25.00	4.33

Probit value t 95% positive is 6.65

log C95= 3.90
LOD (Copy/ml)= 8.03E+03
LOD (Copy/ul)= 8.03E+00
LOD (Copy/rxn)= 2.01E+02

mecA

Sample	Quantity (Copy/ml)	Log Quantity	Number of Rreplicates	number of positives	% positive	Probit value
M1	4.98E+03	3.70	11	9	81.82	5.92
M2	2.49E+03	3.40	11	8	72.73	5.58
M3	1.24E+03	3.09	11	8	72.73	5.58
M4	6.22E+02	2.79	11	3	27.27	4.05
M5	3.11E+02	2.49	11	1	9.09	3.66
M6	1.55E+02	2.19	12	3	25.00	4.33
M7	7.77E+01	1.89	12	0	0.00	
M8	3.89E+01	1.59	12	0	0.00	

Probit value t 95% positive is 6.65

log C95= 3.98
LOD (CFU/ml)= 9.53E+03
LOD (CFU/ul)= 9.53E+00
LOD (CFU/rxn)= 2.38E+02

CoA

Sample	Quantity (Copy/ml)	Log Quantity	Number of Rreplicates	number of positives	% positive	Probit value
M1	4.98E+03	3.70	11	8	72.73	5.58
M2	2.49E+03	3.40	11	6	54.55	5.93
M3	1.24E+03	3.09	11	8	72.73	5.58
M4	6.22E+02	2.79	11	1	9.09	3.66
M5	3.11E+02	2.49	11	0	0.00	
M6	1.55E+02	2.19	12	4	33.33	4.56
M7	7.77E+01	1.89	12	0	0.00	
M8	3.89E+01	1.59	12	0	0.00	

Probit value t 95% positive is 6.65

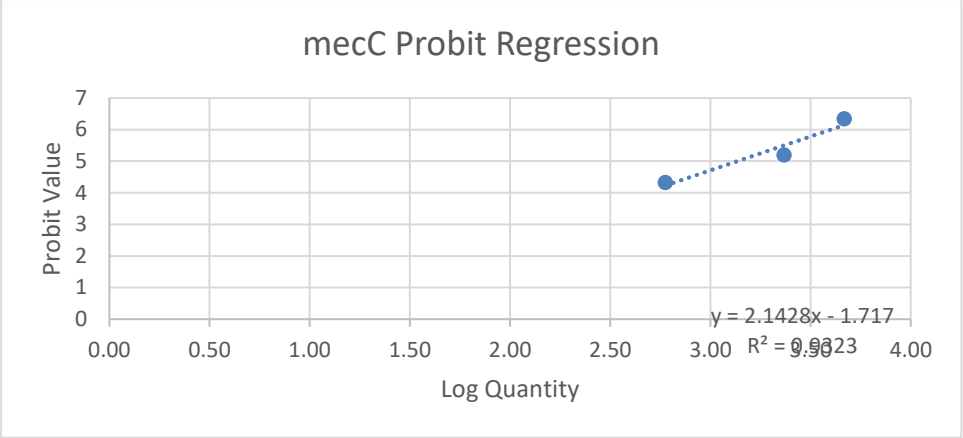
log C95=	3.52
LOD (CFU/ml)=	3.29E+03
LOD (CFU/ul)=	3.29E+00
LOD (CFU/rxn)=	8.23E+01

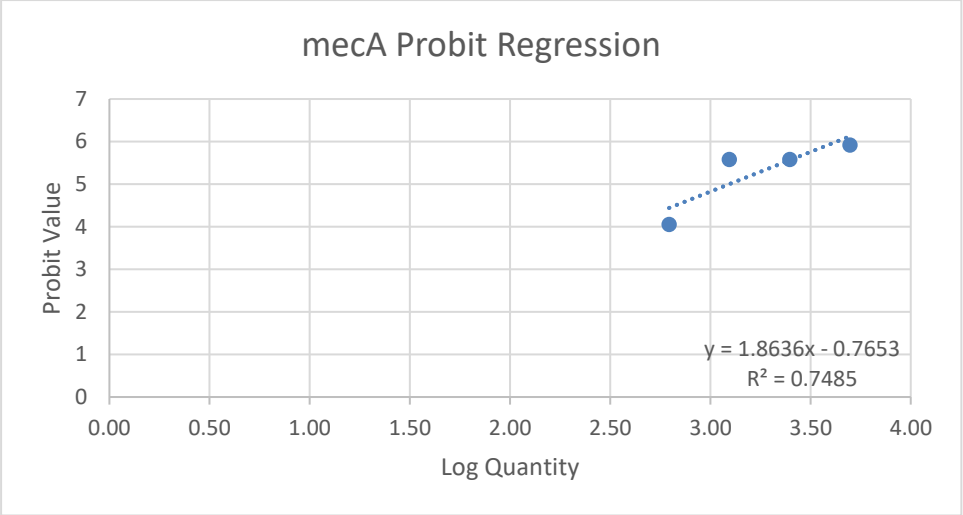
nuc

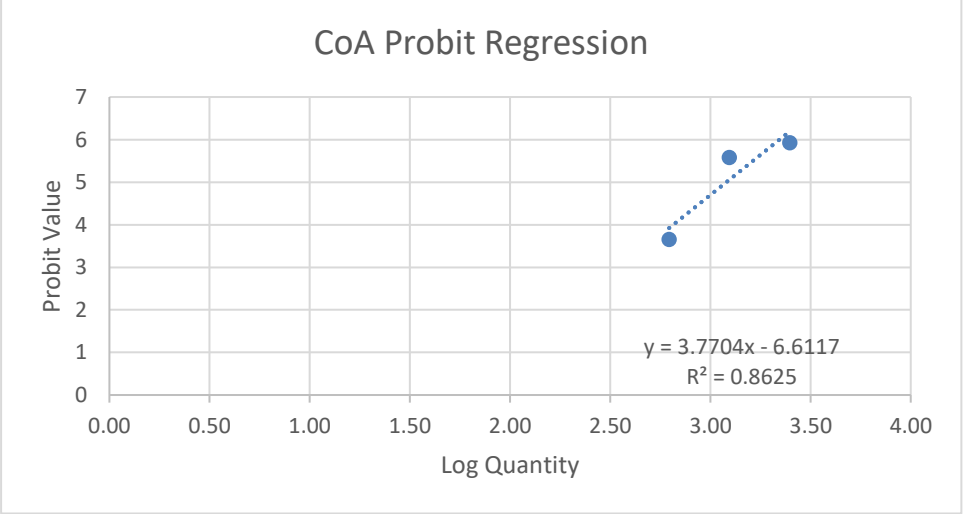
Sample	Quantity (Copy/ml)	Log Quantity	Number of Rreplicates	number of positives	% positive	Probit value
M1	4.98E+03	3.70	11	9	81.82	5.88
M2	2.49E+03	3.40	11	5	45.45	4.9
M3	1.24E+03	3.09	11	6	54.55	5.13
M4	6.22E+02	2.79	11	3	27.27	4.39
M5	3.11E+02	2.49	11	2	18.18	4.08
M6	1.55E+02	2.19	12	0	0.00	
M7	7.77E+01	1.89	12	0	0.00	
M8	3.89E+01	1.59	12	2	16.67	4.05

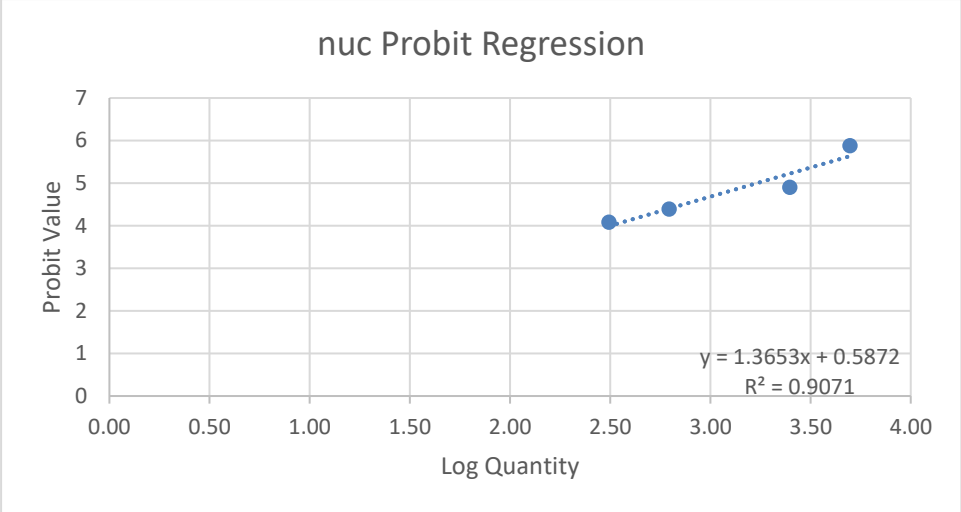
Probit value t 95% positive is 6.65

log C95=	4.44
LOD (CFU/ml)=	2.76E+04
LOD (CFU/ul)=	2.76E+01
LOD (CFU/rxn)=	6.90E+02


mecC Probit Regression
Probit Value
Log Quantity
y = 2.1428x - 1.717
R² = 0.9323
0
1
2
3
4
5
6
7
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00


mecA Probit Regression
Probit Value
Log Quantity
y = 1.8636x - 0.7653
R² = 0.7485
0
1
2
3
4
5
6
7
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00


CoA Probit Regression
Probit Value
Log Quantity
y = 3.7704x - 6.6117
R² = 0.8625
0
1
2
3
4
5
6
7
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00


nuc Probit Regression
Probit Value
Log Quantity
y = 1.3653x + 0.5872
R² = 0.9071
0
1
2
3
4
5
6
7
0.00
0.50
1.00
1.50
2.00
2.50
3.00
3.50
4.00

APPENDIX H: Real-Time PCR Clinical Validation Results

mecC

Sample	Quantity (Copy/ml)	Log Quantity	Run 1			Run 2			Run 3			Run 4			Run 5			Mean	SD
			8/15/2018			1/28/2019			2/6/2019			2/7/2019			2/8/2019				
			Ct1	Ct2	Ct3	Ct4	Ct5	Ct6	Ct7	Ct8	Ct9	Ct10	Ct11	Ct12	Ct13	Ct14	Ct15		
MECC 11	5.96E+10	10.78	11.69	11.41	11.83	10.76	10.55	10.84	10.77	10.80	10.64	11.32	11.45	11.57	11.37	11.25	11.50	11.18	0.41
MECC 10	5.96E+09	9.78	15.42	15.81	15.70	14.56	14.52	14.96	14.85	14.82	15.30	15.27	15.12	15.44	15.32	15.54	15.77	15.23	0.41
MECC 9	5.96E+08	8.78	18.99	18.91	18.92	17.95	18.41	18.37	18.29	18.70	18.59	18.88	19.23	19.16	18.84	19.39	19.49	18.81	0.43
MECC 8	5.96E+07	7.78	22.21	22.27	22.15	21.28	21.52	21.49	21.84	21.83	21.90	22.20	22.34	22.26	22.16	22.41	22.49	22.02	0.36
MECC 7	5.96E+06	6.78	26.49	26.34	26.42	25.33	25.31	25.42	25.48	25.67	25.75	25.70	25.71	25.86	25.83	25.90	25.97	25.81	0.37
MECC 6	5.96E+05	5.78	29.77	29.36	29.32	28.38	28.32	28.27	28.55	28.47	28.65	29.32	29.19	29.10	28.64	NEG	28.39	28.84	0.49
MECC 5	5.96E+04	4.78	33.18	33.32	33.58	31.97	31.98	32.19	31.88	32.69	32.86	32.41	32.93	33.77	32.89	33.88	33.50	32.87	0.67
MECC 4	5.96E+03	3.78	37.36	36.21	37.57	36.13	34.92	35.32	NEG	35.54	36.05	35.18	34.94	36.12	35.47	35.56	NEG	35.87	0.83
MECC 3	5.96E+02	2.78	36.38	NEG	NEG	35.98	NEG	NEG	NEG	NEG	NEG	36.23	NEG	NEG	NEG	NEG	NEG	36.20	0.20
MECC 2	5.96E+01	1.78	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	#DIV/0!	#DIV/0!
MECC 1	5.96E+00	0.78	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	#DIV/0!	#DIV/0!

mecA

Sample	Quantity (CFU/ml)	Log Quantity	Run 1			Run 2			Run 3			Run 4			Run 5			Mean	SD
			1/27/2019			1/28/2019			2/6/2019			2/7/2019			2/8/2019				
			Ct1	Ct2	Ct3	Ct4	Ct5	Ct6	Ct7	Ct8	Ct9	Ct10	Ct11	Ct12	Ct13	Ct14	Ct15		
PC8	95000000	7.98	19.86	19.88	19.94	20.12	20.57	20.38	20.51	20.59	20.64	19.52	19.58	19.39	19.13	19.25	19.22	19.90	0.54
PC7	9500000	6.98	22.42	22.17	22.09	22.83	NEG	20.96	22.99	22.69	22.61	22.56	22.46	22.38	22.92	22.58	22.42	22.43	0.50
PC6	950000	5.98	27.39	27.28	27.45	27.91	28.14	27.76	27.97	27.75	28.13	28.05	28.09	28.17	28.01	27.61	27.86	27.84	0.29
PC5	95000	4.98	30.89	30.69	30.47	31.14	31.17	31.16	32.31	30.85	30.76	32.53	31.62	31.21	31.10	31.38	31.33	31.24	0.56
PC4	9500	3.98	33.19	33.67	33.31	35.46	34.82	33.98	34.28	35.44	36.51	36.97	35.07	35.64	34.15	33.88	33.95	34.69	1.14
PC3	950	2.98	NEG	35.94	35.98	NEG	NEG	36.43	NEG	NEG	NEG	NEG	NEG	NEG	NEG	36.76	NEG	36.28	0.39
PC2	95	1.98	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	#DIV/0!	#DIV/0!
PC1	9.5	0.98	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	#DIV/0!	#DIV/0!

CoA

Sample	Quantity (CFU/ml)	Log Quantity	Run 1			Run 2			Run 3			Run 4			Run 5			Mean	SD
			1/27/2019			1/28/2019			2/6/2019			2/7/2019			2/8/2019				
			Ct1	Ct2	Ct3	Ct4	Ct5	Ct6	Ct7	Ct8	Ct9	Ct10	Ct11	Ct12	Ct13	Ct14	Ct15		
PC8	95000000	7.98	20.86	20.90	20.83	20.42	20.88	20.65	20.81	20.98	20.94	19.74	19.82	19.60	19.23	19.37	19.25	20.28	0.69
PC7	9500000	6.98	23.20	23.20	23.00	23.13	NEG	21.74	23.31	23.11	22.81	22.91	22.84	22.60	23.00	22.84	22.59	22.88	0.39
PC6	950000	5.98	28.24	28.10	28.44	28.11	28.34	28.28	28.22	28.33	28.39	28.61	28.45	28.56	28.15	27.94	28.16	28.29	0.18
PC5	95000	4.98	31.43	32.14	31.67	31.11	31.44	31.62	32.66	31.49	31.48	31.73	32.13	31.65	31.42	31.47	31.15	31.64	0.40
PC4	9500	3.98	35.06	35.12	36.00	35.88	34.14	34.90	34.37	37.29	34.97	35.20	35.04	35.65	33.98	34.78	34.28	35.11	0.85
PC3	950	2.98	37.60	NEG	NEG	NEG	NEG	NEG	NEG	NEG	35.63	NEG	NEG	35.75	NEG	NEG	NEG	36.32	1.10
PC2	95	1.98	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	#DIV/0!	#DIV/0!
PC1	9.5	0.98	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	#DIV/0!	#DIV/0!

nuc

Sample	Quantity (CFU/ml)	Log Quantity	Run 1			Run 2			Run 3			Run 4			Run 5			Mean	SD
			1/27/2019			1/28/2019			2/6/2019			2/7/2019			2/8/2019				
			Ct1	Ct2	Ct3	Ct4	Ct5	Ct6	Ct7	Ct8	Ct9	Ct10	Ct11	Ct12	Ct13	Ct14	Ct15		
PC8	95000000	7.98	21.61	21.47	21.50	21.88	22.14	21.86	21.63	21.68	21.67	20.14	20.10	19.70	19.69	19.80	19.69	20.97	0.97
PC7	9500000	6.98	23.98	23.72	23.66	24.41	NEG	22.72	24.10	23.80	23.37	23.31	23.16	22.87	23.74	23.41	22.95	23.51	0.49
PC6	950000	5.98	28.93	28.82	29.15	29.49	29.71	29.28	28.82	28.69	29.03	28.83	28.67	28.64	28.54	28.24	28.41	28.88	0.40
PC5	95000	4.98	32.39	32.43	31.98	32.58	32.44	33.14	32.88	32.30	32.27	33.12	32.40	31.47	31.98	32.25	31.27	32.33	0.52
PC4	9500	3.98	34.64	35.61	34.48	36.45	34.80	36.53	35.41	35.32	34.99	35.57	35.93	34.24	34.37	33.70	34.28	35.09	0.84
PC3	950	2.98	NEG	NEG	NEG	NEG	NEG	NEG	NEG	36.82	NEG	NEG	NEG	NEG	NEG	NEG	NEG	36.82	#DIV/0!
PC2	95	1.98	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	#DIV/0!	#DIV/0!
PC1	9.5	0.98	NEG	NEG	37.67	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	37.67	#DIV/0!

APPENDIX I: Real-Time PCR Clinical Validation Results

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S1	NEG	True Negative	37.25	NEG	NEG	0.00
S2	NEG	True Negative	24.02	NEG	NEG	0.00
S3	NEG	True Negative	NEG	NEG	NEG	0.00
S4	NEG	True Negative	21.49	26.71	29.05	7.56
S5	NEG	True Negative	NEG	NEG	NEG	0.00
S6	NEG	True Negative	23.78	NEG	NEG	0.00
S7	NEG	True Negative	32.63	NEG	NEG	0.00
S8	NEG	True Negative	33.29	NEG	NEG	0.00
S9	NEG	True Negative	29.95	NEG	NEG	0.00
S10	NEG	True Negative	35.85	NEG	NEG	0.00
S11	NEG	False Positive	33.34	32.03	33.25	0.09
S12	NEG	True Negative	27.42	NEG	NEG	0.00
S13	NEG	True Negative	26.00	NEG	NEG	0.00
S14	NEG	True Negative	28.36	36.91	39.22	9.71
S15	NEG	True Negative	22.52	NEG	NEG	0.00
S16	NEG	False Positive	28.61	27.35	28.22	0.83

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S17	NEG	True Negative	22.94	NEG	NEG	0.00
S18	NEG	True Negative	33.28	NEG	NEG	0.00
S19	NEG	True Negative	NEG	NEG	NEG	0.00
S20	NEG	False Positive	24.46	25.99	25.47	1.27
S21	NEG	True Negative	NEG	NEG	NEG	0.00
S22	NEG	True Negative	NEG	NEG	NEG	0.00
S23	NEG	True Negative	29.50	NEG	NEG	0.00
S24	NEG	True Negative	24.79	NEG	NEG	0.00
S25	NEG	True Negative	NEG	NEG	NEG	0.00
S26	NEG	True Negative	37.84	33.16	35.88	3.32
S27	NEG	True Negative	NEG	NEG	NEG	0.00
S28	NEG	True Negative	22.59	NEG	NEG	0.00
S29	NEG	True Negative	34.34	NEG	NEG	0.00
S31	NEG	True Negative	NEG	NEG	NEG	0.00
S32	NEG	True Negative	22.99	NEG	NEG	0.00
S33	NEG	True Negative	NEG	26.92	27.79	0.00

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S34	NEG	True Negative	20.67	NEG	NEG	0.00
S35	**POS**	True Positive	28.37	28.56	29.99	0.00
S36	**POS**	True Positive	28.42	27.58	29.47	0.00
S37	NEG	True Negative	NEG	NEG	NEG	0.00
S38	NEG	True Negative	27.80	NEG	NEG	0.00
S39	NEG	True Negative	36.48	NEG	38.19	0.00
S40	NEG	True Negative	NEG	25.70	27.32	0.00
S41	NEG	True Negative	34.91	22.88	24.29	11.32
S42	NEG	True Negative	32.70	NEG	NEG	0.00
S43	NEG	True Negative	14.97	NEG	NEG	0.00
S44	NEG	True Negative	35.39	NEG	NEG	0.00
S45	NEG	True Negative	27.83	NEG	NEG	0.00
S46	NEG	True Negative	30.60	NEG	NEG	0.00
S47	NEG	False Positive	22.31	23.39	23.96	1.37
S48	NEG	True Negative	NEG	NEG	NEG	0.00
S50	NEG	True Negative	33.05	35.21	36.46	2.79

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S51	NEG	True Negative	28.63	NEG	38.82	0.00
S52	NEG	True Negative	30.12	NEG	NEG	0.00
S53	NEG	True Negative	27.91	29.68	30.87	2.36
S54	NEG	True Negative	NEG	NEG	NEG	0.00
S55	NEG	True Negative	31.95	NEG	NEG	0.00
S56	NEG	True Negative	NEG	NEG	NEG	0.00
S58	**POS**	True Positive	32.60	31.40	32.98	0.00
S59	**POS**	True Positive	27.32	26.20	28.90	0.00
S60	**POS**	True Positive	27.77	26.38	28.95	0.00
S61	NEG	True Negative	22.92	NEG	NEG	0.00
S62	NEG	True Negative	30.87	NEG	NEG	0.00
S63	NEG	True Negative	NEG	37.47	NEG	0.00
S64	NEG	True Negative	24.94	NEG	NEG	0.00
S65	NEG	True Negative	35.08	NEG	NEG	0.00
S66	NEG	True Negative	26.50	NEG	NEG	0.00
S67	NEG	True Negative	24.38	NEG	NEG	0.00

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S68	NEG	True Negative	NEG	NEG	NEG	0.00
S69	NEG	True Negative	27.91	NEG	NEG	0.00
S70	NEG	True Negative	27.24	NEG	NEG	0.00
S71	NEG	True Negative	NEG	28.70	30.00	0.00
S72	NEG	True Negative	NEG	NEG	NEG	0.00
S73	NEG	True Negative	27.42	NEG	NEG	0.00
S74	NEG	True Negative	33.25	36.95	NEG	0.00
S75	NEG	True Negative	NEG	NEG	NEG	0.00
S76	NEG	True Negative	31.31	NEG	NEG	0.00
S77	NEG	True Negative	34.32	NEG	NEG	0.00
S78	NEG	True Negative	29.76	NEG	36.69	0.00
S79	NEG	True Negative	21.21	NEG	NEG	0.00
S80	NEG	True Negative	21.17	NEG	NEG	0.00
S81	NEG	True Negative	NEG	33.71	34.94	0.00
S82	NEG	True Negative	35.90	NEG	NEG	0.00
S83	NEG	True Negative	NEG	NEG	NEG	0.00

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S84	NEG	True Negative	35.38	NEG	NEG	0.00
S85	NEG	True Negative	17.17	NEG	NEG	0.00
S86	NEG	True Negative	32.61	NEG	NEG	0.00
S87	**POS**	True Positive	29.44	28.08	29.68	0.00
S88	NEG	True Negative	32.16	34.46	34.64	2.39
S89	NEG	True Negative	24.22	NEG	NEG	0.00
S90	NEG	True Negative	NEG	NEG	NEG	0.00
S91	NEG	True Negative	34.59	NEG	38.20	0.00
S92	NEG	True Negative	35.37	NEG	37.15	0.00
S93	NEG	True Negative	34.60	NEG	NEG	0.00
S94	NEG	True Negative	28.60	NEG	NEG	0.00
S95	NEG	True Negative	26.89	NEG	NEG	0.00
S96	NEG	True Negative	34.07	NEG	NEG	0.00
S97	NEG	True Negative	23.21	NEG	NEG	0.00
S98	NEG	True Negative	30.89	NEG	NEG	0.00
S99	NEG	True Negative	30.95	NEG	NEG	0.00

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S100	NEG	True Negative	24.51	NEG	NEG	0.00
S101	NEG	True Negative	NEG	NEG	NEG	0.00
S102	NEG	True Negative	NEG	NEG	NEG	0.00
S103	NEG	True Negative	35.32	NEG	NEG	0.00
S104	NEG	True Negative	NEG	NEG	NEG	0.00
S105	NEG	True Negative	26.66	NEG	39.99	0.00
S106	NEG	True Negative	36.14	NEG	NEG	0.00
S107	NEG	True Negative	31.61	NEG	NEG	0.00
S108	NEG	True Negative	NEG	NEG	NEG	0.00
S109	NEG	True Negative	28.18	NEG	NEG	0.00
S110	NEG	True Negative	28.35	34.80	36.63	7.37
S111	NEG	True Negative	33.56	NEG	NEG	0.00
S112	NEG	True Negative	NEG	NEG	NEG	0.00
S113	NEG	True Negative	27.21	NEG	NEG	0.00
S114	NEG	True Negative	29.93	NEG	NEG	0.00
S115	NEG	True Negative	30.68	NEG	NEG	0.00
S116	NEG	True Negative	35.12	NEG	NEG	0.00
S117	NEG	True Negative	NEG	NEG	NEG	0.00
S118	NEG	True Negative	NEG	33.52	23.43	0.00
S119	NEG	True Negative	30.95	35.76	36.75	5.31
S120	NEG	True Negative	30.68	34.46	35.31	4.21
S121	NEG	True Negative	NEG	NEG	NEG	0.00
S122	NEG	True Negative	28.84	NEG	NEG	0.00

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S123	NEG	True Negative	29.17	NEG	NEG	0.00
S124	NEG	True Negative	29.83	NEG	NEG	0.00
S125	NEG	True Negative	30.08	28.68	NEG	0.00
S126	**POS**	True Positive	29.32	28.13	29.72	0.00
S127	NEG	True Negative	NEG	NEG	NEG	0.00
S128	NEG	True Negative	22.24	NEG	NEG	0.00
S129	NEG	True Negative	33.32	32.97	NEG	0.00
S130	NEG	True Negative	24.70	NEG	NEG	0.00
S131	NEG	True Negative	29.63	36.78	39.20	8.36
S132	NEG	True Negative	25.93	22.54	23.55	2.88
S133	NEG	True Negative	NEG	36.48	38.30	0.00
S134	NEG	True Negative	31.76	NEG	38.83	0.00
S135	NEG	True Negative	36.61	NEG	NEG	0.00
S136	NEG	True Negative	20.54	NEG	NEG	0.00
S137	NEG	True Negative	21.84	NEG	NEG	0.00
S138	NEG	True Negative	26.93	29.66	31.26	3.54
S139	NEG	True Negative	34.24	NEG	NEG	0.00
S140	NEG	True Negative	35.52	NEG	NEG	0.00
S141	NEG	True Negative	32.66	NEG	NEG	0.00
S142	NEG	True Negative	NEG	NEG	NEG	0.00
S143	NEG	True Negative	31.24	NEG	NEG	0.00
S144	NEG	True Negative	26.15	NEG	NEG	0.00
S145	NEG	True Negative	36.94	NEG	NEG	0.00
S146	NEG	True Negative	32.60	NEG	38.69	0.00
S147	NEG	True Negative	32.33	NEG	NEG	0.00
S148	NEG	True Negative	31.44	NEG	NEG	0.00
S149	NEG	True Negative	20.98	NEG	NEG	0.00

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S150	NEG	True Negative	28.10	NEG	NEG	0.00
S151	NEG	True Negative	21.44	NEG	NEG	0.00
S152	NEG	True Negative	21.74	NEG	NEG	0.00
S153	NEG	True Negative	25.15	26.96	28.15	2.40
S154	NEG	True Negative	25.41	NEG	NEG	0.00
S156	NEG	True Negative	28.44	NEG	NEG	0.00
S157	NEG	True Negative	NEG	26.48	28.41	0.00
S158	NEG	True Negative	NEG	NEG	NEG	0.00
S159	NEG	True Negative	21.54	NEG	33.79	0.00
S160	NEG	True Negative	30.05	32.18	33.86	2.97
S161	NEG	False Positive	31.39	30.06	31.54	0.58
S162	NEG	True Negative	33.71	NEG	NEG	0.00
S163	NEG	True Negative	33.90	NEG	NEG	0.00
S164	NEG	True Negative	35.27	NEG	NEG	0.00
S165	NEG	True Negative	37.25	NEG	NEG	0.00
S166	NEG	True Negative	25.16	NEG	NEG	0.00
S167	**POS**	False Negative	18.81	NEG	37.55	18.74
S168	NEG	False Positive	24.74	26.10	26.96	1.79
S169	NEG	True Negative	20.96	NEG	NEG	0.00
S170	NEG	True Negative	29.15	NEG	NEG	0.00
S171	NEG	True Negative	NEG	NEG	NEG	0.00
S172	**POS**	True Positive	30.85	30.43	30.00	0.63
S173	**POS**	True Positive	30.83	31.32	30.64	0.15
S174	**POS**	True Positive	24.65	25.08	24.44	0.11
S175	**POS**	True Positive	26.31	26.68	26.13	0.10
S176	**POS**	True Positive	28.41	28.43	27.76	0.31
S177	**POS**	True Positive	29.58	30.72	30.35	0.95

Sample	Chromogenic Agar MRSA Result	Qualitative Result	mec Ct	coa Ct	nuc	Ct Difference
S178	**POS**	False Negative	29.07	34.51	32.15	4.26
S179	**POS**	False Negative	21.19	30.53	33.11	10.63
S180	**POS**	True Positive	19.19	19.69	20.26	0.79
S181	**POS**	True Positive	28.63	28.92	30.06	0.86
S182	**POS**	True Positive	16.60	17.20	15.98	0.01
S183	**POS**	True Positive	21.50	21.67	20.19	0.57
S184	**POS**	True Positive	26.33	32.07	31.78	5.60
S185	**POS**	False Negative	25.59	33.69	33.15	7.83
S186	**POS**	True Positive	26.03	24.80	26.25	0.50
S187	**POS**	True Positive	22.78	28.45	27.61	5.25
S188	**POS**	True Positive	23.24	23.80	22.62	0.03
S189	**POS**	True Positive	26.72	27.08	26.75	0.19
S190	**POS**	True Negative	NEG	NEG	NEG	0.00
S191	**POS**	True Positive	29.31	31.56	31.39	2.16
S192	**POS**	True Positive	24.52	27.33	27.07	2.68
S193	**POS**	True Positive	30.42	32.33	31.54	1.52
S194	**POS**	True Positive	29.13	31.29	31.28	2.16
S195	**POS**	True Positive	25.32	26.18	24.98	0.26
S196	**POS**	True Positive	29.43	31.78	31.52	2.22
S197	**POS**	True Positive	23.75	24.19	23.73	0.21
S198	**POS**	True Positive	26.97	27.70	27.34	0.55
S199	**POS**	False Negative	27.73	30.64	32.28	3.73
S200	**POS**	False Negative	30.55	35.03	36.63	5.27
S201	**POS**	True Positive	22.36	21.95	24.22	0.73
S202	**POS**	True Positive	33.32	32.62	35.16	0.57
S203	**POS**	True Positive	23.49	23.37	25.11	0.75
S204	**POS**	True Positive	25.50	25.26	27.32	0.79