Journal of Microbiological Methods 164 (2019) 105673

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A 13-Plex PCR for high-resolution melting-based screening of clinically important *Staphylococcus* species in animals

Zafer Ata[a,*], Esra Buyukcangaz[b]

[a] Military Veterinary School and Educational Central Commandership, Gemlik, Bursa, Turkey
[b] Bursa Uludag University, Faculty of Veterinary Medicine, Department of Microbiology, Nilufer, 16059, Gorukle, Bursa, Turkey

ARTICLE INFO

Keywords:
High-resolution melting
nuc gene
PCR
Species identification
Staphylococcus

ABSTRACT

A single-tube multiplex real-time PCR targeting the nuclease (*nuc*) gene and subsequent high-resolution melting analysis (HRMA) were used to identify 13 different *Staphylococcus* species. The *nuc* gene was targeted due to its low intraspecies variation and the greater interspecies variation than the 16S rRNA gene in *Staphylococcus*. We used HRMA software that can store and compare HRMA profiles from different runs as long as the runs contain the same reference reaction. Thus, we reduced the 14 PCRs to 2 different PCRs, one targeting the unknown sequence and the other targeting the reference sequences to screen 13 different *Staphylococcus* species. The specificity of the developed method was tested on 16 different *Staphylococcus* reference strains and 115 different field strains that were isolated from the milk of cattle with subclinical mastitis. We conclude that the method can be used to quickly and cost-effectively differentiate *Staphylococcus aureus* (*S. aureus*) from other *Staphylococcus* species (*S. epidermidis, S. lugdunensis, S. schleiferi, S. hyicus, S. chromogenes, S. lentus, S. haemolyticus, S. xylosus, S. saprophyticus, S. warneri, S. simulans and S. hominis*).

1. Introduction

Staphylococcus, a member of the family *Staphylococcaceae*, is a facultative anaerobic, gram-positive, catalase-positive bacterium, containing 55 known and many unnamed species (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) (Becker et al., 2014). *Staphylococcus* bacteria are naturally found in the mucous membrane and skin flora of warm-blooded animals; cheese, meat, milk or other environmental sources such as soil, sand, air and water (Kloos and Schleifer, 1986). *Staphylococcus* species have also been isolated from indoor environments, such as fitness centers (Mukherjee et al., 2014; Mukherjee et al., 2016), kitchen and bathroom surfaces (Scott et al., 2009), office environments (Hewitt et al., 2012), and hospital settings (Kembel et al., 2012). *Staphylococcus* species can cause a wide variety of diseases in humans and animals (Tenover, 2000).

S. aureus is known to be a major pathogen causing infections in humans and animals (Tenover, 2000). Clinically important *Staphylococcus* subtypes in animals have been identified and characterized in previous reports, namely, *S. aureus, S. intermedius, S. pseudintermedius, S. hyicus, S. aureus* subsp. *anaerobius, S. delphini,* and *S. schleiferi* subsp. *coagulans* are defined as coagulase–positive and *S. chromogens, S. epidermidis, S. haemolyticus, S. hominis, S. lentus, S. saprophyticus, S. simulans, S. sciuri, S. warneri, S. cohnii, S. xylosus, S. caseolyticus, S. arlettae, S. equorum* are defined as coagulase-negative (CoNs) (Haesebrouck and K.H.L.A.D.F., 2004; Quinn et al., 2000; Songer and Post, 2005; Tenover, 2000). Coagulase activates thrombin, which subsequently converts fibrinogen to fibrin. Some Staphylococci produce coagulase, while most do not (Becker et al., 2014). The dogma that coagulase production distinguishes pathogenic from nonpathogenic species held until coagulase-negative staphylococci (CoNs) were recognized as a major cause of wound infections and infections associated with foreign bodies, including catheters, prosthetic heart valves, joint prostheses, and pacemaker electrodes; thus, coagulase is no longer an exclusive indicator of pathogenicity (Songer and Post, 2005). Since 1980, CoNs have been increasingly recognized as nosocomial pathogens in human and animal hospitals. Most important in the pathogenesis of nosocomials is the ability of CoNs to colonize the surface of the device by the formation of a thick multilayered biofilms (Becker et al., 2015). CoNs are also associated with mastitis in animals, fermented food and dairy products (Zell et al., 2008).

Traditionally, the identification of bacterial samples has been carried out using conventional methods, including culture, cell morphology, staining, and biochemical profiling (Murray and Baron, 2007). A large number of phenotypic identification methods have been developed and validated using clinical isolates from human infections for the protection of human health (Zadoks and Watts, 2009). The API

* Corresponding author.
E-mail address: atazafervet@hotmail.com (Z. Ata).

https://doi.org/10.1016/j.mimet.2019.105673
Received 5 February 2019; Received in revised form 15 July 2019; Accepted 19 July 2019
Available online 23 July 2019

Staph® strip represents an overnight method for the manual identification of staphylococci for health care and product safety applications (Becker et al., 2015). Biochemical identification methods for *Staphylococcus* species, including microbial identification systems such as the BD BBL Crystal Rapid Gram-Positive Identification panel, and the Sensititre GPID Identification system, are fairly successful in differentiating *S. aureus, S. epidermidis,* and *S. saprophyticus,* while the accurate identification of less common species is variable (Renneberg et al., 1995). Microbial identification systems may fail to distinguish commonly encountered staphylococcal species, particularly if phenotypic variants or isolates recovered from livestock and food are tested (Becker et al., 2015). The application of matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-tof MS) of whole bacterial cells has reduced the time needed to identify bacteria and fungi grown in culture and is increasingly being deployed in clinical laboratories (Nolte, 2015). Most studies report the identification of staphylococci at the species level at greater than 97% accuracy by MALDI-tof MS. Nevertheless, for nonhuman-associated or newly described CoNs species, the expansion of MALDI-tof MS databases are needed (Becker et al., 2015; Moon et al., 2013). These methods may be less reliable for the identification of *Staphylococcus* isolates obtained from animals due to the lack of sufficient data on the isolates in the reference databases (Jousson et al., 2007).

High-resolution melting analysis (HRMA) is a fast (post-PCR) high-throughput molecular method to scan for sequence variations in a target gene (Mazi et al., 2015). HRMA for sequence matching was made possible by 1) double-stranded "DNA dyes that detect heteroduplexes 2)-modified real-time PCR instruments with increased melting resolution and 3) software for data normalization, curve shape comparison and sequence type clustering. The sequence-specific melting properties of double-stranded DNA results in specific melting curve profiles that can be compared with a designated reference sample and allow the determination of a single base difference between the target amplicons (Krypuy et al., 2006).

HRMA has been compared with other analytical methods (Farrar and Wittwer, 2017). HRMA is more sensitive than denaturing high-pressure liquid chromatography. When blood cultures are analyzed for bacteria, the concordance between HRMA and mass spectrometry is high. Massively parallel sequencing has similar sensitivity, but HRMA is faster, less expensive, and easier to use for specific targets. In studies comparing HRMA with exonuclease probes and allele-specific PCR, concordance is high, and all three assays perform well.

Real-time PCR coupled with HRMA has been used in bacterial genotyping (Cheng et al., 2006; Yang et al., 2009). Direct characterization of amplicons is performed in less than 30 min in a closed system with a reduced risk of contamination (Ngui et al., 2012). Since this method is nondestructive, amplicons can still be analyzed on a gel if ambiguous results are obtained (Vossen et al., 2009).

Slany et al. (2010) tested the potential of HRM analysis to distinguish 12 different species of the *Staphylococcus* genus using a broad-range PCR within the 16S rRNA gene where sequence differences are minimal. HRM analysis of *S. saprophyticus* and *S. xylosus* resulted in indistinguishable profiles because of their identical sequences in the analyzed 16S rRNA region. The remaining species were fully differentiated using the developed HRMA-based method.

To overcome the resolution problem of 16S rRNA-targeted HRMA for the differentiation of *Staphylococcus* species, a single-tube multiplex real-time PCR followed by HRMA-targeting the nuclease (*nuc)* gene was developed in this study. The *nuc* gene of *Staphylococcus* was targeted due to its low intraspecies variation and higher interspecies variation than the 16S rRNA gene in *Staphylococcus* (Hirotaki et al., 2011). We also used HRMA software that can store and compare HRMA profiles from the different runs as long as the runs contain the same reference reaction. The use of the advanced HRMA software reduced the 14 necessary PCRs for the previous HRM-based methods to 2 PCRs for the differentiation of 13 *Staphylococcus* species.

Table 1
The reference strains used in this study.

No	Name of *Staphylococcus* species	Reference strain ID
1	*Staphylococcus aureus*	CECT 435 (ATCC 25923)
2	*Staphylococcus aureus* (MRSA)	LIOFILCHEM (ATCC 33591)
3	*Staphylococcus aureus* (MRSA)	CECT 5190 (ATCC 43300)
4	*Staphylococcus aureus* (MRSA)	LIOFILCHEM (ATCC 700699)
5	*Staphylococcus epidermidis*	CECT 232 (ATCC 14990)
6	*Staphylococcus lugdunensis*	DSM 4804 (ATCC 43809)
7	*Staphylococcus schleiferi* subsp. schleiferi	DSM 4807 (ATCC 43808)
8	*Staphylococcus hyicus*	DSM 20459 (ATCC 11249)
9	*Staphylococcus chromogenes*	DSM 20454 (ATCC 43764)
10	*Staphylococcus lentus*	DSM 20352 (ATCC 29070)
11	*Staphylococcus haemolyticus*	CECT 4900 (ATCC 29970)
12	*Staphylococcus xylosus*	CECT237 (ATCC 29971)
13	*Staphylococcus saprophyticus* subsp. *saprophyticus*	CECT 235 (ATCC 15305)
14	*Staphylococcus warneri*	CECT 236 (ATCC 27836)
15	*Staphylococcus simulans*	CECT 4538 (ATCC 27848)
16	*Staphylococcus hominis* subsp. *hominis*	CECT 234 (ATCC 27844)

2. Materials and methods

2.1. Culture collection

In this study, 16 different reference strains (Table 1) from 13 different *Staphylococcus* species were used. The strains were obtained from Liofilchem, Colleccion Espanolla De Cultivos Tipo (CECT) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) reference culture collections. The *Staphylococcus* strains were cultivated in tryptic soy agar / broth (TSA, TSB) (Merck, 1.05458.0500, 1.05459.0500), nutrient agar / broth (NA, NB) (Merck, 1.05450.0500; Oxoid, CM0001) and sheep blood agar (Merck, 1.10455.0500) under aerobic conditions at 35–37 °C for 24–48 h in accordance with the manufacturer's protocols.

A total of 115 field strains (*Staphylococcus aureus*-100 / *S. chromogenes*-4 / *S. xylosus*-4 / *S. hyicus*-2 / *S. epidermidis*-4 / *S. saprophyticus*-1) were isolated from the milk of cattle with subclinical mastitis between 2010 and 2012 by Uludag University Veterinary Faculty, Department of Microbiology. The isolation was carried out via inoculation on egg yolk tellurite Baird-Parker agar (Oxoid, CM0275) and blood agar (Merck, 1.10455.0500), and the suspected colonies were identified using an API Staph Kit (bioMérieux). The species identity of the field isolates was also confirmed via *nuc* gene DNA sequencing. The isolates were stored at −25 °C in glycerin broth and cultivated on nutrient agar before molecular analyses.

2.2. Primer design

The *nuc* gene sequences from the reference *Staphylococcus* strains were obtained by using the NCBI tools (www.ncbi.nlm.nih.gov). The sequences were aligned by using Clustal Omega (www.ebi.ac.uk). The species-specific primers targeting intraspecies conserved and interspecies variable regions were designed manually based on the principles detailed by Dorak (Dorak, 2007). The specificity of the primers was tested using the "Primer BLAST" tool of NCBI (www.ncbi.nlm.nih.gov). The primer sequences are given in Table 2.

2.3. DNA extraction

A Bio-Speedy® Bacterial DNA Isolation Kit (Bioeksen R&D Tech. Ltd., Turkey) was used for DNA isolation. A single *Staphylococcus* colony was suspended in 400 µl of binding solution (6 M guanidine thiocyanate, 20 mM Tris-HCl, pH 8). After 10 min of incubation at 95 °C, 400 µl of 2-propanol was mixed with the sample and loaded into the silica column. The column was centrifuged at 13000 rpm for 1 min and washed twice with washing buffer (20 mM NaCl, 2 mM Tris-HCl,

Table 2
Primers targeting the *nuc* gene of *Staphylococcus* spp.

Primer	5′-3′ Sequence	Target bacteria
S. aureus F	CATGTTCAAAGAGTTGTGGATGGTG	*Staphylococcus aureus*
S. aureus R	TTCGGTTTCACCGTTTCTGG	*Staphylococcus aureus*
S. xylosus F	TGTTGACGCACGCCTCTTTT	*Staphylococcus xylosus*
S. xylosus R	TTTTAGCCTGTGTTTCAGCTTGTTT	*Staphylococcus xylosus*
S. warneri F	AAAGGCTCAAAATGAAGCTAAGAAAAA	*Staphylococcus warneri*
S. warneri R	TTCAAAATTCAAAGTATCGGTGTGG	*Staphylococcus warneri*
S. simulans F	CCGAATGGCAAGTACCGAGA	*Staphylococcus simulans*
S. simulans R	TGGTCCGACTGAACGTCAAA	*Staphylococcus simulans*
S. schleiferi F	CGTGTGCGTATGATTGGTGTTG	*Staphylococcus schleiferi*
S. schleiferi R	TTTTGTGCTTGTTCGATTTGAGG	*Staphylococcus schleiferi*
S. saprophyticus F	TTGTACCGGCGTATTGGGTTT	*Staphylococcus saprophyticus*
S. saprophyticus R	CGTGTTGTAGATGGAGATACATTCGTT	*Staphylococcus saprophyticus*
S. lugdunensis F	TCCAATGACGGATTTTAACGAAGA	*Staphylococcus lugdunensis*
S. lugdunensis R	TGCTAAGAAAAATGCAAAACGTGAA	*Staphylococcus lugdunensis*
S. hyicus F	TGATAGGGGTAGATACTCCCGAAACTG	*Staphylococcus hyicus*
S. hyicus R	TTGAGCTTGTTTTTGTGCTTGTTCT	*Staphylococcus hyicus*
S. hominis F	CGTTGATCGGGTTGTAGATGGA	*Staphylococcus hominis*
S. hominis R	TGAAGGTGCTGACGAAAAAGAGAA	*Staphylococcus hominis*
S. haemolyticus F	TGTTCTCATCTAACCACACATAAGCAA	*Staphylococcus haemolyticus*
S. haemolyticus R	TGCAACAAATAGCGAAGGGAAA	*Staphylococcus haemolyticus*
S. epidermidis F	CCATTCTGGACCGTTTAGTGGTT	*Staphylococcus epidermidis*
S. epidermidis R	TTTGATGCGTGAGATACTTCTTCGT	*Staphylococcus epidermidis*
S. chromogenes F	TCTAGCGGACAAACGAATGGA	*Staphylococcus chromogenes*
S. chromogenes R	TCGGTTTGACTGTTTCTGGTGTG	*Staphylococcus chromogenes*
S. lentus F	TGAACCATCAGGCGAAGTAGTAGATG	*Staphylococcus lentus*
S. lentus R	AATGGGTTATCTTGGTTTGGAGTTG	*Staphylococcus lentus*

pH 8; 80% *v*/v ethanol). DNA in the column was eluted with 100 µl of nuclease-free deionized water (pH 7.0). The purity and quantity of the extracted DNA was assessed via spectrophotometric measurements at 260 and 280 nm. The DNA was stored at −20 °C until further analyses.

2.4. Real-time PCR and melting curve analysis

An Applied Biosystems® 7500 Fast Real-Time PCR System and Bio-Speedy® 2× qPCR HRM Fusion Mix (Bioeksen R&D Tech. Ltd. Co., Turkey) was used for all real-time PCRs. The final reaction mixes included 0.1–100 ng of template DNA, 0.5 µM of each primer given in Table 2, 1x reaction buffer, 1x HRMA dye and 2.5 µM $MgCl_2$. The following thermal cycling program was used: 5 min at 95 °C; 35 cycles of 10 s at 95 °C, 10 s at 55 °C, 30 s at 72 °C followed by fluorescence acquisition. The real-time PCR amplicon specificity was checked via melt curve analysis between 60 °C and 95 °C with fluorescence acquisition at every 0.05 °C increase. The results were analyzed using 7500 Fast SDS v1.4.1 software (Applied Biosytems, CA, USA). The software was also used to transfer the melting curve data to a Microsoft Excel 2010 sheet that was used as the input for the HRMA software.

2.5. HRM analysis

Bio-Speedy® HRM Analysis and Database Creation Software (Bioeksen R&D Tech. Ltd. Co., Turkey) were used to process the melting curve data. The example user interface and analysis result is given in Figs. 1 and 2. The software reads the data file saved in MS Excel and plots the data on a 2D coordinate system. The user manually selects premelting and postmelting regions and the reference curve. In this study, the reference melting curves were obtained from the genomic DNA of *S. aureus* ATCC 25923. The software converts the melting curve to relative signal values on the y axis at a scale of 0–100 and limits the x axis between the pre- and postmelting temperatures. The software calculates the difference curve by subtracting all normalized curves from the normalized reference curve. The clustering is performed by calculating the Euclidean distance between the difference curves. The error margin required for the clustering is manually set by the user based on the experimental data obtained from the replicate sample analyses.

The HRM profile database was prepared by entering the obtained HRM profiles and the related species information into the software. The "Create Database" tool of the software was used to finalize the database construction. When a new HRM profile was obtained against the reference melt curve, the new profile was analyzed by the "Database Match" tool of the software. If the new HRM profile was 99% or more similar to the profiles in the database, the software grouped the new profile with the stored profiles.

2.6. DNA sequencing

The PCR amplicons were purified using a Bio-Speedy® PCR Product Purification Kit. The purified DNA was sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA). The sequences were analyzed in Chromas (www.technelysium.com) and manually checked for read errors. Homology searches of the sequences in DNA databases were performed with BLAST (www.ncbi.nlm.nih.gov).

3. Results

Singleplex PCRs containing the species-specific primer pairs (Table 2) were positive for the target DNA extracts and negative for the nontarget DNA extracts. Each PCR primer pair was checked versus all other species. The multiplex PCR containing all species-specific primer pairs was applied to each DNA extract from the reference strains and field isolates. The multiplex PCR-amplified *nuc* genes were both sequenced and subjected to the HRMA approach developed in this study. All analyses were repeated for 0.1, 1, 10 and 100 ng DNA templates. Homology searches of the sequences in DNA databases revealed that all of the obtained sequences were 100% identical to the target sequences (Table 3). The analysis of the obtained HRM profiles against the stored profiles from the reference sequences resulted in the specific species estimation for each target (Table 3). The DNA template concentration made no difference to the HRMA results.

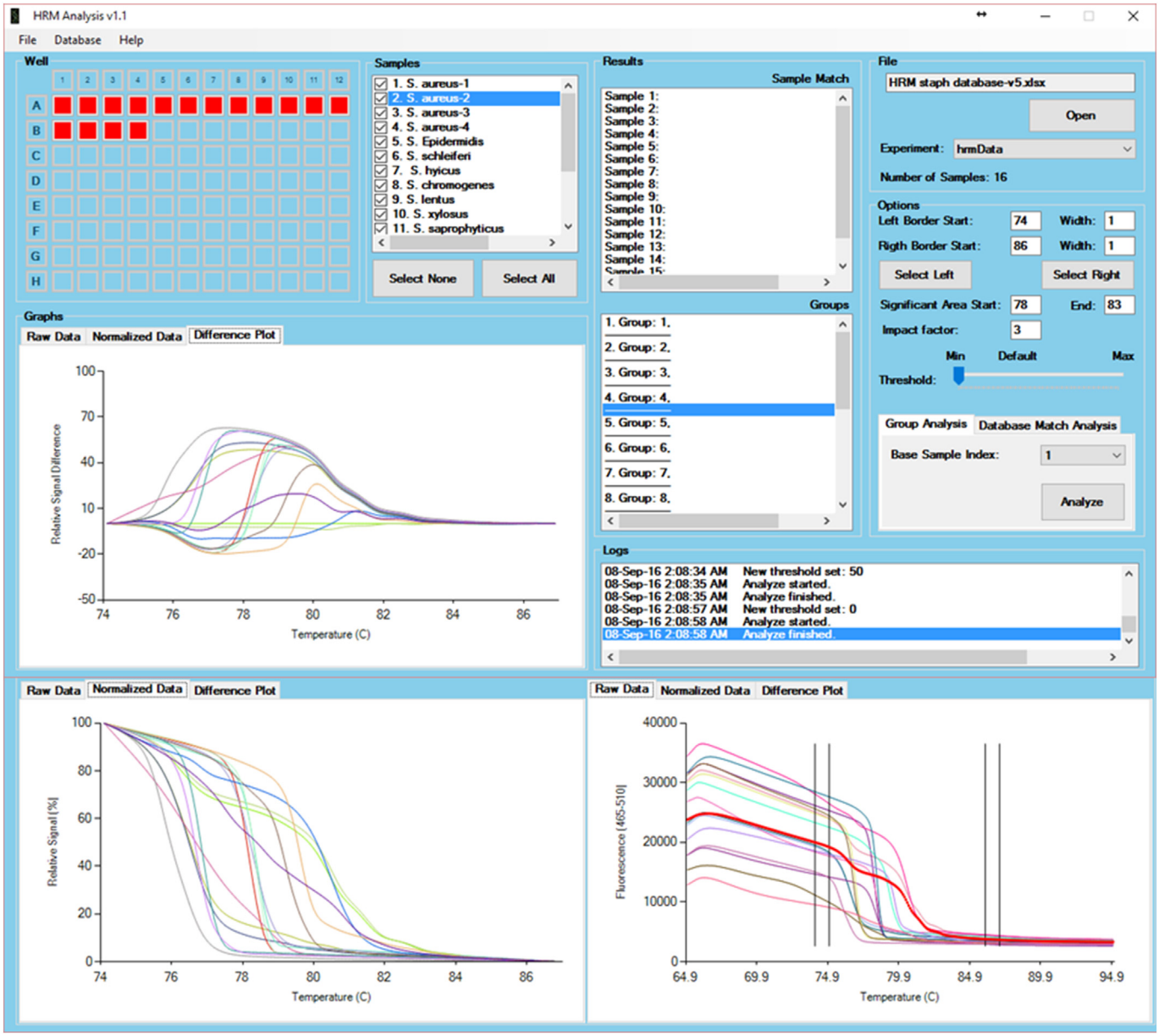

Fig. 1. HRM analysis and database creation software user interface and an example of the database creation using the reference strains.

4. Discussion and conclusions

Slany et al. (2010) tested the potential of 16S rRNA-targeted HRMA to distinguish 12 different species of the *Staphylococcus* genus. HRMA of *S. saprophyticus* and *S. xylosus* resulted in indistinguishable profiles because of their high 16S rRNA homology. Many bacterial identification assays based on HRMA have been developed (Cheng et al., 2006; Ozbak et al., 2012) targeting the 16S rRNA gene (Yang et al., 2009). While all of these studies were able to identify the target bacteria, most of the published approaches require further steps for the precise identification of species, and none of the approaches employ a multiplex technique.

In this study, 115 field strains (*Staphylococcus aureus*-100 / *S. chromogenes*-4 / *S. xylosus*-4 / *S. hyicus*-2 / *S. epidermidis*-4 / *S. saprophyticus*-1) that were isolated from the milk of cattle with subclinical mastitis between 2010 and 2012 were tested using a single-tube multiplex real-time PCR combined with subsequent HRMA to identify the species. It should be noted however that *S. agnetis*, first reported in 2012 (Taponen et al., 2012), associated with subclinical and mild clinical bovine mastitis is genetically very similar to *S. hyicus*, making it difficult to differentiate from *S. hyicus* with routine phenotypic and genotypic tests. Although we did not isolate this strain from the milk of cattle with subclinical mastitis, a multiplex PCR has been developed to differentiate *S. hyicus, S. agnetis,* and *S. aureus* by targeting the *aroD* gene fragment (Adkins et al., 2017). Also *S. argenteus* is a novel staphylococcal species closely related to *S. aureus*, and is considered as a part of *S. aureus* complex (also including *S. schweitzeri*) (Jiang et al., 2018). The method used in our study was not used to differentiate these phenotypically similar species. We believe that future studies will shed light on the differentiation of such strains by selecting different genes with this method, which cannot be distinguished by routine tests.

The *nuc* gene is a target used in the differentiation of *Staphylococcus* species (Hirotaki et al., 2011; Javid et al., 2018; Montazeri et al., 2015; Velasco et al., 2014). The *nuc* gene stands out among other candidate genes, such as *16S rRNA*, *rpoB*, *hsp60*, *sodA* and *dnaJ*, due to its low intra- and high interspecies variation (Drancourt and Raoult, 2002; Hirotaki et al., 2011; Kwok and Chow, 2003; Poyart et al., 2001; Shah et al., 2007). In this study, the *nuc* gene and the advanced HRMA

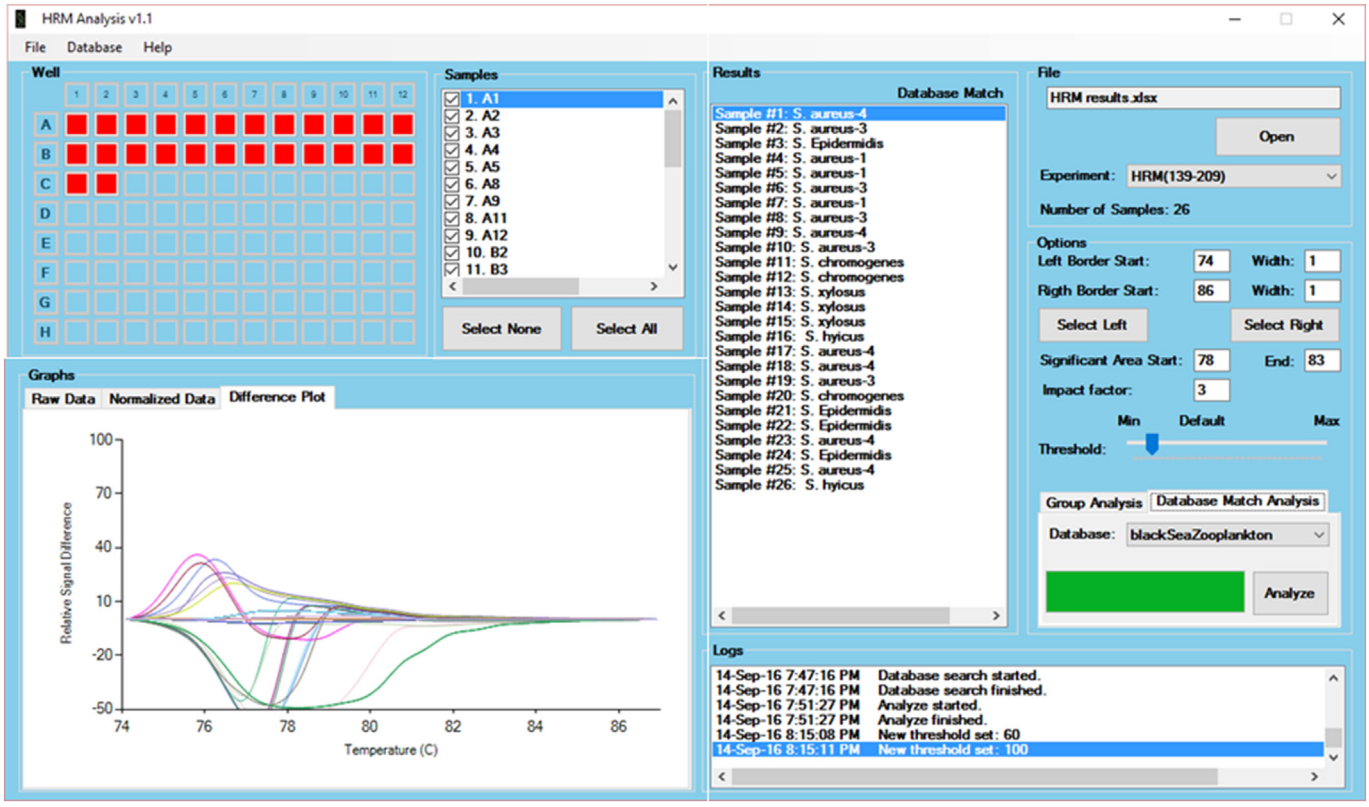

Fig. 2. An example of the HRMA of the field isolates.

software allowed us to develop a 13-plex HRMA methodology. The previous 16S rRNA-targeted HRMA-based methods necessitate 14 PCRs in the same run, one targeting the unknown sequence and the rest targeting the reference sequences to screen the 13 different *Staphylococcus* species. We reduced the 14 PCRs to 2 different PCRs, one targeting the unknown sequence and the other targeting the reference sequences.

The majority (87%) of our field strains were *S. aureus,* which is the major staphylococcal species isolated from cows with mastitis (Gandhale et al., 2017; Saglam et al., 2017). We did not have field strains for all the species that were included in the method. The missing field strains were *S. lugdunensis, S. schleiferi, S. lentus, S. haemolyticus, S. warneri, S. simulans,* and *S. hominis*. Furthermore, there are other studies (Acton, 2012; Ajitkumar et al., 2013) reporting that *S. chromogenes*, *S. xylosus*, *S. simulans*, and *S. sciuri* have multiple genotypes, which complicates their resolution by HRMA. In this study, we did not test many replicates of most species; therefore, we are unable to determine if there are multiple genotypes using the *nuc* method. This is the major drawback of our study.

Another drawback of our study is the exclusion of *S. agnetis* which is phylogenetically closely related to *S. hyicus*. The BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the *nuc* gene sequence that we obtained from the field sequence resulted in 100% match with *S. hyicus nuc* gene (CP008747.1) (Table 3) and 93% match with *S. agnetis nuc* gene (CP009623.1). There are 19 bp mismatches between the two sequences. In-silico melting curve analysis using UMELT (https://www.dna.utah.edu/umelt/um.php) resulted in the melting temperatures of 84 °C and 84.5 °C for the *nuc* genes of *S. hyicus* and *S. agnetis*

Table 3
Nucleic acid homology search and HRMA results of the multiplex PCR-amplified *nuc* gene sequences.

Staphylococcus strains from which partial *nuc* gene sequences were obtained		100% similar NCBI BLAST hit		Predicted species using HRMA
Organism	Strain No.	Organism	Accession No.	
S. aureus	ATCC 25923; ATCC 33591; 82 of the *S. aureus* field isolates	*S. aureus*	LT598688.1	*S. aureus*
S. aureus	ATCC 43300; 19 of the *S. aureus* field isolates	*S. aureus*	CP009361.1	*S. aureus*
S. aureus	ATCC 700699	*S. aureus*	LT598688.1	*S. aureus*
S. epidermidis	ATCC 14990; 3 of the *S. epidermidis* field isolates	*S. epidermidis*	LT571449.1	*S. epidermidis*
S. lugdunensis	ATCC 43809	*S. lugdunensis*	CP014023.1	*S. lugdunensis*
S. schleiferi	ATCC 43808	*S. schleiferi*	CP010309.1	*S. schleiferi*
S. hyicus	ATCC 11249; 2 of the *S. hyicus* field isolates	*S. hyicus*	CP008747.1	*S. hyicus*
S. chromogenes	ATCC 43764; 4 of the *S. chromogenes* field isolates	*S. chromogenes*	AB465333.1	*S. chromogenes*
S. lentus	ATCC 29070	*S. lentus*	AY485196.1	*S. lentus*
S. haemolyticus	ATCC 29970	*S. haemolyticus*	CP013911.1	*S. haemolyticus*
S. xylosus	ATCC 29971; 4 of the *S. xylosus* field isolates	*S. xylosus*	CP007208.1	*S. xylosus*
S. saprophyticus	ATCC 15305; 1 of the *S. saprophyticus* field isolates	*S. saprophyticus*	CP014057.1	*S. saprophyticus*
S. warneri	ATCC 27836	*S. warneri*	AB598385.1	*S. warneri*
S. simulans	ATCC 27848	*S. simulans*	AB465331.1	*S. simulans*
S. hominis	ATCC 27844	*S. hominis*	AJ343912.1	*S. hominis*

respectively. Overall bioinformatics analyses revealed that, *nuc* genes of *S. hyicus* and *S. agnetis* are divergent enough to be differentiated via the simple melting curve analysis. On the other hand, we did not prove our in-silico bioinformatics analyses via the laboratory tests using the *S. agnetis* reference strains. Therefore we couldn't conclude that our method can reliably differentiate *S. hyicus* from *S. agnetis*.

It takes less than 30 min to extract nucleic acid from a *Staphylococcus* isolate by using our extraction methodology. Real-time PCR amplification takes less than 1.5 h, and HRMA takes less than 40 min for the sequence-specific analysis of staphylococcal *nuc* genes. The species identification results can be obtained in less than 2 h and 40 min by using the HRMA approach in this study. The developed method differentiates 13 different reference types of *Staphylococcus* and can be used in a reliable, fast and cost-effective way to identify *S. aureus*. However, the method needs to be validated by using more field strains for other species (*S. epidermidis S. lugdunensis, S. schleiferi, S. hyicus, S. chromogenes, S. lentus, S. haemolyticus, S. xylosus, S. saprophyticus, S. warneri, S. simulans, S. hominis*). As a future prospect, our high-throughput and low-cost HRMA method will allow us to screen many more field isolates to increase the frequency of the species types and genotypes in our database beyond those of *S. aureus*.

Acknowledgments

This research was financially supported by the TUBITAK 115O262 project.

References

Acton, A.Q., 2012. Endopeptidases: Advances in Research and Application.
Adkins, P.R.F., Middleton, J.R., Calcutt, M.J., Stewart, G.C., Fox, L.K., 2017. Species identification and strain typing of Staphylococcus agnetis and Staphylococcus hyicus isolates from bovine Milk by use of a novel multiplex PCR assay and pulsed-field gel electrophoresis. J. Clin. Microbiol. 55, 1778–1788.
Ajitkumar, P., Barkema, H.W., Zadoks, R.N., Morck, D.W., van der Meer, F.J., De Buck, J., 2013. High-resolution melt analysis for species identification of coagulase-negative staphylococci derived from bovine milk. Diagn. Microbiol. Infect. Dis. 75, 227–234.
Becker, K., Heilmann, C., Peters, G., 2014. Coagulase-negative staphylococci. Clin. Microbiol. Rev. 27, 870–926.
Becker, K., Skov, R.L., von Eiff, C., 2015. Staphylococcus, Micrococcus, and other catalase-positive cocci. In: Manual of Clinical Microbiology, Eleventh Edition. American Society of Microbiology, pp. 354–382.
Cheng, J.-C., Huang, C.-L., Lin, C.-C., Chen, C.-C., Chang, Y.-C., Chang, S.-S., Tseng, C.-P., 2006. Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR. Clin. Chem. 52 1997–2004.
Dorak, M.T., 2007. Real-Time PCR. Taylor & Francis.
Drancourt, M., Raoult, D., 2002. Rpob gene sequence-based identification of Staphylococcus species. J. Clin. Microbiol. 40, 1333–1338.
Farrar, J.S., Wittwer, C., 2017. High-Resolution Melting Curve Analysis for Molecular Diagnostics, Molecular Diagnostics (Third Edition). Elsevier, pp. 79–102.
Gandhale, D., Kolhe, R., Nalband, S., Deshpande, P., Jagtap, U., Dhandore, C., Bhave, S., Jadhav, S., Muglikar, D., Kolhe, S., 2017. Molecular types and antimicrobial resistance profile of Staphylococcus aureus isolated from dairy cows and farm environments. Turk. J. Vet. Anim. Sci. 41, 713–724.
Haesebrouck, K.H.L.A.D.F, 2004. In: C. L. G. J. F. P. J. G. S. C. O. Thoen (Ed.), Pathogenesis of Bacterial Infections in Animals, Third ed. Blackwell Publishing.
Hewitt, K.M., Gerba, C.P., Maxwell, S.L., Kelley, S.T., 2012. Office space bacterial abundance and diversity in three metropolitan areas. PLoS One 7, e37849.
Hirotaki, S., Sasaki, T., Kuwahara-Arai, K., Hiramatsu, K., 2011. Rapid and accurate identification of human associated staphylococci by multiplex PCR. J. clin. microbiol. JCM. 00411–00488.
Javid, F., Taku, A., Bhat, M.A., Badroo, G.A., Mudasir, M., Sofi, T.A., 2018. Molecular typing of Staphylococcus aureus based on coagulase gene. Vet. world. 11, 423.
Jiang, B., You, B., Tan, L., Yu, S., Li, H., Bai, G., Li, S., Rao, X., Xie, Z., Shi, X., Peng, Y., Hu, X., 2018. Clinical Staphylococcus argenteus develops to small Colony variants to promote persistent infection. Front. Microbiol. 9, 1347.
Jousson, O., Di Bello, D., Vanni, M., Cardini, G., Soldani, G., Pretti, C., Intorre, L., 2007. Genotypic versus phenotypic identification of staphylococcal species of canine origin with special reference to Staphylococcus schleiferi subsp. coagulans. Vet. Microbiol. 123, 238–244.
Kembel, S.W., Jones, E., Kline, J., Northcutt, D., Stenson, J., Womack, A.M., Bohannan, B.J., Brown, G.Z., Green, J.L., 2012. Architectural design influences the diversity and structure of the built environment microbiome. ISME J. 6, 1469–1479.
Kloos, W., Schleifer, K., 1986. In: Genus IV. Staphylococcus, P (Ed.), Bergey's Manual of Systematic Bacteriology. Williams & Wilkins, Baltimore, MD, pp. 1013–1035.
Krypuy, M., Newnham, G.M., Thomas, D.M., Conron, M., Dobrovic, A., 2006. High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer. BMC Cancer 6, 295.
Kwok, A.Y., Chow, A.W., 2003. Phylogenetic Study of Staphylococcus and Macrococcus Species Based on Partial hsp60 Gene Sequences. International Journal of Systematic and Evolutionary Microbiology. 53. pp. 87–92.
Mazi, W., Sangal, V., Sandstrom, G., Saeed, A., Yu, J., 2015. Evaluation of spa-typing of methicillin-resistant Staphylococcus aureus using high-resolution melting analysis. Int. J. Infect. Dis. 38, 125–128.
Montazeri, E.A., Khosravi, A.D., Jolodar, A., Ghaderpanah, M., Azarpira, S., 2015. Identification of methicillin-resistant Staphylococcus aureus (MRSA) strains isolated from burn patients by multiplex PCR. Burns. 41, 590–594.
Moon, H.W., Lee, S.H., Chung, H.S., Lee, M., Lee, K., 2013. Performance of the Vitek MS matrix-assisted laser desorption ionization time-of-flight mass spectrometry system for identification of Gram-positive cocci routinely isolated in clinical microbiology laboratories. J. Med. Microbiol. 62, 1301–1306.
Mukherjee, N., Dowd, S.E., Wise, A., Kedia, S., Vohra, V., Banerjee, P., 2014. Diversity of bacterial communities of fitness center surfaces in a U.S. metropolitan area. Int. J. Environ. Res. Public Health 11, 12544–12561.
Mukherjee, N., Sulaiman, I.M., Banerjee, P., 2016. Characterization of methicillin-resistant Staphylococcus aureus isolates from fitness centers in the Memphis metropolitan area. Tenn. Am J Infect Control. 44, 1681–1683.
Murray, P.R., Baron, E.J., 2007. Manual of Clinical Microbiology. ASM Press, Washington, D.C.
Ngui, R., Lim, Y.A., Chua, K.H., 2012. Rapid detection and identification of human hookworm infections through high resolution melting (HRM) analysis. PLoS One 7, e41996.
Nolte, F.S., 2015. In: Jorgensen, M.A.P.J.H., Carroll, K.C., Funke, G., Landry, M.L., Richter, S.S., Warnock, D.W. (Eds.), Manual of Clinical Microbiology. ASM press, Washington DC, pp. 54–90.
Ozbak, H., Dark, P., Maddi, S., Chadwick, P., Warhurst, G., 2012. Combined molecular gram typing and high-resolution melting analysis for rapid identification of a syndromic panel of bacteria responsible for sepsis-associated bloodstream infection. J. Mol. Diagn. 14, 176–184.
Poyart, C., Quesne, G., Boumaila, C., Trieu-Cuot, P., 2001. Rapid and accurate species-level identification of coagulase-negative staphylococci by using the sodA gene as a target. J. Clin. Microbiol. 39, 4296–4301.
Quinn, P.J., C.M.E, Markey, B., Carter, G.R., 2000. Section 2: Bacteriology, 8: Staphylococcus Species, Clinical Veterinary Microbiology. Mosby, Harcourt publishers limited, pp. pp, pp. 118–126.
Renneberg, J., Rieneck, K., Gutschik, E., 1995. Evaluation of staph ID 32 system and staph-Zym system for identification of coagulase-negative staphylococci. J. Clin. Microbiol. 33, 1150–1153.
Saglam, A.G., Sahin, M., Celik, E., Celebi, O., Akca, D., Otlu, S., 2017. The role of staphylococci in subclinical mastitis of cows and lytic phage isolation against to Staphylococcus aureus. Vet World. 10, 1481–1485.
Scott, E., Duty, S., McCue, K., 2009. A critical evaluation of methicillin-resistant Staphylococcus aureus and other bacteria of medical interest on commonly touched household surfaces in relation to household demographics. Am. J. Infect. Control 37, 447–453.
Shah, M.M., Iihara, H., Noda, M., Song, S.X., Nhung, P.H., Ohkusu, K., Kawamura, Y., Ezaki, T., 2007. DnaJ gene sequence-based assay for species identification and phylogenetic grouping in the genus Staphylococcus. Int. J. Syst. Evol. Microbiol. 57, 25–30.
Songer, J., Post, K., 2005. Bacterial and Fungal Agents of Animal Disease. Veterinary Microbiology. Elsevier Saunders, St Louis, pp. 84–91.
Taponen, S., Supre, K., Piessens, V., Van Coillie, E., De Vliegher, S., Koort, J.M., 2012. Staphylococcus agnetis sp. nov., a coagulase-variable species from bovine subclinical and mild clinical mastitis. Int. J. Syst. Evol. Microbiol. 62, 61–65.
Tenover, F.C., 2000. The Epidemiology of Staphylococcus Aureus. Gram-Positive Pathogens. pp. 414–421.
Velasco, V., Sherwood, J.S., Rojas-García, P.P., Logue, C.M., 2014. Multiplex real-time PCR for detection of Staphylococcus aureus, mecA and Panton-valentine Leukocidin (PVL) genes from selective enrichments from animals and retail meat. PLoS One 9, e97617.
Vossen, R.H., Aten, E., Roos, A., den Dunnen, J.T., 2009. High-resolution melting analysis (HRMA)—more than just sequence variant screening. Hum. Mutat. 30, 860–866.
Yang, S., Ramachandran, P., Rothman, R., Hsieh, Y.-H., Hardick, A., Won, H., Kecojevic, A., Jackman, J., Gaydos, C., 2009. Rapid identification of biothreat and other clinically relevant bacterial species by use of universal PCR coupled with high-resolution melting analysis. J. Clin. Microbiol. 47, 2252–2255.
Zadoks, R.N., Watts, J.L., 2009. Species identification of coagulase-negative staphylococci: genotyping is superior to phenotyping. Vet. Microbiol. 134, 20–28.
Zell, C., Resch, M., Rosenstein, R., Albrecht, T., Hertel, C., Gotz, F., 2008. Characterization of toxin production of coagulase-negative staphylococci isolated from food and starter cultures. Int. J. Food Microbiol. 127, 246–251.