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Research Article

IDENTIFICATION OF *Staphylococcus aureus* CHEESE ISOLATES WITH RESPECT TO VIRULENCE PROPERTIES, GENETIC RELATEDNESS AND ANTIBIOTIC RESISTANCE PROFILES

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ABSTRACT

The problems on identification of *Staphylococcus aureus* isolates from cheese samples were investigated by phenotypic and genotypic tests in this study. Among 207 *Staphylococcus* spp. isolated from 31 cheese samples, 23 isolates that were Gram positive, catalase and slide coagulase positive, with 1 isolate that was latex agglutination test negative showed different phenotypic properties. Polymerase chain reaction (PCR) and quantitative PCR (qPCR) analyses showed that DNase test and target genes (*nuc*, *coa*) regarded as gold standard regions for *S. aureus* were not found to be unique for identification of *S. aureus*. The toxin genes (SEA-SEE) were not detected by PCR. Antibiotic resistance profiles of *S. aureus* isolates demonstrated that two isolates were resistant to penicillin G. This study showed that the unique phenotypic and genotypic test was not adequate for identification of *S. aureus* isolates. There was no correlation between the presence of the *nuc* gene and toxin genes. The presence of *nuc* gene which was used for detection of *S. aureus* was also found to be present in other *Staphylococcus* isolates. As a conclusion, the results revealed that biochemical tests could lead to false positive results for identification of *S. aureus*. The presence of *nuc* gene is not correlated with the presence of *toxin* genes.

Keywords: *Staphylococcus aureus*, PCR, Identification, Antibiotic resistance

Introduction

Staphylococcus aureus (*S. aureus*) is one of the most significant bacterial pathogens for human health and commonly involved in bacterial infections and food poisoning outbreaks worldwide (Chapaval et al., 2008; Ertas et al., 2010). Heat stable enterotoxins produced by specific strains of *S. aureus* are significant agents in staphylococcal food poisoning cases (Guven et al., 2010). The pathogenesis of *S. aureus* infection could be related to secretion of extracellular toxins and enzymes such as coagulase, DNase, thermonuclease etc (Kong et al., 2016). Milk and dairy products are pasteurized to eliminate high contamination levels of *S. aureus*; however, toxins produced by the bacterium are not inactivated in this process (Peles et al., 2007; Akineden et al., 2008). Several antibiotics are used to eliminate the diseases in animals and the bacterial intoxication cases. The common antibiotic use for treatment of animals and preservation of milk has caused development of antibiotic resistance (Alian et al., 2012).

Molecular methods can distinguish differences among closely related species as demonstrated by many researchers (Gičová et al., 2014; Villarreal et al., 2013; Kabadjova et al., 2002). Molecular methods can be used for identification of *S. aureus* to control the invasiveness of this bacterium among human, animal, and food (André et al., 2008). Surface proteins, invasions, toxins, biochemical properties, and inherent and acquired resistance to antimicrobial agents are the main virulence factors of *S. aureus* (Franklin and Lowy 1998; Stutz et al., 2011). Staphylococcal enterotoxins are the important virulence factors involved in pathogenicity of *S. aureus* (Huong et al., 2010). Staphylococcal food poisoning is caused by ingestion of foods contaminated with *S. aureus* that include one or more enterotoxins (Vasconcelos and Cunha 2010). Therefore, it is significant to detect and identify *S. aureus* in food samples. The presence of the *nuc* gene coding thermostable nuclease enzyme was used as an indication of *S. aureus* contamination in several studies (Alarcón et al., 2006; Aprodu et al., 2011; Hein et al., 2001; Lem et al., 2001). The *nuc* gene was used together with *coa* gene for identification of enterotoxigenic *S. aureus* strains by analyzing with PCR method (Cremonesi et al., 2007). PCR amplification of *coa* gene was regarded as a gold standard when compared to tube coagulase test (Tiwari et al., 2008). The genes encoding 23S rRNA, 16S to 23S rRNA spacer region, and 16S rRNA were used to confirm the biochemical test results for identification of *S. aureus* (Akineden et al., 2008; Gomez et al., 2007; Phuektes et al., 2003).

The identification of *S. aureus* can be carried out inaccurately based on unique phenotypic or genotypic tests. Studies on *S. aureus* showed that there were some contradictory results on identification of *S. aureus*. The phenotypic and genotypic tests can lead to misidentification by the impact of environmental factors on gene expression (Gandra et al., 2005).

Although several studies have been reported on the isolation and identification of the isolated *S. aureus* strains from Turkey, there have been only limited numbers of studies on the investigation of this bacterium from western part of the country. Another distinguishing point in our study is the comprehensive evaluation of latex agglutination test, tube coagulase and DNase activity tests with the presence of *nuc* and *coa* genes. The main objective of this study is to carry out the molecular and biochemical identification of *S. aureus* strains isolated from white cheese samples from three different locations in western part of Turkey. In addition, antibiotic sensitivities and toxin production properties were also characterized. Genetic relatedness of the isolates was determined by sequencing of the 16S rDNA region. Antibiotic resistance profiles of the isolates were obtained by performing the antibiotic susceptibility tests of the isolates to the 31 antibiotics and by searching the presence of *mecA* gene by PCR analysis.

Materials and Methods

Isolation and Identification of Strains

A total of 207 strains were purified from 31 unpackaged cheese samples purchased from local markets in western Turkey (cities of İzmir, Manisa, and Aydın). Twent five gram cheese samples were homogenized in 225 mL of sterile 0.1% buffered peptone water (Merck, Darmstadt, Germany). Serial dilutions were prepared up to 10^{-3} and 0.1mL aliquots were plated on Baird-Parker agar (BD-Difco, Sparks, Maryland) supplemented with egg yolk tellurite (BD-BBL, Sparks, Maryland) and incubated at 37ºC for 24–48 h. The typical and atypical bacterial colonies isolated from the incubated plates were transferred into tryptic soy broth medium for enrichment. The enriched bacteria were subcultured using streak plate technique. Gram staining, catalase, latex agglutination and tube coagulase, DNase activity and mannitol fermentation tests were performed. DNase activity test was performed by inoculating the culture to the DNase test agar and grown for 24h at 37ºC. 37 % HCl solution was poured onto the colonies for 5 minutes and observed for the clear zone around the colonies. Coagulase test was performed in two ways as tube coagulase test and latex

agglutination test. Latex agglutination test was carried out by using latex agglutination test kit. Tube coagulase test was performed for the determination of free coagulase production of the isolates, for this, coagulase plasma (0.5 mL) in the clean test tube was mixed with the tested isolate. The test tube was incubated at 37ºC and observed every 30 minutes for clotting by gently shaking the tube (Sperber and Tatini, 1975; Kateete et al., 2010). All purified isolates were stored at -80°C for further analysis. *S. aureus* RSKK 1009 was used as positive control in the study.

Bacterial DNA Extraction

Overnight tryptic soy broth culture (0.2 mL) of each isolate was transferred to eppendorf tubes and centrifuged at 15.000 x g for 5 min. The pellet was homogenized with 45 µL of sterile deionized water. The cells were treated with lysostaphin (100 µg mL^{-1}) and incubated at 37°C for 1 h. Following this, 15 µL of proteinase K (100 µg mL^{-1}) and 150 µL of Tris-HCl (0.1 M, pH 7.5) were added. Cell suspensions were incubated at 37°C for 1 h and subsequently held in boiling water for 5 min. These cell lysates were stored at -20°C (Sudagidan et al., 2008).

Quantitative PCR Analysis

The primers and probe targeting *nuc* gene were used as reported by Alarcón et al. (2006). The TaqMan probe was labeled with 6-carboxy-fluorescein (FAM) and with 6-carboxy-tetramethyl-rhodamine (TAMRA) in 5′ and 3′ ends, respectively. The size of the amplified *nuc* gene product was expected to be 124 bp in length. Amplification assay of TaqMan based qPCR included in a total volume of 20 µL. This mixture composed of 10X probes master, 500 nM of each primer, 200 nM probe and 5 µL of template DNA. The thermal cycling programme started with 95°C for 10 min of incubation. 50 cycles of amplification included 95°C for 15 s denaturation step, annealing at 60°C for TaqMan probe. The reaction ended with extention step at 72°C for 1 s. The data analyses were carried out using LightCycler® 480 Instrument software version 1.5 (Roche Diagnostics, Basel, Switzerland).

PCR Amplification of the Targeted Genomic Loci

S. aureus strains were genotyped by PCR amplification targeting 23S rDNA (Straub et al., 1999), the spacer region between 16S-23S (Forsman et al., 1997), clumping factor (*clfA*), X and IgG binding regions of the protein A and *coa*gulase (*coa*) using the PCR as described previously (Akineden et al., 2008). *femA* and *sau* regions were used as internal amplification controls in PCR analyses (Mehrotra et al., 2000; Holochová et al., 2010). PCR programme of amplification was given in Table 1.

Table 1. PCR amplification conditions

Target gene	Amplification Program
23S rDNA	Pre-denaturation 5 min at 94ºC, 37 cycles of denaturation at 94ºC for 40 s, annealing at 64ºC for 60 s, extension at 72ºC for 75 s and a final extension of 3.5 min at 72ºC.
16 S- 23 S rDNA	Pre-denaturation 5 min at 94ºC, 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, extension at 72ºC for 30 s and a final extension of 3.5 min at 72ºC.
Clf A	Pre-denaturation 5 min at 94ºC, 35 cycles of denaturation at 94ºC for 60 s, annealing at 57ºC for 60 s, extension at 72ºC for 60 s and a final extension of 3.5 min at 72ºC.
Nuc	Pre-denaturation 5 min at 94ºC, 37 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 30 s, extension at 72ºC for 30 sec and a final extension of 3.5 min at 72ºC.
Coa, *Spa Igg*	Pre-denaturation 5 min at 94ºC, 30 cycles of denaturation at 94ºC for 60 s, annealing at 58ºC for 60 s, extension at 72ºC for 60 s and a final extension of 3.5 min at 72ºC.
Spa X	Pre-denaturation 5 min at 94ºC, 30 cycles of denaturation at 94ºC for 60 s, annealing at 60ºC for 60 s, extension at 72ºC for 60 s and a final extension of 3.5 min at 72ºC.
femA	Pre-denaturation 5 min at 94ºC, 35 cycles of denaturation at 94ºC for 2 min, annealing at 57ºC for 2 min, extension at 72ºC for 60 s and a final extension of 7 min at 72ºC.
Sau	Pre-denaturation 3 min at 94ºC, 30 cycles of denaturation at 94ºC for 45 s, annealing at 56ºC for 60 s, extension at 72ºC for 90 s and a final extension of 7 min at 72ºC.

Detection of Toxin Production and Toxin Genes in S. aureus Strains

Enterotoxin production was investigated for all *S. aureus* strains isolated using SET-RPLA Toxin kit (Oxoid, Hampshire, UK) according to manufacturer's instructions. The enterotoxin genes (SEA-SEE) were amplified using the primers reported previously (Akineden et al., 2008). *S. aureus* reference strains with SEA (619/93), SEB (62/92), SEC (1229/93), SED (1644/93), SEE (FRI 918) were used as toxin positive controls. The PCR program was performed following 30 cycles of 94°C for 5 min, 94°C for 120 sec, 55°C annealing temperature for toxins A, B and E, 50°C annealing temperature for toxins C and D, 72 °C for 60 and final extension of 72°C for 3.5 min. The amplification was carried out with thermal cycler (Bio-Rad, California, USA).

Antimicrobial Disc Susceptibility Test and Detection of mecA Gene

The isolates were tested for antibiotic susceptibility using agar disc diffusion method using Mueller Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI 2006). The antibiotics included were amoxycillin/clavulanate, ampicillin/sublactam, cefoxitin, cephazolin, clindamycin, chloramphenicol, ciprofloxacin, clarithromycin, fusidic acid, gentamycin, imipenem, kanamycin, levofloxacin, linezolid, moxifloxacin, neomycin, norfloxacin, ofloxacin, oxacillin, penicillin G, piperacillin/tazobactam, quinupristin/dalfopristin, rifampicin, teicoplanin, tetracycline, ticarcillin/clavulanate, tigecycline, tobramycin, trimethoprim-sulfamethazole, vancomycin, enrofloxacin (Oxoid, Hampshire, United Kingdom). Twenty of the antibiotics were in the critically important, 7 were selected from highly important, 4 antibiotics were selected from important class of antibiotics. The inhibition zone diameters were classified susceptible, intermediate or resistant according to CLSI (2006) and Comite de'Antibiogramm de la Societe Francaise de Microbiologie (for fusidic acid) (2001). The primers and PCR method given by Lem et al. (2001) were used for the detection of methicillin resistance gene (*mecA*). PCR consisted of 40 cycles starting with an initial incubation of 95°C for 5 min followed by 95°C for 20 sec, 63°C for 45 sec annealing, 72°C for 45 sec extension and final incubation of 72°C for 5 min.

Sequence Analysis

The bacterial strains were identified by using the primers amplifying 350 bp fragment of 16S ribosomal DNA gene. The primers were: Forward primer: 5′ AGAGTTTGATCCTGGCTCAG-3′ Reverse primer: 5′-CCCACTGCTGCCTCCCGTAG-3′

The reaction conditions and primers were used as described by Riyaz-Ul-Hassan et al. (2008). The amplified products were purified and sequenced with Genetic Analyzer 3130 XL (Applied Biosytems, California, USA). One forward primer was used for sequencing. The sequences obtained were compared with the sequences in the NCBI database with BLAST Analysis. The sequences were aligned with ClustalW program adapted to Mega 5.2 program (Tamura et al. 2011). Phylogenetic distance tree was constructed with Maximum Likelihood method with phylogency test of Bootstrap method with 1000 replications to investigate the similarity between different isolates.

Results and Discussion

Identification of the Isolates

Due to the importance of the *S. aureus* as an important foodborne pathogen, it is necessary to characterize *S. aureus* strains isolated from white cheese samples and to investigate these strains by toxin typing. For this purpose 207 strains were obtained from 31 different cheese samples. Twenty four (24) isolates that were Gram positive and catalase positive and gave at least one positive reaction to DNase activity, mannitol fermentation and latex agglutination tests were further investigated for the presence of *coa* and *nuc* genes and sequence analyses. The presence of *nuc* gene was examined with qPCR analysis. A total of 3 of the isolates were identified as *S. aureus* according to all biochemical test, PCR, qPCR and sequence analysis results with higher than 93% sequence identity. The test results of the isolates to these analyses are given in Table 2.

Previously, it was reported that there is no single test that can definitely identify *S. aureus* (Kateete et al., 2010). Biochemical tests are not enough for reliable identification of *S. aureus* strains. For this reason both biochemical and genetic tests were carried out for correct identification of the isolated strains. Comparative analyses such as latex agglutination test, tube coagulase test, and *coa* and *nuc* gene presence were examined to choose the gold standard method for identification of *S. aureus*. Tube coagulase test has been used for differentiation of *S.aureus* in most of the studies (Malathi et al., 2009; Akineden et al., 2011). In one of these studies; latex agglutination test, Slidex Staph plus test and tube coagulase test were compared. Analysing the presence of *coa* gene by PCR was used as a gold standard for detection of *S. aureus* and tube coagulase test was recommended as routine test to correctly differentiate *S. aureus* from coagulase negative staphylococci (Tiwari et al., 2008). However, it is important that coagulase negative strains of *S. aureus* have also been reported. In these studies, the isolates gave negative reaction to tube coagulase test, but they all carried *coa* gene

when amplified with PCR (Vandenesch et al., 1994; Akineden et al., 2011).

qPCR amplification of *nuc* gene has been used as a gold standard for the detection of *S.aureus* in many studies (Hein et al., 2001, Alarcón et al., 2006, Esan et al., 2009). The *nuc* gene was reported to have *S. aureus* species specific sequences (Asfour and Darwish, 2011). In this study, the results showed that 3 isolates (16, 20, 21) identified as *Staphylococcus* spp. that tested negative in tube coagulase test were positive for the *coa* gene. Also 2 of the isolates (15, 20) that were tube coagulase negative had the *nuc* gene. These isolates gave positive reaction to latex agglutination test.

The isolates harbouring the *nuc* gene could not be identified as *S. aureus* by sequence analyses. The common property of these isolates was that they did not show DNase activity. The sequence analyses were performed to investigate the genetic similarity of the isolates using 16S rDNA gene sequences. The distance tree showing the genetic relatedness of the isolates is given in Figure 1. But there were no definite clusters among the *S. aureus* isolates and other isolates. The isolates 1, 3, 4, 5 and *S. aureus* RSKK 1009 which was used as positive control (PC) were found closer to each other under the same branch of the tree. The isolate 18 which was sequenced as *S. carnosus* was grouped with *S. hyicus* and *S. intermedius* apart from the other isolates.

Virulence Properties of the Isolates

The virulence properties of *S. aureus* isolates were investigated by PCR analysis. Several target regions including the 23S rDNA, the spacer region between 16S-23S rDNA, *coa*, *clf*, *spa*X, and *spa*IgG were amplified in the bacterial genome using PCR method. *Sau* and *fem*A regions were used as internal controls in several studies for confirmation of the presence of *S. aureus* (Mehrotra et al., 2000; Holochová et al., 2010). The results of the PCR experiments are given in Table 3. As the results indicated, except for 16S-23S region, all of the target regions tested positive to the isolated strains. Also, in correlation to our study, 5 of the 64 isolates which were confirmed as *S. aureus* tested negative for the 16S-23S rDNA intergenic spacer region in a previous study (Akineden et al., 2008).

Table 2. Biochemical test results, PCR, qPCR and sequence analyses results of the isolates.

Sample code	Gram staining	Catalase test	Latex agglutination test	DNase activity	Mannitol ferm.	Tube *coa*gulase test	*coa*	*nuc*	Sequence
PC	+	+	+	+	+	+	+	+	*S. aureus* (97%)
1	+	+	+	+	+	+	+	+	*S. aureus* (94%)
2	+	+	+	+	+	+	+	+	*S. aureus* (98%)
3	+	+	+	+	+	+	+	+	*S. aureus* (90%)
4	+	+	+	-	+	+	+	+	*S.pasteuri* (85%)
5	+	+	+	-	+	+	+	+	*S.saprophyticus* (83%)
6	+	+	+	-	-	-	-	-	*Staphylococcus spp.*(97%)
7	+	+	-	-	+	-	-	-	*S. epidermidis* (99%)
8	+	+	+	-	-	-	-	-	*Macrococcus* spp. (92%)
9	+	+	+	-	-	-	-	-	*Staphylococcus* spp.(87%)
10	+	+	+	-	-	-	-	-	*Staphylococcus* spp.(93%)
11	+	+	+	+	-	-	-	-	*S. carnosus* (86%)
12	+	+	+	+	-	-	-	-	*S. carnosus* (99%)
13	+	+	+	-	+	-	-	-	*S. aureus* (83%)
14	+	+	+	-	-	-	-	-	*S.carnosus* (85%)
15	+	+	+	-	+	-	-	+	*S. xylosus* (88%)
16	+	+	+	-	-	-	+	-	*Uncultured bacterium (91%)*
17	+	+	+	+	+	-	-	-	*S. sciuri* (83%)
18	+	+	+	-	+	-	-	-	*S. carnosus* (81%)
19	+	+	+	-	+	-	-	-	*S. saprophyticus* (86%)
20	+	+	+	-	+	-	+	+	*S. equorum* (81%)
21	+	+	+	-	-	-	+	-	*S. carnosus* (99%)
22	+	+	+	-	+	-	-	-	*S. xylosus* (98%)
23	+	+	+	-	+	-	-	-	*S. saprohyticus* (88%)
24	+	+	+	+	+	-	-	-	*Staphylococcus* spp. (84%)

PC: Positive control (*S. aureus* RSKK 1009)

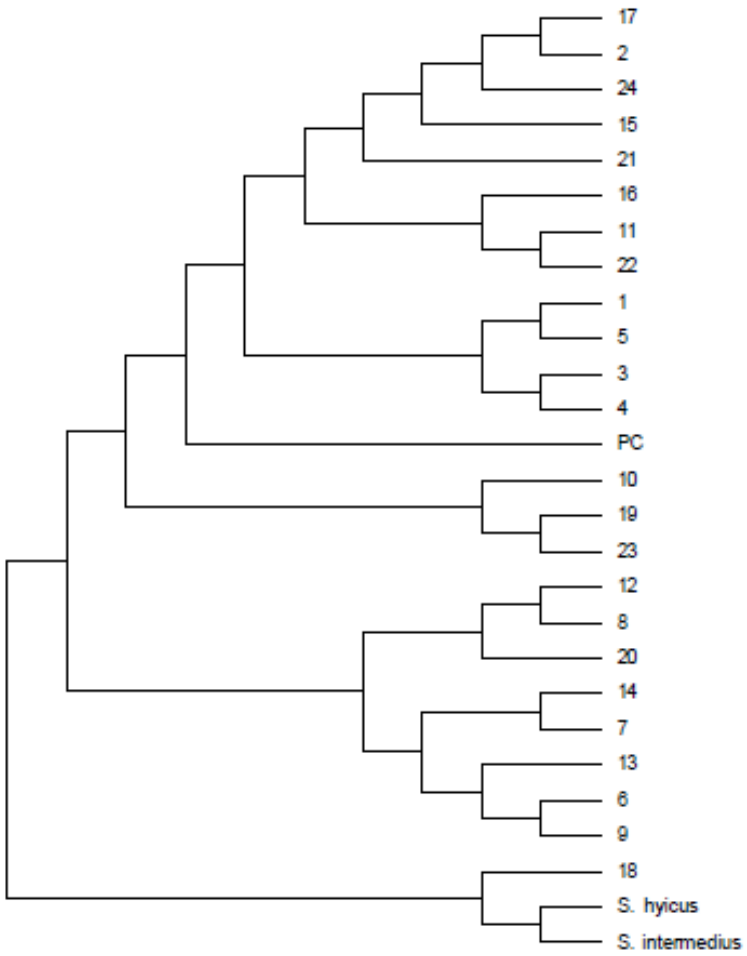

Figure 1. Genetic relatedness of *Staphylococcus* isolates

Toxin Production Ability of the Isolates

The presence of the enterotoxin genes were also investigated by application of PCR targeting SEA, SEB, SEC, SED and SEE, but none of the strains were found to contain these toxin genes in their genomes. This result was in accordance with the results obtained using the toxin detection kit.

Staphylococcus strains produce thermonuclease that degrades both DNA and RNA. The *nuc* gene encoding thermonuclease protein has species-specific sequences (Brakstad et al., 1992). Detection of toxin genes does not necessarily indicate that the organism produces biologically active molecules or toxins. In a food system, PCR detection of toxin genes coupled with the specific detection of the producing species (*nuc*-PCR) represents the potential of toxin formation in food and hazardous food products due to the level of contamination (Ercolini et al., 2004). In this study, the correlation between the presence of *nuc* gene and toxin genes was not found. The production of toxins was also tested by toxin test kit, but the results were in accordance with the PCR analyses of toxin genes.

Antibiotic Resistance Profiles of the Isolates

Antibiotic resistance is an important issue for transmission of *S. aureus* isolates to humans and the use of antibiotics as therapeutic purposes or growth promoters in animal husbandry (Alian et al., 2012). In this study, susceptibilities of the 3 isolates to 31 different antibiotics were investigated by agar disc diffusion method. Antibiotic resistance profiles of these isolates are shown in Table 4. All of the isolates were found to be susceptible to amoxycillin, ampicillin, cephazolin, chloramphenicol, ciprofloxacin, clindamycin, gentamycin, imipenem, kanamycin, levofloxacin, linezolid, ofloxacin, oxacillin, rifampicin, teicoplanin, tetracycline, tobramycin, trimethoprim-sulfamethazole, vancomycin, en-

rofloxacin. In our study, it was found that 2 (2, 3) of the isolates were found to be resistant to Penicillin G. This can be related to the common use of penicillin for treatment of infections in humans and animals (Yucel et al., 2011). One isolate (1) showed intermediate resistance to fusidic acid. Similarly Sudagidan et al. (2010) investigated the antibiotic susceptibilites of *S. aureus* strains isolated from 1070 food samples and found that most of the strains were resistant to penicillin G from the samples collected from Marmara region of Turkey. In another study, antibiotic resistance tests of 138 *S. aureus* strains isolated from 413 food samples obtained from Eskisehir and Kütahya provinces in Turkey illustrated that many of the strains showed high resistance to penicillin G (Guven et al., 2010).

Table 3. PCR analysis results of *S. aureus* isolates.

Sample code no	23S rDNA	16S-23S	*coa*	*clf*	*spa X*	*spa Igg*	*femA*	*sau*
1	+	-	+	+	+	+	+	+
2	+	-	+	+	+	+	+	+
3	+	-	+	+	+	+	+	+
PC	+	+	+	+	+	+	+	+

PC: Positive control (*S. aureus* RSKK 1009)

Table 4. Antibiotic resistance profiles of *S. aureus* isolates.

		Isolates			Zone diameters		
Antibiotics Name	**Code**	**1**	**2**	**3**	**R**	**I**	**S**
Amoxycillin/clavulanate	AMC30	43	29	30	≤19		≥20
Ampicillin/sublactam	SAM20	40	20	25	≤11	12-14	≥15
Cefoxitin	FOX30	33	33	33	≤21		≥22
Cephazolin	KZ30	38	35	27	≤14	15-17	≥18
Chloramphenicol	C30	31	25	29	≤12	13-16	≥17
Ciprofloxacin	CIP5	33	32	31	≤15	16-18	≥19
Clarithromycin	CLR15	34	28	29	≤13	14-17	≥18
Clindamycin	DA2	35	29	28	≤14	15-20	≥21
Fusidic acid	FD10	20	32	34	≤15	16-21	
Gentamycin	CN120	40	31	31	≤12	13-14	
İmipenem	IPM10	51	50	52	≤13	14-15	
Kanamycin	K30	33	24	25	≤13	14-17	
Levofloxacin	LEV5	31	33	33	≤15	16-18	
Linezolid	LZD30	36	31	33			≥21
Moxifloxacin	MXF5	33	34	35	≤20	21-23	≥24
Neomycin	N30	30	23	24			
Norfloxacin	NOR10	29	30	30	≤12	13-16	≥17
Ofloxacin	OFX5	28	31	30			
Oxacillin	OX1	13	24	26	≤10	11-12	≥13
Penicillin G	P10	33	21	22	≤28		≥29
Piperacillin/tazobactam	TZP110	38	27	27	≤17		≥18
Quinupristin/dalfopristin	QD15	33	27	28	≤15	16-18	≥19
Rifampicin	RD5	40	33	35	≤10	17-19	≥20
Teicoplanin	TEC30	21	19	20	≤10	11-13	≥14
Tetracycline	TE30	43	34	35	≤14	15-18	≥19
Ticarcillin/clavulanate	TIM85	35	34	33	≤22		≥23
Tigecycline	TGC15	36	28	30			≥20
Tobramycin	TOB10	34	24	25	≤12	13-14	≥15
Trimethoprim-sulfamethazole	SXT25	37	33	34	≤10	11-15	≥16
Vancomycin	VA30	23	20	21			≥15
Enrofloxacin	ENR5	31	33	33	≤13	14-22	

R: Resistant I: Intermediate Resistance S: Susceptible zone diameter standards reported by Clinical and Laboratory Standards Institute.

As reported by Alian et al. (2012), *S. aureus* strains isolated from milk samples were most commonly resistant to ampicillin (54.3%), followed by oxacillin (28.3%), tetracycline (26.1%), penicillin G (23.9%), erythromycin (23.9%), trimethoprim-sulfamethoxazole (17.4%) and cephalotin (2.2%). It was evident that the isolates were resistant to ß-lactams which were in accordance with our findings. Miranda et al. (2009) investigated the antibiotic resistance profiles of *S. aureus* isolated from conventional and organic cheeses and concluded that raw and pasteurized milk conventional cheese samples showed higher levels than pasteurized milk organic cheese samples for ciprofloxacin, penicillin, oxacillin and rifampicin. *Mec*A gene that is highly conserved in methicillin resistant *S. aureus* strains provides resistance to methicillin and all other ß-lactam antibiotics (Chambers, 1997). The susceptibility results to oxacillin, vancomycin, and erythromycin in the disc diffusion test were supported by PCR analysis of *mec*A gene which reveals that none of the isolates were resistant to methicillin.

Conclusions

The results indicated that the target genes (*coa*, *nuc*) that were regarded as gold standard regions for *S. aureus* were not found to be unique for the identification of *S. aureus*. The DNase activity which was used as a discriminatory test for *S. aureus* was not unique to *S. aureus* isolates. In addition, this study revealed that the presence of *nuc* gene did not correlate with the DNase activity. No correlation was observed between the *nuc* gene and enterotoxigenecity. Three isolates were confirmed as *S. aureus* by using phenotypic tests, genotypic tests, and sequencing. These isolates were found to be resistant to Penicillin G only with slight resistance to fusidic acid. In conclusion, sequencing of the ribosomal DNA solely and only using phenotypic tests in the identification of *S. aureus* was not enough for correct identification of the isolates. In order to identify correctly all the genetic and phenotypic markers should be evaluated together.

Compliance with Ethical Standard

Conflict of interests: The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

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References

Akineden, Ö., Hassan, A.A., Schneider, E., Usleber, E. (2008). Enterotoxigenic properties of *Staphylococcus aureus* isolated from goats' milk cheese. *International Journal of Food Microbiology*, 124, 211-216.

Akineden, Ö., Hassan, A.A., Schneider, E., Usleber, E. (2011). A *coa*gulase-negative variant of *Staphylococcus aureus* from bovine mastitis milk. *Journal of Dairy Research,* 78, 38-42.

Alarcón, B., Vicedo, B., Aznar, R. (2006). PCR-based procedures for detection and quantification of *Staphylococcus aureus* and their application in food. *The Society of Applied Microbiology, Journal of Applied Microbiology,* 100, 352-364.

Alian, F., Rahimi, E., Shakerian, A., Momtaz, H., Riahi, M., Momeni, M. (2012). Antimicrobial resistance of *Staphylococcus aureus* isolated from bovine, sheep and goat raw milk. *Global Veterinaria,* 8, 111-114.

André, C.M.D.P.B., Campos, M.R.H., Borges, L.J., Kipnis, A., Pimenta, F.C., Serafini, A.B. (2008). Comparison of *Staphylococcus aureus* isolates from food handlers, raw bovine milk and Minas Frescal cheese by antibiogram and pulsed-field gel electrophoresis following SmaI digestion. *Food Control*, 19, 200-207.

Aprodu, I., Walcher, G., Schelin, J., Hein, I., Norling, B., Rådström, P., Nicolau, A., Wagner, M. (2011). Advanced sample preparation for the molecular quantification of *Staphylococcus aureus* in artificially and naturally contaminated milk. *International Journal of Food Microbiology*, 145, 61-65.

Asfour, H.A.E., Darwish, S. H. (2011). Phenotypic and genotypic detection of both *mecA* and *blaZ* genes mediated β-lactam resistance in Staphylococcus strains isolated from bovine mastitis. *Global Veterineria,* **6**, 39-50.

Brakstad, O.G, Aasbakk, K., Maeland, J.A. (1992). Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *Journal of Clinical Microbiology*, 30, 1654-1660.

Chapaval, L., Moon, D.H., Gomes, J.E., Duarte, F.R., Tsai, S.M. (2008). An alternative method for *Staphylococcus aureus* DNA isolation. *Arquivo Brasileiro Medicina Veterinária e Zootecnia*, 60*,* 299-306.

Chambers H.F. (1997). Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clinical Microbiology Reviews,* 10(4), 781-791.

CLSI (Clinical Laboratory Standards Institute). (2006). *Performance Standards for Antimicrobial disc susceptibility testing*, sixth ed. Pennsylvania, USA, p 188.

Comite de'Antibiogramm de la Societe Francaise de Microbiologie Report (2000−2001). (2001). *MIC and zone diameter interpretive standards and interpretive reading rules for Staphylococcus spp.*, Paris.

Cremonesi, P., Perez, G., Pisoni, G., Moroni, P., Morandi, S., Luzzana, M., Brasca, M., Castiglioni, B. (2007). Detection of enterotoxigenic *Staphylococcus aureus* isolates in raw milk cheese. *The Society of Applied Microbiology, Letters in Applied Microbiology,* 45, 586–591.

Ercolini, D., Blaiotta, G., Fusco, V., Coppola, S. (2004). PCR-based detection of enterotoxigenic *Staphylococcus aureus* in the early stages of raw milk cheese making. *Journal of Applied Microbiology*, 96, 1090-1096.

Ertas, N., Gonulalan, Z., Yildirim, Y., Kum, E. (2010). Detection of *Staphylococcus aureus* enterotoxins in sheep cheese and dairy desserts by multiplex PCR technique *International Journal of Food Microbiology,* 142, 74-77.

Esan, C.O., Famurewa, O., Lin, J., Shittu, A.O. (2009). Characterization of *Staphylococcus aureus* isolates obtained from health care institutions in Ekiti and Ondo States, South-Western Nigeria. *African Journal of Microbiology Research*, 3, 962-968.

Forsman, P., Tissala-Timisjärvi, A., Alatossava, T. (1997). Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S–23S rRNA spacer regions. *Microbiology*, 143, 3491-3500.

Franklin, D., Lowy, M.D. (1998). *Staphylococcus aureus* infections. *The New England Journal of Medicine*, 339, 520-532.

Gandra, E.Á., Silva, J.A., Macedo, M.R.P., Araújo M.R., Mata, M.M., Silva, W.P. (2005). Differentiation between *Staphylococcus aureus*, *S. intermedius* and *S. hyicus* using phenotypical tests and PCR. *Alimentos e Nutrição Araraquara,* 16, 99-103.

Gičová, A., Oriešková, M., Oslanecová, L., Drahovská, H., Kaclíková, E. (2014). Identification and characterization of *Cronobacter* strains isolated from powdered infant foods. *Letters in Applied Microbiology*, 58, 242-247.

Gomez, C., Pinal, L., Franco, J., Carrillo, J.M., Ramírez, J. (2007). Identification of *Staphylococcus aureus* strains negative for enterotoxins A, B and C isolated from bovine mastitis in México. *Veterinary Immunology and Immunopathology*, 117, 249-253.

Guven, K., Mutlu, M.B., Gülbandılar, A., Cakır, P. (2010). Occurrence and characterization of *Staphylococcus aureus* isolated from meat and dairy products consumed in Turkey. *Journal of Food Safety*, 30, 196-212.

Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E., Wagner, M. (2001). Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheeses. *Applied and Environmental Microbiology*, 67, 3122-3126.

Holochová, P., Růžičková, V., Dostálová, L., Pantůček, R., Petráš, P., Doškař, J. (2010). Rapid detection and differentiation of the exfoliative toxin A-producing Staphylococcus aureus strains based on ϕETA prophage polymorphisms. *Diagnostic Microbiology and Infectious Disease*, 66, 248-252.

Huong, B.T.M., Mahmud, Z.H., Neogi, S.B., Kassu, A., Nhien, N.V., Mohammad, A., Yamato, M., Ota, F., Dao, N.T.L.H.T.A. and Khan, N.C. (2010). Toxigenicity and genetic diversity of *Staphylococcus aureus* isolated from Vietnamese ready-to-eat foods. *Food Control*, 21, 166-171.

Kabadjova, P., Dousset, X., Le Cam, V., Prevost, H. (2002). Differentiation of closely related *Carnobacterium* food isolates based on 16S–23S ribosomal DNA intergenic spacer region polymorphism. *Applied and Environmental Microbiology*, 68, 5358-5366.

Kateete, D.P., Kimani, C.N., Katabazi, F.A., Okeng, A., Okee, M.S., Nanteza, A., Joloba, M.L., Najjuka, F.C. (2010). Identification of *Staphylococcus aureus*:

DNase and mannitol salt agar improve the efficiency of the tube coagulase test. *Annals of Clinical Microbiology and Antimicrobials*, 9, 23-29.

Kong, C., Neoh H., Nathan S. (2016). Targeting *Staphylococcus aureus* toxins: a potential form of anti-virulence therapy. *Toxins (Basel),* 8(3), 72.

Lem, P., Spiegelman, J., Toye, B. and Ramotar K. (2001). Direct detection of *mecA, nuc* and 16S rRNA genes in BacT/Alert blood culture bottles. *Diagnostic Microbiology and Infectious Disease*, 41, 165-168.

Malathi, J., Sowmiya, M., Margarita, S., Madhavan, H.N., Lily Therese, K. (2009). Application of PCR based-RFLP for species identification of ocular isolates of methicillin resistant Staphylococci (MRS). *Indian Journal of Medical Research*, 130, 78-84.

Mehrotra, M., Wang, G., Johnson, W.M. (2000). Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *Journal of Clinical Microbiology*, 38, 1032-1035.

Miranda, J.M., Mondragón, A., Vázqueza, B.I., Fentea, C.A., Cepedaa, A., Francoa, C.M. (2009). Microbiological quality and antimicrobial resistance of *Escherichia coli* and *Staphylococcus aureus* isolated from conventional and organic ‘‘Arzúa-Ulloa’’ cheese. *CyTA – Journal of Food.* **7**, 103-110.

Peles, F., Wagner, M., Varga, L., Hein, I., Rieck, P., Gutser, K., Keresztúri, P., Kardos, G., Turcsányi, I., Béri, B., Szabó, A. (2007). Characterization of *Staphylococcus aureus* strains isolated from bovine milk in Hungary. *International Journal of Food Microbiology,* 118, 186-193.

Phuektes, P., Browning, G.F., Anderson, G., Mansel, P.D. (2003). Multiplex polymerase chain reaction as a mastitis screening test for *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* in bulk milk samples. *Journal of Dairy Research*, 70, 149-155.

Riyaz-Ul-Hassan, S., Verma, V., Qazi, G.N. (2008). Evaluation of three different molecular markers for the detection of *Staphylococcus aureus* by polymerase chain reaction. *Food Microbiology*, 25, 452-459.

Sperber W.H., Tatini S.R. (1975). Interpretation of the tube coagulase test for identification of *Staphylococcus aureus. Applied Microbiology,* 29(4), 502-505.

Straub, J.A., Hertel, C., Hammes, W.P. (1999). A 23S rRNA targeted polymerase chain reaction-based system for detection of *Staphylococcus aureus* in meat starter cultures and dairy products. *Journal of Food Protection*, 62, 1150–1156.

Stutz, K., Stephan, R., Tasara, T. (2011). *SpA, ClfA*, and *FnbA* Genetic variations lead to staphaurex test-negative phenotypes in bovine mastitis *Staphylococcus aureus* isolates. *Journal of Clinical Microbiology*, 49, 638-646.

Sudagidan, M., Çavuşoğlu, C., Bacakoğlu, F. (2008). Investigation of the virulence genes in methicillin-resistant *Staphylococcus aureus* strains isolated from biomaterial surfaces. *Mikrobiyoloji Bülteni*, 42, 29-39.

Sudagidan, M., Aydin, A. (2010). Virulence properties of methicillin-susceptible *Staphylococcus aureus* food isolates encoding Panton–Valentine Leukocidin gene. *International Journal of Food Microbiology*, 138, 287-291.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731-2739.

Tiwari, H.K., Sapkota, D., Sen, M.R. (2008). Evaluation of different tests for detection of *Staphylococcus aureus* using coagulase (*coa*) gene PCR as the gold standard. *Nepal Medical College Journal*, 10, 129-131.

Vandenesch, F., Lebeau, C., Bes, M., Mcdevitt, D., Greenland, T., Novickj, R.P., Etienne, J. (1994). Coagulase deficiency in clinical isolates of *Staphyococcus aureus* invoIves both transcriptional and post-transcriptional defects. *Journal of Medical Microbiology*, 40, 344-349.

Vasconcelos, N.G., Cunha, M.L.R.S. (2010). Staphylococcal enterotoxins: Molecular aspects and detection methods. *Journal of Public Health and Epidemiology*, 2, 29-42.

Villarreal, M.L.M., Padilha, M., Vieira, A.D.S., Franco, B.D.G.M., Martinez, R.C.R., Saad, S.M.I. (2013). Advantageous direct quantification of viable closely related probiotics in petit-suisse cheeses under in vitro gastrointestinal conditions by propidium monoazide-qPCR. *PloS One*, 8, 1-11.

Yucel, N. Citak, S., Bayhun, S. (2011). Antimicrobial resistance profile of *Staphylococcus aureus* isolated from clinical samples and foods of animal origin. *Foodborne Pathogens and Disease*, 8, 427-431.