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Duplex real-time PCR assay for rapid identification of *Staphylococcus aureus* isolates from dairy cow milk

Rachel Pilla, Gustavo G M Snel, Michela Malvisi and Renata Piccinini*

Department of Veterinary Sciences and Public Health, University of Milan, Via Celoria 10, 20133 Milano, Italy

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Staphylococcus aureus isolates from dairy cow mastitis are not always consistent with the characteristic morphology described, and molecular investigation is often needed. The aim of the study was to develop a duplex real-time PCR assay for rapid identification of *Staph. aureus* isolates, targeting both *nuc* and *Sa442*. Overall, 140 isolates collected from dairy cow mastitis in 90 different herds, were tested. All strains had been identified using morphological and biochemical characteristics. DNA from each strain was amplified in real-time PCR assay, to detect *nuc* or *Sa442*. Thereafter, a duplex real-time PCR assay was performed, and specificity of the amplified products was assessed by high resolution melting curve analysis. Out of 124 *Staph. aureus* isolates, 33 did not show the typical morphology or enzymic activity; in 118 strains, the two melt-curve peaks consistent with *nuc* and *Sa442* were revealed, while 2 isolates showed only the peak consistent with *Sa442*. Four isolates bacteriologically identified as *Staph. aureus*, were PCR-negative and were further identified as *Staph. pseudintermedius* by sequencing. *Staph. pseudintermedius* and coagulase-negative staphylococci did not carry *nuc* or *Sa442*. The results showed the correct identification of all isolates, comprehending also coagulase—or nuc-negative *Staph. aureus*, while other coagulase-positive Staphylococci were correctly identified as non-*Staph. aureus*. Both sensitivity and specificity were 100%. High resolution melting analysis allowed easy detection of unspecific products. Finally, the duplex real-time PCR was applied directly to 40 milk samples, to detect infected mammary quarters. The assay confirmed the results of bacteriological analysis, on *Staph. aureus*-positive or—negative samples. Therefore, the proposed duplex real-time PCR could be used in laboratory routine as a cost-effective and powerful tool for high-throughput identification of atypical *Staph. aureus* isolates causing dairy cow mastitis. Also, it could be applied directly to milk samples, to detect *Staph. aureus* mammary infections avoiding bacteriological analysis.

Keywords: *Staphylococcus aureus*, mastitis, duplex real-time PCR, dairy cow.

Staphylococcus aureus is a major pathogen causing dairy cow mastitis. In control programmes, it is essential to promptly identify positive animals, to avoid the spread of infection. Bacterial identification is based on phenotypic criteria, but *Staph. aureus* isolates are often not consistent with the characteristic morphology described (Boerlin et al. 2003). Typically, the colonies of growth are pigmented, β-haemolytic and coagulase positive (Hogan et al. 1999). Nevertheless, many isolates show week haemolysis and some do not express coagulase (Hogan et al. 1999). In addition, coagulase-negative staphylococci (CNS) are often morphologically similar to *Staph. aureus* isolates, and display α-haemolysis (Boerlin et al. 2003).

*For correspondence; e-mail: renata.piccinini@unimi.it

As a consequence, further investigation of suspected colonies using molecular techniques is often needed. Several species-specific markers have been described, to confirm *Staph. aureus* isolates, including the coagulase gene (*coa*), the factor essential for expression of methicillin resistance gene (*femA*), the thermonuclease gene (*nuc*), and a chromosomal DNA insertion of 442 bp (*Sa442*). Both *coa* and *femA* show high polymorphism, and are unsuitable for diagnostic purposes (Schwarzkopf & Karch, 1994; Jayaratne & Rutherford, 1997). To the contrary, *nuc* is frequently used as a species-specific marker both in human (Costa et al. 2005) and veterinary isolates (Gao et al. 2011), being highly conserved among *Staph. aureus* strains (Brakstad et al. 1992). Nonetheless, some isolates have been reported as *nuc* negative by partial deletion or mutation of the *nuc* gene (Costa et al. 2005; Van Leeuwen et al. 2008). Recently, *Sa442* targeting has been proposed as an

Table 1. Primer sequence, amplicon size and PCR conditions used in single-gene real-time PCR (†) and duplex assay (‡)

Gene	Primer sequence (5′–3′)	Amplicon size	PCR conditions
Nuc	*fw* GCGATTGATGGTGATACGGTT *rv* AGCCAAGCCTTGACGAACTAAAGC Brakstad et al. 1992 (†, ‡)	270 bp	95 °C for 2 min; 35 cycles including 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s.
Sa442	*fw* GTCGGGTACACGATATTCTTCACG *rv* CTCGTATGACCAGCTTCGGT Grisold et al. 2002 (†)	178 bp	95 °C for 2 min; 40 cycles including 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 15 s
	fw AATCTTTGTCGGTACACGATATTCTTCACG *rv* CGTAATGAGATTTCAGTAGATAATACAACA Martineau et al. 1998 (‡)	108 bp	95 °C for 2 min; 35 cycles including 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s.

alternative method to culture assays (Martineau et al. 1998) even though partial deletion is recognized in *Sa442* among a few human strains (Klaassen et al. 2003; Heilman et al. 2004). As a consequence, the use of one gene as single marker may lead to misidentification, while incorporation of an additional species-specific marker could increase strain coverage and assay robustness (Van Leeuwen et al. 2008).

Therefore, the present study was aimed at the development of a species-specific duplex real-time PCR assay for rapid identification of *Staph. aureus* isolates, using both *nuc* and *Sa442* as target genes.

Materials and Methods

Overall 140 strains, out of them 124 *Staph. aureus* and 16 CNS, from the culture collection of the Department, were tested. Bacterial isolates had been collected from quarter milk samples of dairy cow mastitis in 90 different herds, which were undergoing a control programme for contagious pathogens. Quarter milk (10 µl) had been plated on blood agar plate (5% bovine blood; Oxoid, Cambridge, UK) and incubated overnight at 37 °C. Colonies of growth had been isolated; the large and haemolytic ones, that were catalase and coagulase positive, were identified as *Staph. aureus*, as indicated elsewhere (Hogan et al. 1999). The non-haemolytic and coagulase-positive isolates, as well as the coagulase-negative isolates morphologically resembling *Staph. aureus*, and all CNS were further identified by API ID32 Staph (Biomerieux, Marcy l'Etoile, France). Strains were maintained at − 80 °C in Microbank Bacterial Preservation System (Thermo Fisher Scientific Inc., Waltham MA, USA) until needed. In addition, three ATCC strains were included: *Staph. aureus* ATCC 25923 and *Staph. aureus* ATCC 29213 as positive controls, *Staphylococcus epidermidis* ATCC 12228 as a negative control.

After thawing, strains were cultured on blood agar plates (Oxoid, Cambridge, UK); then one colony of growth was inoculated in brain heart infusion broth (Oxoid, Cambridge, UK) and grown overnight at 37 °C. DNA was extracted using Wizard Genomic DNA extraction kit (Promega Inc., Madison WI, USA) following the manufacturer's instructions, with the addition of lysostaphin (5 mg/ml; Sigma-Aldrich, St Louis MO, USA) for bacterial lysis. DNA amount and purity were tested with a ND-100 Spectrophotometer (NanoDrop Technologies Inc., Wilmington DE, USA).

As a preliminary step, all samples were amplified in single-gene real-time PCR assay, to detect presence or absence of *nuc* or *Sa442* genes. Real-time PCR was performed on an Opticon 2 (MJ Research, Bio-Rad, Carlsbad CA, USA), following conditions listed in Table 1. Each 20-µl reaction mixture consisted of 10 µl SsoFast EvaGreen Supermix (Bio-Rad), 0·075 µM of *Sa442* primers or 0·1 µM of *nuc* primers, and 20 ng of genomic DNA. Specificity of the amplified PCR product was assessed by analysis of melting curves.

Coagulase-negative *Staph. aureus* strains and the strains that were negative for one or both marker genes, were further characterized by sequencing a fragment of RNA polymerase (*rpoB*) gene, as described elsewhere (Drancourt & Raoult, 2002). Sequences were aligned using GenBank BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and identification was assessed by >97% similarity with one of the database *rpoB* sequences.

The duplex PCR assay was performed using an Eco Real-time PCR System (Illumina Inc., San Diego CA, USA), and choosing a new primer pair for *Sa442,* in order to produce amplicons of sufficiently different melting temperatures. PCR conditions are listed in Table 1. Each 18-µl reaction mixture consisted of 9 µl Sso Fast EvaGreen Supermix (Bio-Rad), 0·4 µM of *Sa442* primers, 0·1 µM of *nuc* primers and 20 ng of DNA template. Specificity of the amplified PCR product was assessed by performing high resolution melting curve analysis.

Thereafter, 40 quarter milk samples were randomly selected from 5 herds, to evaluate the possible application of the method to detect *Staph. aureus* directly in the milk. Half of the samples tested had been previously identified as positive for *Staph. aureus* by bacteriological procedures, as described above. All other samples were either bacteriologically negative, or positive for other micro-organisms. Bacterial DNA was extracted according to Cremonesi et al. (2006), and duplex PCR assay was performed as described above.

Results

Overall, out of 124 *Staph. aureus* strains, 33 did not show the typical morphology or enzymic activity: 5 were

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Table 2. Results of the identification of coagulase-positive or coagulase-negative strains tested in the study, by single-gene real-time PCR and duplex assay

			Single-gene PCR		Duplex PCR	
Identification	Coagulase	Number of isolates (%)	*nuc*	*Sa442*	*nuc*	*Sa442*
Staph. aureus	+	113 (80·7)	+	+	+	+
Staph. aureus	−	5 (3·6)	+	+	+	+
Staph. aureus	+	2 (1·4)	−	+	−	+
Staph. pseudintermedius	+	4 (2·9)	−	−	−	−
CNS	−	16 (11·4)	−	−	−	−

coagulase-negative, 17 were weakly haemolytic and 11 were non-haemolytic. Out of 16 CNS isolates, 7 different species were identified: 3 isolates were classified as *Staph. haemolyticus*, 3 as *Staph. chromogenes*, 3 as *Staph. xylosus*, 2 as *Staph. warneri*, 2 as *Staph. sciuri*, 1 as *Staph. epidermidis*, 1 as *Staph. lentus*. Finally, 1 isolate was a *Micrococcus sp*.

Out of 124 *Staph. aureus* isolates, 118 isolates (95·2%) carried *nuc*, while 6 (4·8%) were negative. The amplicons revealed a peak at a melting temperature of 79·4 °C. Not one CNS harboured the *nuc* gene. When *Sa442* was considered, 120 (96·8%) showed a peak in the melting temperature at 77·6 °C, while 4 strains (3·2%) gave negative results. No CNS carried the *Sa442* gene.

Further characterization of coagulase-negative *Staph. aureus* strains and of the strains that were negative for one or both marker genes, was performed by sequencing a fragment of *rpoB* gene. All coagulase-negative *Staph. aureus* strains, and 2 strains carrying only *Sa442*, were confirmed as *Staph. aureus*. To the contrary, 4 isolates previously identified as *Staph. aureus* by phenotypic and biochemical characteristics, that were PCR-negative for both genes, presented 99–100% similarity with *Staph. pseudintermedius* (GenBank accession number CP002478·1), aligning within the *rpoB* gene sequence. Those strains were therefore reclassified as *Staph. pseudintermedius*.

According to single-gene PCR results, duplex PCR assay revealed two amplicons for 118 *Staph. aureus* strains, showing two peaks in the melting curve at 74·5 °C (*Sa442*) and 79·4 °C (*nuc*). In 2 strains, only the peak consistent with *Sa442* was detected. *Staph. pseudintermedius* and CNS isolates showed weak to no amplification, with peaks in the melting curve outside the expected range of temperature, and were therefore considered negative for both genes.

Results of single-gene and duplex PCR assays are summarized in Table 2. Since all staphylococcal isolates tested in the study were correctly identified by duplex real-time PCR assay, calculated sensitivity and specificity were 100%.

When the assay was applied to quarter milk samples, 17 out of 20 samples previously diagnosed as bacteriologically positive for *Staph. aureus*, presented two melt-curve peaks, which were compatible with *nuc* and *Sa442* genes; 3 samples revealed only the peak compatible with *Sa442* gene. All the samples bacteriologically negative for *Staph. aureus* were lacking both genes. Out of such samples, 11 were sterile when tested by bacteriological analysis; CNS were detected in 3 samples; *Prototheca* sp. in 2 samples; *Streptococcus* sp. or *Enterococcus faecalis* in 2 samples and *Serratia* sp. in 1 sample; finally, 1 sample presented mixed bacterial growth and was considered as contaminated.

Discussion

It has been stated that the diagnosis of *Staph. aureus* based on the detection of a single species-specific gene could lead to misidentification, favouring the spread of atypical strains (Van Leeuwen et al. 2008). Furthermore, the occurrence of *Staph. aureus* strains lacking the *nuc* gene has been previously reported in milk samples collected in Northern Italy (Cremonesi et al. 2005). The results of the present study showed that 2 *Staph. aureus* isolates would not have been identified as such, if tested by single-gene PCR for *nuc*. Interestingly, one of these atypical strains had been isolated in a herd during a control programme for contagious micro-organisms. In this herd, the strain detected at the beginning of the programme was a typical one, carrying both *nuc* and *Sa442*; thus, it was hypothesized that in the course of the programme a deletion of *nuc* gene occurred.

Even though all *Staph. aureus* tested in this study harboured *Sa442*, isolates lacking *Sa442* have been reported in human clinical samples (Klaassen et al. 2003; Heilman et al. 2004); it is then presumable that such strains could also be causative agents of dairy cow mastitis.

We also reported the bacteriological misidentification of 4 *Staph. pseudintermedius* strains, which were morphologically and biochemically consistent with the species *aureus*, but tested negative for both *nuc* and *Sa442*. The consequence of such false-positive results in routine laboratory diagnosis could have been the segregation or even culling of the cows, if the herds were managing a strict control programme for contagious pathogens. Currently, no data are available in the literature regarding *Staph. pseudintermedius* prevalence in bovine milk. Nonetheless, the scarce data on *Staph. intermedius* indicate that bacteria from *Staph. intermedius* group are not important mastitis pathogens (Roberson et al. 1996).

When applied to bacterial DNA extracted directly from milk samples, the assay correctly identified all the samples

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diagnosed as positive for *Staph. aureus* by bacteriological examination and duplex real-time PCR assay. To the contrary, no amplification curve was observed in bacteriologically negative milk or in the samples positive for a variety of other micro-organisms. As a result, the method showed 100% sensitivity and specificity also when applied to milk samples.

The use of high resolution melting analysis applied to real-time PCR with EvaGreen dye, allowed easy detection of unspecific amplifications. Therefore, this assay could be used in laboratory routine as a cost-effective and powerful tool for high-throughput identification of atypical *Staph. aureus* isolates causing dairy cow mastitis. Also, it could be directly applied to milk samples to detect *Staph. aureus* mammary infections, avoiding bacteriological analysis.

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