Development of a real-time PCR assay for the detection and identification of *Staphylococcus capitis*, *Staphylococcus haemolyticus* and *Staphylococcus warneri*

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Staphylococcus capitis, *Staphylococcus haemolyticus* and *Staphylococcus warneri* are coagulase-negative staphylococci. Each species has different characteristics, and a difference in pathology is also seen in compromised hosts. Therefore, the development of a species-specific simple detection method for the identification of these staphylococci is important. Here, a species-specific real-time PCR assay is reported that targets the superoxide dismutase A-encoding gene of these bacteria. Primers were designed with a base that was non-complementary with regard to the other bacteria. This base was at the 3' end of the primer (3' mismatch primer) and conferred high specificity. These primers were then evaluated using real-time PCR. They reacted only with the target bacterium. In addition, stable quantitative reactions were observed when experiments were performed using genomic DNA extracted from varying numbers of staphylococci cells (10^1 – 10^7 cells). These results indicate that this method is useful for the identification and quantitative analysis of *S. capitis*, *S. haemolyticus* and *S. warneri*.

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INTRODUCTION

Staphylococcus capitis, *Staphylococcus haemolyticus* and *Staphylococcus warneri* are coagulase-negative staphylococci (CNS) that inhabit human skin (Kloos & Schleifer, 1975). Although infection with these staphylococci is rare compared with *Staphylococcus aureus*, staphylococci infection increases gradually in compromised hosts (Buttery *et al.*, 1997; Wang *et al.*, 1999; Otto, 2004; Raponi *et al.*, 2005; Stollberger *et al.*, 2006). In addition, these bacteria have been reported to possess drug resistance (Froggatt *et al.*, 1989; Monsen *et al.*, 1999; Wang *et al.*, 1999; Raponi *et al.*, 2005; de Allori *et al.*, 2006). Therefore, accurate detection is important (Pfaller & Herwaldt, 1988).

A conventional technique for the identification of staphylococci is the sugar fermentation test. However, this method often results in false positives (Skulnick *et al.*, 1989; Bannerman *et al.*, 1993; Perl *et al.*, 1994; Lee & Park, 2001). In contrast, results obtained using PCR are more accurate; the 16S rRNA gene, a housekeeping gene, is generally targeted when species-specific detection by PCR is performed. However, the 16S rRNA gene of *Staphylococcus epidermidis* is highly similar to that in other CNS. In order to solve this problem, it is possible to use alternative single-copy target sequences that exhibit a higher sequence

divergence than the 16S rRNA gene. Recently, the use of the highly conserved ubiquitous *sodA* gene of CNS, which encodes the manganese-dependent superoxide dismutase, was reported (Poyart *et al.*, 2001). Therefore, to develop a species-specific detection method, we targeted the superoxide dismutase A-encoding (*sodA*) gene, another housekeeping gene useful for the identification of staphylococci (Poyart *et al.*, 2001; Sivadon *et al.*, 2004, 2005). We designed primers for *S. capitis*, *S. haemolyticus* and *S. warneri* each having a base that was non-complementary with regard to the two other bacteria. This base was at the 3' end of the primer (3' mismatch primer) for the *sodA* gene. Furthermore, to perform quantitative analysis, we adopted real-time PCR using SYBR Green chemistry. The result was a species-specific detection and quantification method for *S. capitis*, *S. haemolyticus* and *S. warneri*.

METHODS

Procedures for the design of species-specific primers. We searched the sequences of the *sodA* genes of each strain registered in GenBank [GenBank accession nos: AJ343896 and AJ343939–AJ343941 (*S. capitis*); AJ343910, AJ343949 and AJ343950 (*S. haemolyticus*); and AJ343932, AJ343958, AY795889–AY795891 and AY485204 (*S. warneri*)] and an alignment analysis was conducted using the CLUSTAL W program. PRIMEREXPRESS (Applied Biosystems) was used to design appropriate specific primers.

Abbreviation: CNS, coagulase-negative staphylococci.

Table 1. PCR primers for species-specific detection and quantification using the *sodA* gene of *S. capitis*, *S. haemolyticus* and *S. warneri*

Species	Primer	Sequence (5'→3')	Amplicon size (bp)	Annealing temp. (°C)
<i>S. capitis</i>	ScapF	GCTAATTTAGATAGCGTACCTTCA	208	59
	ScapR	CAGATCCAAAGCGTGCA		
<i>S. haemolyticus</i>	ShaeF	GTTGAGGGAACAGAT	85	50
	ShaeR	CAGCTGTTTGAATATCTT		
<i>S. warneri</i>	SwarF	TGTAGCTAACTTAGATAGTTCCTTCT	63	60
	SwarR	CCGCCACCGTTATTTCCT		

Bacterial strains and preservation conditions. The following type strains were used in the present study: *S. epidermidis* JCM 2414^T, *S. warneri* JCM 2415^T, *S. haemolyticus* JCM 2416^T, *Staphylococcus cohnii* JCM 2417^T, *Staphylococcus xylosus* JCM 2418^T, *S. capitis* JCM 2420^T, *Staphylococcus intermedius* JCM 2422^T, *Staphylococcus simulans* JCM 2424^T, *Staphylococcus saprophyticus* JCM 2427^T, *S. aureus* JCM 2874^T, *Staphylococcus schleiferi* JCM 7470^T, *Staphylococcus saccharolyticus* GTC 181^T, *Staphylococcus auricularis* GTC 326^T, *Staphylococcus caprae* GTC 378^T, *Staphylococcus lugdunensis* GTC 458^T and *Staphylococcus hominis* GTC 485^T. In addition, laboratory and clinically isolated strains of the following were prepared: 29 *S. capitis* strains, 5 *S. haemolyticus* strains, 4 *S. warneri* strains, 134 *S. epidermidis* strains, 14 *S. hominis* strains, 2 *S. cohnii* strains, 4 *S. saprophyticus* strains, 14 *S. caprae* strains, 2 *S. lugdunensis* strains, 34 *S. aureus* strains and 3 *Micrococcus* sp. strains. Bacteria were maintained by cultivation in tryptic soy agar medium (Difco).

Extraction of DNA. Bacteria were cultivated with shaking in tryptic soy broth (Difco) for 16 h at 37 °C. After centrifugation of each bacterial culture medium, the pellets were washed twice with PBS. To extract DNA, a miniprep kit (Qiagen) was used and the DNA was dissolved in 50 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Human genomic DNA was obtained from Applied Biosystems.

Conventional (qualitative) PCR procedures. For qualitative PCR, an ABI Prism 7700 (Applied Biosystems) was used without a fluorescence monitor. AmpliTaq Gold PCR master mix (Applied Biosystems) and a 100 nM concentration of each primer were used. The reaction mix volume was 25 µl. The reaction was performed for 35 cycles of 15 s at 94 °C, 30 s at the appropriate annealing temperature (Table 1) and 30 s at 72 °C. A first step of 10 min at 94 °C was included for activation of the PCR enzyme. After the reaction was complete, PCR products were detected by agarose gel electrophoresis followed by visualization under a UV transilluminator. Experiments were performed at least three times.

Real-time PCR procedures. An ABI Prism 7700 was used with SDS software v1.7 and the procedure was conducted according to the manufacturer's manual. Power SYBR PCR mix (Applied Biosystems) and a 100 nM concentration of each primer were used. The volume of each reaction mix was 50 µl. Genomic DNA prepared from staphylococci cells (0–10⁷ cells) was used. The reaction was performed for 40 cycles of 10 s at 95 °C, 30 s at the appropriate annealing temperature (Table 1) and 45 s at 72 °C. A first step of 10 min at 94 °C was included for activation of the PCR enzyme. Melting point analysis was then performed using dissociation curve analysis software. Experiments were performed at least three times.

RESULTS AND DISCUSSION

We first designed species-specific primers for the *sodA* gene (Table 1). The lengths of the amplicons were 208, 85 and

63 bp (Table 1). We determined experimentally that suitable annealing temperatures for the primers for *S. capitis*, *S. haemolyticus* and *S. warneri* were 59, 50 and 60 °C, respectively (Table 1). When PCR was performed using these primers and standard genomic DNA from *S. capitis*, *S. haemolyticus* or *S. warneri*, an amplification product of the expected length was observed in each case (data not shown). To evaluate the specificity of these primers, a test using other staphylococci and human genomic DNA was performed. In this test, no amplification products were observed (Table 2).

Next, quantitative analysis of *S. capitis*, *S. haemolyticus* and *S. warneri* DNA was performed using these primers. Each set of primers targeted a specific bacterium and the results of real-time PCR showed the same specificity as conventional PCR (Table 2). The melting point of the amplicons

Table 2. Results of testing of 18 species using a species-specific assay for *S. capitis*, *S. haemolyticus* and *S. warneri*

Species	Total no. of strains	No. of strains identified using species-specific assay:		
		<i>S. capitis</i>	<i>S. haemolyticus</i>	<i>S. warneri</i>
<i>S. epidermidis</i>	135	0	0	0
<i>S. hominis</i>	15	0	0	0
<i>S. capitis</i>	30	30	0	0
<i>S. haemolyticus</i>	6	0	6	0
<i>S. warneri</i>	5	0	0	5
<i>S. cohnii</i>	3	0	0	0
<i>S. saprophyticus</i>	5	0	0	0
<i>S. xylosus</i>	1	0	0	0
<i>S. auricularis</i>	1	0	0	0
<i>S. caprae</i>	15	0	0	0
<i>S. intermedius</i>	1	0	0	0
<i>S. lugdunensis</i>	3	0	0	0
<i>S. saccharolyticus</i>	1	0	0	0
<i>S. schleiferi</i>	1	0	0	0
<i>S. simulans</i>	1	0	0	0
<i>S. aureus</i>	35	0	0	0
<i>Micrococcus</i> sp.	3	0	0	0
<i>Homo sapiens</i>	1	0	0	0

Fig. 1. Melting curve analysis of amplicons obtained using primers specific for *S. capitis* (a), *S. haemolyticus* (b) and *S. warneri* (c). The y axis indicates the negative derivative of fluorescence (F) with respect to temperature (T). Representative results are shown.

was 75.8, 71.6 and 72.4 °C, respectively (Fig. 1). The specificity of these primers for other staphylococci and human genomic DNA was also evaluated. No amplification products (Table 2) or primer dimers were observed (Fig. 1). We calculated the regression line based on the results of quantitative PCR using genomic DNA extracted from *S. capitis*, *S. haemolyticus* and *S. warneri* cells (10^1 – 10^7 cells) and found linearity within the range of concentrations tested (Fig. 2).

This study reports a simple species-specific PCR method for studying *S. capitis*, *S. haemolyticus* and *S. warneri*. The primers designed in the present study were successful not only with conventional PCR but also with real-time PCR using SYBR Green chemistry.

PCR is usually considered to be a good method for bacterial detection as it is simple, sensitive and specific. However, it does have limitations. Although the 16S rRNA gene is generally targeted for the design of species-specific PCR primers for identification, designing primers is difficult when the sequences of the homologous genes have high similarity. In the case of staphylococci, therefore, ribotyping, internal transcribed spacer PCR and various other methods have been studied (Welsh & McClelland, 1992; Gribaldo *et al.*, 1997; Gaszewska-Mastalarz *et al.*, 1998;

Edwards *et al.*, 2001; Lee & Park, 2001; Sakai *et al.*, 2004; Dobbins *et al.*, 2002). However, the development of a species-specific quantitative PCR methodology has proved difficult.

In this study, 3' mismatch primers for the *sodA* gene made the selective identification of *S. capitis*, *S. haemolyticus* and *S. warneri* possible. The 3' mismatch primer is frequently used for highly specific detection of single-nucleotide polymorphisms (Ayyadevara *et al.*, 2000; Papp *et al.*, 2003). Each primer designed in the present study for *S. capitis*, *S. haemolyticus* and *S. warneri* was specific and reacted only with the target bacteria. No primer-dimer formation was observed. Moreover, these primers were also useful for species-specific quantitative analysis by real-time PCR using SYBR Green chemistry. The SYBR Green method is a new quantitative detection method in which the PCR product is detected directly, and quantified by measuring the increase in fluorescence caused by the binding of the SYBR Green dye to double-stranded DNA (Arya *et al.*, 2005). When genomic DNA extracted from *S. capitis*, *S. haemolyticus* and *S. warneri* cells (0 – 10^7 cells) was used, the reactions were stable and quantitative in the range of 10^1 – 10^7 cells. Therefore, the present method is simple, sensitive and specific.

Fig. 2. Mean threshold cycle (Ct) values from three replicates tested using primers specific for *S. capitis* (a), *S. haemolyticus* (b) and *S. warneri* (c) on DNA from the respective bacteria. The plot of Ct values and DNA inputs fits a linear function: (a) $r^2=0.998$, (b) $r^2=0.998$, (c) $r^2=0.996$.

Our results indicate that the primers designed in the present study are useful for the identification of individual colonies and for the quantitative analysis of *S. capitis*, *S. haemolyticus* and *S. warneri*. In addition, this technique may be applied to other bacteria that are difficult to identify by standard methods.

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REFERENCES

- Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N. & Patel, H. R. (2005). Basic principles of real-time quantitative PCR. *Expert Rev Mol Diagn* 5, 209–219.
- Ayyadevara, S., Thaden, J. J. & Shmookler Reis, R. J. (2000). Discrimination of primer 3'-nucleotide mismatch by *Taq* DNA polymerase during polymerase chain reaction. *Anal Biochem* 284, 11–18.
- Bannerman, T. L., Kleeman, K. T. & Kloos, W. E. (1993). Evaluation of the Vitek systems Gram-positive identification card for species identification of coagulase-negative staphylococci. *J Clin Microbiol* 31, 1322–1325.
- Buttery, J. P., Easton, M., Pearson, S. R. & Hogg, G. G. (1997). Pediatric bacteremia due to *Staphylococcus warneri*: microbiological, epidemiological, and clinical features. *J Clin Microbiol* 35, 2174–2177.
- de Allori, M. C., Jure, M. A., Romero, C. & de Castillo, M. E. (2006). Antimicrobial resistance and production of biofilms in clinical isolates of coagulase-negative *Staphylococcus* strains. *Biol Pharm Bull* 29, 1592–1596.
- Dobbins, B. M., Kite, P., Kindon, A., McMahon, M. J. & Wilcox, M. H. (2002). DNA fingerprinting analysis of coagulase negative staphylococci implicated in catheter related bloodstream infections. *J Clin Pathol* 55, 824–828.
- Edwards, K. J., Kaufmann, M. E. & Saunders, N. A. (2001). Rapid and accurate identification of coagulase-negative staphylococci by real-time PCR. *J Clin Microbiol* 39, 3047–3051.
- Froggatt, J. W., Johnston, J. L., Galetto, D. W. & Archer, G. L. (1989). Antimicrobial resistance in nosocomial isolates of *Staphylococcus haemolyticus*. *Antimicrob Agents Chemother* 33, 460–466.
- Gaszewska-Mastalarz, A., Bartoszewicz-Potyrala, M., Przondo-Mordarska, A., Mordarski, M. & Zakrzewska-Czerwinska, J. (1998). Characterization of coagulase-negative staphylococci by primer-specific polymerase chain reaction and ribotyping. *Clin Microbiol Infect* 4, 27–32.
- Gribaldo, S., Cookson, B., Saunders, N., Marples, R. & Stanley, J. (1997). Rapid identification by specific PCR of coagulase-negative staphylococcal species important in hospital infection. *J Med Microbiol* 46, 45–53.
- Kloos, W. E. & Schleifer, K. H. (1975). Isolation and characterization of staphylococci from human skin. *Int J Syst Bacteriol* 25, 62–79.
- Lee, M. K. & Park, A. J. (2001). Rapid species identification of coagulase-negative staphylococci by rRNA spacer length polymorphism analysis. *J Infect* 42, 189–194.
- Monsen, T., Ronnmark, M., Olofsson, C. & Wistrom, J. (1999). Antibiotic susceptibility of staphylococci isolated in blood cultures in relation to antibiotic consumption in hospital wards. *Scand J Infect Dis* 31, 399–404.
- Otto, M. (2004). Virulence factors of the coagulase-negative staphylococci. *Front Biosci* 9, 841–863.
- Papp, A. C., Pinsonneault, J. K., Cooke, G. & Sadee, W. (2003). Single nucleotide polymorphism genotyping using allele-specific PCR and fluorescence melting curves. *Biotechniques* 34, 1068–1072.
- Perl, T. M., Rhomberg, P. R., Bale, M. J., Fuchs, P. C., Jones, R. N., Koontz, F. P. & Pfaller, M. A. (1994). Comparison of identification systems for *Staphylococcus epidermidis* and other coagulase-negative *Staphylococcus* species. *Diagn Microbiol Infect Dis* 18, 151–155.
- Pfaller, M. A. & Herwaldt, L. A. (1988). Laboratory, clinical, and epidemiological aspects of coagulase-negative staphylococci. *Clin Microbiol Rev* 1, 281–299.
- 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.
- Raponi, G., Ghezzi, M. C., Gherardi, G., Dicuonzo, G., Caputo, D., Venditti, M., Rocco, M., Micozzi, A. & Mancini, C. (2005). Antimicrobial susceptibility, biochemical and genetic profiles of *Staphylococcus haemolyticus* strains isolated from the bloodstream of patients hospitalized in critical care units. *J Chemother* 17, 264–269.
- Sakai, H., Procop, G. W., Kobayashi, N., Togawa, D., Wilson, D. A., Borden, L., Krebs, V. & Bauer, T. W. (2004). Simultaneous detection of *Staphylococcus aureus* and coagulase-negative staphylococci in positive blood cultures by real-time PCR with two fluorescence resonance energy transfer probe sets. *J Clin Microbiol* 42, 5739–5744.
- Sivadon, V., Rottman, M., Quincampoix, J. C., Avettand, V., Chaverot, S., de Mazancourt, P., Trieu-Cuot, P. & Gaillard, J. L. (2004). Use of *sodA* sequencing for the identification of clinical isolates of coagulase-negative staphylococci. *Clin Microbiol Infect* 10, 939–942.
- Sivadon, V., Rottman, M., Chaverot, S., Quincampoix, J. C., Avettand, V., de Mazancourt, P., Bernard, L., Trieu-Cuot, P., Feron, J. M. & other authors (2005). Use of genotypic identification by *sodA* sequencing in a prospective study to examine the distribution of coagulase-negative *Staphylococcus* species among strains recovered during septic orthopedic surgery and evaluate their significance. *J Clin Microbiol* 43, 2952–2954.
- Skulnick, M., Patel, M. P. & Low, D. E. (1989). Evaluation of five commercial systems for identification of coagulase-negative staphylococci to species level. *Eur J Clin Microbiol Infect Dis* 8, 1001–1003.
- Stollberger, C., Wechsler-Fordos, A., Geppert, F., Gulz, W., Brownstone, E., Nicolakis, M. & Finsterer, J. (2006). *Staphylococcus warneri* endocarditis after implantation of a lumbar disc prosthesis in an immunocompetent patient. *J Infect* 52, e15–e18.
- Wang, S. M., Liu, C. C., Tseng, H. W., Yang, Y. J., Lin, C. H., Huang, A. H. & Wu, Y. H. (1999). *Staphylococcus capitis* bacteremia of very low birth weight premature infants at neonatal intensive care units: clinical significance and antimicrobial susceptibility. *J Microbiol Immunol Infect* 32, 26–32.
- Welsh, J. & McClelland, M. (1992). PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. *Mol Microbiol* 6, 1673–1680.