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Rapid and Accurate Identification of Human-Associated Staphylococci by Use of Multiplex PCR▽

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Although staphylococci are identified by phenotypic analysis in many clinical laboratories, these results are often incorrect because of phenotypic variation. Genetic analysis is necessary for definitive species identification. In the present study, we developed a simple multiplex-PCR (M-PCR) for species identification of human-associated staphylococci, which were as follows: *Staphylococcus aureus*, *S. capitis*, *S. caprae*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. saprophyticus*, and *S. warneri*. This method was designed on the basis of nucleotide sequences of the thermonuclease (*nuc*) genes that were universally conserved in staphylococci except the *S. sciuri* group and showed moderate sequence diversity. In order to validate this assay, 361 staphylococcal strains were studied, which had been identified at the species levels by sequence analysis of the *hsp60* genes. In consequence, M-PCR demonstrated a sensitivity of 100% and a specificity of 100%. By virtue of simplicity and accuracy, this method will be useful in clinical research.

Staphylococci are normal inhabitants of human skin and mucous membranes. *Staphylococcus aureus* is a common cause of invasive and life-threatening infections, but coagulase-negative staphylococci (CNS) cause mainly nosocomial infections, such as catheter-related bloodstream infections, prosthetic valve endocarditis, central nervous system shunt infections and prosthetic joint infections (17, 31, 42). Unlike other CNS, however, *S. lugdunensis* is highly pathogenic and can cause aggressive skin and soft tissue infections, bone and joint infections, and native valve endocarditis (13, 31, 42). In addition, the oxacillin MIC breakpoints to determine methicillin resistance differ among staphylococcal species. Therefore, it is important to identify staphylococci at the species levels.

A variety of methods have been reported for species identification of staphylococci. Although conventional biochemical assays are employed in many clinical laboratories, they are frequently imprecise due to phenotypic variation (5, 16, 19, 26). Real-time PCR has been developed, but some staphylococcal species are indistinguishable, and/or interpretation of results is intricate (12, 37). Sequence analyses of several target genes are the most reliable methods (10, 25, 28, 36). However, these are expensive, laborious, and time-consuming. Thus, a simple and reliable assay is needed for identification of staphylococcal species.

The purpose of the present study was to develop a rapid and accurate multiplex-PCR (M-PCR) for species identification of human-associated staphylococci, which contained the following: *S. aureus*, *S. capitis*, *S. caprae*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. saprophyticus*, and *S. warneri*. This method was based on nucleotide sequences of the thermonuclease (*nuc*) genes that had been used for staphylococcal identification (4, 35).

MATERIALS AND METHODS

Bacterial strains and species identification. A total of 24 staphylococcal strains, which included 5 species with whole-genome sequences (14, 23, 24, 32, 40), were used for phylogenetic analysis of the *nuc* genes (Table 1). A total of 361 staphylococcal strains were studied to assess the utility of M-PCR for species identification of human-associated staphylococci, which were as follows: *S. aureus* ($n = 60$), *S. epidermidis* ($n = 104$), *S. lugdunensis* ($n = 26$), *S. haemolyticus* ($n = 24$), *S. saprophyticus* ($n = 23$), *S. warneri* ($n = 23$), *S. hominis* subsp. *hominis* ($n = 8$), *S. hominis* subsp. *novobiosepticus* ($n = 14$), *S. capitis* subsp. *capitis* ($n = 7$), *S. capitis* subsp. *ureolyticus* ($n = 24$), *S. caprae* ($n = 16$), *S. auricularis* ($n = 1$), *S. arlettae* ($n = 1$), *S. carnosus* ($n = 1$), *S. chromogenes* ($n = 1$), *S. cohnii* subsp. *cohnii* ($n = 1$), *S. cohnii* subsp. *urealyticum* ($n = 1$), *S. croceolyticus* ($n = 1$), *S. delphini* ($n = 1$), *S. devriesei* ($n = 1$), *S. equorum* ($n = 1$), *S. felis* ($n = 1$), *S. fleurettii* ($n = 1$), *S. gallinarum* ($n = 1$), *S. hyicus* ($n = 1$), *S. intermedius* ($n = 1$), *S. kloosii* ($n = 1$), *S. lentus* ($n = 1$), *S. lutrae* ($n = 1$), *S. nepalensis* ($n = 1$), *S. muscae* ($n = 1$), *S. pasteuri* ($n = 1$), *S. pettenkoferi* ($n = 1$), *S. piscifermentans* ($n = 1$), *S. pseudintermedius* ($n = 1$), *S. saccharolyticus* ($n = 1$), *S. schleiferi* ($n = 1$), *S. sciuri* ($n = 1$), *S. simiae* ($n = 1$), *S. simulans* ($n = 1$), *S. succinus* ($n = 1$), *S. vitulinus* ($n = 1$), *S. xylosus* ($n = 1$).

All strains used in this study were identified at the species levels by sequence analysis of the *hsp60* genes (25), which were determined by high sequence similarity (>95%).

DNA extraction. DNA was extracted by using the procedure described previously (35). A single colony was suspended to a 1.0 McFarland standard in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) with 10 U of achromopeptidase (Wako Chemical, Co. Ltd.). The suspension was heated at 55°C for at least 10 min until it became transparent. The supernatant was used as template DNA for PCR.

Sequence analysis of the *nuc* genes. The *nuc* genes were sequenced by using the procedure described previously (35). Primers Nuc-alF1 (5′-CCNAAYACN CCNGTNCARCCN-3′) and Nuc-alR (5′-NADCCANACRTANGCNARNGT-3′) were used to amplify the conserved regions of the *nuc* genes. PCR products were cloned into plasmid pCR-4 I-TOPO (Invitrogen, Life Technologies, Carlsbad, CA) and were transformed into *Escherichia coli* TOP10 cells (Invitrogen). Insert DNA of the recombinant plasmid was sequenced by using a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA) with an ABI Prism 3100 genetic analyzer (Applied Biosystems). The 5′ and 3′ regions were obtained by inverse PCR, and the complete sequences of the *nuc* genes were determined. For the species for which a degenerate PCR of the *nuc* genes was inadequate, degenerate primers Nuc-AsdF (5′-WRNCKRTTCATN ARRTAYTT-3′) and AsdR (5′-ACNTAYMGNGARATGMGNGAR-3′) were

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TABLE 1. Strains used for sequence analysis of the *nuc* genes

Species	Strain	Source	Size of ORF (bp)	GenBank accession no. for sequence of *nuc*	Reference
S. aureus	N315	Human	534	BA000018	23
S. epidermidis	RP62A	Human	537	CP000029	14
S. haemolyticus	JCSC 1435	Human	537	AP006716	40
S. saprophyticus	ATCC 15305T	Human	531	AP008934	24
S. lugdunensis	ATCC 43809T	Human	543	AB598384	This study
S. warneri	ATCC 27836T	Human	531	AB598385	This study
S. caprae	CCM 3573T	Goat	537	AB598386	This study
S. capitis subsp. *capitis*	ATCC 27840T	Human	537	AB598387	This study
S. capitis subsp. *ureolyticus*	JCSC 4868	Human	537	AB598388	This study
S. hominis subsp. *hominis*	ATCC 27844T	Human	546	AB598389	This study
S. hominis subsp. *novobiosepticus*	JCSC 3392	Human	546	AB598390	This study
S. pseudintermedius	LMG 22219T	Cat	507	AB327164	34
S. delphini group A	LMG 22190T	Dolphin	516	AB327167	34
S. delphini group B	P-27B	Pigeon	507	AB327166	34
S. intermedius	ATCC 29663T	Pigeon	507	AB327165	34
S. schleiferi subsp. *schleiferi*	P-45C	Pigeon	504	None	35
S. schleiferi subsp. *coagulans*	JCM 7470T	Dog	504	AB465334	35
S. hyicus	JCM 2423T	Pig	510	AB465332	35
S. felis	JCM 7469T	Cat	507	AB465335	35
S. chromogenes	P-29B	Pigeon	507	AB465333	35
S. xylosus	h-2B	Horse	537	AB465329	This study
S. kloosii	P-40	Pigeon	543	AB465330	This study
S. simulans	JCM2424T	Human	534	AB465331	This study
S. carnosus	TM300	Dry sausage	543	AM295250	32

used to amplify the conserved regions of the aspartate kinase genes, which were located about 2 to 8 kbp upstream of the *nuc* genes. Downstream sequences were analyzed by inverse PCR in order to determine the complete sequences of the *nuc* genes. Multiple alignments were carried out by using the CLUSTAL W program (41). Construction of the phylogenetic tree was performed by the neighbor-joining method (33).

M-PCR for species identification of human-associated staphylococci. As previously described (35), the primer pairs for M-PCR were designed on the basis of nucleotide sequences of the *nuc* and adjacent genes, and they were specific for each species (Table 2). For M-PCR, the reaction mixture contained 2 μl template DNA, 0.2 μM each primer, 200 μM each deoxynucleotide triphosphate, Ex *Taq* buffer, and 2 U Ex *Taq* polymerase (Takara Co., Ltd., Kyoto, Japan), in a final volume of 50 μl. A Takara PCR thermal cycler was used for amplification, with an initial denaturation step (95°C, 5 min); 30 cycles of denaturation (95°C, 30 s), annealing (58°C, 30 s), and extension (72°C, 70 s); and a final elongation step at 72°C for 2 min. PCR products were visualized by electrophoresis in 1× Tris-acetate-EDTA on a 1% agarose gel stained with ethidium bromide.

Nucleotide sequence accession numbers. GenBank accession numbers for the sequence of the *nuc* genes determined in this study are as follows: AB598384 for *S. lugdunensis*, AB598385 for *S. warneri*, AB598386 for *S. caprae*, AB598387 for *S. capitis* subsp. *capitis*, AB598388 for *S. capitis* subsp. *ureolyticus*, AB598389 for *S. hominis* subsp. *hominis*, AB598390 for *S. hominis* subsp. *novobiosepticus*, AB465329 for *S. xylosus*, AB465330 for *S. kloosii*, and AB465331 for *S. simulans*.

RESULTS

Development of M-PCR for species identification of human-associated staphylococci. M-PCR successfully amplified the DNA fragments corresponding in size to each species as follows: *S. hominis*, 177 bp; *S. epidermidis*, 251 bp; *S. aureus*, 359 bp; *S. haemolyticus*, 434 bp; *S. capitis*, 525 bp; *S. lugdunensis*, 695 bp; *S. saprophyticus*, 843 bp; *S. warneri*, 999 bp; and *S.*

TABLE 2. Oligonucleotide primers for M-PCR for species identification of human-associated staphylococci

primer	Sequence (5′–3′)	Size of PCR product (bp)	Species	Reference
hom-F	TACAGGGCCATTTAAAGACG	177	*S. hominis*	This study
hom-R	GTTTCTGGTGTATCAACACC			
epi-F	TTGTAAACCATTCTGGACCG	251	*S. epidermidis*	This study
epi-R	ATGCGTGAGATACTTCTTCG			
aur-F	TCGCTTGCTATGATTGTGG	359	*S. aureus*	35
aur-R	GCCAATGTTCTACCATAGC			
hae-F	TAGTGGTAGGCGTATTAGCC	434	*S. haemolyticus*	This study
hae-R	ACGATATTTGCCATTCGGTG			
cap-F	ACTACGCCTATGATTATTGC	525	*S. capitis*	This study
cap-R	GAYGCTTCTTTACCATAGGG			
lug-F	TCCAATGATGGTAACGAGGC	695	*S. lugdunensis*	This study
lug-R	TTTTGCGCCTCGTTTTGTGC			
sap-F	TTTTGGATGCGATAGATTGG	843	*S. saprophyticus*	This study
sap-R	TCTTCAGACTTTTCAAAGGC			
war-F	CGTTTGTAGCAAAACAGGGC	999	*S. warneri*	This study
war-R	GCAACGAGTAACCTTGCCAC			
rae-F	TTGTTCTWGCACTYATTGCG	1,227	*S. caprae*	This study
rae-R	TTTTATAGAACAGGGTCGAC			

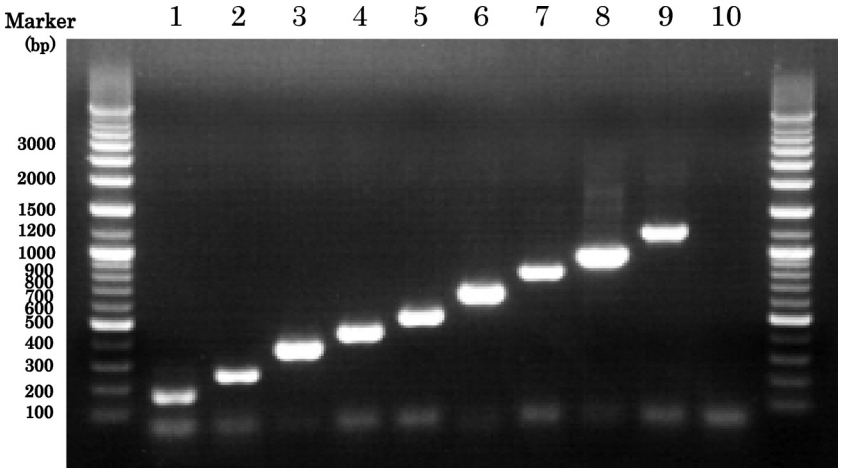

FIG. 1. Electrophoresis after multiplex PCR for species identification of human-associated staphylococci on a 1.0% agarose gel. Lanes: 1, *S. hominis*; 2, *S. epidermidis*; 3, *S. aureus*; 4, *S. haemolyticus*; 5, *S. capitis*; 6, *S. lugdunensis*; 7, *S. saprophyticus*; 8, *S. warneri*; 9, *S. caprae*; 10, water (negative control).

caprae, 1,227 bp (Fig. 1). In order to validate this assay, 361 staphylococcal strains, which had been identified at the species levels by sequence analysis of the *hsp60* genes, were studied. For nine human-associated staphylococcal species, the sizes of the amplified DNA fragments matched those predicted for each species, and others showed negative results. The 177-bp fragments contained *S. hominis* subsp. *hominis* and *S. hominis* subsp. *novobiosepticus*, the 525-bp fragments contained *S. capitis* subsp. *capitis* and *S. capitis* subsp. *ureolyticus*, and the 843-bp fragments contained *S. saprophyticus* subsp. *saprophyticus* and *S. saprophyticus* subsp. *bovis*, so this method could not identify these three species at the subspecies levels. Consequently, M-PCR had a sensitivity of 100% and a specificity of 100% in the identification of human-associated staphylococci at the species levels.

Phylogenetic analysis of the *nuc* genes of staphylococci. We constructed the phylogenetic tree based on amino acid sequences of the *nuc* genes of 24 staphylococcal strains (staphylococcal thermonuclease group), staphylococcal nuclease family proteins of Gram-positive bacteria (staphylococcal nuclease group), and thermonuclease family proteins of thermophilic bacteria (thermophilic bacterial thermonuclease group) (Fig. 2).

There were the *nuc* genes at specific gene loci in 24 staphylococcal strains, which were located about 2 to 8 kbp downstream of the aspartate kinase genes (Table 1). We could not detect this gene in any *S. sciuri* group (*S. sciuri*, *S. lentus*, and *S. vitulinus*) strains. The staphylococcal thermonuclease group formed two main clusters (Fig. 2). One included *S. aureus*, and the other included *S. intermedius*. Phylogenetic relationships of the *nuc* genes among staphylococci were analogous mostly to those of the *hsp60* genes (25). The sequence identity of the *nuc* genes among staphylococci ranged from 59.6 to 99.6% (mean, 67.2%). The sequence similarity of these genes was 99.6% between two subspecies of *S. schleiferi*, 98.4% between two subspecies of *S. hominis*, 89.9% between two subspecies of *S. capitis*, and 79.5% between *S. delphini* group A and *S. delphini* group B. It was 95.9% between *S. delphini* group B and *S. pseudintermedius* and 87.7% between *S. delphini* group B and *S. intermedius*, so the *nuc* gene sequence of *S. delphini* group B was more similar to that of *S. pseudintermedius* and *S. intermedius* than that of *S. delphini* group A (34, 35).

There were staphylococcal nuclease genes in Gram-positive bacteria (2, 21–23, 27, 29, 32, 40) and *nuc* genes in thermophilic bacteria (1, 9, 30, 38, 43) whose complete genome sequences had been determined. Each group formed a distinct cluster from the staphylococcal thermonuclease group, and some genes were on plasmids or a prophage (Fig. 2).

DISCUSSION

Staphylococci are identified by phenotypic analysis in many clinical laboratories. Although many phenotypic identification methods are commercially available, diagnostic accuracy has been reported to be 36.7 to 93.6% (5, 16, 19, 26). This inaccuracy is problematic in clinical practice. Because CNS are closely related to prosthetic device infections, CNS isolated from blood cultures of previously healthy individuals may be regarded as contaminants. However, *S. lugdunensis* can cause native valve endocarditis, and community-acquired *S. lugdunensis* bacteremia has been associated with endocarditis (7, 44). Failure to identify *S. lugdunensis* might lead to delayed or inadequate treatment with an increase in morbidity and mortality.

Currently, the breakpoint for oxacillin resistance is $\geq$4 μg/ml in *S. aureus* and *S. lugdunensis*, whereas it is $\geq$0.5 μg/ml in other staphylococci (8). If other staphylococcal strains are misidentified as *S. aureus* or *S. lugdunensis*, those showing oxacillin MICs of 0.5 to 2 μg/ml are incorrectly considered to be sensitive, which will result in usage of ineffective antimicrobial agents. If *S. aureus* or *S. lugdunensis* strains are misidentified as other staphylococci, those showing oxacillin MICs of 0.5 to 2 μg/ml are falsely considered to be resistant. This indicates that antibacterial agents for methicillin-resistant *S. aureus*, such as vancomycin, can be used for treatment of methicillin-sensitive *S. aureus* infection. This is also problematic since vancomycin was previously reported to be inferior to beta-lactam antibiotics in the treatment of methicillin-sensitive

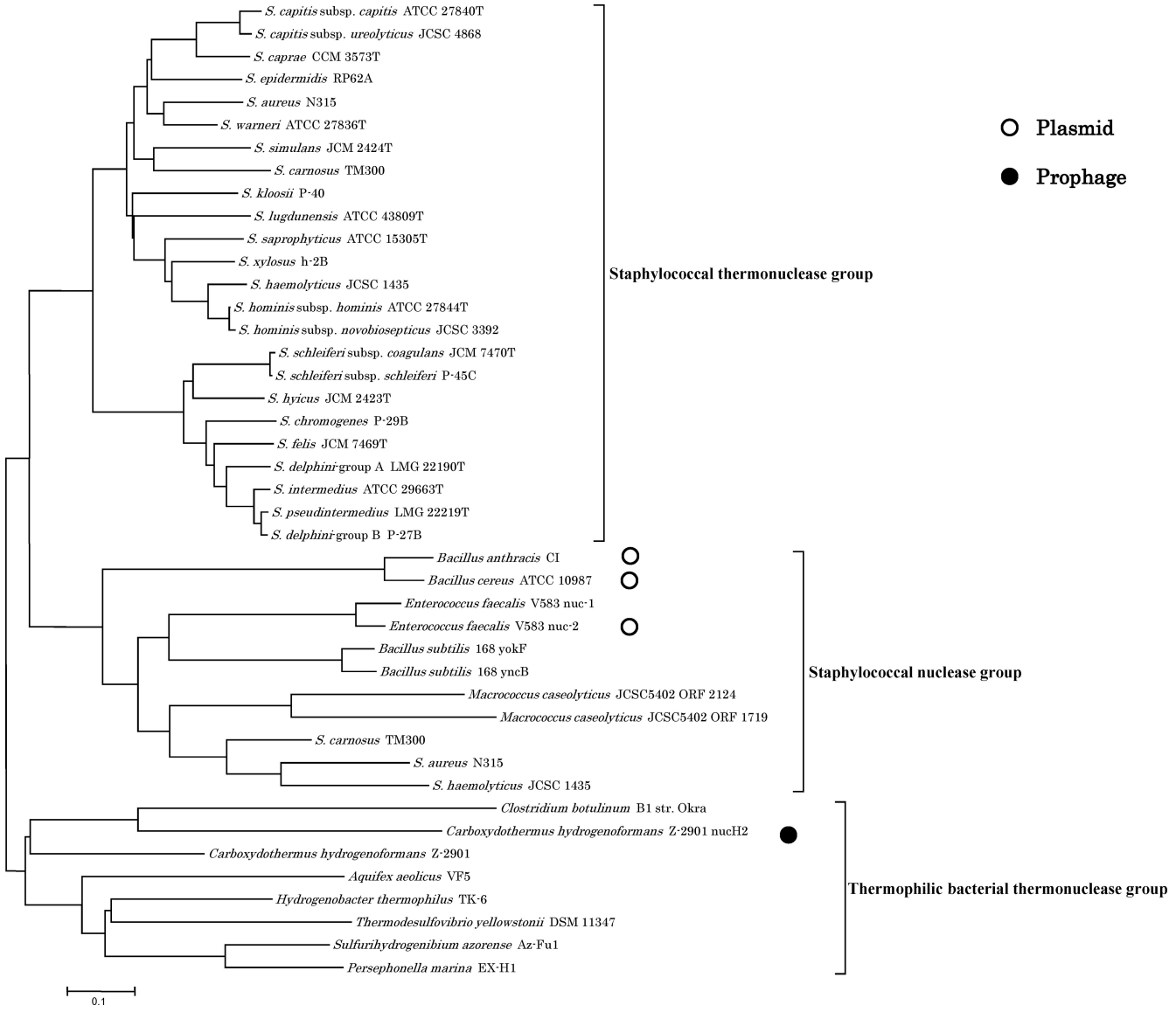

FIG. 2. The phylogenetic tree based on amino acid sequences of the *nuc* genes of staphylococci (staphylococcal thermonuclease group), staphylococcal nuclease family proteins of Gram-positive bacteria (staphylococcal nuclease group), and thermonuclease family proteins of thermophilic bacteria (thermophilic bacterial thermonuclease group). The tree was constructed by the neighbor-joining method using CLUSTAL W. The genes on plasmids and a prophage are indicated by open circles and a filled circle, respectively.

S. aureus bacteremia (6, 20, 39). In addition, it has been reported that isolates with oxacillin MICs of 0.5 to 2 μg/ml are *mecA* negative, particularly in *S. saprophyticus* and *S. warneri* (15, 18). This can lead to inappropriate use of antibiotics, such as vancomycin. For these species, the new oxacillin MIC breakpoints need to be determined for methicillin resistance. As a result, it is necessary to precisely identify staphylococci at the species levels.

Little is known about the ecology of CNS despite commensal bacteria. It is thought that *S. capitis* is seen mostly on the head and that *S. saprophyticus* is a member of the normal rectal or urogenital flora of some women (31, 42). However, isolates have been identified not genotypically but phenotypically. Although matrix-assisted laser desorption ionization–time of flight mass spectrometry, which is the novel phenotypic method, appears to be sensitive and specific (11), the results are unreliable without the protein extraction step (3). Genetic analysis is needed for definitive species identification. Our assay can also be helpful to elucidate the ecology of CNS in humans.

Sequence analyses of several target genes have been reported for staphylococcal identification, and the mean sequence similarity of 16S rRNA, *rpoB*, *hsp60*, *sodA*, and *dnaJ* genes was 97%, 86%, 82%, 81%, and 77%, respectively (10, 25, 28, 36). Since the *nuc* genes showed moderate sequence diversity (mean similarity, 67%) and were generally found in staphylococci except the *S. sciuri* group, we could develop an M-PCR for species identification of staphylococci based on nucleotide sequences of the *nuc* and adjacent genes.

The *nuc* genes were present at specific gene loci in 24 staph-

ylococcal strains but absent in the *S. sciuri* group. In whole-genome analysis of a *Macrococcus caseolyticus* strain (2), which is a species closely related to the genus *Staphylococcus* (25), this strain also did not have the *nuc* gene. Additionally, there were *nuc* genes in thermophilic bacteria, and one was on a prophage. These results suggest that the *nuc* genes might have been derived from thermophilic bacteria and might have been acquired by the common ancestor of staphylococci after divergence from the *S. sciuri* group.

In summary, our M-PCR has been proved to be a rapid and accurate method for species identification of human-associated staphylococci. This is simple and easy to interpret. This will be useful mainly in clinical research and help to elucidate the ecology of human-associated staphylococci.

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