Multiplex-PCR Method for Species Identification of Coagulate-Positive Staphylococci

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In veterinary medicine, coagulate-positive staphylococci (CoPS) other than Staphylococcus aureus have frequently been misidentified as being S. aureus strains, as they have several phenotypic traits in common. There has been no reliable method to distinguish among CoPS species in veterinary clinical laboratories. In the present study, we sequenced the thermonuclease (nuc) genes of staphylococcal species and devised a multiplex-PCR (M-PCR) method for species identification of CoPS by targeting the nuc gene locus. To evaluate sensitivity and specificity, we used this M-PCR method on 374 staphylococcal strains that had been previously identified to the species level by an hsp60 sequencing approach. We could successfully distinguish between S. aureus, S. hyicus, S. schleiferi, S. intermedius, S. pseudintermedius, and S. delphini groups A and B. The present method was both sensitive (99.8%) and specific (100%). Our M-PCR assay will allow the routine species identification of CoPS isolates from various animal species for clinical veterinary diagnosis.

The genus Staphylococcus is present in skin and nasal flora and causes opportunistic infections in humans and various animals. To date, seven species of coagulate-positive staphylococci (CoPS) have been identified: Staphylococcus aureus, S. intermedius, S. schleiferi subsp. coagulans, S. hyicus, S. lutrae, S. delphini, and S. pseudintermedius (10, 12). In addition to S. aureus, the other CoPS species can cause severe infections compared with those caused by coagulate-negative staphylococci (CoNS) (2, 13, 16, 22, 25, 28, 32).

It is known that staphylococcal species exhibit host specificity, and the species of CoPS isolated from clinical specimens differ with host animal species; for example, the predominant species in ruminants, pigs, dogs, and pigeons (Columbia livia) are S. aureus, S. hyicus, S. pseudintermedius, and S. intermedius, respectively (11, 28). MIC breakpoints of oxacillin to determine methicillin resistance differ with species. Therefore, the identification of CoPS to the species level is an important task for veterinary diagnostic laboratories.

It is difficult to discriminate between CoPS species based on phenotypic differences because there is a lack of unique biochemical markers for species identification (12, 28). Although various molecular methods have been reported (1, 3, 6, 7, 19, 20, 24, 28), they are costly and/or time-consuming, and interpretation of the results is complicated. Thus, a simple and precise method for discriminating among CoPS species is highly desirable.

In the present study, we performed a sequence analysis of nuc genes in CoPS and related species and developed a multiplex-PCR (M-PCR) method for the species identification of the CoPS-targeted nuc gene locus.

MATERIALS AND METHODS

Bacterial strains and species identification. As shown in Table 1, eight CoPS strains and six closely related CoNS species were used for phylogenetic analysis based on thermonuclease (nuc) genes. To evaluate the sensitivity and specificity of M-PCR for the species identification of CoPS, 374 staphylococcal strains derived from various animal species were used in the present study (Table 2).

All strains used in this study were identified to the species level by sequencing analysis based on the hsp60 gene (19). The discrimination among S. delphini groups A and B was performed by using a nuc gene sequencing method reported previously (28). The identification of S. schleiferi to the subspecies level was performed by a coagulate test using rabbit serum (Eiken Chemical Co., Ltd., Tokyo, Japan).

Strains were stored in 10% skim milk at −80°C until use and were maintained on Trypticase soy agar II with 5% sheep blood (BD Japan, Co., Ltd., Tokyo, Japan).

DNA extraction. 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 achrromopeptidase (Wako Chemical Co., Ltd., Japan), and the suspension was incubated at 55°C for 10 min. Supernatants were used as crude DNA extracts for PCR.

Amplification and sequence analysis of the nuc gene. In order to amplify the conserved regions of nuc genes, degenerate primers were designed by multiple alignments of amino acid sequences of the staphylococcal nuc genes, which were available from NCBI databases. Primers NucaF1 (5′-CCNAAYACNCNGTNCARCCN-3′) and NucaR (5′-NADCCANACRTANGCNARNGT-3′) were used. The reaction mixture for the PCR consisted of 2 μl of DNA extract in a total volume of 50 μl composed of 2 U of Ex Taq (Takara Co., Ltd., Kyoto, Japan), 30 pmol each primer, 0.2 mM deoxynucleoside triphosphate mixture, and 1× reaction buffer (Takara). Reaction mixtures were thermally cycled once at 95°C for 2 min; 30 times at 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s; and then once at 72°C for 2 min. The PCR product was cloned into pGemini pCR4-TOPO (Invitrogen, Life Technologies, Carlsbad, CA) and was transformed into Escherichia coli TOP10 cells (Invitrogen). Insert DNA of the recombinant plasmid was sequenced by using a Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA) with an ABI Prism 3100...
M-PCR targeting the nuc gene locus for species identification of CoPS. By using this M-PCR method, seven species of CoPS (S. aureus, S. intermedius, S. schleiferi subsp. coagulans, S. delphini group A, S. hyicus, S. pseudintermedius, and S. delphini group B) showed a successful amplification of internal fragments with the expected sizes (359 bp, 430 bp, 526 bp, 661 bp, 793 bp, 926 bp, and 1,135 bp, respectively) with the primer pairs specific for each species (Fig. 2). Among the tested CoPS strains, all strains other than an S. pseudintermedius strain were correctly identified to the species level. This S. pseudintermedius strain had a 1.6-kbp insertion mutation (78% nucleotide identity with a partial sequence of the IS1181 transposase) within the nuc gene open reading frame (ORF). In addition, S. schleiferi subsp. coagulans strains, all S. schleiferi subsp. schleiferi strains had 526-bp fragments amplified by the M-PCR method. Consequently, our method had no discriminating power at the subspecies level for S. schleiferi. We also applied this method to S. lutrae CCUG 38494 and 29 CoNS species other than S. schleiferi subsp. schleiferi, and no false-positive result was observed. Consequently, this method is both sensitive (99.8%) and specific (100%).

RESULTS

Sequence analysis of nuc genes of staphylococci. As shown in Table 1, all staphylococcal species analyzed in this study had kept the nuc gene. With regard to the nuc phylogenetic tree (Fig. 1), the relationship among CoPS species other than S. delphini group B agreed with that determined by the 16S rRNA and hsp60 genes (14, 19). The nuc sequence for S. delphini group B was more closely related to that of S. pseudintermedius LMG 22219 than to that of S. delphini LMG 22190 (S. delphini group A), as previously reported (28). Five S. schleiferi subsp. coagulans strains were phylogenetically indistinguishable from 31 S. schleiferi subsp. schleiferi strains (data not shown).

The nucleotide identity of the nuc genes among the CoPS and closely related CoNS species ranged from 60.0 to 95.9% (mean, 71.7%). The most similar pair was S. pseudintermedius and S. delphini group B (95.9%).

DISCUSSION

The species identification of CoPS needs to be performed accurately in veterinary clinical laboratories for two reasons. The first reason is that the MIC breakpoints of oxacillin to determine methicillin resistance in staphylococci differ with species. According to Clinical and Laboratory Standards Institute (CLSI) guidelines, MIC cutoff values for oxacillin for S. aureus and S. lugdunensis are 4 µg/ml and differ from the values for other species (0.5 µg/ml) (8). It was previously reported that some strains exhibited oxacillin MICs of 0.5 to <4 µg/ml among mecA-positive S. pseudintermedius strains (4, 9, 27). If such strains are not identified as S. pseudintermedius strains but are identified as S. aureus strains, they could be misidentified as being methicillin-susceptible strains. Such inadequate species identification could lead to suboptimal or inappropriate treat-
ment decisions for methicillin-resistant staphylococcal infections (5, 23, 27).

The second reason is the public health issue of whether methicillin-resistant staphylococcal isolates from pet and farm animals are *S. aureus* strains. The isolation of methicillin-resistant *S. aureus* (MRSA) strains is also now increasingly common in veterinary medicine. There are significant concerns about the potential for household pets, horses, and food-producing animals to act as reservoirs of MRSA, with subsequent transmission to humans (21, 31). Therefore, a precise diagnosis of MRSA colonization or infection in animals is a necessary social mission for clinical veterinarians. Our M-PCR method will allow the routine identification of CoPS isolates from various animal species in veterinary clinical laboratories and will provide important clues for approaching the issue.

Ghebremedhin et al. previously reported a comparative analysis of interspecific similarity values of 16S rRNA, hsp60, rpoB, sodA, tuf, and gap gene sequences in staphylococci (90 to 99%, 74 to 93%, 71.6 to 93.6%, 81.5 to 98%, 86 to 97%, and 24 to 96%, respectively) (14), which indicate the ranges of nucle-
otide identity scores. The nuc gene has been well conserved and has shown moderate diversity among members of the genus Staphylococcus. Therefore, we considered this gene to be a suitable PCR target for species identification.

To date, there has been no reliable method to distinguish among CoPS species in veterinary clinical laboratories. Recently, Bannoehr et al. and Blaiotta et al. reported molecular identification methods for CoPS species by PCR-restriction fragment length polymorphism (PCR-RFLP) targeting the partial pta (encoding phosphotransacetylase) and katA (encoding catalase) genes, respectively (3, 7). Compared to these approaches, the present M-PCR method is excellent in terms of rapidity, simplicity, and cost and is better suited for clinical veterinary applications.

We previously reported that phenotypically identified St. intermedius strains were reclassified as being S. intermedius, S. pseudintermedius, and S. delphini strains by DNA-DNA hybridization and phylogenetic analysis based on partial sodA and hsp60 gene sequences. In addition, S. delphini strains were divided into two clusters (S. delphini groups A and B) by nuc sequencing analysis (28). Although S. delphini group B strains were more closely related to S. pseudintermedius strains than to S. delphini group A strains (belonging to S. delphini LMG 22190T) upon nuc phylogenetic analysis (28), Blaiotta et al. recently reported that S. delphini group B strains (strains h-2C and P-27B) were more closely related to S. intermedius than to S. delphini group A strains according to katA gene analysis (7). S. delphini group B strains may therefore represent a unique evolutionary path among staphylococci.

In conclusion, we developed a single-PCR method for the species identification of CoPS, the sensitivity and specificity of which were confirmed by using other molecular-based methods such as hsp60 and nuc sequencing approaches. The present method will contribute to future clinical and research findings for staphylococcal infections in veterinary medicine.

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