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Development of a PCR-denaturing Gradient Gel Electrophoresis Method Targeting the *tuf* Gene to Differentiate and Identify *Staphylococcus* Species

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ABSTRACT

Staphylococcal intoxications are often associated with staphylococcal enterotoxins produced by *Staphylococcus aureus*. However, several studies revealed that other *Staphylococcus* species, such as *S. intermedius* and *S. warneri,* could produce enterotoxins as well. To facilitate the identification of a mixed culture while tracing the causative staphylococci of food poison, a polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method was developed to differentiate *Staphylococcus* species using *Staphylococcus*-specific primers targeting the elongation factor Tu gene (*tuf* gene). Eleven tested species were differentiated to 10 separated patterns. Variance of the patterns between strains within a species was analyzed using 9 strains of *S. aureus* and several strains of other species. It was shown that strains within a species migrated the same distance in the DGGE assay. When mixed cultures of different *Staphylococcus* species in milk were subjected to DNA extraction and PCR-DGGE, the resulted patterns faithfully corresponded to the species of the mixed cultures, including those of potential to secret enterotoxins.

Key words: *staphylococcus* species, staphylococcal poisons, identification, DGGE, *tuf* gene

INTRODUCTION

The bacteria in genus *Staphylococcus* are associated with the normal skin flora and mucous membranes of human beings and can be isolated from many other sources such as meat, milk, cheese, soil, sand, water, and air. Currently, there are more than 30 species in the genus *Staphylococcus*, including coagulase-positive staphylococci (CPS) and coagulase-negative staphylococci (CNS). It was generally recognized that *S. aureus* strains would be able to produce several related staphylococcal enterotoxins (SEs). These SEs belong to the large family of staphylococcal and streptococcal pyrogenic exotoxins (PT), which cause toxic shock-like syndromes and have been implicated in food poisons. The heat-stable SEs produced by staphylococci are the major causative agents of staphylococcal food-borne illness. Nowadays, staphylococcal intoxication is one of the most common causes of food poisoning in many countries[1] and in most cases, staphylococcal food poisoning was thought to be associated with *S. aureus*. Only limited and conflicting data are available regarding enterotoxins production of non-*S. aureus* staphylococcal species[2-6]. Recently researches of *S. intermedius* isolates revealed their potential to produce enterotoxins based on the genomic and immunological level[5]. Moreover, lines of evidence of the noninvasive zoonotic transmission of *S. intermedius* from animal hosts to humans have been documented[7], assuming the intoxigenic role of *S. intermedius* in staphylococcal food poisoning via contamination of food products. Since non-*S. aureus* staphylococci, which present in a variety of foodstuffs and food ingredients, are able to produce SEs as well, it is reasonable to consider that SEs present in foodstuffs are not solely produced by *S. aureus* alone. As a result, a variety of the *Staphylococcus* species responsible for the staphylococcal toxin would be assumed and needs to be disclosed. Therefore, a method for the rapid identification of the *Staphylococcus* species on foods would be necessary when tracing staphylococcal outbreaks.

There are several commercial kits developed for the identification of staphylococci, such as API identification systems (bioMérieux Vitek) and MicrodScan systems (Dade Behring Inc., Deerfield, Ill.). Those kits are based on the phenotypic characteristics. Although the sensitivity in the detection of *S. aureus* was between 76 and 87%[8], the overall accuracy of these systems ranges only 50 to 70%[9-10]. For a more rapid and correct identifica-

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tion, several genotypic methods to identify *Staphylococcus* isolates have been developed, including PCR-based methods[11-14] and enzyme immunoassay[15]. In addition, a number of PCR methods for the species-specific detection of non-*S. aureus* species have been reported[16-17]. During the last decade, 16S rDNA was thought to be the most suitable target gene for the identification of eubacteria. However, the identical 16S rDNA sequences of closely related species and the divergent 16S rDNA sequences of a single organism remain problems. To solve these problems, alternative mono-copy target sequences are persistently being developed, including *tuf* gene[13], tRNA gene intergenic spacer[18], *rpoB* gene[19], heat shock protein 60 (HSP60) gene[20], *femA* gene[21], and *sodA* gene[10]. Several target genes revealed high divergence in *Staphylococcus* species and could serve as suitable alternative molecular markers. In this study, the *tuf* gene was used as the target gene for the PCR-DGGE analyses.

Denature gradient gel electrophoresis (DGGE) is a technique used in the resolution of similar DNA fragments by the different endurance of the DNA fragments to the denaturant concentration. In recent years, this culture-independent DGGE technique has been employed in monitoring the microbial populations dynamics of fermented foods, such as silica cheese, fermented Italian sausages, and fermented cassava dough, and provided fast and reliable data[22]. In this study, we attempted to use this technique combining with *Staphylococcus*-specific PCR for the identification of individual *Staphylococcus* species in mixed cultures

MATERIALS AND METHODS

I. *Bacterial Strains and Culture Condition*

Bacterial strains used in this study were listed in Table 1. All *Staphylococcus*, *Citrobacter*, and *Bacillus* strains were cultured at 37°C in tryptic soy broth (TSB broth; Difco, Sparks, Maryland, USA) and subcultured on tryptic soy agar aerobically. The *Klebsiella oxytica* strain was cultured on LB broth (USB, Cleveland, Ohio, USA) at 37°C.

II. *Rapid DNA Isolation*

Rapid DNA extraction from the cultures of each strain was done as follows: 1mL of overnight culture was transferred to an eppendorf tube and was centrifuged at 13000 rpm for 2 min. The collected bacterial pellet was resuspended with 250 μL of Solution D (4M guanidine thiocyanate, 25mM sodium citrate, 0.5% sarcosyl) and 500 μL of phenol/chloroform (1:1). After vortexing for 3 min and centrifugation for 5 min at 10,000 rpm, DNA was recovered from the supernatant and precipitated with ammonium acetate and isopropanol. The addition of 1 μL of RNase (1 mg/1mL) would help to degrade residual RNA. To extract bacterial DNA from milk, the bacteria were collected by centrifugation and resuspended in 100 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The bacterial suspension was treated with 3 μL of lysostaphin (20 mg/mL) (Sigma, St. Louis, Missouri, USA) and 3 μL of lysozyme (40 mg/mL) (Amresco, Solon, Ohio, USA), and incubated at 37°C for 20 min, followed by treatment with proteinase K (10 mg/mL) (Worthington, Lakewood, New Jersey, USA) at 65°C for 20 min. The suspension was thus adjusted to 500 μL with TE buffer, and subjected to DNA extraction as above.

Table 1. Bacterial strains used in this study

Species	Strain[a]
Staphylococcus aureus	BCRC 14958
Staphylococcus aureus	BCRC 12654
Staphylococcus aureus	BCRC 12656
Staphylococcus aureus	BCRC 10823
Staphylococcus aureus	BCRC 12653
Staphylococcus aureus	BCRC 13825
Staphylococcus aureus	BCRC 13826
Staphylococcus aureus	BCRC 13827
Staphylococcus aureus	BCRC 13831
Staphylococcus capitis	BCRC 15231
Staphylococcus capitis	BCRC 12161
Staphylococcus chromogenes	BCRC 12924
Staphylococcus epidermidis	BCRC 15245
Staphylococcus epidermidis	BCRC 11030
Staphylococcus haemolyticus	BCRC 15240
Staphylococcus haemolyticus	BCRC 15241
Staphylococcus intermedius	BCRC 15235
Staphylococcus lentus	BCRC 12926^T
Staphylococcus saprophyticus	BCRC 15283
Staphylococcus saprophyticus	BCRC 13978
Staphylococcus sciuri	BCRC 15242
Staphylococcus warneri	BCRC 15220
Staphylococcus warneri	BCRC 15221
Staphylococcus xylosus	BCRC 15251
Bacillus subtilis	BCRC 14716
Klebsiella oxytoca	BCRC 13985
Citrobacter koseri	BCRC 14804

[a]BCRC: Bioresource Collection and Research center, Hsinchu, Taiwan

For the detection of mixed *Staphylococcus* spp. in milk, each bacterial suspension was diluted with TSB broth until OD_{660} value reached 1. One milliliter of the suspension was centrifuged at 10,000 rpm for 5 min to obtain bacterial pellets. The bacterial sediments were thus resuspended and mixed in sterilized milk and subjected to DNA extraction as above. For the sensitivity test, *S. aureus* BCRC 14958 in the logarithmic phase of growth was collected and diluted serially 10-fold in commercial milk. After centrifugation of 1 mL of the dilutions at 10,000 rpm for 5 min, the bacterial sediment was resuspended in 100 μL TE buffer and used for DNA extraction from milk as above. In parallel, the actual number of colony-forming units (CFU) for each dilution was determined by culturing onto TSB agar plate at 37°C for 24 hrs.

III. *Staphylococcus-specific PCR Protocol*

Twenty four *Staphylococcus* strains and three other bacterial strains (Table 1) were used for the specificity test. PCR reactions were carried out on a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, California, USA.). For the specific amplification of partial *tuf* genes of staphylococci, each PCR tube consisted of 25 μL mixture of 0.2 mM deoxyribonucletide triphosphate (dNTP), 2.5 μL 10X TBR reaction buffer, 1U Prozyme polymerase (Protech, Taipei, Taiwan), and 25 pmol of each *Staphylococcus*-specific primer (TstaG422 and Tstag765) described by Martineau *et al.*[13]. For PCR-DGGE, a GC clamp sequence (CGCCCGCCGCGCGCG- GCGGGC-GGGGCGGGGGCACGGGGGG) was added to the 5' end of the reverse primer Tstag765. The amplification program was 94°C for 5 min, 30 cycles of 30s at 94°C, 30s at 55°C, and 20s at 72°C, followed by 7 min at 72°C. The PCR products were resolved by 2% agarose gel electrophoresis, followed by staining with ethidium bromide and examining visually under UV transillumination. The reaction sensitivity were measured by using purified genomic DNA with serial dilution and the specificity was verified using genomic DNA from several Gram-positive bacteria close to the genus *Staphylococcus*.

IV. *DGGE*

After PCR amplification, the amplicons were subsequently applied to DGGE analysis. DGGE was performed on DCode^{TM} System (Bio-Rad, Hercules, CA, USA),by running a 6.4% acrylamide gel (160 × 160 × 1 mm) in 1X TAE buffer with a denaturing gradient from 25 to 40% [A 100% denaturing solution contained 40% (vol/vol) formamide and 7.0 M urea.] in the direction of parallel electrophoresis. The electrophoresis was conducted with a constant voltage of 130 V at 60°C for 5 h. After electrophoresis, gels were stained with 1X TAE buffer containing 25 μg/ mL ethidium bromide for 5-10 min, destained in 1X TAE buffer for 20 min, and then visualized under UV transillumination.

RESULTS

I. *PCR Amplification with Staphylococcus-specific Primers*

Genomic DNA prepared from bacteria listed in Table 1 was used in *Staphylococcus*-specific PCR and the PCR products were examined on agarose gel under UV light. From all 18 *Staphylococcus* strains generated a band about 410-bp, the similar size to the predicted size of 370-bp fragment of the amplified *tuf* genes plus a 40-bp GC clamp (Figure. 1). The specificity was confirmed by using purified genomic DNA of other bacteria as templates of PCR. As expected, bacteria from other genus remained negative with the set of primers. The result was in accordance with the original research of the primers [13].

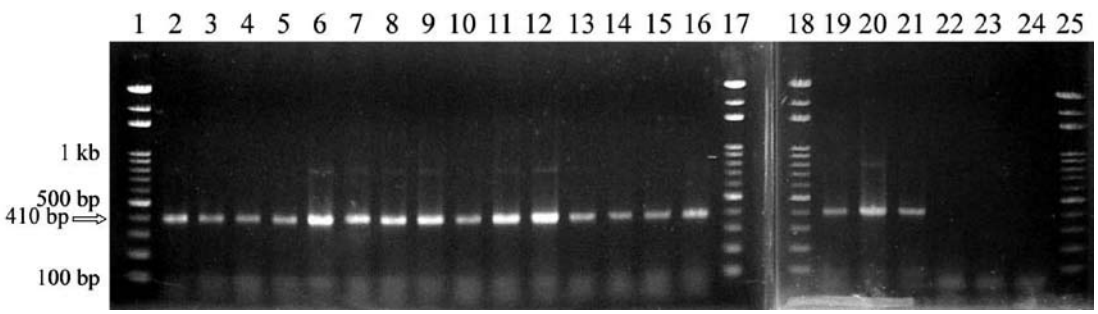

Figure 1. Agarose gel electrophoresis of *Staphylococcus*-specific PCR. From left to right: DNA marker, *S. chromogenes* BCRC 12924, *S. lentus* BCRC 12926, *S. intermedius* BCRC 15235, *S. haemolyticus* BCRC 15240, *S. haemolyticus* BCRC 15241, *S. sciuri* BCRC 15242, *S. aureus* BCRC 14958, *S. aureus* BCRC 12654, *S. aureus* BCRC 12656, *S. warneri* BCRC 15220, *S. warneri* BCRC 15221, *S. capitis* BCRC 15231, *S. capitis* BCRC 12161, *S. epidermidis* BCRC 11030, *S. epidermidis* 15245, DNA marker, DNA marker, *S. saprophyticus* BCRC 15283, *S. saprophyticus* BCRC 13978, *S. xylosus* BCRC 15251, *Bacillus subtilis* BCRC 14716, *Klebsiella oxytoca* BCRC 13985, *Citrobacter koseri* BCRC 14804. The DNA marker was purchased from Protech (Taipei, Taiwan). The sizes of the marker bands were 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700bp, 800 bp, 900 bp, 1 kb, 1.5 kb, 2 kb, and 3kb (from bottom to top).

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II. *DGGE*

After PCR amplification, the products were subjected to DGGE analysis directly. A total of 18 strains belonging to 11 *Staphylococcus* species generated ten migration patterns. All *Staphylococcus* species tested in this research were successfully differentiated except for *S. intermedius* and *S. haemolyticus* (Figure. 2). Compared to previous DGGE analysis targeting the 16S V2-V3 region[23], a better resolution was achieved here, especially in the differentiation of *S. xylosus* and *S. haemolyticus*. According to another PCR-DGGE study targeting the V3 region to differentiate *Staphylococcus* species[11], 23 species were differentiated to 15 patterns. However, *S. aureus*, *S. epidermidis* and *S. lentus* belonged to the same pattern and could not be distinguished. Meanwhile, *S. saprothyticus* and *S. warneri* belonged to another pattern and could not be separated either. As a result, when targeting the V3 region, the capability of DGGE in the identification of staphylococci would be restricted, since suspected nosocomial pathogens such as *S. aureus*, *S. epidermidis, S. saprophyticus* and *S. warneri*[13, 24] could not be definitely identified. Contrastingly, *S. aureus*, *S. epidermidis*, *S. saprothyticus* and *S. warneri* could be differentiated in a single PCR-DGGE assay (Figure. 2 and Figure. 5), where partial *tuf* gene was taken as the target gene in this study. Though *S. intermedius* and *S. haemolyticus* could not be separated in the DGGE pattern, the two species could be discriminated by a further coagulase test. This DGGE results demonstrated that a broad range of *Staphylococcus* species could be identified, including not only those of clinical significance but those commonly recovered from foodstuffs.

III. *Conservation of the DGGE Profiles within Staphylococcus Species*

To confirm the conservation of the PCR-DGGE method in *Staphylococcus* strains belonging to the same species, several strains of the same species were exam-

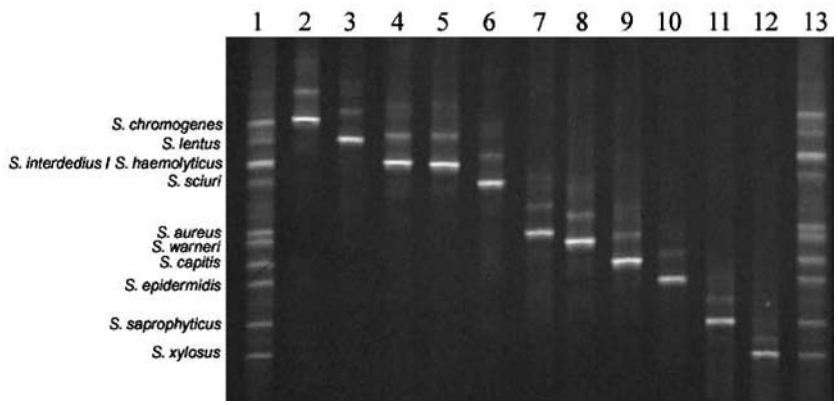

Figure 2. DGGE profiles of 11 *Staphylococcus* species. Lane 1 and 13, DNA ladder of the PCR products of the *Staphylococcus* species; lane 2, *S. chromogenes*; lane 3, *S. lentus*; lane 4, *S. intermedius*; lane 5, *S. haemolyticus*; lane 6, *S. sciuri*; lane 7, *S. aureus*; lane 8, *S. warneri*; lane 9, *S. capitis*; lane 10, *S. epidermidis*; lane 11, *S. saprophyticus*; lane 12, *S. xylosus*.

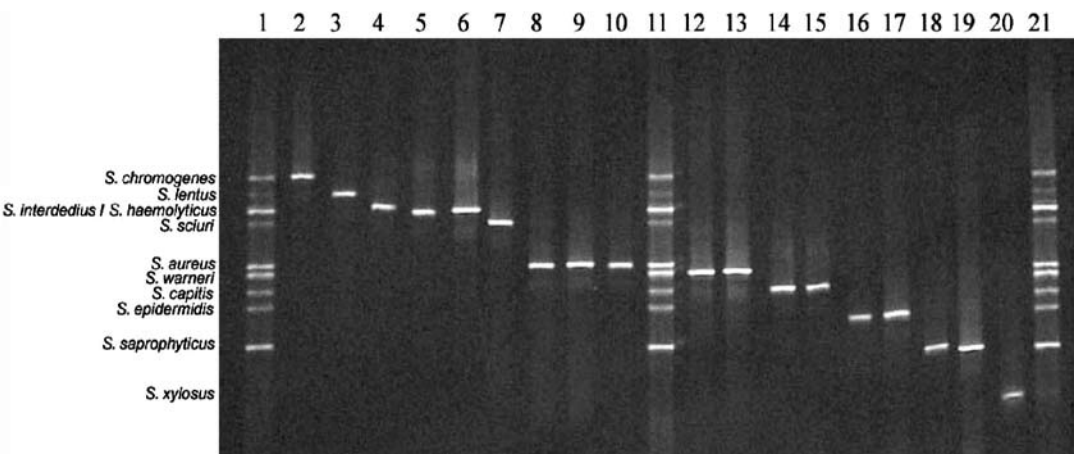

Figure 3. DGGE profiles of 18 staphylococcal strains belonging to 11 species. Lane 1, 11, and 21, DNA ladder; lane 2, *S. chromogenes* BCRC 12924; lane 3, *S. lentus* BCRC 12926; lane 4, *S. intermedius* BCRC 15235; lane 5 and 6, *S. haemolyticus* BCRC 15240 and BCRC 15241; lane 7, *S. sciuri* BCRC 15242; lane 8-10, *S. aureus* BCRC 14958, BCRC 12654,and BCRC 12656; lane 12 and 13, *S. warneri* BCRC 15220 and BCRC 15221; lane 14 and 15, *S. capitis* BCRC 15231 and BCRC 12161; lane 16 and 17, *S. epidermidis* BCRC 11030 and BCRC 15245; lane 18 and 19, *S. saprophyticus* BCRC 15283 and BCRC 13978; lane 20, *S. xylosus* BCRC 15251.

ined in PCR-DGGE. A total of 18 strains were used for PCR-DGGE assay, including one *S. chromogene*, one *S. lentus*, one *S. intermedius*, two *S. haemolyticus*, one *S. sciuri*, three *S. aureus*, two *S. warneri*, two *S. capitis*, two *S. epimidis*, two *S. saprophyticus*, and one *S. xylosus* strains. It was shown that DNA from various strains of the same species migrated the same distances (Figure. 3), indicating the highly conserved taxa of the partial *tuf* gene intra-species. In addition, other 6 strains of *S. aureus* were tested in another PCR-DGGE assay in order to explicate the accuracy of the *S. aureus* identification (Figure. 4). The DNA of all *S. aureus* strains tested migrated the same distances, suggesting the high reproductivity and accuracy in the identification of *S. aureus*.

IV. *Identification of Individual Species in the Staphylococcus Mixed Cultures*

The TstaG422 and Tstag765 primers were examined in the amplification of the partial *tuf* gene of all representative staphylococcal pathogens. Since opportunistic infections[25] and food contamination may sometimes be due to more than one species of staphylococci, the practicability of this PCR-DGGE method was evaluated to identify individual species in the mixed cultures of *Staphylococcus* spp.. Genomic DNA of different *Staphylococcus* species were mixed and applied to PCR-DGGE. All tested species migrated different distances and could be resolved. No false-negative reaction was found. At least five common *Staphylococcus* species could be diagnosed in one assay (Figure. 5). Staphylococcal pathogens commonly isolated from nosocomially infected patients such as *S. aureus*, *S. epidermidis* and *S. saprophyticus* could be detected and identified in spite of the existence of other species (Figure. 5).

V. *Detection of Staphylococcus Species in Milk*

Eleven species of staphylococcal cultures, including those cause mastitis such as *S. aureus*, *S. chromogenes*, *S. epidermidis*, and *S. xylosus*[26], were randomly chosen and added to pasteurized milk. Before mixing, the OD_{660} value of each bacterial culture was adjusted to unity, and 1 mL of the bacterial suspension was taken for the mixture. Each milk sample was designed to consist of four *Staphylococcus* species. PCR-DGGE results showed that all tested species migrated the anticipated distances in the DGGE pattern. *S. aureus*, *S. chromogenes*, *S. epidermidis*, and *S. xylosus* could be detected and differentiated in a single PCR-DGGE assay (Figure. 6). The results indicated the efficiency and applicability of PCR-DGGE in the detection of mixed staphylococcal cultures in milk sample.

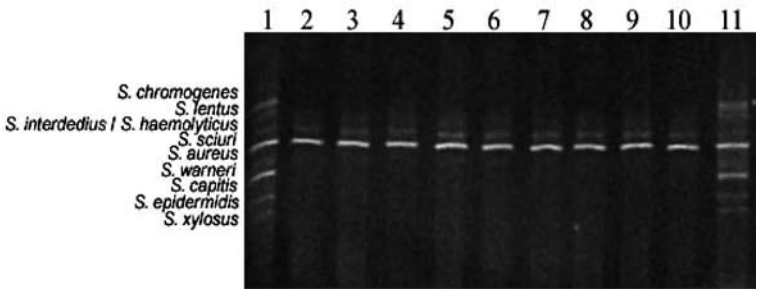

Figure 4. A total of 9 strains of *S. aureus* were subjected to PCR-DGGE and observed a conservative profile. Lane 1 and 11: DNA ladder of staphylococci. Lane 2-10: *S. aureus* BCRC 14985, 12654, 12656, 10823, 12653, 13825, 13826, 13827, 13831.

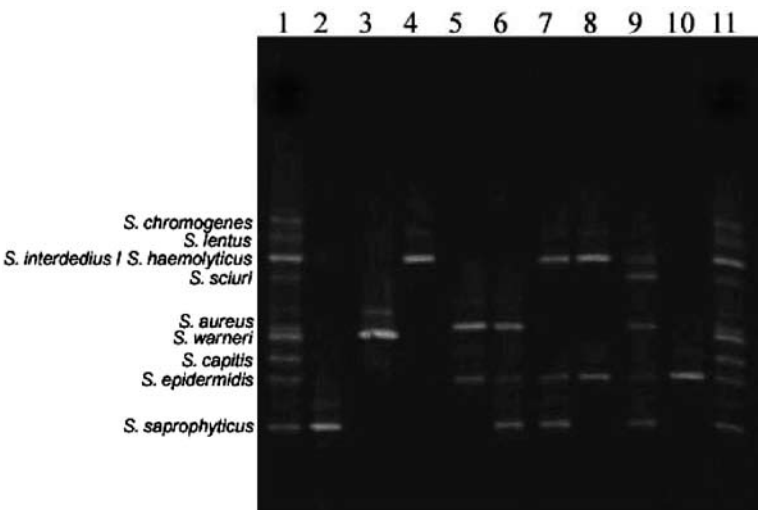

Figure 5. PCR-DGGE profiles using mixed purified DNA of staphylococci as templates. Lane 1 and 11, DNA ladder of staphylococci; lane 2-4, *S. saprophyticus*, *S. warneri*, and *S. haemolyticus* respectively; lane 5, mixed DNA of *S. aureus* and *S. epidermidis*; lane 6, mixed DNA of *S. aureus*, *S. epidermidis* and *S. saprophyticus*; lane 7, mixed DNA of *S. epidermidis*, *S. saprophyticus*, and *S. haemolyticus*; lane 8, mixed DNA of *S. epidermidis* and *S. haemolyticus*; lane 9, mixed DNA of *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus* and *S. sciuri*; lane 10, *S. epidermidis*.

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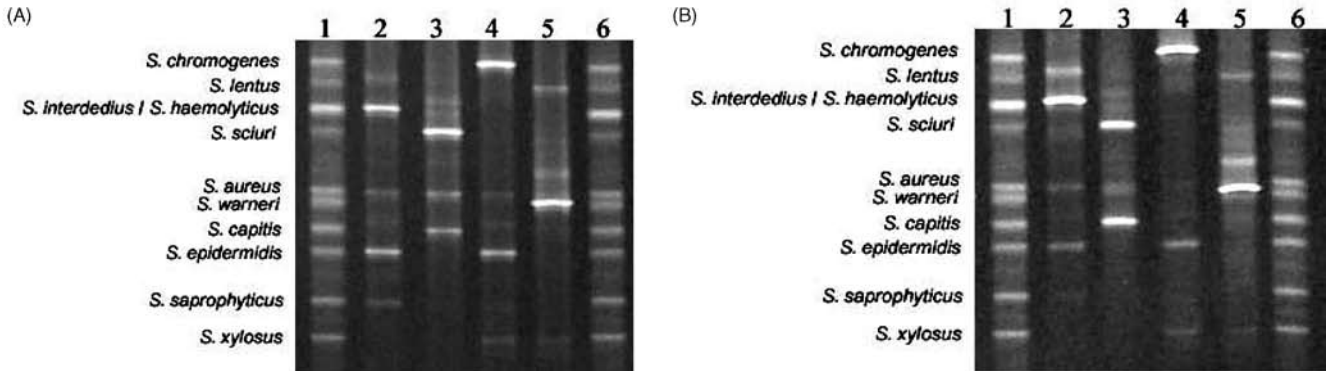

Figure 6. PCR-DGGE analysis of mixed cultures of *Staphylococcus* species in (A) TSB medium and (B) pasteurized milk. Lane L: DNA ladder of *Staphylococcus* species. Lane 1 and 6, DNA ladder. Lane 2: mixed cultures of *S. aureus*, *S. epidermidis*, *S. intermedius* and *S. saprophyticus*. Lane 3: mixed cultures of *S. aureus*, *S. capitis*, *S. haemolyticus* and *S. sciuri*. Lane 4: mixed cultures of *S. aureus*, *S. chromogenes*, *S. epidermidis* and *S. xylosus*. Lane 5: mixed cultures of *S. aureus*, *S. lentus*, *S. warneri* and *S. xylosus*.

VI. *Sensitivity of PCR-DGGE*

Cultures of *S. aureus* BCRC14958 were collected and serially diluted in pasteurized milk. Sensitivity tests revealed that a 30-cycle PCR-DGGE could detect 1.12×10^4 CFU/mL while a 40-cycle PCR-DGGE could detect 1.12×10^3 CFU/mL (Figure. 7). In addition, serial dilutions of extracted DNA were subjected to PCR. For a 40-cycle PCR, the PCR assay was able to detect about 15 copies of genome of *Staphylococcus aureus* (deduced from the genome size of *S. aureus*) (Figure. 7). This result was similar to that described by Martineau *et al.*[13].

DISSCUSSION

Recently, the high occurrence of staphylococcal food poisoning among many countries accentuates the demands of a rapid identification method. Further, the importance of CNS strains as major causes of nosocomial infection draw more attention. Although *S. epidermidis* accounts for the majority of infections caused by CNS, many other species have also been identified in association with human infections[27]. As a result, a rapid, simple and cost-less method is required in the detection and identification of staphylococci, not only to detect the nosocomially infectious staphylococci but to trace the causative staphylococci responsible for staphylococcal food poisoning.

In this study, a PCR-DGGE method was developed to differentiate staphylococcal species, including *S. aureus* and important nosocomial CNS. A total of 11 tested species were differentiated by the PCR-DGGE method except for *S. intermedius* and *S. haemolyticus*. Significantly infectious staphylococci, such as *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus* could be identified in this PCR-DGGE method. Six staphylococcal species that comprise two or three strains and six more *S. aureus* strains were subjected to PCR-DGGE to test the intra-species conservation. The conservation of the intra-species DGGE profiles was proven since strains in the same species produced the same DGGE profiles.

The PCR reaction targeted the staphylococcal *tuf* gene, which is the gene of the bacterial elongation factor. The *tuf*-based PCR assay developed by Martineau *et al*[13]. for staphylococcal species identification has shown excellent sensitivity and specificity, and could be adapted for the direct detection of staphylococci from contaminated bloods or from normally sterile clinical specimens such as blood or urine. Unlike the multi-copy numbers of ribosome rDNA, the *tuf* gene exists single copy in Gram-positive bacteria. This makes *tuf* gene more ascendant when applied in DGGE since multi-copy genes may be indefinite and confuse in the DGGE profiles. Moreover, the sequences of the *tuf* gene have been analyzed and considered to be partially in agreement with phylogenetic studies performed with 16S rRNA in *Staphylococcus* species[13], *Lactobacillus* species and *Bifidobacterium* species[28,29]. In our previous study, a PCR-DGGE method targeting the *tuf* gene of *Lactobacillus* had been developed and actually revealed a better resolution than that targeting the 16S V2-V3 region (unpublished data). In this study, a *Staphylococcus*-specific PCR that targets the *tuf* gene was combined with DGGE to differentiate *Staphylococcus* species, and a considerable resolution and species-conservation were obtained in the result. The value of this study lied in that not only monoclonal but also mixed cultures of clinically significant staphylococci could be differentiated in a single assay.

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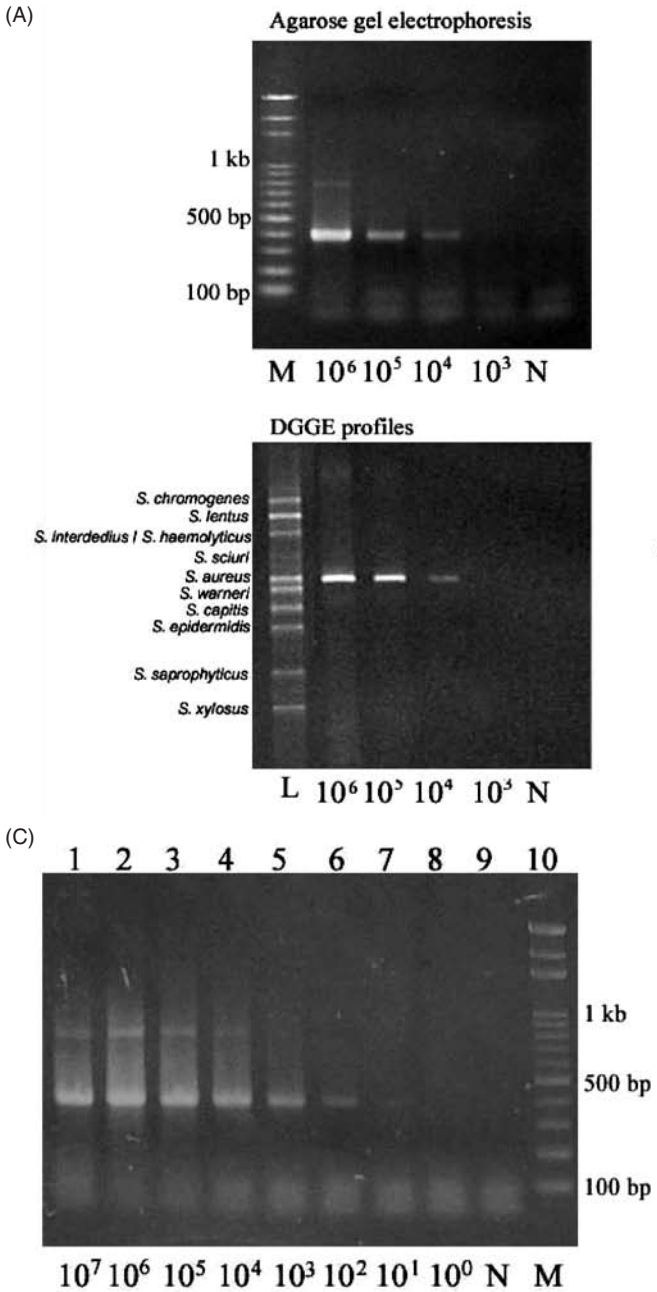

Figure 7. Sensitivity tests of PCR-DGGE using *S. aureus* 14958 as target microorganism. (A) A 30-cycle PCR could detect 104 cells in milk. (B) A 40-cycle PCR could detect 103 cells in milk. (C) A 40-cycle PCR could detect 101 genome copies using serial diluted DNA as templates. M: DNA marker. L: DNA ladder of staphylococci. The sizes of the marker bands were 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700bp, 800 bp, 900 bp, 1 kb, 1.5 kb, 2 kb, and 3kb (from bottom to top).

REFERENCES

1. Fueyo, J. M., Martin, M. C., Gonzalez-Hevia, M. A. and Mendoza, M. C. 2001. Enterotoxin production and DNA fingerprinting in *Staphylococcus aureus* isolated from human and food samples. Relations between genetic types and enterotoxins. Int. J. Food Microbiol. 67: 139-145.
2. Becker, K., Keller, B., Von Eiff, C., Bruck, M., Lubritz, G., Etitenne, J. and Peters, G. 2001. Enterotoxigenic potential of *Staphylococcus intermedius*. Appl. Environ. Microbiol. 67: 5551-5557.
3. Carmo, L. S., Dias, R . S., Linardi, V. R., De Sena, M. J., Santos, D. A., de Faria, M. E., Pena, E. C., Jett, M. and Heneine, L. G. 2002. Food poisoning due to enterotoxigenic strains of Staphylococcus present in Minas cheese and rawmilk in Brazil. Food Microbiol. 19: 9-14.
4. Chou, C. C. and Chen, L. F. 1997. Enterotoxin production by *Staphylococcus warneri* CCRC 12929, a coagulase-negative strain. J. Food Prot. 60: 923-927.
5. Futagawa-Saito, K., Suzuki, M., Ohsawa, M., Ohshima, S., Sakurai, N., Ba-Thein, W. and Fukuyasu, T. 2004. Identification and prevalence of an enterotoxin-related gene, se-*int*, in *Staphylococcus intermedius* isolates from dogs and pigeons. J. Appl. Microbiol. 96: 1361-1366.
6. Vernozy Rozand, C., Mazuy, C., Prevost, G., Lapeyre,

C., Bes, M., Brun, Y. and Fleurette, J. 1996. Enterotoxin production by coagulase-negative staphylococci isolated from goats' milk and cheese. Int. J. of Food Microbiol. 30: 271-280.

7. Guardabassi, L., Loeber, M. E. and Jacobson, A. 2004. Transmission of multiple antimicrobial-resistant *Staphylococcus* intermedius between dogs affected by deep pyoderma and their owners. Vet. Microbiol. 98: 23-27.
8. Zschöck, M., Nesseler, A. and Sudarwanto, I. 2005. Evaluation of six commercial identification kits for the identification of *Staphylococcus aureus* isolated from bovine mastitis. J. Appl. Microbiol. 98: 450-455.
9. Ieven, M., Verhoeven, J., Pattyn, S. R. and Goossens, H. 1995. Rapid and economical method for species identification of clinically significant coagulase-negative staphylococci. J. Clin. Microbiol. 33: 1060-1063.
10. Poyart, C., Quesne, G., Boumaila, C. and 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.
11. Blaiotta, G., Pennacchia, C., Ercolini, D., Moschetti, G. and Villani, F. 2003. Combining denaturing gradient gel electrophoresis of 16S rDNA V3 Region and 16S-23S rDNA spacer region polymorphism analyses for the identification of staphylococci from Italian fermented sausages. Syst. Appl. Microbiol. 26: 423-433.
12. Chesneau, O., Morvan, A., Aubert, S. and el Solh, N. 2000. The value of rRNA gene restriction site polymorphism analysis for delineating taxa in the genus *Staphylococcus*. Int. J. Syst. Evol. Microbiol. 50: 689-697.
13. Martineau, F., Picard, F. J., Ke, D., Paradis, S., Roy, P., Ouellette, M. and Bergeron, M. G. 2001. Development of a PCR assay for identification of staphylococci at genus and species levels. J. Clin. Microbiol. 39: 2541-2547.
14. Mason, W. J., Blevins, J. S., Beenken, K., Wibowo, N., Ojha, N. and Smeltze, M. S. 2001. Multiplex PCR protocol for the diagnosis of staphylococcal infection. J. Clin. Microbiol. 39: 3332-3338.
15. Yazdankhah, S. P., Hellemann, A. L. , Rønningen, K. and Olsen, E. 1998. Rapid and sensitive detection of *Staphylococcus* species in milk by ELISA based on monodisperse magnetic particles. Vet. Microbiol. 62: 17-26.
16. Martineau, F., Picard, F. J., Roy, P. H., Ouellette, M. and Bergeron, M. G. 1996. Species-specific and ubiquitous DNA-based assays for rapid identification of *Staphylococcus epidermidis*. J. Clin. Microbiol. 34: 2888-2893.
17. Zakrzewska-Czerwinska, J., Gaszewska-Mastalarz, A., Pulverer, G. and Mordarski, M. 1992. Identification of *Staphylococcus epidermidis* using a 16S rRNA-directed oligonucleotide probe. FEMS Microbiol. Lett. 79: 51-58.
18. Maes, N., De Gheldre, Y., De Ryck, R., Vaneechoutte, M., Meugnier, H., Etifnne, J. and Struelens, M. J. 1997. Rapid and accurate identification of *Staphylococcus* species by tRNA intergenic spacer length polymorphism analysis. J. Clin. Microbiol. 35: 2477-2481.
19. Drancourt, M. and Raoult, D. 2002. *rpoB* gene sequence-based identification of *Staphylococcu*s species. J. Clin. Microbiol. 40: 1333-1338.
20. Goh, S., Santucci, Z., Kloos, W. E., Faltyn, M., George, C. G., Driedger, D. and Hemmingsen, S. M. 1997. Identification of *Staphylococcus* species and subspecies by the chaperonin 60 gene identification method and reverse checkerboard hybridization. J. Clin. Microbiol. 35: 3116-3121.
21. Vannuffel, P., Heusterspreute, M., Bouyer, M., Vandercam, B., Philippe, M. and Gala, J. L. 1999. Molecular characterization of *femA* from *Staphylococcus hominis* and *Staphylococcus saprophyticus*, and *femA*-based discrimination of staphylococcal species. Res. Microbiol. 150: 129-141.
22. Ercolini, D. 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. J. Microbiol. Methods 56: 297-314.
23. Rantsiou, K., Iacumin, L., Canton, C., Comi, G. and Cocolin, L. 2005. Ecology and characterization by molecular methods of *Staphylococcus* species isolated from fresh sausages. Int. J. Food Microbiol. 97: 277-284.
24. Kamath, U., Singer, C. and Isenberg, H. D. 1992. Clinical Significance of *Staphylococcus warneri* Bacteremia. J. Clin. Microbiol. 30: 261-264.
25. Seifert, H., Oltmanns, D., Becker, K., Wisplinghoff, H. and von Eiff, C. 2005. *Staphylococcus lugdunensis* Pacemaker-related infection. Emerg. Infect. Dis. 11: 1283-1286.
26. Forsman, P., Tilsala-Timisjarvi, A. and Alatossava, T. 1997. Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S-23S rRNA spacer regions. Microbiology 143: 3491-3500.
27. Couto, I., Pereira, S., Miragala, M., Sanches, I.S. and De Lencastre, H. 2001. Identification of clinical staphylococcal isolates from humans by internal transcribed spacer PCR. J. Clin. Microbiol. 39: 3099-3103.
28. Chavagnat, F., Haueter, M., Jimeno, J. and Casey, M. G. 2002. Comparison of partial *tuf* gene sequences for the identification of lactobacilli. FEMS Microbiol. Lett. 217: 177-183.
29. Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T. R. and Zink, R. 2003. Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. Appl. and Environ. Microbiol. 69: 6908-6922.