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Full Length Research Paper

Application of PCR-RFLP of *gap* gene method as a molecular typing tool for coagulase negative Staphylococci from bovine and human origin identified with VITEK 2

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The aim of this study was to apply the Restriction Fragment Length Polymorphism (RFLP) of Glyceraldehyde-3-Phosphate Dehydrogenase encoding gene (*gap*) for testing its performance as a molecular typing tool in coagulase negative staphylococci (CNS) isolates from bovine mastitis (n = 59) and human clinical cases (n = 13) identified with VITEK 2. According to the phenotypic identification results, bovine mastitis isolates were *Staphylococcus haemolyticus*, *Staphylococcus simulans*, *Staphylococcus auricularis*, *Staphylococcus warneri*, *Staphylococcus hominis*, *Staphylococcus capitis*, *Staphylococcus xylosus*, *Staphylococcus epidermidis* and *Staphylococcus cohnii*. Although most of those isolates were generated PCR amplicons with *gap* gene specific primers, PCR amplification of *gap* gene failed in 29 from 72 isolates. The samples that did not produce amplicons were reamplified with Staphylococcal 16S rRNA gene specific primers. After PCR amplifications, amplicons were produced in 17 from 29 samples. Three different restriction endonucleases (AluI, MseI and RsaI) were used for PCR-RFLP analysis, among these AluI has been found the most discriminatory power for identification in species. The results of the RFLP of *gap* gene provide a support for the misidentification problem associated with VITEK 2 system for *S. simulans*, *S. auricularis* and *S. capitis* species. Moreover, more frequent failure in *gap* gene amplification for bovine isolates which were phenotypically identified as *S. simulans, S. auricularis, S. capitis*, *S. xylosus* and *S. cohnii* was not clear. In addition, the method verified the phenotypic identification for *S. haemolyticus*, *S. warneri*, *S. hominis* and *S. epidermidis* isolates with different rates at 100, 33.3, 57.1, and 66.7%, respectively.

Key words: Coagulase negative staphylococci, *gap* gene, PCR-RFLP.

INTRODUCTION

Coagulase negative staphylococci (CNS) represent a heterogeneous group and some of them recently become significant as potential pathogens. Moreover, they have been frequently isolated from bovine mastitis cases (Taponen et al., 2006; Sawant et al., 2009) and caused persistent intramammary infections (Aarestrup et al., 1999). However, accurate identification of the members of this group at species level is challenging. Most of the times, phenotypic identification methods with manual and automated systems are not reliable for correct

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Table 1. Results of *gap* gene amplifications and misidentification percentages.

Organisms (number of isolates)	Isolates	*Gap* gene positive	*Gap* gene negative	Number (%) of misidentified isolates
S. haemolyticus (15)	4-8-33-36-41-42-44-56-70-84-85-88-91-92-96	14	1	14 (0)
S. simulans (12)	15-28-31-40-43-45-53-68-69-86-87-99	7	5	7 (100)
S. hominis (11)	46-48-50-51-79-83-107*-108*-109*-101*-113*	7	4	3 (42.9)
S. auricularis (2)	47-49-59-61-62-63-74-90-94-97	2	8	2 (100)
S. epidermidis (9)	13-102*-103*-104*-105*-106*-110*-111*-112*	6	3	2 (33.3)
S. warneri (7)	12-54-64-67-77-80-81	6	1	4 (66.7)
S. capitis (5)	1-75-76-78-82	1	4	1 (100)
S. xylosus (2)	93-100	0	2	nd
S. cohnii (1)	60	0	1	nd
Total (72)		43	29	

*Clinical isolates; nd (not detected).

identification (Layer et al., 2006; Kim et al., 2008). Recently many PCR-based molecular methods have been developed to provide alternative ways for accurate identification. Several molecular targets such as *gap* (Yugueros et al., 2000), *sod*A (Poyart et al., 2001) and 16S rRNA (Boerlin et al., 2003) genes have been used for the molecular identification of *Staphylococcus* species. Finally, more sophisticated methods such as real-time PCR and melt curve analysis were also utilized for identification of CNS species (Skow et al., 2005). Among those molecular methods, 16S rRNA sequence analysis is considered to be a reliable tool for both species and subspecies level identification of CNS and therefore is regarded as the definitive identification way in many studies which aim to investigate the accuracy of different phenotypic methods (Kim et al., 2008).

PCR-RFLP analysis of *gap* gene has been suggested as a powerful method for identification of *Staphylococcus* species (Yugueros et al., 2000; Yugueros et al., 2001). Basis of this method was the presence of a 42 kDa transferrin binding protein (Tpn) which has been found within the cell wall of *S. aureus* and the other CNS species including *S. epidermidis*, *S. capitis*, *S. haemolyticus* and *S. hominis* (Modun et al., 1994). This protein is a member of the multifunctional cell wall-associated glyceraldehyde-3-phosphate dehydrogenases which catalyzes the conversion of glyceraldehyde- 3-phosphate to 1,3-diphosphoglycerate and incorporates binding sites for human transferrin (Modun et al., 1998). Layer et al. (2007) improved the original method using reference strains and tested the accuracy of the improved method on the clinical isolates, which consisted of *S. aureus*, *S. epidermidis* and *S. haemolyticus*. However, the method has not been extensively evaluated on the clinical isolates belonging to other *Staphylococcus* species. Therefore, the aims of the study were to use PCR-RFLP of *gap* gene method as a molecular typing tool to differentiate the CNS isolates from two different origins (bovine and human) which identified using VITEK 2 phenotypic system and to determine discriminatory power of different endonucleases for PCR-RFLP analysis.

MATERIALS AND METHODS

Bacterial strains

The 72 CNS isolates were identified using both conventional methods and VITEK 2 identification system. According to phenotypic identification, bovine mastitis isolates (n = 59) were more diverse group including nine different species (*S. haemolyticus*, *S. simulans*, *S. auricularis*, *S. warneri*, *S. hominis*, *S. capitis*, *S. xylosus*, S. *epidermidis* and *S. cohnii*), however clinical isolates (n = 13) were representative of two different species (*S. epidermidis* and *S. hominis*) (Table 1). Bovine isolates were isolated and phenotypically identified in a previous study from our laboratory (unpublished data). On the other hand, phenotypically identified clinical isolates were obtained from another laboratory.

DNA extraction

Whole cellular DNA from bovine CNS isolates was prepared for PCR amplifications with some modifications of the method described by Bell et al. (1998). Briefly, following to overnight growth of the isolates at 37°C in 4 ml of nutrient broth, 1 ml was transferred into an eppendorf tube and centrifuged at 10000 rpm for 2 min. Then, the pellet was washed with 1 ml of TE buffer (10 mM Tris-HCl, 1 mm EDTA, pH 8.0) and re-centrifuged. The second washing of the pellet was done with 200 µl of TE buffer and suspension was incubated at 95°C for 20 min. After this incubation, another centrifugation at 4°C was performed for 2 min. Following the last centrifugation, supernatant was collected and used for PCR amplifications. DNA from clinical isolates were obtained with a different method since preferred method for routine applications was different at the laboratory where clinical isolates were obtained. Overnight grown cultures were adjusted to 5 McFarland with sterile ddH_2O and the culture was transferred to a fresh eppendorf tube.

Then, centrifugation was done at 13000 rpm for 3 min. Following

centrifugation, pellets were suspended with 1 ml of TE and the tubes were incubated at 100°C for 10 min. At the end of incubation, 100 µl of glass beads was added for mechanical disruption in the Mickle Cell Desintegrator (The Mickle Laboratory Engineering, UK). Samples were shaken at maximum speed for 7 min. After mechanical disruption, another centrifugation was performed at 12000 rpm for 5 min. Finally, 200 - 500 µl of upper phase was transferred into a fresh tube and used for PCR amplifications.

PCR

The *gap* gene was amplified with the previously published primers (Yugueros et al., 2000). PCR components and cycling conditions were slightly modified. Each PCR reaction was performed in 50 µl containing 5 µl of target DNA, 200 µM deoxynucleotide triphosphates (dNTP), 30 pmol of each primer, 5 µl of 10X PCR buffer (500 mM KCl, 15mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0), and 1 unit of Taq DNA Polymerase (MBI Fermentas). Amplifications were initiated with a denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 40 s, and polymerization at 72°C for 1 min in a thermal cycler (Eppendorf Mastercycler® Gradient, Germany). A final extension was at 72°C for 5 min. Another PCR was performed with the previously published primers for Staphylococcal 16S rRNA gene on the samples that failed to amplify with *gap* primers (Kohner et al., 1999). The components of PCR amplifications targeting both genes were essentially the same except final concentration of each primer and dNTP in Staphylococcal 16S rRNA gene amplifications which were 60 pmol and 250 µM, respectively. Amplifications were initiated with a denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and polymerization at 72°C for 1 min in a thermal cycler (Eppendorf Mastercycler® Gradient, Germany). A final extension was at 72°C for 2 min. Amplicons were separated on 1.5% agarose gel electrophoresis and visualized on gel documentation system (Vilber Lourmat, France).

Restriction digests

The 933 bp PCR amplicon corresponding to the *gap* gene was digested with three different restriction endonucleases, AluI, MseI and RsaI, respectively. The restriction endonucleases were selected based on the previously published partial *gap* gene sequences for *Staphylococcus* species (Layer et al., 2007). Digestions were performed in a 50 µl reaction volume containing 10 µl of PCR sample. Patterns of AluI digested fragments observed in the original method for test strains have been used as reference for molecular typing of the studied strains (Yugueros et al., 2000; Yugueros et al., 2001; Spanu et al., 2003).

PCR-RFLP analysis

AluI digested PCR-RFLP fragments were separated on standard agarose and denaturing polyacrylamide gels. AluI, MseI and RsaI digested fragments were resolved on 1.5% standard agarose gels and visualized on gel documentation system (Vilber Lourmat, France). Electrophoresis of AluI digested PCR-RFLP samples on 6% polyacrylamide gel containing 7 M urea was also performed in 1X TBE (90 mM tris-base, 90 mM boric acid, 2 mM EDTA, pH 8.0) at 85 watt constant power. Bands were visualized with silver staining as described by Bassam et al. (1991).

RESULTS

The *gap* gene was amplified twice from 72 samples with *gap* gene specific primers, GF1 and GR2, twice. In order to optimize PCR conditions, analysis of PCR were repeated under two different conditions. In the first condition, high numbers of samples were produced amplicons. In the second condition, components of PCR reaction were slightly changed. For example, final concentration of $MgCl_2$ and each primer was 2.5 mM and 100 pmol, respectively. Under the second condition, more primer dimer and non-specific bands formation were observed. In addition, less number of samples showed amplification under this condition (data not shown). Therefore, the first PCR conditions were used for PCR amplifications and *gap* gene was amplified in 43 out of 72 samples (Table 1; Figure 1).

Another PCR with Staphylococcal 16S rRNA primers was performed on the samples, which were not amplified with *gap* gene specific primers. At the end of this second PCR amplifications, 17 out of 29 samples produced amplicons (Figure 2). This result indicated that most of those samples which fail to amplified with *gap* primers have enough DNA both quantitatively and qualitatively for PCR amplifications. Moreover, their identification was verified for genus level.

PCR-RFLP patterns after AluI digestion on agarose gels were found to be similar to the previously reported patterns for reference strains of *S. haemolyticus*, *S. warneri*, *S. hominis* and *S. epidermidis* species (Figure 3). However, patterns obtained for all *S. haemolyticus* isolates were similar to that of the reference strains for *S. haemolyticus* and *S. cohnii* subsp. *cohnii* species in the original report (Yugueros et al., 2000; 2001). Therefore, partial sequence of 16S rRNA gene from the representative of *S. haemolyticus* phenotypic bovine isolate was obtained for discrimination between two species (unpublished data). Sequence from this isolate was similar to sequences from *S. haemolyticus* found in the GenBank. Therefore, isolates producing the same pattern were identified as *S. haemolyticus*. AluI digested PCR-RFLP fragments were also resolved in denaturing polyacylamide gels in order to search the difference in appearance of PCR-RFLP pattern observed in agarose gels. Although higher numbers of fragments were observed on denaturing acylamide gels, patterns corresponding to four species (*S. haemolyticus*, *S. warneri*, *S. hominis* and *S. epidermidis*) were in agreement in two different gel systems (Figure 4). Therefore, it can be concluded that resolving power of agarose gel electrophoresis is satisfactory for strain identification. However, denaturing acylamide gels could be useful for accurate detection of the intraspecies RFLP variation. In addition, the identical band pattern observed for all *S. haemolyticus* isolates in this gel system further supported the correct discrimination of *S. haemolyticus* isolates. Figure 5 represents

Figure 1. *Gap* gene amplifications of all CNS isolates. Lane M, 100-bp marker. Numbers correspond to the isolate numbers same as presented in Table 1.

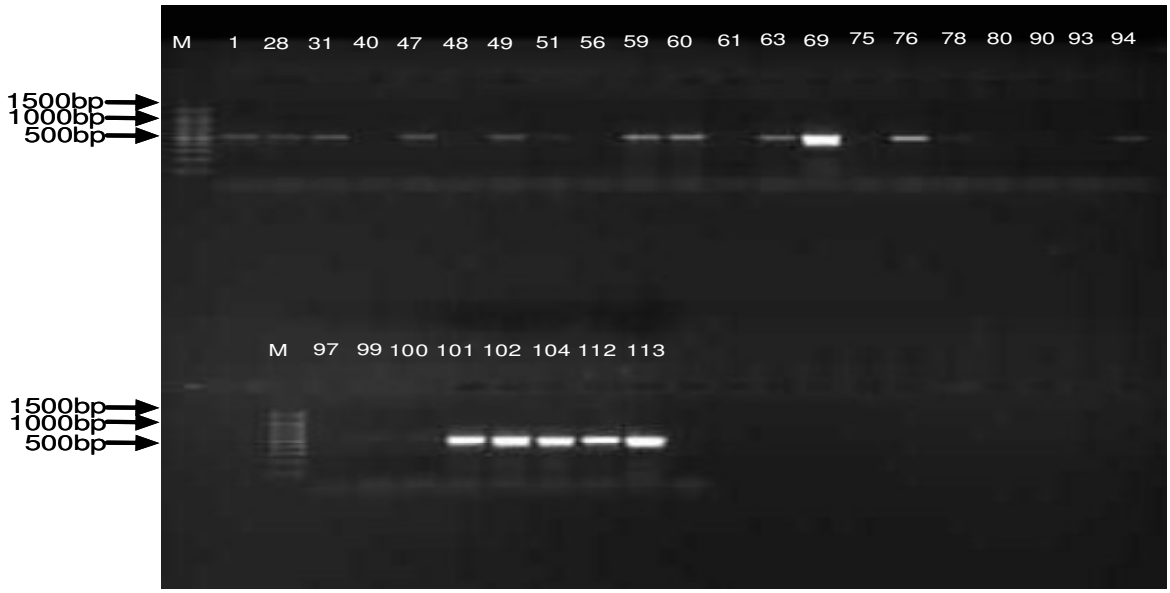

Figure 2. PCR amplifications with Staphylococcal 16S rRNA primers of isolates which failed in *gap* gene amplifications. Lane M, 100-bp marker. Numbers correspond to the isolate numbers same as presented in Table 1.

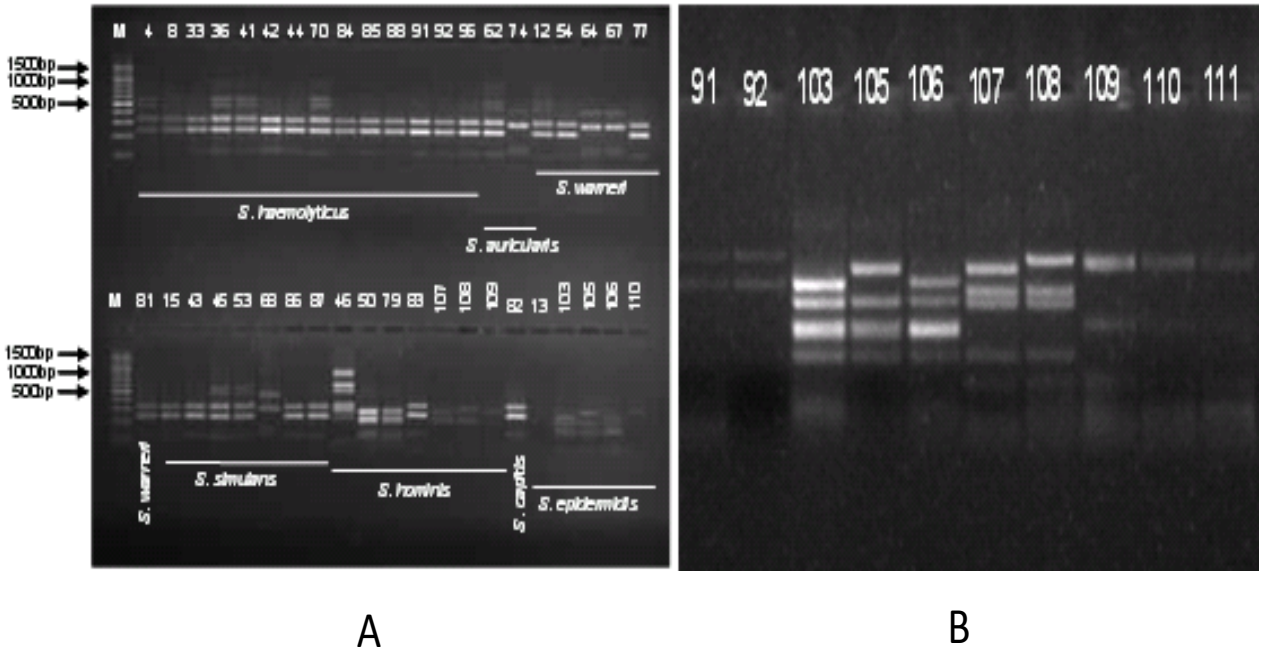

Figure 3. AluI digestion of *gap* PCR products separated on agarose gel. Lane M, 100-bp marker. A represents all samples, B represents a larger view of some samples depicted in A.

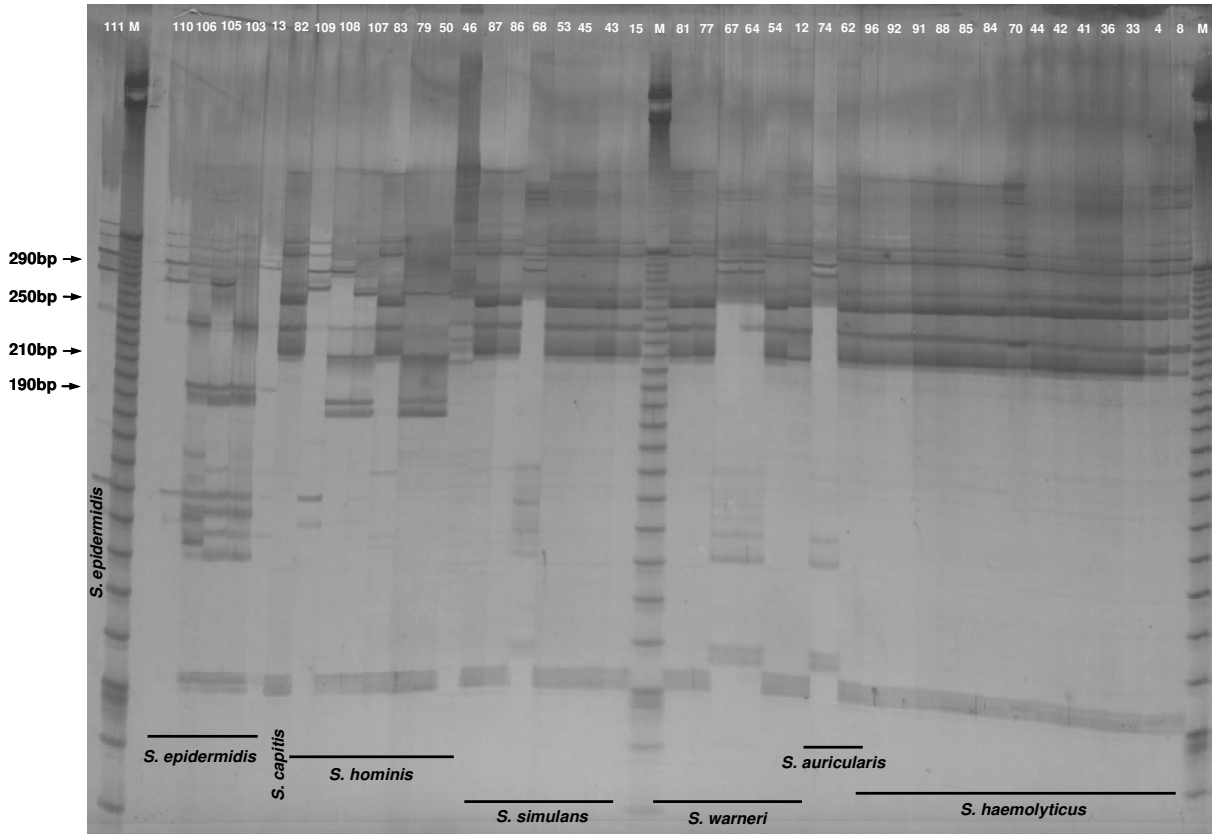

Figure 4. AluI digestion of *gap* PCR products separated on denaturing acrylamide gel. Lane M, 10-bp DNA ladder.

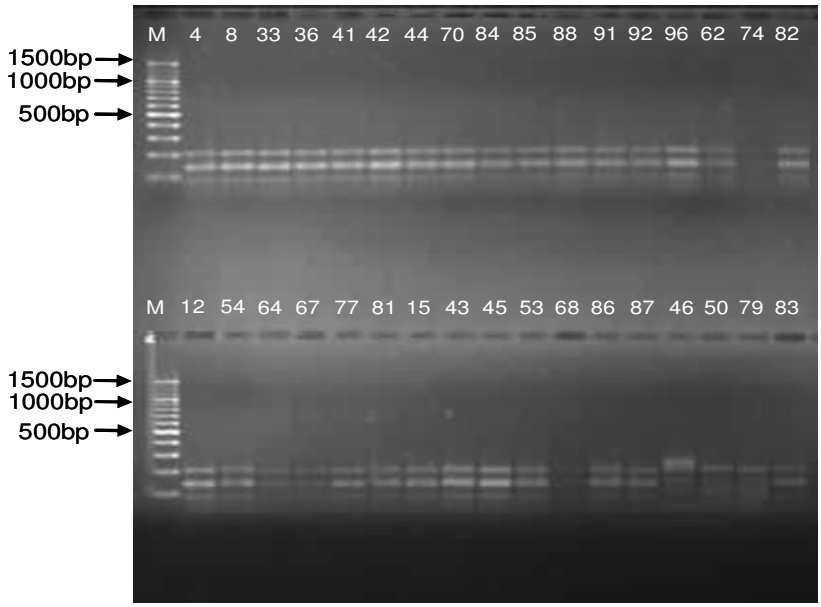

Figure 5. MseI digestion of *gap* PCR products. Lane M, 100-bp marker.

results of PCR-RFLP analysis on standard agarose gel after MseI digestion. The PCR-RFLP pattern from different CNS species was similar to each other; therefore, MseI digestion of *gap* PCR amplicons was not suitable for identification at species level. PCR-RFLP pattern of MseI digestion only allowed discrimination of two *S*. hominis isolates (50 and 79) from the rest of the samples. When RsaI enzyme was used for restriction endonuclease on *gap* gene amplicons, more complex PCR-RFLP pattern was observed on even agarose gels (data not shown).

Similar to the results obtained with MseI enzyme, RsaI enzyme had limited power to discriminate strains belonging to different species and only allowed discrimination of *S. warneri* isolates from the rest of the samples.

DISCUSSION

Several studies tested PCR-RFLP and T-RFLP of *gap* gene for identification of CNS from clinical rather thananimal cases (Layer et al., 2007; Ghebremedhin et al., 2008). In addition, *S. haemolyticus* and *S. epidermidis*, which are the most encountered CNS species in human infections, represented the major part of the isolates in those studies (Layer et al., 2007). Therefore, we aimed to apply the method on 72 CNS isolates representing nine different species identified phenotypically as *S. haemolyticus*, *S. simulans*, *S. auricularis*, *S. warneri*, *S. hominis*, *S. capitis*, *S. xylosus*, *S. epidermidis* and *S. cohnii*.

It seems that failure of amplifications in *gap* gene occurred more frequently for *S. auricularis, S. capitis*, *S. xylosus* and *S. cohnii*. In addition, comparison of PCR-RFLP profiles with phenotypic identification results showed that misidentification occurred more frequently for *S. simulans*, *S. auricularis* and *S. capitis* bovine isolates identified with VITEK 2 system. In fact, several studies were addressed reliability of VITEK 2 system for identification of CNS species mostly on human isolates instead of veterinary isolates. In one of those studies, the system has been found to offer rapid, reliable and accurate species level identification from bloodstream infections, however the necessity of improvement for identification of certain CNS species, especially *S. hominis* which was indicated (Spanu et al., 2003). More recently, Moon et al. (2007) used VITEK system to identify bovine mastitis isolates, but the verification of phenotypic identification with molecular methods was not applied. In our study, AluI digested PCR-RFLP pattern of all of the *S. simulans* isolates, except for isolate 68, were found to be similar to that found for *S. haemolyticus* isolates. Congruent with our results, single clinical *S. simulans* isolate was misidentified as *S. haemolyticus*, however, phenotypic misidentification with VITEK 2 was tested only for 4 clinical *S. simulans* isolates (Delmas et al., 2008). The results of that study also indicated misidentification of *S. xylosus*, *S. sciuri* and *S. auricularis* and requirement of complementary test for confirmation of species level identification. In agreement with the results of that study, our results clearly demonstrated

misidentification problems for *S. auricularis* isolates. *S. auricularis* isolate 62 had PCR-RFLP pattern of *S. haemolyicus* and *S. auricularis* isolate 74 had PCR-RFLP pattern of *S. warneri.* Moreover, the results of our study also showed misidentification for *S. warneri* and *S. capitis* bovine isolates. Three *S. warneri* isolates (12, 54 and 77) were also identified as *S. haemolyticus* after PCR-RFLP analysis of *gap* gene. However, percentage of correctly identified *S. haemolyticus*, *S. hominis* and *S. epidermidis* isolates were higher than the other species in this study. This was in agreement with findings of other studies (Spanu et al., 2003; Kim et al., 2008).

Intraspecies RFLP pattern was detected in *S. epidermidis*, *S. simulans* and *S. hominis* isolates (Yugueros et al., 2000). Similar to their results, we have detected intraspecies RFLP variation among *S. epidermidis* and *S. hominis* isolates (Figure 3 and 4). However, no intraspecies variation was detected for *S. warneri* and *S. haemolyticus* isolates. Moreover, bands at 185 and 210 bp region was commonly seen for most of *S. hominis* isolates, but not in the other species, therefore it might be used as species level identification. A similar species specific bands were detected at 145 and 200 bp regions for most of the *S. epidermidis* isolates (Figure 4). In addition, *S. hominis* isolates, isolate 46 had a PCR-RFLP pattern similar to the reference strain of *S. simulans* (Yugueros et al., 2000), however fragment sizes were different from the reference strain. Therefore, this isolate was considered as nonidentified *Staphylococcus* spp., but further examination of this isolate with other molecular methods is needed.

Bovine mastitis isolate 50 and 51 were phenotypically identified as *S. hominis* with confidence levels, of 56 and 91%, respectively. Although isolate 50 was identified with lower confidence level in VITEK 2 system, *gap* gene of this isolate was amplified with *gap* specific primers and PCR-RFLP pattern after AluI digestion was similar to the pattern of the reference strain of *S. hominis* (Yugueros et al., 2000). On the other hand, isolate 51 did not yield amplification product with *gap* specific primers, although it was identified with higher confidence level in VITEK 2 system. Another *S. hominis* isolate 79 was identified with a considerable high level of confidence level (97%) and its *gap* PCR-RFLP profile was similar to that obtained for isolate 50. This might suggest that confidence level during the identification is not necessarily related with accurate phenotypic identification as stated by the results of other studies (Yugueros et al., 2000; Kim et al., 2008).

In conclusion, PCR-RFLP of *gap* gene has been successfully used for molecular identification of *S. haemolyticus*, *S. warneri*, *S. hominis* and S. *epidermidis* isolates from bovine and human origin; however correctly identified isolates were 100, 33.3, 57.1, and 66.7%, respectively. Based on the results of molecular typing, phenotypic misidentification rate should be determined more precisely for bovine isolates phenotypically identified as *S. simulans*, *S. auricularis* and *S. capitis*.

Moreover, accuracy of phenotypic identification should be further investigated for *S. xylosus* and *S. cohnii* bovine isolates, which were represented, with low numbers of isolates in this study, before the cause for the failure in *gap* gene amplifications should be elicited.

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