Biyoloji Bilimleri Araştırma Dergisi 8 (1): 01-04, 2015
ISSN: 1308-3961, E-ISSN: 1308-0261

Rapid Identification of Coagulase Negative *Staphylococcus* Isolates from Mastitic Milk by RFLP Analysis of PCR-Amplified *gro*EL Gene (HSP60)

Safiye Elif KORCAN[1] Beytullah KENAR[2] Sevim Feyza ERDOĞMUŞ[3*]
[1]Afyon Kocatepe University, Faculty of Science and Literature, Biology Department, Afyonkarahisar, Turkey
[2]Afyon Kocatepe University, Faculty of Veterinary, Microbiology Department, Afyonkarahisar, Turkey
[3]Afyon Kocatepe University, Department of Laboratory and Veterinary Health, Technical Vocational School of Higher Education of Bayat, Afyonkarahisar, Turkey

*Corresponding Author:
E-mail: sfeyza@aku.edu.tr

Received: January 03, 2015
Accepted: February 12, 2015

Abstract

The aim of this study was to evaluate of reliability the technique of restriction fragment length polymorphism (RFLP-PCR) of *gro*EL gene for Coagulase Negative *Staphlococcus* (CNS) of animal origin. For this reason, 47 CNS species were identified by the API Staph test kit. Genotypic characterization, based on RFLP-PCR of the *gro*EL gene, was performed on 20 CNS isolates. The most commonly identified CNS species were *Staphylococcus epidermidis* 21(44,7%) and *Staphylococcus haemolyticus* 11 (23,4%) followed by *Staphylococcus xylosus* 7 (14,9%). Acording to PCR-RFLP profiles, 5 different species were identified: *Staphylococcus cohnii, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hyicus* and *Staphylococcus xylosus*. In conclusion, PCR-RFLP of the *gro*EL gene is a potentially precius assay for accurate molecular identification of CNS species. Most of the commercial identification sistems are not disigned to identify veterinary pathogen. This method must be developed for CNS of animal origin.

Key words: Coagulase Negative Staphlococcus (CNS), groEL gene, PCR-RFLP, bovine subclinic mastitis.

INTRODUCTION

Currently, coagulase-negative staphylococci (CNS) are the most prevalent microorganisms causing mastitis [1]. Sixteen CNS species (some common species include S. *chromogenes, S. haemolyticus, S. epidermidis, S. simulans,* and *S. sciuri*) have been previously isolated from cows with clinical and subclinical mastitis [2]. Researchers are generally in agreement that microbiological examination of milk samples is vital for implementation of effective mastitis control. CNS infection can damage udder tissue and lead to decreased milk production [1, 3, 4]. In lactation, CNS infection is associated with an increased milk somatic cell count (SCC), which can result in economic losses and decreased milk production [5]. Also according to Interpreting Milk Culture Reports, some CNS species may be more pathogenic than previously thought [6]. Thus, CNS identification at the species level is important [7]. Unfortunately, the taxonomic classification and species identification of these microorganisms remain problematic [8]. Mastitis diagnosis programs still lack a rapid and reliable identification method that can discriminate between CNS species isolated from bovine intramammary infections (IMI) [7]. Very few clinical or research laboratories routinely use classical identification methods because of time constraints and cost.

A number of rapid and simple commercial identification kits and diagnostic schemes have been developed to speciate staphylococci. But these methods have an accuracy of 70–90%, although additional tests were also required for final identification [8].

Nowadays, genotypic methods are used to define new species, such as, ribotyping, sequencing of the 16SrRNA gene and sequencing of additional housekeeping genes [9]. The *gro*EL was proven to be an ideal universal DNA target for identification to the species level because it has well conserved DNA sequences. Santos et al.[10] show that the PCR-RFLP of the *gro*EL gene is a potentially valuable tool for accurate identification of *Staphylococcus*.

The aims of this study were to compare phenotypic and genotypic identification methods and to evaluate the technique of PCR-RFLP of the *gro*EL gene for the differentiation of CNS species isolated from dairy cows with mastitis Fort this reason, CNS isolates were first phenotypically identified by means of the API Staph ID test. Furthermore, the same 20 isolates were subjected to RFLP-PCR of the staphylococcal *gro*EL gene.

MATERIALS AND METHODS

Bacterıal Strains and Biochemical İdentification

The study was carried out with a total of 47 CNS isolates. They were collected from lactating bovines on in the Middle Western Anatolia between April 2010 and June 2011. Milk samples were aseptically collected into sterile vials and transported to laboratory. Samples were cultured following National Mastitis Council recommendations [11]. Grown colonies were analyzed based on colony morphology, Gram-staining, coagulase test and susceptibility to novobiocin (5 µg) to distinguish CNS colonies and CNS species further identification was performed based on conventional biochemical techniques described by Holt et al. [12] and using the API Staph test

kit (BioMerieux, France) as described by the manufacturer. 20 isolates, the API web assigned a percent of identification (ID%) over 92%, were used for molecular identification.

Extraction of Genomic DNA

All CNS isolates were grown in Brain Heart Infusion Broth (BHI) and subcultured overnight on BHI agar plates for examination of purity and colony characteristics. A single colony was picked for extraction. DNA extractions of isolates were carried out by using GF-1 Bacterial DNA Extraction Kit (Vivantis) according to manufacturer's instructions.

PCR Amplıfication and RFLP

PCR amplification was performed using degenerate PCR primers for *gro*EL genes previously described [10, 13]. The primers for the amplification were the following: H279 5'-GAIIIIGCIGGIGA(TC)GGIACIACIAC-3' and H280 5'-(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCIGGIGC(TC)TT-3'. Each PCR mixture contained: 10 µl 10X PCR buffer, 50 mM KCl_2, 25 mM (each) deoxynucleoside triphosphate (dNTP), 2 µl of genomic (total) DNA, 0.5 µl of *Taq* DNA polymerase (Promega), and 2.7 µl of each of the degenerate HSP60 primers, in a final volume made up to 50 µl with distilled H_2O.

The PCR conditions for the amplification of *gro*EL encoding genes consisted of an initial cycle of 3 min at 95 ^oC, followed by 40 cycles of: denaturation at 94 ^oC for 1 min, annealing at 37 ^oC for 2 min, and extension at 72 ^oC for 5 min. The amplified products were analyzed on 1% agarose gels stained with ethidium bromide and photographed with UV illumination [10]. 20 µl of each PCR-amplified product was digested with 10 U of *Alu*I restriction enzyme at 37°C for 16 h. Digested PCR products were separated by electrophoresis at 80 V on 2% agarose gel. The gel was stained with ethidium bromide, and photographed under UV illuminator. A double digestion with *Hind*III (10 U) and *Pvu*II (10 U) was also performed for the identification of the isolates. The size of the fragments was determinated by comparision with the 1161 bp-DNA ladder (Vivantis). *S. aureus* ATCC 12600 was used as a positive control [14]. Our results were analyzed using the Santos et al. results [10]. for the differentiation of CNS species (Table 2).

RESULTS

As shown Table 1, 47 CNS species were isolated and identified. The most commonly identified CNS species were *S. epidermidis* 21(44,7%) and *S. haemolyticus* 11 (23,4%) followed by *S. xylosus* 7 (14,9%). Twenty isolates were identified [(ID%) over 92%] by the API Staph test kit as *S. cohnii* (n=4), *S. epidermidis* (n=4), *S. haemolyticus* (n=7), *S. xylosus* (n=4), *S. hominis* subsp. novobiosepticus (n=1) (Table 2). 20 isolates (ID% over 92%) were by the API Staph test kit applied to RFLP-PCR of the staphylococcal *gro*EL gene.

Table 1. Species of coagulase-negative staphylococci isolated from subclinical mastitis milk samples. API Staph test kit.

Bacterial spp.	Number of isolated bacteria (%)
S. cohnii,	4(8,5)
S. epidermidis,	21(44,7)
S. haemolyticus,	11(23,5)
S. xylosus	7(14,9)
S.hominis novobiosopticus	2(4,3)
S. simulans	1(2,1)
S. warneri	1(2,1)
n	47(100)

*gro*EL PCR product of 600 bp was successfully amplified with degenerate primers from all strains examined. Amplicons were digested restriction enzymes and analyzed by agorose gel electrophoresis. Each *Alu*I digested pattern consisted of one to three DNA fragments ranging in size from approximately 107 bp to 438 bp. There was no band below 105 due to the used of 2% agarose gel. Four isolates were classified as *S. xylosus.* Four isolates showed the same PCR–RFLP profile of *S. cohnii.* Six isolates identified as *S. haemolyticus* and four isolates were classified as *S. epidermidis*. Only one isolate showed the same PCR–RFLP profile of *S. hyicus* (Table 2).

Table 2. Compair of PCR-RFLP results of CNS isolates by Santos et al.(2008)

PCR-RFLP of *gro*EL gene						
Our RFLP patterns of the staphylococ				RFLP patterns of the staphylococcal control strains [10]		
n	API Staph test kit	Size of fragment		Control strains	Size of fragments	
		*Alu*I	*Hind*III and *Pvu*II		*Alu*I	*Hind*III and *Pvu*II
	-	-	-	*S. chromogenes*	75, 107, 371	180, 374
4	*S. epidermidis*	173- 189-191	-	*S. epidermidis*	173, 189, 191	-
	-	-	-	*S. capitis*	9, 11, 66, 107, 360	15, 66, 114, 360
6	*S. haemolyticus*	107-438	-	*S. haemolyticus*	9, 107, 438	-
	-	-	-	*S. sciur*	14, 93, 446	-
4	*S. xylosus*	123-258	-	*S. xylosus*	20, 75, 78, 123, 258	-
	-	-	-	*S. caprae*	182, 372	-
4	*S. cohnii*	267	-	*S. cohnii*	2, 24, 65, 90, 93, 267	-
	-	-		*S. hominis*	5, 547	-
1	*Staphylococcus hyicus*	117, 324	-	*S. hyicus*	10, 100, 117, 324	14, 539
1	*Staphylococcus sp.*	ND	ND			
1	*S. aureus (ATCC*12600*)*	204-258	-	*S. aureus*	14, 78, 204, 258	-

ND: Not Determined

DISCUSSION

Different CNS species have been isolated from mastitic bovine milk samples. Most commonly reported species are *S. chromogenes* and *S. simulans* [15]. *S. hyicus* and *S. epidermidis* have also frequently been isolated [8, 16]. In Turkey, many studies have shown the importance of clinical and subclinical bovine mastitis. Kırkan et al. [17]. reported that 20 (33.33%) CNS were identified as *S. hyicus*, 16 (26.66%), *S. chromogenes*, 9 (15.00%), *S. epidermidis*, 5 (8.33%), *S. haemolyticus*, 4 (6.66%), *S. sciuri*, 3 (5.00%), *S. lentis* and 3 (5.00%) as *S. cohnii* subsp. *cohnii*. In the study of Thorberg et al. [8], *S. epidermidis* and *S. chromogenes* were the species mainly found in cows with subclinical mastitis.

67 CNS were isolated from CMT positive milk samples in Afyonkarahisar by Kenar et al. (2012). In total, 11 CNS species: *S. epidermidis* (n=18), *S. simulans* (n=14), *S. warneri* (n=10), *S. hominis* (n=5), *S. chromogenes* (n=4), *S. caprae* (n=4), *S. xylosus* (n=3), *S. haemolyticus* (n=3), *S. hyicus* (n=3), *S. cohnii* (n=2), and *S. capitis* (n=1) were identified. The most commonly identified CNS species were determinated as *S. epidermidis* (26.8%), *S. simulans* (20.8%) and *S. warneri* (14.9%) [18]

In our sudy, 47 coagulase negative staphylococci were identified by the API Staph test kit as *S. cohnii*, 4(8,5%)*; S. epidermidis*, 21(44,7%)*; S. haemolyticus*, 11(23,5%); *S. xylosus*, 7(14,9%); *S.hominis ss. novobiosopticus*, 2(4,3%) (Table I).

Most of the commercial identification systems are not designed to identify important veterinary pathogens. These methods were primarily developed for human strains. Their insufficient performance for identifying CNS strains of animal origin is limited number of veterinary strains in databases [19]. The accuracy of conventional methods for species identification of staphylococci based on phenotypic characteristics is limited. Genotypic methods have got higher specificity and sensitivity than commercial identification systems and they may provide a better alternative for the identification of animal staphylococcal isolates [20].

PCR-RFLP approach has already been used in other studies for *Staphylococcus* identification. PCR-RFLP analysis of the *gap* gene using *Alu*I rendered distinctive patterns that allowed the identification of *Staphylococcus* spp. but *S. saprophyticus* and *S. warneri*, important staphylococcal pathogens, could not be identified [9]. *tuf* gene, which was used for the identification of the 11 most common staphylococcal species, but 4 restriction enzymes were used [21].

The *gro*EL gene, which encodes a 60-kDa polypeptide (known as *gro*EL, 60-kDa chaperonin, or HSP60 for heat shock protein 60) has the potential to serve as a general phylogenic marker because of its ubiquity and conservation in nature [22]. This gene was proven to be an ideal universal DNA target for identification to the species level because it has well-conserved DNA sequences within a given species [23]. Our attention has been focused on *gro*EL gene because several authors have described its use as a tool for the identification of *Staphylococcus* species isolated from cows with mastitis [13, 14, 24].

In this study *Alu*I was used in PCR-RFLP due to their high resolution in determining them 20 CNS were isolated from raw milk samples collected. The identification to species level of each isolate was confirmed by API Staph test kit. Four isolates were classified as *S. xylosus.* Four isolates showed the same PCR RFLP profile of *S. cohnii.* Six isolates identified as *Staphylococcus haemolyticus* and four isolates were classified as *S. epidermidis*. . Only one isolate showed the same PCR–RFLP profile of *S. hyicus* (Table 2).

We should emphasize that the PCR-RFLP with the partial *gro*EL gene is edequate for identification of most common CNS species. PCR-RFLP of the *gro*EL gene proved to be an adequate tool for the identification of the most common animal staphylococcal pathogens, independent of their phenotypic characteristics [10].

Mastitis remains the most costly disease for dairy farmers worldwide despite all efforts to control its spread. One key component of better control of this disease is identification of the causative bacterial agent during udder infections in cows. Onni et al. [25] reported that the PCR-RFLP assay of *gro*EL gene is a more reliable method than the API Staph ID test for the identification of CNS causing goats mastitis. To identify staphylococcal isolates involved in bovine mastitis. We have found similar results with those obtained by Santos et al. [10]. This study showed that genotypic identification based on the PCR-RFLP of the *gro*EL gene (used only *Alu*I restriction enzyme,) might be a useful for the suitable identification of frequently isolated CNS isolates of animal origin. This method must be developed for animal strains.

Acknowledgments

This study was supported by a project of the Afyon Kocatepe University Research Foundation, project no: 11.FENED.03.

REFERENCES

[1] Pyorala, S., Taponen, S. 2009. Coagulase-negative staphylococci emerging mastitis pathogens. Veterinary Microbiology. 134: 3–8.

[2] Capurro, A., Artursson, K., Waller, K.P., Bengtsson, B., Ericsson-Unnerstad, H., Aspán, A. 2009. Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and tuf gene sequence-based genotyping for species-level identification of coagulase-negative staphylococci isolated from cases of bovine mastitis. Veterinary Microbiology, 134:327–333.

[3] Pitkala, A., Haveri, M., Pyorala, S., Myllys, V., Honkanen-Buzalski, T. 2004. Bovine mastitis in Finland 2001—prevalence, distribution of bacteria and antimicrobial resistance. Journal of Dairy Sciences. 87: 2433–2441.

[4] Ribeiro, M.E.R., Petrini, L.A., Aita, M.F., Balbınotti, M., Stumpf, W., Gomes, J.F., Schramm, R.C., Martins, P.R., Barbosa, R.S. 2003. Relation between clinical, subclinical infectious and noninfectious mastitis in milk production units in the southern region of the Rio Grande do Sul State. Revista Brasileria Agrociência., 9: 287–290.

[5] De Vliegher, S., Barkema, H.W., Stryhn, H., Opsomer, G., De Kruif, A. 2005. Impact of early lactation somatic cell count in heifers on milk yield over the first lactation. Journal of Dairy Sciences. 88, 938–947.

[6] Interpreting Milk Culture Reports: Coagulase-negative Staphylococcus, NMC Newsletter, "Udder Topics" August, 2000. http://www.nmconline.org/articles/coagnegbtm.htm

[7] Tomazi, T., Gonçalves, J. L., Barreiro, J.R., Braga, C., P.A., Silva, L.F.P., Eberlin, M.N., dos Santosa M.V.

2014. Identification of coagulase-negative staphylococci from bovine intramammary infection by matrix-assisted laser desorption ionization–time of flight mass spectrometry, Journal of Clinical Microbiology. 52 (5): 1658 –1663.

[8] Thorberg, B.M., Brandstrom, B. 2000. Evaluation of two commercial systems and a new identification scheme based on solid substrates for identifying coagulase-negative staphylococci from bovine mastitis. Journal of Veterinary Medicine. 47: 683–691.

[9] Yugueros, J., Temprano, A., Sanchez, M., Luengo, J.M., Naharro, G. 2001. Identification of *Staphylococcus* spp. by PCR-restriction fragment length polymorphism of *gap* gene. Journal of Clinical Microbiology. 39: 3693–3695.

[10] Santos, O.C., Barros, E.M., Brito M.A., Bastos, M., Dos Santos, K.R., Giambiagi-Demarval, M. 2008. Identification of coagulase-negative staphylococci from bovine mastitis using RFLP-PCR of the *gro*EL gene. Veterinary Microbiology. 130: 134–140.

[11] National Mastitis Council-NMC: Microbiological procedures for the diagnosis of udder infection. 3rd ed. Arlington, VA. National Mastitis Council Inc.1990.

[12] Holt, J.G., Krieg, N.R., Sneath, P.H.A., Stanley, J.T., Williams, T.S. 1994. Bergeys manual of determinative bacteriology, William and Wilkıns (eds), Baltimore 9, Maryland, USA, pp. 532.

[13] Goh, S.H., Santucci, Z., Kloos, W.E., Faltyn, M., George, C.G., Drıedger, D., Hemmingsen, S.M. 1997. Identification of *Staphylococcus* species and subspecies by the chaperonin 60 gene identification method and reverse checkerboard hybridization. Journal of Clinical Microbiology. 35:3116–3121.

[14] Barros, E.M., Iorıo, N.L., Bastos, M.F.B., Dos Santos K.R.R, Gıambıagi-de Marval M. 2007. Species-level identification of clinical staphylococcal isolates based on polymerase chain reaction-restriction fragment length polymorphism analysis of a partial *gro*EL gene sequence. Diagnostic Microbiology and Infectious Disease 59:157–251.

[15] Trinidad ,P., Nickerson, S.C., Adkinson, R.W. 1990Histopathology of staphylococcal mastitis in unbred heifers. Journal of Dairy Sciences. 73: 639-47.

[16] Myllys, V. 1995. Staphylococci in heifer mastitis before and after parturition. Journal of Dairy Sciences. 62: 51–60.

[17] Kırkan, S., Göksoy, E.O., Kaya, O. 2005. Identification and antimicrobial susceptibility of *Staphylococcus aureus* and coagulase negative staphylococci from bovine mastitis in the Aydin region of Turkey. Turkish Journal of Veterinary Animal Sciences. 29: 791-796.

[18] Kenar, B., Kuyucuoğlu, Y., Şeker, E. 2012. Antibiotic susceptibility of soagulase-negative *Staphylococci* isolated from bovine subclinical mastitis in Turkey. Pakistan Veterinary Journal. 32(3):390-393.

[19] Bes, M., Guérin-Faublée, V., Meugnier, H., Etienne, J., Freney, J. 2000. Improvement of the identification of staphylococci isolated from bovine mammary infections using molecular methods. Veterinary Microbiology. 71: 287–294.

[20] Taponen, S., Simojoki, H., Haveri, M., Larsen, H.D., Pyorala, S. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. Veterinary Microbiology. 115: 199–207.

[21] Kontos, F., Petınaki, E., Spiliopoulou, I., Maniati, M., Maniatis, A.N. 2003. Evaluation of a novel method based on PCR restriction fragment length polymorphism analysis of the tuf gene for the identification of *Staphylococcus* species. Journal of Microbiological Methods. 55: 465-469.

[22] Segal, G., Ron, E.Z. 1996. Regulation and organization of the *groE* and *dnaK* operons in eubacteria. FEMS Microbiology Letters. 138: 1–10.

[23] Goh, S.H., Potter, S., WOOD, J.O., Hemmıngsen,S.M., Reynolds, R.P., Chow A.W. 1996. Hsp60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. Journal of Clinical Microbiology. 34: 818–823.

[24] Kwok, A.Y., Chow, A.W. 2005. Phylogenetic study of *Staphylococcus* and *Macrococcus* species based on partial hsp60 gene sequences. International Journal of Systematic and Evolutionary Microbiology. 53: 87-92.

[25] Onni, T., Vidili, A., Bandino, E., Marogna, G., Schianchi, S., Tola S. 2012. Identification of coagulase-negative staphylococci isolated from caprine milk samples by PCR-RFLP of *gro*EL gene. Small Ruminant Research. 104: 185– 190.