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Genotyping of *Staphylococcus aureus* Strains Isolated from Bovine Mastitis in Turkey by using ERIC-PCR Method

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ABSTRACT

Mastitis is an intramammary infection that leads to important economic losses in dairy industry such as increasing the amount of waste milk by affecting the quality and quantity of the milk, removing cows from herd or sending them for slaughter early. *Staphylococcus aureus* has an important place in the fight against mastitis. In this study, it is aimed to genotyping of the 98 *S. aureus* isolates that were isolated from bovine mastitis by the polymerase chain reaction of enterobacterial repetitive intergenic consensus (ERIC-PCR). ERIC primers generated DNA products in sizes ranging between ~8000 bp and 250 bp. According to the Nei homology, a dendrograme was obtained by the assessment of 28 bands in total. ERIC-PCR grouped 98 *S. aureus* isolates into this clusters (I–VI) showing 64 ERIC genotypes. This method, which enables the *S. aureus* strains to be described and classified successfully, could be used in forming a database by formulation of genotypes, determining the origin of the epidemic, studies of vaccine development and developing effective protection strategies against mastitis since it is easy, fast and reliable. At the same time, this study would contribute to the comprehension of *S. aureus* epidemiology and ecology in dairy herds.

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EA conceived and designed the project. EGM and EA executed the experimental work. EGM wrote the article. EA helped in preparation of manuscript.

Key words
Bovine, Enterobacterial repetitive intergenic consensus PCR, Genotyping, mastitis, *Staphylococcus aureus*.

INTRODUCTION

Mastitis is an inflammatory anomaly causing economic losses and milk safety problems in dairy industry (Arslan *et al.*, 2009a; Ibarra-Velázquez *et al.*, 2011; Qayyum *et al.*, 2016). The infection threats human health by consumption of raw milk and milk products. Mastitis, which is seen in all of the domestic animals in many countries of the world, is particularly known to be a significant problem in dairy cows (Gudding *et al.*, 1984; Han *et al.*, 2000).

Mastitis is generally caused by bacterial (*Staphylococcus aureus, Escherichia coli, Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus pyogenes, Corynebacterium pyogenes*), mycotic (*Candida, Cryptococcus, Trichosporon, Aspergillus, Penicillium*) and viral pathogens (herpes-type viruses). *Staphylococcus aureus* (*S. aureus*) is the most prevalent pathogen among the bacterial factors (isolation rate from clinical mastitis: 60-65%, isolation rate from subclinical mastitis: 80-85%) causing mastitis in ruminants. But non-infectious (trauma, hot-cold, chemical agents) mastitis can be shaped depending on traumatic cause. *S. aureus* is in the first place among the bacterial factors (Singh and Singh, 1968; Erganiş *et al.*, 1995). It was reported that phenotypic and genotyping characteristics of the factorneed to be known for an active fight (Aarestrup *et al.*, 1995). Moreover, it was stated that genotypic typing methods are more valuable than phenotypic typing methods in epidemiological studies (Olive and Bean, 1999). In recent two decades, phenotyping and genotyping methods have been improved and applied to study mastitis-causing bacteria of dairy cattle at species, subspecies and strain level. Many genotyping methods have existed to characterize bovine mastitis-causing pathogens (Tenover *et al.*, 1995; Struelens, 1996; van Belkum *et al.*, 2007; Zadoks *et al.*, 2011). Comparative typing methods based on electrophoretic banding patterns are increasingly used in veterinary diagnostic laboratories, bringing the use of molecular epidemiology for outbreak- and farm- investigations within reach of dairy veterinarians and farm advisors (Zadoks *et al.*, 2011).

Molecular methods have become an important tool in revealing origins of the infections concerning public health or hospital outbreaks. In order for the genotypic methods to be used in routine, they should be easy, cheap, fast, and reliable for application and should distinguish between microorganisms that are similar but unmatched microorganisms and provide more information than traditional methods (Tenover *et al.*, 1994).

Precise and effective use of epidemiological typing system is required in limitation and monitoring the spread

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of intra-herd and inter-herd *S. aureus* strains. DNA-based methods are used to compare *S. aureus* isolates from staphylococci infections with human and animal origin and to describe epidemiologically (Kapur *et al.*, 1995; Zadoks *et al.*, 2000; Reinoso *et al.*, 2004). Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC) method has been determined to be reliable in identification of *Staphylococcus* and *Streptococcus* strains with human and animal origin (del Vecchio *et al.*, 1995; van Belkum *et al.*, 1996; Wieser and Busse, 2000; Reinoso *et al.*, 2007). ERIC-PCR has become a powerful tool for molecular genetic analysis of bacteria and bacterial taxonomy since it allows individual fingerprints of genus, species and strains and helps to determine the phylogenetic relationships (de Brujin, 1992).

Studies about epidemiology, ecology, pathogenesis and strain variations of important *Staphylococcus* species significant udder pathogens in many countries and herds are limited. In this study, it is aimed to type advanced-level 98 *S. aureus* isolates isolated from the bovine mastitis by ERIC-PCR method.

MATERIALS AND METHODS

Bacterial strains

A total of 98 isolates from Konya Region were isolated from bovine subclinical mastitis milk samples. All isolates were identified as *S. aureus* by a standart microbiological procedure (Holt *et al.*, 1994) and confirmed by use of identification test with the VITEK 2 system (bioMerieux).

DNA isolation and PCR amplifications

Bacterial strains were incubated in Brain Heard Infusion Broth at 37 °C for 18 hours. Genomic DNA isolation was made modifying the method described by Ausubel *et al*. (1991).

DNA samples were amplified by PCR for the repetitive element sequence using the primer ERIC-PCR: ERIC1R: 5'-ATGTAAGCTCCTGGGGATTCAC-3', ERIC2: 5'-AAGTAAGTGACTGGGGTGAGC-3' (Louws *et al.*, 1994; Versalovic *et al.*, 1991).

Each 25µl of the reaction contained 50ng of genomic DNA, 10XPCR reaction buffer (50mM KCl, 10mM Tris-HCl, pH=9, %0.1 TritonX-100), 3mM $MgCl_2$, 2.5mM dNTP, 75pmol of each primer and 2.5U of *Taq* DNA polymerase (Fermentas). PCR was performed in a Mastercycler Gradient thermal cycler (Eppendorf). The initial step of 95°C for 7 min was followed by 30 cycles of 94°C for 1 min, annealing 52 °C for 1 min, 65°C for 8 min, and a final cycle at 65°C for 16 min. The amplification products were separated by gel electrophoresis in 2% agarose gels, stained with ethidium bromide, photographed on a UV transilluminator. Gel image has been transferred to the computer with DNA imaging system (Vilber Lourmat).

Data analysis

Fingerprints were scored visually as present (1) or absent (0). Genetic similarity among *S. aureus* strains was estimated based on Nei homology using Bio1D++ computer programme. Cluster analysis was performed using the UPGMA.

RESULTS

Genetic relationship within all *S. aureus* isolates was analyzed by ERIC-PCR using the ERIC1R and ERIC2 primers. ERIC-PCR produced genomic profiles consisted of 8 to 14 bands, with a size of range of~ 8000 bp and 250 bp.

According to the dendrogram obtained by the assessment of 28 bands in total (Fig.1), a differentiation was formed by analyzing the dendrograme with a similarity coefficient of 68% and considered to define six clusters namely I to VI. ERIC-PCR grouped 98 *S. aureus* isolates into these clusters I–VI showing 64 ERIC genotypes with discrimination indexes (*D*) of 0.96. While this result showed that genotypes were highly similar between *S. aureus* isolates, the discriminatory power by ERIC-PCR method was determined to be high. In the entire *S. aureus* isolates worked on, it was observed that two band patterns with sizes <6000 bp and 500 bp are common (monomorphic). In sizes 3000 bp, 1000 bp and 250 bp, common bands were observed in most isolates. It was found that the isolates in cluster VI exhibited their specific band profiles (2000 bp and 7500bp).

DISCUSSION

In identification of bacteria strains phenotypic characteristics such as biotyping, phage typing, serotyping and antibiotic resistance are used. However, more recently, molecular approaches are beneficial in genotypic distinction of bacteria strains. Restriction fragment lenght polymorphism (RFLP) (Owen, 1989), Pulsed field gel electrophoresis (PFGE) (Murray *et al.*, 1990; Gardella *et al.*, 2005), plasmid profiles (Litwin *et al.*, 1991; Arslan *et al.*, 2009a), repetitive extragenic palindromic–polymerase chain reaction (REP-PCR) (Versalovic *et al.*, 1994; Louws *et al.*, 1996; Arslan *et al.*, 2009b; Aruna *et al.*, 2009; Nordin *et al.*, 2010; Manga and Vyletělová, 2012; Njage *et al.*, 2013; Kang and Dunne, 2003) and random amplified polymorphic DNA-Polymerase chain reaction (RAPD-PCR) (Arslan *et al.*, 2005; Gardella *et al.*, 2005) can be given as examples of some molecular genotyping methods.

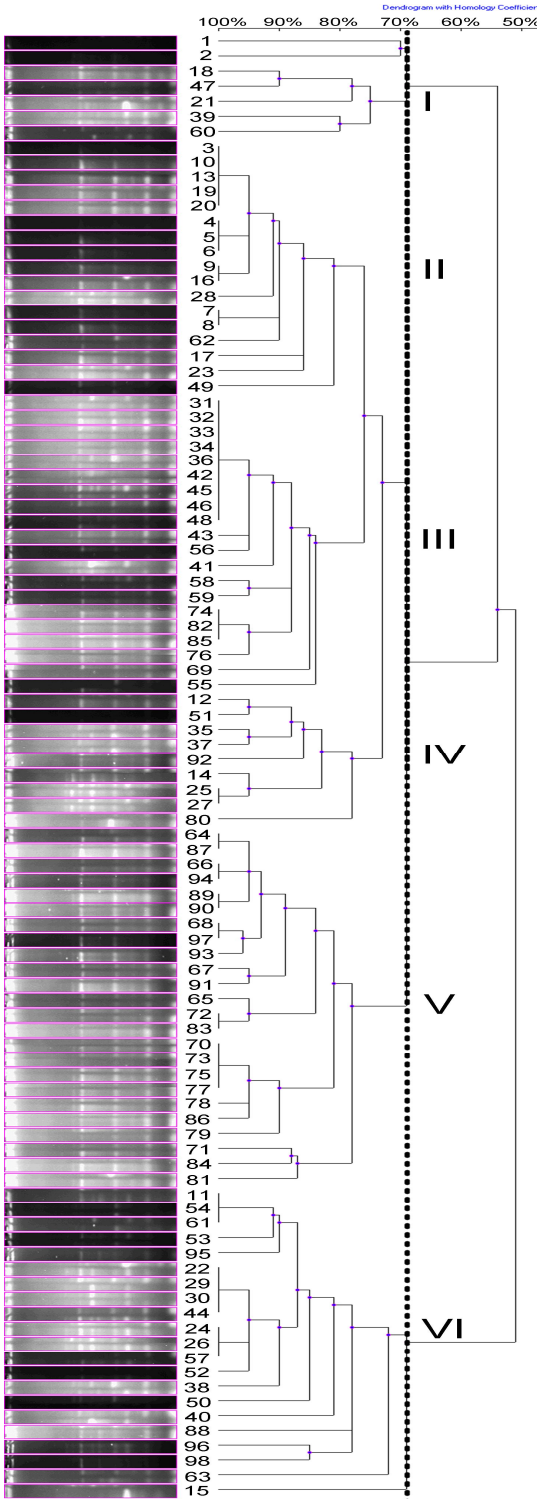

Fig. I. Genetic relationship among the *S. aureus* strains estimated by clustering analysis of DNA fingerprints generated by ERIC -PCR methods.

ERIC-PCR have been successfully used by many researchers in distinction of gram (-) (Versolavic *et al.*, 1991; Rodriguez-Barradas *et al.*, 1995; Kerouanton *et al.*, 1996; Appuhamy *et al.*, 1997; Diab and Al-Turk, 2011) and gram (+) (van Belkum *et al.*, 1993; Lipman *et al.*, 1996; Kang and Dunne, 2003) bacteria strains. Lipman *et al.* (1996), by genotyping of *S. aureus* strains from isolated bovine mammary glands, determined origin of outbreak of *S. aureus* in herd. It was emphasized that ERIC-PCR allow the clear differentiation of *Staphylococcus epidermidis* and *Staphylococcus hominis* strains which have a problem in biochemical tests by exhibiting species specific banding patterns (Wieser and Busse, 2000). By separating to 11 types with ERIC-PCR of 67 *S. aureus* strains, it was shown that ERIC-PCR technique provides a reliable tool for investigating epidemiology and tracking the spread of *S. aureus* strains in the hospital environment (Candan *et al.*, 2013). But in this study, ERIC-PCR showed better discrimination power with 64 genotypes of 98 *S. aureus* strains. The result of a previous study exhibiting 75 ERIC-types in 90 *S. aureus* was also in concordance with our data (Abdollahi *et al.*, 2014). As similar with our discrimination indexes (*D*) of 0.96, Ye *et al.* (2012) classified 35 *S. aureus* isolates into 28 ERIC types with discrimination indexes (*D*) of 0.984. It was shown that PFGE allowed better discrimination of *S. epidermidis* isolates than REP-PCR and RAPD methods (Begović *et al.*, 2013). Gardella *et al.* (2005) determined that ERIC method is as good as PFGE technique.

CONCLUSION

According to these studies, the ERIC–PCR method has been successfully applied for typing and distinguishing of *Staphylococcus* species and strains. In conclusion, it will be useful to create a fingerprint database through this rapid and reliable molecular method (ERIC-PCR) in finding the source of the epidemic causing the disease, determining the pathogen routinely and in vaccine development studies. In this way, rapid diagnosis will be possible and the treatment process will be shortened by providing useful data in developing effective protection strategies against the mastitis.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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