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Quantitative Analysis of Various Virulance Genes (*PVL, LukED, γ-hemoliz*) by Real-Time PCR of *Staphylococcus aureus* Strains Isolated from Bovine Mastitis

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ABSTRACT

In 98 *Staphylococcus aureus* (*S. aureus*) strains isolated from bovine mastitis, presence / absence of virulence genes such as Panton-Valentine leukocidin (*PVL*), *γ-hemolysis* and *LukED* and quantitative analysis were conducted by Real-Time PCR. According to Real-Time PCR *PVL*, *γ-hemolysis* and *LukED* genes of 98 *S. aureus* strains were found as 70.40%, 90.81% and 66.32%, respectively. As a result of the quantitative analysis of virulence genes according to strains, they are grouped into 3 groups as 40<-10,000, 10,000-100,000 and 100,000< IU/ml considering viral gene amount. The amount of PVL gene among these groups was determined as 27.55%, 9.18% and 32.65% respectively, and *γ-hemolysis* as 48.97%, 39.79% and 2.04% and *LukED as* 45.91%, 14.28% and 6.12%. As a result, in *S. aureus* strains isolated from bovine mastitis the prevalence of these virulence genes were higher. Therefore it is deduced that *PVL*, *γ-hemolysis* and *LukED* may be important virulence factors in the pathogenesis of *S. aureus*.

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Authors' Contributions
ER collected, isolated and identified *Staph. aureus.* SL isolated DNA. SL, EA and UA performed real-time PCR. SL wrote the article.

Key words
Real-Time PCR, PVL, *γ- hemolysis*, LukED, *Staphylococcus aureus* mastitis

INTRODUCTION

Mastitis, which leads to decrease in milk yield, milk quality deterioration (Pyorala and Syvajarvi, 1987; Philpot and Nickerson, 1991), early removal of cows from the herd (Nickerson, 1989; Han *et al.*, 2000) and significant economic loss, is a disease characterized by inflammation (Pyorala and Syvajarvi, 1987; Philpot and Nickerson, 1991). This infection also affects the human health adversely with the consumption of raw milk and dairy products (Han *et al.*, 2000, Idrees et al. 2011).

Mastitis is a disease of complex etiology, usually formed by the bacteria. Thus far, bacteria have been discovered to be a vast majority of factors that form mastitis in studies conducted both in our country and several countries in the world (Mathews *et al.*, 1992). According to many researches, most cases of mastitis at dairy enterprises are caused by *Staphylococcus* and *Streptococcus* factors (Biggadike, 2001; Rişvanlı and Kalkan, 2002). Among these bacterial factors *Staphylococcus aureus* is in the first place. *S. aureus* may lead to various infections in many animal species and in humans. These infections, abscesses and purulent cases observed in many animals are mastitis cases observed in various degrees in cows (Alaçam *et al.*, 1994; Erganiş *et al.*, 1995; Hadimli *et al.*, 2001).

Pathogenesis of *S. aureus* infections, are in relation with bacterial surface complements containing connected matrix molecules (*e.g.*, klamping factor, fibronectin binding protein) recognising various surface complements (*e.g.*, capsular polysaccharide and protein A) and extracellular proteins (*e.g.*, coagulase, hemolysis [α (Hla), β, γ (HLG), δ], enterotoxins, toxic shock syndrome toxin (TST), exfoliatins and PVL) (Tomita and Kamio, 1997; Archer, 1998).

Gamma toxin is a two-component protein that inflicts damage to delicate cell membranes. These proteins play a role together in membrane damage (Küplülü *et al.*, 1995). Another important toxin synthesized by most of *S. aureus* isolates that originate from human and animal is *PVL* (lukS-PV ve lukF-PV) leukocidin (Lina *et al.*, 1999; Rainard *et al.*, 2003; Fueyo *et al.*, 2005). This toxin, targets only human and rabbit polymorphnuclear leukocytes and macrophages, only has cytolytic effect on these cells and contributes to virulance by prevent phagocytosis. *PVL* is composed by two separate protein components F and S. These have little or no effect separately, but act synergistically (Fox *et al.*, 2000). *LukED* is as effective as *PVL* in rabbit skin samples for dermonecrosis but does not have hemolytic activity or has weak leukocytolitic activity (Kaneko and Kamio, 2004).

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Clinical diagnosis is usually not sufficient in the diagnosis of mastitis. Isolation and identification of the agent causing mastitis is required. A wide variety of methods are needed for the control of mastitis caused by *S. aureus.* Phenotypic techniques (phage typing, serotyping, biotyping, antibiogram) have low typing capability for bovine origin *Staphylococcus* (Matthews *et al.*, 1992; Tenover *et al.*, 1994). Rapid epidemiological typing is very important for identification of the source and has provided detailed information for the development of bacteria in spread of infection (Del Vecchio *et al.*, 1995).

Determination of the numerical value of viral genome is of great importance in detection of the degree of impact of the disease and the treatment. Real-Time PCR is an efficient, reliable and fast method. Very important microbial pathogens can be described with this technology and some species that are resistant to antibiotics are also determined. Amplification and analysis is carried out together in Real-Time PCR (Wittwer and Kusukawa, 2004). Since the fluorescent dye used in this method is only connected to double-stranded DNA, in parallel with an increase in the amount of DNA, the amount of fluorescence read is increased at the same time in Real-Time PCR. And this enables a wide range of applications, originality and accurate quantitative analyses (Higuchi *et al.*, 1993).

In this study, it was aimed to determine the incidence and amount of *PVL, γ-hemolysis* and *LukED* genes in *Staph.. aureus* isolated from bovine mastitis with Real-Time Light Cycler PCR

MATERIALS AND METHODS

Bacterial isolates

Bacterial strains that consist of *S. aureus* isolates were identified from milk samples of bovine mastitis between 2000-2003 at Microbiology Department, Faculty of Veterinary, Selçuk University.

DNA isolation

The bacteria that was stored in -86°C was activated in tryptic soy agar (TSA) medium and was incubated at 37°C in 18 h in brain heart infusion for DNA isolation. DNA isolation was done as described by Ausubel *et al.* (1991). The reference strains with *γ-hemolysis* positive and negative control were provided by M.C. Martin at XX Aniversario de la fundación del Instituto de Productos Lácteos de Asturias (IPLA). *LukED* and *PVL*-positive and negative control strains were provided from Microbiology culture collection, Selçuklu Faculty of Medicine, Selçuk University.

Amplification of real-time PCR

Oligonucleotide primers of virulence genes were used in sequences in the previous study (Table I). Each 15 µl mixture contained 1 µl of Light Cycler 480 DNA Master SYBR GREEN I (Roche), 5 pmol of ech primer and 100 ng of DNA template. Light Cycler program for the amplification of *PVL, γ-hemolysis* and *LukED,* include an initial denaturation step (10 min at 95ºC) followed by 35 cycles of 95ºC for 1min, 56ºC for 1min and 72ºC for 1min, with single fluorescence acquisition at the end of each extension step. Melting was performed from 60 to 95ºC at 0.1ºC/s with a smooth curve stting averaging 1 point.

Quantitation range of virulence genes was determined as 4x10-1.85x10^{14}IU/ml and above 40 IU / ml was considered as positive for all three virulence genes.

RESULTS

Quantitive analysis of PVL

While *PVL* positive control isolate (ATCC 49775) gave amplification products of 465 bp in length in Real-Time PCR, *PVL* negative control isolate did not have an amplification at ATCC 2913 (Fig. 1a). Melting point (Tm) of *PVL* positive amplification control occurred in every run at 78±2.5ºC and in 10^{th} cycle. The melting point of 75 *PVL* positive strains was amplified at 77.8±2.5ºC. 76.53% of all *S. aureus* strains have been found to be *PVL* positive. *PVL* gene amounts at the lowest standard value 40 IU/ml and above were considered to be positive. The quantitative values of *PVL* virulence gene obtained for each strain were evaluated by separating into three groups as 40<-10.000, 10.000-100.000 and 100.000< IU/ml. *PVL* gene amount of strains in the range of values of these 3 groups was calculated as 27.55%, 9.18% and 32.65%, respectively. Gene copy number of most S. *aureus* strains was in the range of 100.000< IU/ml *i.e.*, the amount of the gene was observed to be great. As virus copy number increases, the pathogenicity of bacteria will increase and thus the virulence effect of this gene will be high.

Detection and quantitive analysis of γ-hemolysis

While *γ-hemolysis* positive control isolate (NTCT 8325) gave 937 bp-long products, no amplification product at ATCC 9114 which is *γ-hemolysis* negative control isolate was determined (Fig. 1b). Melting point (Tm) of *γ-hemolysis* positive standard amplification control occurred in each run at 74.9±2.5ºC and 27^{th} cycle Melting point of *γ-hemolysis* positive 89 strains was amplified at 73.8±2.5ºC. 90.81% of all S. *aureus* strains were found to be γ-*hemolysis* positive. *γ-hemolysis* gene

Table I.- Real-time PCR primers for amplification of *S. aureus* virulence genes.

Gene	Oligonucleotide sequence (5'-3')	Product	Anneling temperature	Positive Negative control	Reference
PVL	F TTCATTTAGACGCAGCAGGA R TTGAATAGCCGTCCCTTAACG	465 bp	56ºC	ATCC49775/ATCC 2913	Zecconi *et al.* (2006)
γ-hemolysis (hlg)	F GCCAATCCGTTATTAGAAAATGC R CCATAGACGTAGCAACGGAT	937 bp	56ºC	NTCT8325/ATCC 9114	Lina *et al.* (1999)
LukED	F CAGAACTTCATTTTCGGATGTGAAGGG R CTCCAGGATTAGTTTCTTTAGAATCCG	1674 bp	65ºC	ATCC 2913/ATCC49775	Yamada *et al.* (2005)

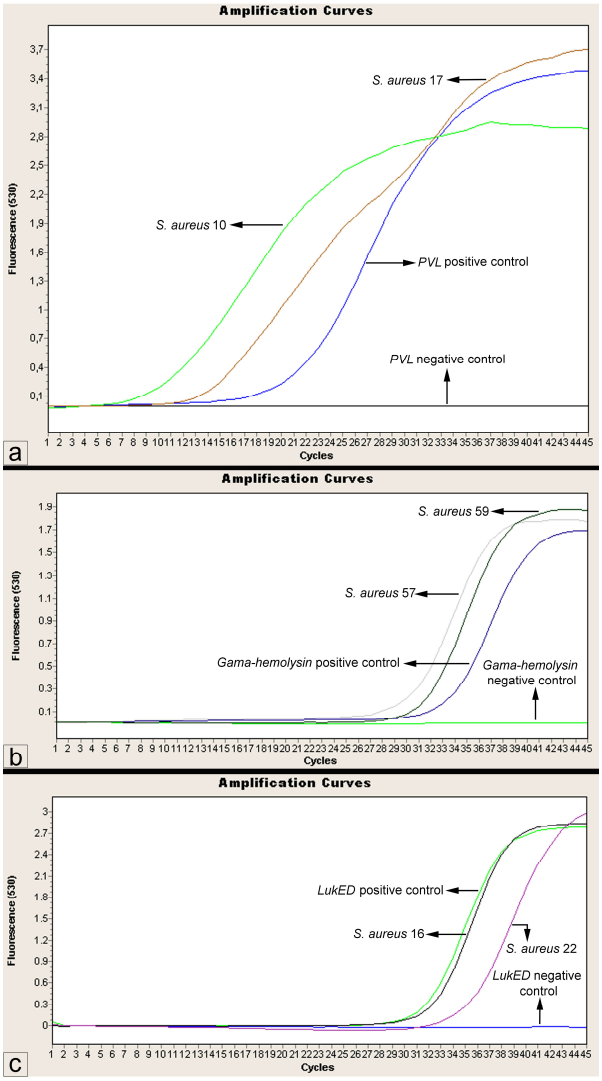

Fig. 1. Melting speaks for Real-time PCR using the LightCycler System with SYBR Green I for detection the PVL gene of *S. aureus*. Lower lines represent profiles obtained with negative controls. **a,** *PVL* gene; **b,** *γ-hemolysis* gene; **c,** *LukED* gene.

amounts above the lowest standard (40 IU/ml) were considered to be positive. Among quantitative values separated into 3 groups as in *PVL*, *y-hemolysis* gene amount of strains resulting in the range of 40<-10.000 IU/ml was calculated as 48.97%, in the range of 10.000-100.000 IU/ml as 39.79% and in the range of 2.04%. Most *S. aureus* strains were found to have gene copy number in the range of 40 <-10 000 UI / ml. Very few bacteria samples (2.04%) included many copies of *γ-hemolysis* gene. And it showed that virulence effect of the gene which has high prevalence is little.

Detection and quantitive analysis of LukED

While *LukED* positive control isolate (ATCC 2913) gave 1674 bp-long amplification product, no amplification product was determined at ATCC 49775 (Fig. 1c). Melting point (Tm) *of LukED* positive standard amplification control occurred in all circumstances at 76.5±2.5ºC and 25th cycle. Melting point of *LukED* positive 65 strains was amplified at 75.8±2.5ºC. 66.32% of all *S. aureus* strains were found to be *LukED* positive. *LukED* gene amounts above the lowest standard value 40 IU/ml were considered to be positive. When we evaluate the quantitative values of *LukED* virulance gene putting them into three groups as 40<-10.000, 10.000-100.000 and 100.000< IU/ml, *LukED* gene amount of strains among the values of the 3 groups was calculated as 45.91%, 14.28% and 6.12%, respectively. Most *S. aureus* strains were found to have gene copy number in the range of 40<-10.000 IU/ml. Very few bacteria samples (6.12%) included too many samples of *LukED* gene. It showed that virulence effect of the gene which has prevalence 66.32 % is little.

DISCUSSION

It was indicated that *S. aureus* caused very serious diseases in humans and animals and was the most common cause of contagious bovine mastitis. A connection was observed between clonal type and putative virulence factors of genetic characterization of *S. aureus* in bovine mastitis (Fitzgerald *et al.*, 2000). It has

been emphasized in recent years that the bacterium includes a variety of virulence factors and evaluating the combination of these virulence factors is important (Zecconi *et al.*, 2006).

Johnsson *et al.* (2004) observed only 0-2% *PVL* positive strains in *S. aureus* originating from human by Real-Time PCR SYBR Green method. It was reported that these results were consistent with other researches originating from human (in rates between 0-4%) (Prévost *et al.*, 1995; Narita *et al.*, 2001; Melles *et al.*, 2006) and *PVL* has a low prevalence. Rainard *et al.* (2003) observed that all strains were *PVL* negative in PVL screening with classical PCR in *S. aureus* originating from animals. However, Dufour *et al.* (2002), Melles *et al.* (2004) and Lina *et al.* (1999) argued that *PVL* has a high prevalence with their rates 21%, 38% and 37.20%, respectively. In contrast to PVL-positive rates of these researches, according to the results of that of Coppie *et al.* (1994), which is in line with our results, found that *PVL* gene was 93% *PVL* positive in necrotic infections such as bouton. In these studies, in *S. aureus* mostly isolated from hospital samples originating from human, it was aimed to search for the presence of *PVL* gene and very low prevalence was observed as expected. As in this study the *PVL* prevalence of community-onset *S. aureus* was found to be high as hoped. The results of this study in which we determined presence of the *PVL* gene that is done for the first time (or very limited) in *S. aureus* isolated from bovine mastitis were quite compatible.

Fueyo *et al.* (2005) tested *S. aureus* strains obtained from milks of subclinical bovine mastitis in terms of some genes with conventional and multiplex PCR and did not find *γ-hemolysis* gene in any isolate. In contrast, in a study of Haveri *et al.* (2007) on *S. aureus* strains with mastitis. İt was observed that the vast majority of the strains (88.8%) carried the *γ-hemolysis* gene.

Yamada *et al.* (2005) screened in their study the *γ-hemolysis* gene in *S. aureus* isolates both in birds and pigs and in ones isolated from cows of two different regions. They observed *γ-hemolysis* gene in all *S. aureus* strains isolated from birds and pigs. They determined 98.1% and 98.5% *γ-hemolysis* gene in *S. aureus* isolated from cows of two different regions. İn some studies it was reported that all *S. aureus* isolated from bovine mastitis contained *γ-hemolysis* (Fitzgerald *et al.*, 2000; Rainard *et al.*, 2003). Our results support this study which advocats that *γ-hemolysis* gene has a high prevalence of 90.81%.

As a result of PCR screening of *S. aureus* strains isolated from birds, pigs and cows from two regions, *S. aureus* isolated from birds and cows carry *LukED* gene by 91.7% and 95.95%, respectively but this gene was not found in pigs (Yamada *et al.*, 2005). Haveri *et al.* (2007) supported these studies finding *LukED* gene positive by 96.6%. Similarly, another study reported that *LukED* gene exists in all of the *S. aureus* isolates from cows (Rainard *et al.*, 2003). In this study, according to the results obtained with Real-Time PCR, the existence of *LukED* by 66.32% was similar to the results of other studies. In contrast to these studies Ikawaty *et al.* (2010) reported that *LukE* gene has a very low prevalence rate of 20% in *S. aureus* strains.

CONCLUSION

This study is important since it helps determine the characterization of virulence gene profiles and the pathogenesis of *S. aureus* strains isolated from bovine mastitis, predict clinical outcomes and identify especially pathogenic strains. That *PVL*, *γ-hemolysis* and *LukED* virulence genes have high prevalence may be due to the fact that they were isolated from animals of the same farm and therefore they are likely to have the same origin.

As a result of rapid identification of these strains by Real-Time PCR SYBR Green method, it will particularly play an important role in the cure of the disease, which is a creeping disease and is hard to cure due to resistance to many antibiotics. At the same time, we think that this study would be useful in the prevention of this disease by also developing a vaccine against the determined pathagonic bacteria of high virulance.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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