DETECTION OF *Staphylococcus aureus* USING QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION AND LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHODS IN TURKISH WHITE CHEESE

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ABSTRACT

DETECTION OF *Staphylococcus aureus* USING QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION AND LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHODS IN TURKISH WHITE CHEESE

The aim of this study was to utilize the quantitative real-time polymerase chain reaction (qPCR) method for direct quantitative detection of *S. aureus* in Turkish white cheese samples and identification of the isolated colonies using phenotypic and molecular methods. The TaqMan and LNA probe-based qPCR analyses were compared by targeting the *nuc* gene of *S. aureus* cells to improve the performance of qPCR analyses to quantify low levels of *S. aureus* cells. TaqMan probe-based assay was found to be more sensitive than LNA probe based assay in *S. aureus* pure culture and artificially contaminated white cheese sample studies. For direct bacterial DNA extraction from cheese samples, four different methods were compared. The detection range of *S. aureus* quantification was improved down to 13.2 CFU/g with the combined DNA extraction method of using trypsin and the food DNA extraction kit in artificially contaminated cheese samples. In the naturally contaminated cheese samples, the bacterial numbers calculated by the qPCR method were higher than the plate counts which can be related to detection of live and dead cells together. Loop-mediated isothermal amplification (LAMP) method was found to have some weak points in giving consistent results with lower efficiency value for detection of *S. aureus*. In the identification part of the study, 207 pure cultures were obtained. Among the 24 phenotypically and genotypically characterized isolates, 3 isolates were found to be *S. aureus* according to the sequence analyses of the 16S ribosomal DNA genomic region.

ÖZET

Staphylococcus aureus'UN TÜRK BEYAZ PEYNİRİNDE GERÇEK ZAMANLI POLİMERAZ ZİNCİR REAKSİYONU VE İLMİĞE DAYALI İZOTERMAL ÇOĞALTMA YÖNTEMLERİ İLE BELİRLENMESİ

Bu çalışmanın amacı kantitatif polimeraz zincir reaksiyonu yöntemini kullanarak *S. aureus* varlığının direkt olarak Türk beyaz peynir örneklerinde belirlenmesi ve peynirlerden izole edilen kolonilerin fenotipik ve moleküler yöntemler kullanılarak tanımlanmasıdır. Bu amaçla, TaqMan ve LNA prob temelli kantitatif PZR analizleri düşük miktarlardaki *S. aureus* varlığını belirlemek amacıyla karşılaştırılmıştır. TaqMan prob temelli kantitatif PZR yöntemi LNA prob temelli yönteme göre hem saf kültür hem de *S. aureus* bulaştırılmış peynir örnekleriyle yapılan çalışmalarda daha hassas bulunmuştur. Peynire sonradan *S.aureus* bulaştırma yapıldıktan sonra direkt bakteriyel DNA izolasyonu için 4 yöntem karşılaştırılmıştır. Tripsin ve kitin birlikte kullanıldığı yeni yöntemde 13.2 kob/g'a kadar *S. aureus* miktarı belirlenebilmiştir. Doğal bulaşmış peynir örneklerinde de kantitatif PZR metodunda klasik koloni sayım yönteminden daha fazla miktarda *S. aureus* sayısına ulaşılmıştır. Bu sonuç kantitatif PZR metodunda ölü ve canlı bakterilerin toplam miktarlarını belirlediği için elde edilmiştir. İlmiğe dayalı çoğaltma (LAMP) yöntemi ile yapılan *S. aureus* belirleme çalışmalarında etkinlik değeri düşük bulunmakla birlikte sonuçlar arasında tutarsızlık gözlemlenmiştir. Bu çalışmanın tanımlama kısmında, 207 izolat saflaştırılmıştır. Fenotipik ve genotipik olarak karakterize edilen 24 izolattan 3 tanesi 16S ribozomal DNA genomik bölgesinin dizi analizi ile *S. aureus* olarak tanımlanmıştır.

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xii

CHAPTER 1. INTRODUCTION 1

CHAPTER 2. CHARACTERIZATION OF *Staphylococcus aureus* 4

2.1. *S. aureus* Microbiology 4

2.2. *S. aureus* Epidemiology 5

2.3. The Thermostable Nuclease (*Nuc*) Gene of *S. aureus* 6

2.4. Biochemical Characterization of *S. aureus* Isolates 7

2.5. Genotypic Characterization of *S. aureus* Isolates 8

2.6. Prevalance of *S. aureus* in Turkish White Cheese 9

CHAPTER 3. DNA EXTRACTION METHODS 11

3.1. DNA Extraction Methods Used for Isolation of *S. aureus* from Food Samples 11

3.1.1. Silica Based DNA Extraction Method 12

3.1.2. Bead Based DNA Extraction Method 13

3.1.3. Chemical Treatment Based DNA Extraction Method 13

CHAPTER 4. QUANTITATIVE DETECTION OF *Staphylococcus aureus* 14

4.1. Rapid Methods 14

4.1.1. Conventional PCR 14

4.2.2. Quantitative Real-Time PCR (qPCR) 15

4.1.2.1. Data Analysis in qPCR 17

4.1.2.1.1. Absolute Quantification 17

4.1.2.2. Probe Chemistries Used in qPCR 19

4.1.2.2.1. TaqMan Probes 19

4.1.2.2.2. LNA Probes 20

CHAPTER 5. LOOP MEDIATED ISOTHERMAL AMPLIFICATION 22
5.1. Loop Mediated Isothermal Amplification (LAMP) Method 22
5.2. Comparison of qPCR and LAMP Methods 26

CHAPTER 6. MATERIALS AND METHODS 27
6.1. Materials 27
6.1.1. Cheese Samples 27
6.1.2. Reference Strains 27
6.2. Methods 28
6.2.1. Isolation of Bacteria 28
6.2.2. Storage of Isolates 28
6.2.3. Biochemical Identification of Isolates 28
6.2.3.1. Gram Staining 28
6.2.3.2. Catalase Test 29
6.2.3.3. DNase Activity Test 29
6.2.3.4. Coagulase Test 29
6.2.3.5. Anaerobic Utilization of Mannitol 30
6.2.4. Genomic DNA Isolation 30
6.2.5. Conventional PCR Amplification Conditions 30
6.2.6. Sequence Analysis 32
6.2.7. qPCR Method 33
6.2.7.1. Preparation of Quantification Standard 33
6.2.7.2. Artificially Contaminated Cheese Samples 33
6.2.7.2.1. DNA Extraction From Artificial Contamination of Cheese 34
6.2.7.3. Naturally Contaminated Cheese Samples 36
6.2.7.4. TaqMan and LNA Probes Based qPCR Assay 37
6.2.8. LAMP Method 38

CHAPTER 7. RESULTS AND DISCUSSION 39
7.1. Quantitative Real-Time PCR Method for Detection and Quantification of *S. aureus* 39
7.1.1. Sensitivity of qPCR Assay with Pure Cultures 40

7.1.1.1. Determination of The Detection Limit and Quantification Range of qPCR Method 40
7.1.1.1.1. Standard Curve Construction by Using Ten-fold Dilutions of DNA 41
7.1.1.1.2. Standard Curve Construction by Using Ten-fold Dilutions of Cell Suspensions of The Pure Culture 43
7.1.2. Specificity of qPCR Assay 45
7.1.3. Comparison of DNA Extraction Strategies for Detection of *S. aureus* in Turkish White Cheese 46
7.1.3.1. DNA Extraction by Using The Commercial Kit.............. 47
7.1.3.2. DNA Extraction by Using The Beads............................. 48
7.1.3.3. DNA Extraction by Using Trypsin 49
7.1.3.4. DNA Extraction by Combined Method of Using Trrypsin and The Commercial Kit 49
7.1.3.5. Comparison of Bacterial DNA Extraction Methods 50
7.1.4. Comparison of TaqMan and LNA Probes for Quantification of *S. aureus* Cells in Artificially Contaminated White Cheese Samples 51
7.1.5. Determination of Sensitivity of the Bacterial DNA Extraction Method Using Trypsin and the Food DNA Extraction Kit with TaqMan probes Based qPCR Method 53
7.1.6. Comparison of TaqMan and LNA probes for quantification of S. aureus cells in naturally contaminated white cheese samples 55
7.2. Detection of *S. aureus* Using LAMP Method 57
7.3. Identification of *S. aureus* Isolates 59
7.3.1. Isolation of Staphylococci 59
7.3.2. Phenotypic Characterizations 59
7.3.2.1. Gram Reaction and Morphology 59
7.3.2.2. Biochemical Tests 60
7.3.3. Genotypic Characterizations 62
7.4. Sequence analyses of *S. aureus* isolates 67

CHAPTER 8. CONCLUSION 70

REFERENCES 71

APPENDIX A. CHEMICALS USED IN THE EXPERIMENTS 85

LIST OF FIGURES

Figure		Page
Figure 2.1.	An image of *S. aureus* cells under light microscope	4
Figure 4.1.	Polymerase Chain Reaction	15
Figure 4.2.	Schematic illustration of qPCR	16
Figure 4.3.	A hypothetical amplification plot in qPCR	18
Figure 4.4.	A schematic standard-curve plot for calculation of PCR efficiency and quantitation	18
Figure 4.5.	Schematic illustration of TaqMan Probe system	20
Figure 4.6.	Schematic presentation of LNA Probe system	21
Figure 5.1.	Primer design of LAMP reaction	24
Figure 5.2.	Starting material production step of LAMP reaction	25
Figure 5.3.	Cycling amplification step of LAMP reaction	25
Figure 6.1.	Methods used for bacterial DNA extraction from cheese samples	36
Figure 7.1.	Standard curve generated with TaqMan probes based qPCR	42
Figure 7.2.	Standard curve generated with LNA probes based qPCR	42
Figure 7.3.	Amplification plot obtained using serial dilutions of DNA	44
Figure 7.4.	Standard curve generated with cell suspensions by TaqMan probes	44
Figure 7.5.	Standard curve generated with cell suspensions by LNA probes	45
Figure 7.6.	Amplification curve plot of positive and negative controls	46
Figure 7.7.	Comparison of four different methods based on qPCR results	51
Figure 7.8.	Standard curve constructed using DNA isolated from artificially inoculated cheese samples generated by TaqMan probes based qPCR assay	52
Figure 7.9.	Standard curve constructed using DNA isolated from artificially inoculated cheese samples generated by LNA probes based qPCR assay	52
Figure 7.10.	qPCR results of serial DNA suspensions obtained with the fourth isolation method	53
Figure 7.11.	Correlation of plate count and qPCR results from cheese samples treated with the fourth method	54
Figure 7.12.	Amplification plot of positive and negative controls using LAMP	58

Figure 7.13. Standard curve constructed using bacterial DNA isolated from serial cell suspensions by LAMP method 58
Figure 7.14. Gram staining image of *S. aureus* cells under light microscope 59
Figure 7.15. Amplification of the genes encoding 23S rDNA and16S-23S rDNA 65
Figure 7.16. Amplification of the genes *Coa* and *Clf* 65
Figure 7.17. Amplification of the genes encoding the *IgG*, *X* region of protein A 66
Figure 7.18. Amplification of the genes *Nuc, femA* and *Sau* 66
Figure 7.19. The distance tree showing the relative position of 24 isolates based on ribosomal gene sequence similarity 69

LIST OF TABLES

Table **Page**

Table 2.1. Typical phenotypic characteristics of *S. aureus* 7
Table 4.1. Studies in which quantification of *S. aureus* was done with qPCR............ 17
Table 6.1. Reference strains used in the study.. 27
Table 6.2. Oligonucleotide primers for amplification of staphylococcal genes 31
Table 6.3. PCR amplification conditions.. 32
Table 6.4. The primers and the TaqMan probe used in qPCR analysis........................ 37
Table 7.1. Plate counts (CFU/ml) and C_t values obtained with the first standard curve method.. 41
Table 7.2. Plate counts (CFU/ml) and C_t values obtained with the second standard curve method 43
Table 7.3. Nanodrop results of DNA extraction methods.. 50
Table 7.4. Plate counts and qPCR enumeration in naturally contaminated cheese samples... 56
Table 7.5. Biochemical test results and PCR analyses of *coa* and *nuc* genes............... 61
Table 7.6. PCR analysis results of *S. aureus* isolates 62
Table 7.7. Sequence analysis results of the isolates.. 68
Table A1. Chemicals used in experiments.. 85

CHAPTER 1

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an important pathogen causing intoxications and infections in humans and animals. The reasons of *S. aureus* infections are related with toxin production, direct invasion and tissue destruction. Food poisoning is one of the main illnesses caused by *S. aureus* in the world (Can and Çelik, 2012). The sources of this bacterium are frequently food handlers and asymptomatic carriers under unhygienic conditions (Alarcón et al., 2006).

Milk and dairy products are proper food products for contamination of *S. aureus* and its enterotoxins (Morandi et al., 2007). Cheese can be an important source of *S. aureus* contamination through raw milk, inadequate pasteurization process and improper conditions after pasteurization or production area (Miranda et al., 2009). *S. aureus* transmitted by contaminated cheese is important for microbiological safety of this food product (Cremonesi et al., 2007). In Turkey, the most widely consumed cheese type is Turkish white cheese with a production rate of 67%. Infections and intoxications related to consumption of contaminated cheese with pathogenic microorganisms have significance for product quality and public health (Temelli et al., 2006). The thermostable nuclease enzyme that is encoded by the *nuc* gene is expressed under similar conditions and shows comparable stability properties with staphylococcal enterotoxins (Brakstad et al., 1992; Meyrand et al., 1999; Tang et al., 2008). Toxins are produced and cause food poisoning when *S. aureus* counts are higher in number. Milk and dairy products are pasteurized to eliminate high contamination levels of *S. aureus* however the toxins produced by the bacterium are not inactivated in this process (Peles et al., 2007; Akineden et al., 2008). The labourious and expensive methods used for detection of enterotoxins in routine analysis of food products can be replaced by the detection of the *nuc* gene (Szabo, 2001).

For evaluating the safety of food production process, it is important to quantify the changes in microbial numbers (Schelin et al., 2011). Traditional microbiological methods have several drawbacks such as long detection time, high cost and low sensitivity (Cremonesi et al., 2007). Quantitative PCR (qPCR) is increasingly used as a

rapid, specific and sensitive method for reliable detection and accurate gene quantification (Ginginzer, 2002). qPCR is the detection and measurement of fluorescence signals generated during exponential phase in a range of cycles (Dorak, 2007). The increase in PCR products is directly proportional to the increase in the amount of template during PCR (Ginzinger, 2002). In this technology, amplification and detection proceeds in a closed-tube. The advantages of qPCR are high sensitivity, high specificity and lower risks of cross contamination (Abdunaser et al., 2009). Several studies have been carried out on detection of *S. aureus* in food samples by qPCR method (Hein et al., 2001; Alarcón et al., 2006; Poli et al., 2007; Fusco et al., 2011). Cheese is a difficult matrix for direct *S. aureus* detection and quantification by qPCR method due to its high fat content (Ercolini et al., 2004). There have been few studies on development of DNA extraction methods directly from food samples for detection of *S. aureus*. Two important chemistries that are used in qPCR systems are TaqMan and LNA probes that specifically bind to the target sequence (Gachon et al., 2004). Several studies have been performed by qPCR to detect and quantify different bacterial cells in food products (Pennacchia et al., 2009; Martínez-Blanch et al., 2009; D'Urso et al., 2009). Locked nucleic acid (LNA) is the analogue of a nucleic acid described by the Wengel and Imanishi laboratories (Letertre et al., 2003). The C3′-endo type sugars are conformationally locked by a methylene bridge between 2′ oxygen and 4′ carbon of the ribose ring (Jepsen et al., 2004). The flexibility of the ribofuranose ring is reduced by this methylene bridge causing the formation of a rigid bicyclic monomer. This also increases the local organization of the phosphate backbone and result in strong hybridization between two DNA strands (Letertre et al., 2003; Reynisson et al., 2006). The incorporation of LNA monomers in DNA increases the thermal stability by changing the conformation of the duplex and increasing melting temperature [T_m] by 1 to 8 °C against DNA (McTigue et al., 2004).

S. aureus genotyping is critical for source tracking of *S. aureus* and characterization of outbreaks (Aires de Souza et al., 2006). Although several studies have been reported on the isolation and identification of the isolated *S. aureus* strains from Turkey there have been only limited numbers of studies on the investigation from the western part of the country.

The main objective of this study is to find the most sensitive direct DNA isolation of *S. aureus* method from Turkish white cheese samples. For this purpose the

performance of TaqMan and LNA probes were compared in the qPCR assay for the detection and quantification of *S. aureus* bacterial cells by targeting species specific *nuc* gene in artificially contaminated Turkish white cheese samples. The molecular and biochemical identification of the *S. aureus* strains isolated from white cheese samples from 3 different locations in western part of Turkey were carried out. The sequence analyses were performed to identify the isolates accurately to correlate the biochemical and genotypical identification methods.

CHAPTER 2

CHARACTERIZATION OF *Staphylococcus aureus*

2.1. *S. aureus* Microbiology

The genus *Staphylococcus* with 49 species and 26 subspecies belongs to the family of *Staphylococcaceae* (Euzéby, 1997). Among them, *Staphylococcus aureus* (*S. aureus*) named by Rosenbach in 1884 is an important human and animal pathogen (ICMSF, 1996).

S. aureus is a spherical bacterium forming irregular “grape-like” clusters as shown in Figure 2.1. This non-motile and non-spore forming bacterium has an average size of 0.5 to 1.5 µm. The temperature range of *S. aureus* growth is between 7 and 48 ºC. The enterotoxin production of *S. aureus* was observed at temperature range from 10 to 46 ºC. *S. aureus* is a gram positive and catalase positive coccus and has facultative anaerobic respiratory metabolism (ICMSF, 1996). In previous studies, *S. aureus* was differentiated from other staphylococci with coagulase test. However, other staphylococcus species such as *S. hyicus* and *S. intermedius* are coagulase positive (Devriese 1980).

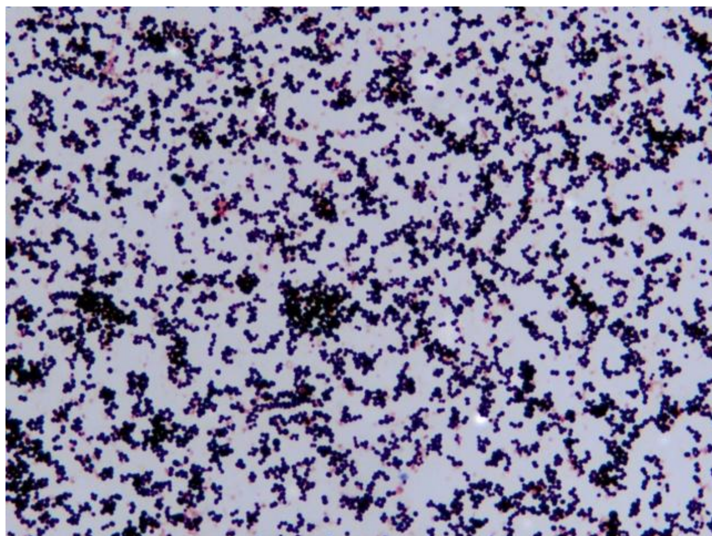

Figure 2.1. An image of *S. aureus* cells under light microscope.

2.2. *S. aureus* Epidemiology

S. aureus is the aetiologic agent of different infectious diseases such as skin and soft tissue infections, severe, life-threatening septicaemia and toxic shock syndrome in humans (Fang and Hedin, 2003). Staphylococcal bacteremia can be the cause some diseases such as endocarditis, metastatic infections or the sepsis syndrome. The onset and spread of infections depend on the relation between *S. aureus* virulence factors and host defence mechanisms (Franklin and Lowy, 1998). *S. aureus* adheres and attaches to host epithelial cells through microbial surface components recognizing adhesive matrix molecules (Liu, 2009).

The niche of the bacterium is the skin flora and nasal cavity of humans (Agius et al., 2007). 30 to 50 of healthy people are naturally colonized with *S. aureus*. The number of staphylococcal infections has increased due to some factors. One of these factors is the use of intravascular devices. Antibiotic usage is another reason of the increased cases of *S. aureus* resistant to methicillin and other antimicrobial agents (Franklin and Lowy 1998). *S. aureus* are common inhabitants of children, patients with HIV and diabetes (Liu, 2009). The report about antibiotic resistance treats in United States revealed that the estimated number of invasive methicillin resistant *S. aureus* infections was 80,461 per year in US. 11,285 deaths occurred in 2011 as reported by centers for disease control and prevention. The main cause of staphylococcal infections was healthcare-associated. Another recent study on 876 pediatric cases showed that the number of invasive community-acquired methicillin resistant *S. aureus* infections increased in five years from 2005 to 2010 (Iwamoto et al., 2013).

In addition to these infections, *S. aureus* causes food poisoning cases related to its toxin-mediated virulence, invasiveness, and antibiotic resistance (Chiang et al., 2008; Tasci et al., 2011). Staphyloccal intoxication is the name of the disease caused by ingestion of enterotoxins produced by some *S. aureus* strains. The staphylococcal food poisoning was first examined by J. Denys in 1894 and later by M.A Barber in 1914. The first food product that contaminated with *S. aureus* was the milk sample. The disease is observed with symptoms such as nausea, vomiting, abdominal cramps and diarrhea and recovered within 3 days completely. Toxin dose of 1.0 microgram and *S. aureus* cell number of 100.000 per gram can produce these symptoms (Bennett and Lancette, 1998).

S. aureus is frequently isolated from food handlers and asymptomatic carriers under unhygienic conditions (Alarcón et al., 2006). Foods that are usually cause of staphylococcal food poisoning are meat and meat products with fermented meat products, ready-to eat foods, raw milk and dairy products (Aydin et al., 2011). Infections and intoxications related to consumption of contaminated cheese with pathogenic microorganisms have significance for microbiological quality of cheese and public health (Temelli et al., 2006). The European Food Safety Authority (EFSA) reported that cheese with mixed or buffet meals were the two main ways involved in food poisoning outbreaks caused by staphylococcal toxins (Schelin et al., 2011).

2.3. The *Thermostable Nuclease* (*Nuc*) Gene of *S. aureus*

S. aureus strains produce an extracellular *thermostable nuclease* (*thermonuclease*) with a molecular mass of 17,000 Da. Thermonuclease is a calcium dependent enzyme. This enzyme is able to degrade both deoxyribonucleic acids (DNA) and ribonucleic acids (RNA) by hydrolysing their phosphodiester bonds. It contains 149 aminoacids with a highly twisted five stranded beta-barrel and three helices (Shortle and Lin 1985). This enzyme demonstrates similar stability patterns with staphylococcal enterotoxins (Brakstad et al., 1992; Meyrand 1999, Tang et al., 2008). The gene of thermostable nuclease has been sequenced and characterized. *Nuc* gene is 966 bp in size as reported by Shortle et al. (1983).

Thermonuclease encoded by *nuc* gene is an important virulence factor of *S. aureus*. In addition, the amplification of *nuc* gene was used to confirm the presence of *S. aureus* (Esan et al., 2009; Kateete et al., 2010). The PCR amplification of the *nuc* gene for detection of *S. aureus* was carried out in several studies (Hein et al., 2001; Alarcón et al., 2006).

The correlation between *nuc* gene and enterotoxin production was reported previously (Cremonesi et al., 2005). The labourious and expensive methods used for detection of enterotoxins in routine analysis of food products can be replaced by the detection of *nuc* gene that represents presence of enterotoxigenic *S. aureus* per gram (Szabo, 2001).

2.4. Biochemical Characterization of *S. aureus* Isolates

S. aureus is the third significant pathogen producing enterotoxins and causing food poisoning cases worldwide. There are also other species of Staphylococcus involved in food poisoning cases by producing enterotoxins mainly *S. hyicus* and *S. intermedius* (Gandra et al., 2005; Rohinishree and Negi, 2011). Biochemical methods are still utilized for phenotypic characterization of *S. aureus.* The production of coagulase is mostly used as a single biochemical test used for identification of *S. aureus* (Vandenesch et al., 1994; Malathi et al., 2009, Akineden et al., 2011). Coagulase enzyme that clots plasma is produced by *S. aureus* in two types. Free coagulase is the enzyme secreted out of the cell and detected by the tube coagulase test. Bound caogulase is the cell associated protein determined by the slide coagulase test. In some studies, coagulase test with thermostable nuclease tests were performed to distinguish *S. aureus* isolates (Rayman et al., 1975; Gudding 1983). Typical phenotypic characteristics of *S. aureus* are given in Table 2.1.

Table 2.1. Typical phenotypic characteristics of *S. aureus*
(Source: Bennett and Lancette, 1998)

Characteristic	***S. aureus***
Catalase activity	+
Coagulase production	+
Thermonuclease production	+
Lysostaphin sensitivity	+
Anaerobic utilization of glucose	+
Anaerobic utilization of mannitol	+

However, *S. hyicus, S. schleiferi* subspecies *coagulans* or *S. intermedius* are the other coagulase positive and *thermostable nuclease* producing species. Otherwise, coagulase-negative *S. aureus* was reported in some studies. The study on examination

of sixty-five clinical isolates of coagulase-negative, methicillin-resistant staphylococci showed that 6.2 % of the strains were classified as coagulase-negative *S. aureus* (Wilkinson et al., 1980). The negative test results of *S. aureus* isolates for the coagulase reaction demonstrated the suspicion of choosing the coagulase test as the critical test for identification of *S. aureus* (Akineden et al., 2011). These studies showed that biochemical tests are subjective and they cannot be used for identification solely.

2.5. Genotypic Characterization of *S. aureus* Isolates

Molecular methods are well distinguishing differences among closely related species as demonstrated by many researchers. The importance of molecular discrimination power for *S. aureus* is to control the invasiness of this bacterium among human, animal and food (Andre et al., 2008). Surface proteins, invasions, toxins, biochemical properties, inherent and acquired resistance to antimicrobial agents are the main virulence factors of *S. aureus* (Franklin and Lowy, 1998; Stutz et al., 2011). These virulence factors of *S. aureus* induce attachment and colonization to surface of the host cell, cause cell–cell interactions, immune evasion and tissue damage (Holden et al., 2004).

Staphylococcal enterotoxins are one of the important virulence factors involved in pathogenicity of *S. aureus* (Huong et al., 2010). Staphylococcal food poisoning is caused by ingestion of foods contaminated with *S. aureus* that include one or more enterotoxins (Vasconcelos and Cunha, 2010). Therefore, it is significant to detect and identify *S. aureus* in food samples. Biochemical tests used for detection of *S. aureus* is time consuming and laborious. Molecular methods are coming into use for replacement of classical microbiological methods (Alarcón et al., 2006). The presence *nuc* gene was used as indication of *S. aureus* contamination in several studies (Lem et al., 2001; Hein et al., 2001; Alarcón et al., 2006; Aprodu et al., 2011). The *nuc* was used together with *coa* gene for identification of enterotoxigenic *S. aureus* strains by analyzing with PCR method (Cremonesi et al., 2007). PCR amplification of *coa* gene was regarded as a gold standard when compared to tube coagulase test (Tiwari et al., 2008).

Genotypic characterization of *S. aureus* strains was performed after isolation from cattle with bovine subclinical mastitis. All isolates revealed same size of amplicon when species-specific part of the gene encoding the 23S rRNA was amplified with

PCR. *S. aureus* isolates has been shown to include the genes encoding staphylococcal coagulase, clumping factor, X region and immunoglobulin G binding region of protein A (Akineden et al., 2001). The genes encoding 23S rRNA, 16S to 23S rRNA spacer region, and 16S rRNA were used to confirm the biochemical test results for identification of *S. aureus* (Phuektes et., 2003; Gomez et al., 2007; Akineden et al., 2008). For *S. aureus* isolates, PCR amplification of 16S to 23S rRNA region was found to be more sensitive than biochemical tests (Phuektes et al., 2003)

Phylogenetic relationships among *S. aureus* isolates were determined by using different methods. Coagulase protein is one of the important virulence factors. Coagulase gene restriction fragment length polymorphism (RFLP) analysis was used to determine the genetic relations of *S. aureus* strains isolated from different bovine mastitic milk and raw milk samples, dairy products and human samples (Su et al., 2000; Morandi et al., 2009). Protein A is a surface protein and an important virulence factor of *S. aureus. Spa* gene encoding surface protein A has been used for typing of *S. aureus* (Aires de Souza et al., 2006; Agius et al., 2007).

2.6. Prevalance of *S. aureus* in Turkish White Cheese

In Turkey, the most widely consumed cheese type is Turkish white cheese with a production rate of 60 - 80 %. Turkish white cheese is salted or ripened in brine with approximately ~12-14 % NaCl solution. It has semi-hard or semi-soft texture as defined by some researchers. This cheese type is classified into 4 groups based on their % fat in dry matter (FDM). These groups are; full fat with minimum 45 % FDM, semi-fat 30-44 % FDM, low fat 20-29 % FDM and non-fat <20 % FDM (Hayaloglu et al., 2002).

Pasteurized cow's milk, sheep, goat are used for cheese making in industry. The composition and quality of white cheese change providing that it is manufactured with raw milk traditionally in small dairy plants. This type of cheese carries health risk for consumers (Çelik and Uysal, 2009).

White cheese samples produced in traditional way are highly contaminated with pathogenic microorganisms such as *S. aureus*. *S. aureus* is a significant public health concern that has to be controlled and understood (Agius et al., 2007). The maximum tolerable microbiological limit of *S. aureus* for cheese was specified as 10^3 CFU/g according to the Turkish Food Codex (Anon, 2009). Classical microbiological methods

were used to detect and quantify *S. aureus* in Turkish cheese samples. Can and Çelik (2012) examined 200 Turkish cheeses collected from Ankara for the presence of *S. aureus* that are enterotoxigenic and resistant to antibiotics. The results showed that 6 % of have *S. aureus*. 1% of the cheese samples were found to contain methicillin resistant *S. aureus*. Among the 12 *S. aureus* isolates, 3 of them were enterotoxigenic. The incidence of *S. aureus* and its enterotoxins in raw milk samples retailed in Kayseri were evaluated in another study by using biochemical tests such as coagulase and catalase tests and enzyme linked immunosorbent assay (ELISA). The results of the study showed that the amount of enterotoxins was over the maximum Turkish Food Codex limit in 37 raw milk samples (Yılmaz and Gönülalan, 2010). Therefore, the rapid, simple and sensitive detection and quantification of this bacterium is crucial for safety and the quality of traditionally produced cheese samples.

CHAPTER 3

DNA EXTRACTION METHODS

3.1. DNA Extraction Methods Used for Isolation of *S. aureus* From Food Samples

Food is a difficult matrix for DNA isolation of the target bacteria. The food components, DNA extraction method and the target bacteria should be considered together in DNA extraction procedure. The DNA extraction procedures begin with cell disruption using chemical or physical methods. The membrane lipids and cell debris are separated by using detergents and centrifuge. Proteinase K and lysostaphin are commonly used for cell lysis and degradation of proteins. Guanidine chloride or guanidine thiocyanate is the chaotropic salt added to lysis solution for protein denaturation and destabilizing. Commercial kits use silica-based technologies, where DNA specifically adsorbs to silica membranes. Washing steps are used to remove the cellular components. High fat and protein contents or the different pH range in food samples hinder the effect of DNA extraction methods (Mertens et al., 2013). Also several compounds such as detergents, lysozyme, NaOH, alcohols have found to have inhibitory effect in DNA extraction method (Rossen et al., 1992).

The detection of *S. aureus* in low level is important because the foods contaminated with this bacterium are frequently ready-to-eat food products or the foods not cooked before consumption. qPCR method is the rapid and specific method for detection and quantification. However, the efficiency of PCR method used for detection is closely related with the appropriate DNA extraction method. Conventional detection methods are time consuming because of the procedures such as enrichment, preparation of media for isolation and biochemical identification. DNA amplification based methods are more sensitive, specific and rapid when compared to these conventional methods. The main drawback of using DNA amplification based methods is inhibition of PCR reaction by the food components or other inhibitory substances extracted with DNA. The purity and amount of DNA are important parameters for successful PCR

analysis (Amagliani et al., 2010). It was emphasized that the performance of DNA extraction method changes with the type of food product, the calibration curves should be constructed according to each food sample.

In theory, the inhibitory substances have effect on every component of PCR reaction involving the template DNA, the nucleotides, the amplification primers, Mg^{2+} and the polymerase enzyme. Ca^{+2} was reported as the most inhibitory divalent ion than monovalent ions (K^{+} and Na^{+}) since the Taq polymerase enzyme was most sensitive to this molecule (Alaeddini, 2012).

3.1.1. Silica Based DNA Extraction Method

The DNA extraction method from food sample is the most critical step for successful PCR analysis. Silica membrane spin columns are used in commercial DNA extraction systems. DNA is bound to the silica membrane spin columns selectively by effect of high concentrations of chaotrophic salts such as sodium iodide, sodium perchlorate, guanidinium thiocyanate. These salts destabilize the water molecules or the hydration layers. These layers are formed by the hydrogen bounds between silanol groups on the silica surface and the water molecule of the DNA (Alaeddini, 2012). The interaction between DNA and silica particles depends on three driving forces which are the shielded intermolecular electrostatic forces, dehydration of the DNA and silica surface and intermolecular hydrogen bond formation in the DNA silica contact layer (Melzak et al., 1996). At washing steps contaminants are removed. DNA is eluted from the silica membrane by using water or low-salt buffer (Esser et al., 2006).

In a study, the commercial membrane cards were used for cell lysis and DNA storage as compared to conventional DNA isolation method. The method worked well for detection of *S. aureus* from milk samples of clinical mastitis cases (Tilsala-Timisjärvi and Alatossava, 2004).

In one of the recent studies, the amino-modified silica coated magnetic nanoparticles were used for detection of *Salmonella* Enteritidis and *Listeria monocytogenes* in artificially contaminated raw milk samples. It was demonstrated that by this method, each of these pathogens could be detected at sensitivities of 8 and 13 CFU/ml and 15 and 25 CFU/ml in single PCR and multiplex PCR assays, respectively (Bai et al., 2013).

3.1.2. Bead Based DNA Extraction Method

In the bead based DNA extraction method, the cell suspension is mixed with the beads and this sample is mixed with vortex in the laboratory. Beads used in this process can be glass, steel or ceramic (Ustok 2007). The chaotropic agent (acid guanidinium thiocyanate), detergents (SDS, N-lauroylsarcosine), chelating agent (EDTA) and a mechanical method were combined for genomic DNA isolation from cheese samples (Bonaïti et al., 2006).

The diffrence in the extraction yields in this method can be related to the large quantity of macromolecules such as casein or fat present in the curd and cheese. These molecules might adsorb detergents, chaotropic agent, chelating agents or prevent them or even beads from accessing to the cells (Bonaïti et al., 2006). The major disadvantage of this method is the problem of glass-bead contamination of DNA samples.

3.1.3. Chemical Treatment Based Extraction Method

Different chemical substances are used for DNA extraction especially in the cell lysis step. Guanidinium isothiocyanate is effective chaotropic agent used for cell lysis and protein denaturaion in DNA adsorption due to its high DNA saturation value (Alaeddini, 2012).

Chemicals can have toxic effects and damage products that are sensitive to them. Some detergents may solubilize the lipid bilayer and damage the cells. The lipoproteins of the cell membrane may be dissolved by the detergents and release the compounds in the cell. These chemicals can denature proteins. They should be eliminated before further steps of extraction (Stansbury and Whitaker, 1987).

CHAPTER4

QUANTITATIVE DETECTION OF *Staphylococcus aureus*

4.1. Rapid Methods

Polymerase chain reaction (PCR) and quantitative real-time PCR have been widely used for detection of different variety of bacteria. These methods are easy to perform, sensitive and specific methods that need less time for analysis over culture dependent methods. There are some advantages of qPCR over classical PCR. Rapidity is one of these advantages, which is reduced by shortening the time for heating the PCR mixture. High sensitivity and specificity are obtained by performing the amplification and detection together with incorporation of intercalating dyes and specific probes. The main advantage is of qPCR is quantification (Gachon et al., 2004).

4.1.1. Conventional PCR

PCR has been invented by Kary Mullis in 1983. This reaction involves the synthesis of DNA initiated by two oligonucleotide primers and DNA polymerase enzyme. The sequence specific primers define the length of amplified region. The reaction consists of denaturation, annealing and extension steps at different temperatures. Main steps of PCR were shown in Figure 4.1. Denaturation is the separation of two DNA strands by heating to high temperatures mostly 94- 95 °C. At these higher temperatures hydrogen bonds between the bases break but the covalent bonds between the deoxyribose and phosphates remain. The annealing step is the binding of primers to template strand. The temperature of annealing is based on the melting temperature of primers. The extension step includes adjusting the optimal temperature for a recombinant thermostable *Taq* DNA polymerase activity for synthesizing new double bond of DNA strand (McPherson and Møller, 2000). PCR has been shown to be more reliable than phenotypic tests in differentiation of *S. aureus* from *S. hyicus* and *S. intermedius* (Gandra et al., 2005). PCR has been also used for

molecular characterization of *S. aureus* (Aslantas et al., 2007; Akineden et al., 2008; Aydin et al., 2011) and for detection purposes (Brakstad et al., 1992; Atanassova et al., 2001; Cremonesi et al., 2005; Yang et al., 2007; Zhang et al., 2009).

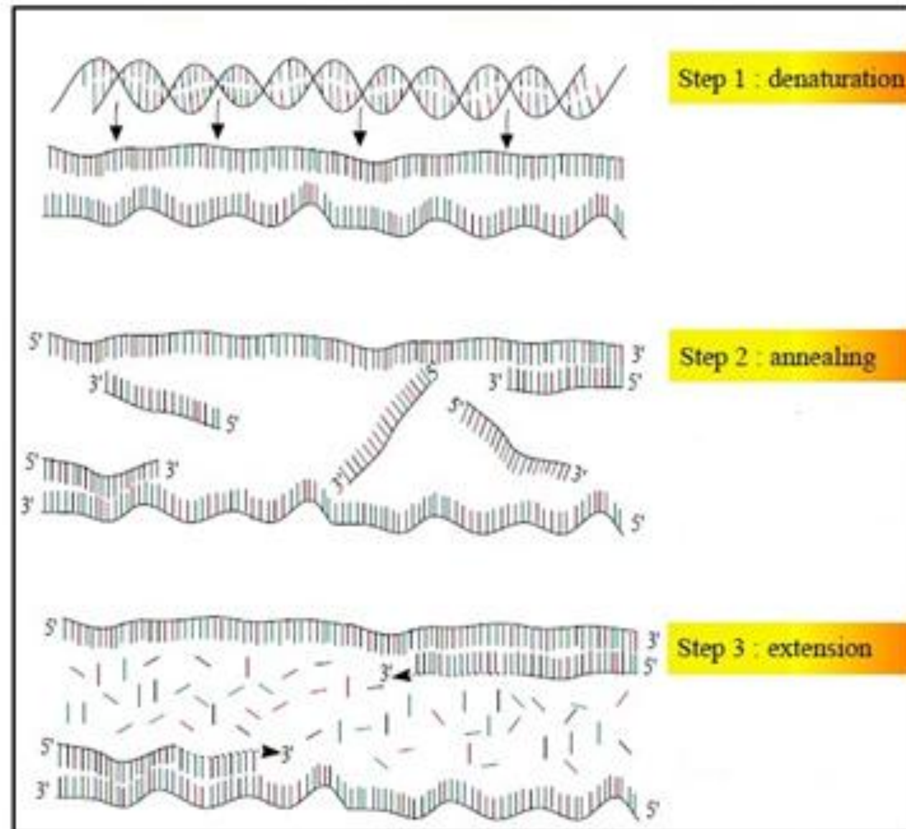

Figure 4.1. Polymerase Chain Reaction
(Source: Vierstraete, 1999)

4.1.2. Quantitative Real-time Polymerase Chain Reaction (qPCR)

Quantitative real-time PCR (qPCR) is the detection of fluorescence signals measured at each exponential phase of the reaction in a range of cycles (Dorak, 2007). The amplification is montitored in real time by using the double stranded dyes or specific probes (Kennedy and Oswalt, 2011). The increase in amplification products is directly proportional to the increase in the amount of template during qPCR (Ginginzer, 2002). Schematic illustration of qPCR method was given by Figure 4.2.

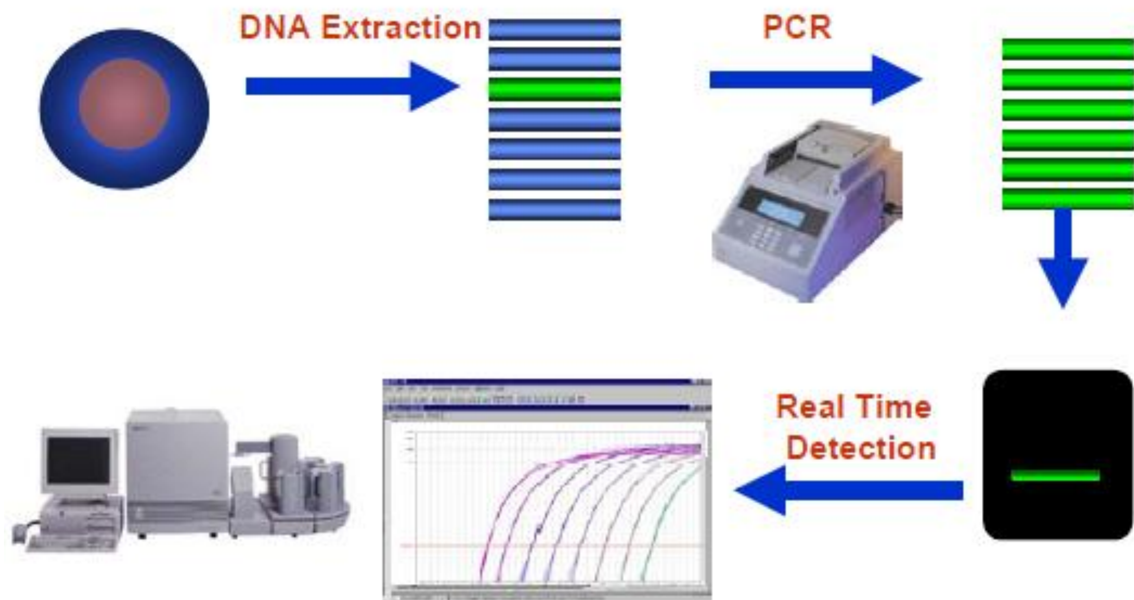

Figure 4.2. Schematic illustration of qPCR
(Source: Applied Biosystems)

qPCR method has been used in several studies for the detection of *S. aureus* in food samples (Hein et al., 2001; Alarcón et al., 2006; Poli et al., 2007; Fusco et al., 2011). Examples of qPCR studies on quantification of *S. aureus* were summarized in Table 4.1. TaqMan and SYBR-Green I based appraoaches were compared targeting to *nuc* gene of *S. aureus*. Sensitivity studies on artificially contaminated cheese samples showed that TaqMan probe gave higher sensitivity results quantifying 1.5 x 10^2 to 6.4 x 10^2 copies of the nuc gene/2 g based on the cheese type (Hein et al., 2001). TaqMan and SYBR-Green I based qPCR assays were performed to quantify *S. aureus* cells using *nuc* specific primers on artificially contaminated beef samples. The results revealed that SYBR-Green based assay was tenfold more sensitive than TaqMan probe wth 4.9 x10^2 CFU/g detection limit (Alarcón et al., 2006). qPCR method was applied targeting *nucA* gene of *S. aureus* in Monte Veronese cheese. *S. aureus* counts were found to be above the allowed quantification limit in 78% of cheese samples. The presence of enterotoxigenic *S. aureus* was investigated with multiplex PCR using toxin specific primers (Poli et al., 2007).

Table 4.1.Studies in which quantification of *S. aureus* was done with qPCR

Food Matrix	qPCR Chemistry	Limit of Quantification	Reference
Cheese	TaqMan	1.5 x 10^2 copies nuc gene/2 g	Hein et al., 2001
UHT Milk	Dua labeled probe	10^3 CFU/ml	Gillespie and Oliver, 2005
Beef	TaqMan SYBR-Green I	490 ± 202 CFU/ml 49 ± 20 CFU/ml	Alarcón et al., 2006
Cheese	SYBR-Green I	10^2 CFU/g	Poli et al., 2007
Raw milk	TaqMan SYBR-Green I	10^4 CFU/ml 10^3 CFU/ml	Fusco et al., 2011

4.1.2.1. Data Analysis in qPCR

Quantification is based on calculation of cycle threshold values within a range of low to high concentrations. Cycle threshold (C_t) is defined as the cycle at which sample fluorescence reaches a threshold above baseline fluorescence is defined as (Gibson et al., 2009). The two main methods used for quantifying the real time PCR data are known as absolute and relative quantification methods.

4.1.2.1.1. Absolute Quantification

The standard curve method sometimes referred to as absolute quantification was used in this study that includes construction of a standard curve with the threshold cycle number (C_t) versus known initial amount of DNA or copy number (Liu and Saint, 2002). The amount of unknown samples can be calculated by using the standard curve. A hypothetical amplification plot in qPCR is shown in Figure 4.3.

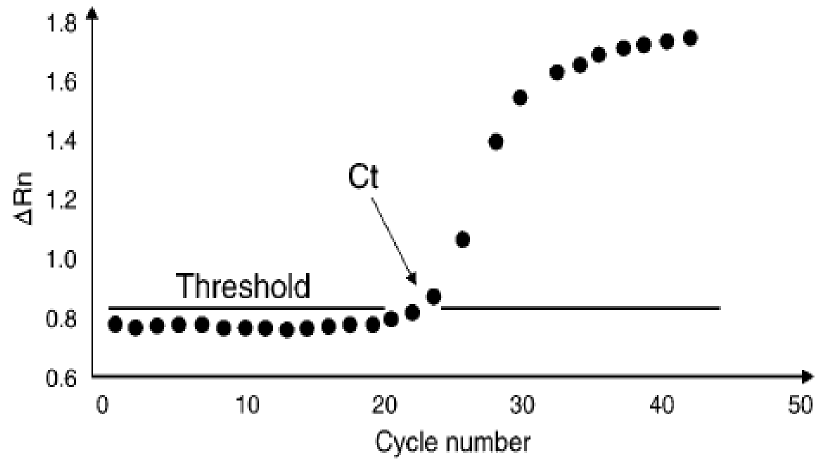

Figure 4.3. A hypothetical amplification plot in qPCR
(Source: Overbergh et al., 2003)

Efficiency is the term used for the indication of the goodness of the PCR process. It is formulated by $E = \left[10^{\left(-\frac{1}{slope}\right)}\right] - 1$ (Dorak, 2007). The slope of the line constructed for standard curve is calculated and used for determining the PCR efficiency (Figure 4.4). The slope of -3.32 indicates 100 % efficient PCR reaction (Ginginzer, 2002).

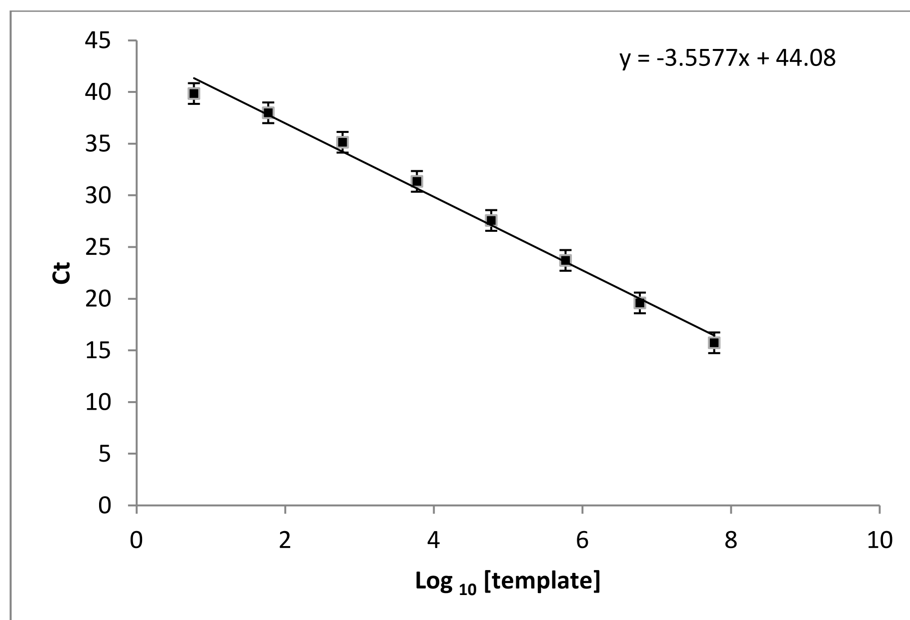

Figure 4.4. A schematic standard-curve plot for calculation of PCR efficiency and quantitation.

4.1.2.2. Probe Chemistries Used in qPCR

There are a number of techniques that are used commonly with PCR such as TaqMan probes, molecular beacons and SYBR Green I intercalating dyes (Ginginzer, 2002). SYBR Green I has high affinity for the minor groove of the double stranded DNA (Dorak, 2007). However, it has a disadvantage that the equal incorporation of this dye into every amplicon can lead to amplification of unspecific sequences preventing the accurate quantification. To overcome this problem, labeled oligonucleotides or probes that specifically bind to the target sequence can be used (Gachon et al., 2004). The fluorescently labeled oligonucleotide between the primers is known as a probe (Dorak, 2007). The two main probes among different probe based systems used recently are TaqMan and LNA probes.

4.1.2.2.1. TaqMan Probes

TaqMan probes include a fluorescent reporter at the 5′end and quencher dyes at the 3′ end. The quencher dye absorbs the fluorescence of the reporter dye due to the fluorescence resonance energy transfer (FRET) in close proximity. By the 5′ to 3′ exonuclease activity of *Taq* polymerase enzyme, the probe is hydrolyzed separating the reporter from quencher. This results in an increase of fluorescence emission which is symbolized with Rn. The software calculates delta Rn by getting the difference of Rn+ which is the fluorescence obtained from the sample at each time and Rn- values that is the fluorescence of the baseline (Giulietti et al., 2001). This fluorescence difference is monitored in real time during amplification and shown by amplification plots.

In a study performed for the identification of *S. aureus* a TaqMan and a SYBR Green qPCR were compared for the reliable identification and quantitative detection of *S.aureus* strains having enterotoxin gene cluster (egc) (Fusco et al., 2011). The results showed that TaqMan based approach resulted in a ten-fold lower sensitivity than SYBR Green I based assay.

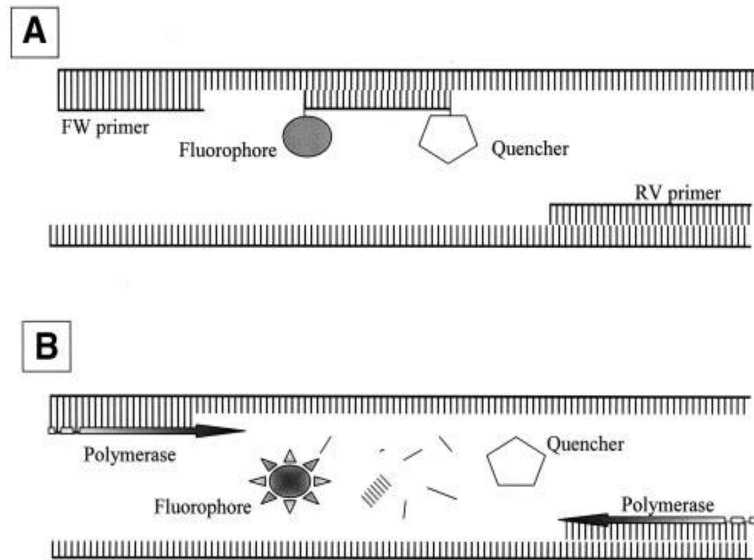

Figure 4.5. Schematic illustration of TaqMan Probe system
(Source: Giulietti et al., 2001).

4.1.2.2.2. LNA Probes

Locked nucleic acid (LNA) is the analogue of a nucleic acid described by the Wengel and Imanishi laboratories (Letertre et al., 2003). The C3´-endo (N-type) sugars are conformationally locked by a methylene bridge between 2´ oxygen and 4´ carbon of the ribose ring (Jepsen et al., 2004). Locked conformation of an LNA nucleotide was described with N-type sugar conformers.The flexibility of the ribofuranose ring is reduced by this methylene bridge causing the formation of a rigid bicyclic monomer. This also increases the local organization of the phosphate backbone and result in strong hybridization between two DNA strands (Letertre et al., 2003; Reynisson et al., 2006). Koshkin et al. (1998), named these oligonucleotides having one or more of 2´-O,4´-C-methylene-β-D-ribofuranosyl nucleosides as LNA. Schematic presentation of LNA probes was shown in Figure 4.6.

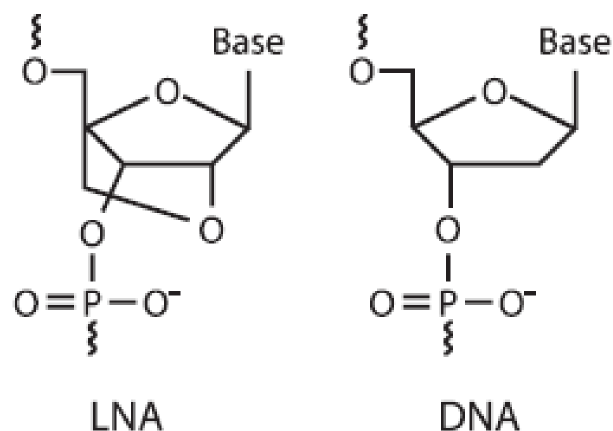

Figure 4.6. Schematic presentation of LNA Probe system
(Source: Kubota et al., 2006)

The incorporation of LNA monomers in DNA increases the thermal stability by changing the conformation of the duplex and increasing melting temperature [T_m] by 1 to 8 °C against DNA (Kubato et al., 2006). The thermodynamic studies have been performed by McTigue et al. (2004) with 100 LNA-DNA duplexes containing a single internal LNA nucleotide by examining hybridization entropy ($\Delta H°$) and enthalpy ($\Delta S°$) and T_m values. These studies demonstrated that LNA pyrimidines provide more stability than LNA purines. The importance 5' and 3' neighbors on LNA incorporation has been emphasized by higher stability of purines. In the study performed on conformation of LNA, it was demonstrated that the stability of the LNA incorporated duplexes is related to restriction of local backbone motions by the LNA nucleotides in single stranded LNA. This decreases entropy loss during duplex formation with a more efficient stacking of the nucleobases.

CHAPTER 5

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

5.1. Loop-mediated Isothermal Amplification (LAMP) Method

Although PCR is commonly used as a standard method due to its high simplicity, it has several limitations. These drawbacks are the requirement of thermal cycler, lower specificity and amplification efficiency, the risk of contamination and the need for electrophoresis step (Notomi et al., 2000; Fang et al., 2008; Mori et al., 2001). qPCR has been developed to deal with the disadvantages of PCR. On the other hand, in qPCR method skilled personnel, expensive equipment and reagents are required (Xu et al., 2011).

LAMP is a novel amplification method originally performed by Notomi et al (2000). This method is based on the amplification of DNA at isothermal condition with four specially designed primers and a DNA polymerase enzyme with strand displacement activity (Fang et al., 2008; Nagamine et al., 2001; Notomi et al., 2000). Bsm DNA polymerase, large fragment is an enzyme that performs autocycling strand displacement. This enzyme is highly similar to *Bst* DNA polymerase in functionality. Strand displacement means that the enzyme displaces the strand by generating a new polymerized strand. Besides this strand displacement activity, this enzyme has 5′→3′ DNA polymerase activity, but it does not show 5′→3′ exonuclease activity (Walker, 1994).

The main property of LAMP is to amplify nucleic acid under isothermal conditions within 60-65 °C for time interval of 15 min to 1 hour. The amplification products are large amount of different structures. The reaction is performed in a microtube which contain 1xLamp buffer, $MgSO_4$, betaine, dNTPs, DNA template, a set of four primers and *Bst* DNA polymerase (Fang et al., 2008). In this method 4 primers are designed targeted to 6 distinct regions on the DNA template which provides high specificity (Mori et al., 2001).

In a LAMP reaction, pyrophosphate ion is formed as the by-product of reaction between DNA with dNTPs by activity of DNA polymerase enzyme. This

pyrophosphate ion precipitates as magnesium pyrophosphate that is white in color and not soluble in the mixture. These precipitate products can be detected indirectly by the turbidity that increases due to a large amount of by-product in the reaction mixture (Mori et al., 2001). Since the increase in the turbidity of the reaction mixture produced by the precipitate correlates with the synthesized amount of DNA, real-time monitoring of the LAMP reaction can be achieved by real-time measurement of turbidity (Hara-Kudo et al., 2007). The amplification of DNA can also be observed by using fluorescence intercalation dye such as ethidium bromide or SYBR-Green I (Notomi et al., 2000). Real time monitoring of LAMP products was carried out by using qPCR instrument with intercalating dyes YO-PRO I and PicoGreen (Aoi et al., 2006; Tian et al., 2012). In another study, the visual detection of LAMP reaction was performed by using ethidium bromide or PicoGreen dye staining under a UV lamp with 100 more sensitivity tham PCR analysis (Tsai et al., 2009). Recently, LAMP assays have been developed for the detection of *Escherichia coli* (Hara-Kudo et al., 2007), *Salmonella* spp. (Hara-Kudo et al., 2005) and *Yersinia enterocolitica* (Gao et al., 2009).

Proper primer design is important and complicated procedure for performing LAMP amplification. Four primers are needed to perform a LAMP reaction. The primers used in LAMP are one pair of inner-primers, and outer-primers in shorter sizes (Savan et al., 2005). Nagamine et al. reported the use of additional loop primers that accelerated the reaction time of LAMP by hybridizing to the stem-loops that differ from the loops hybridized by the inner primers and start DNA with strand displacement activity (Nagamine et al., 2002).

Four primers are designed based on the 6 distinct regions of the target gene. The regions are named by F3c, F2c and F1c at the 3' side and the B1, B2 and B3 at the 5' side. Forward Inner Primer (FIP) consists of the F2 and F1c regions. F3 primer is designed to amplify F3c region. Backward Inner Primer (BIP) consists of the B2 region at the 3' end and B1c region at the 5' end. B3 Primer consists of the B3 region that is designed as a complementary to the B3c region (Notomi et al., 2000). The design of these primers was shown in Figure 5.1.

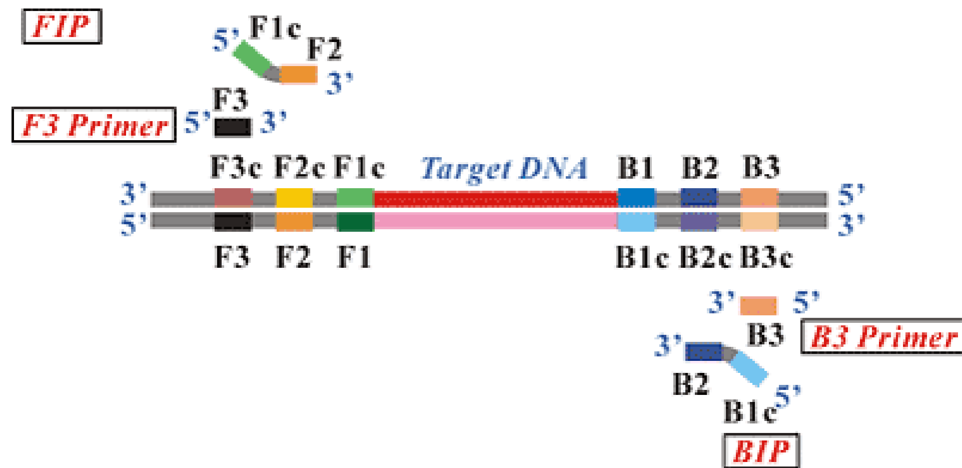

Figure 5.1. Primer design of LAMP reaction
(Source: Eiken genome site, 2007).

The amplification in the LAMP reaction is performed in two steps named as starting material production and cycling amplification. In the starting material production step, at isothermal amplification conditions around 60 °C, FIP first bounds to F2c to initiate DNA synthesis of the double stranded DNA. By strand displacement activity of the DNA polymerase enzyme, the complementary of the template DNA strand is synthesized. This strand is formed as a single stranded template DNA. The F3 primer targeted to the F3c region on the target DNA primes the synthesis of double strand by strand displacement. Because of this displacement, the single strand that includes FIP-linked complementary strand is synthesized. Then, the F1c and F1 complementary regions form a stem-loop structure at the 5' end. This single stranded DNA template is used by BIP and B3 primers. During this process, the DNA is synthesized from the BIP and through the displacement activity of the enzyme; it serves as a template for F3 primer. The BIP-linked complementary strand with a structure with stem-loops at each end is similar to a dumbbell structure. This strand forms the starting structure for the amplification cycle in the LAMP method as shown in Figure 5.2.

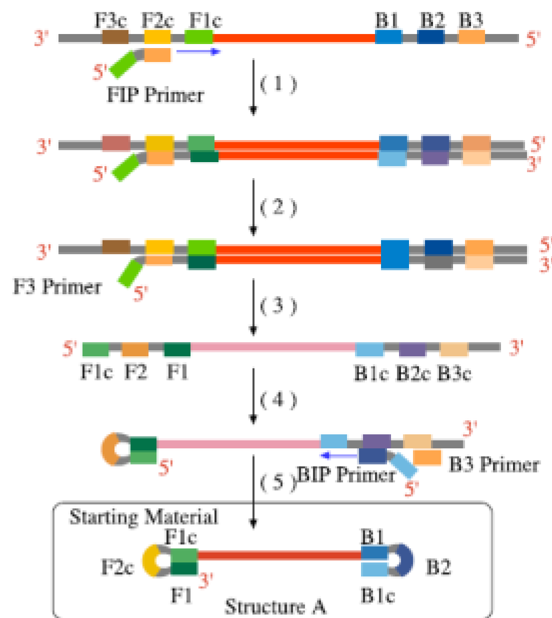

Figure 5.2. Starting material production step of LAMP reaction
(Source: Eiken genome site, 2007).

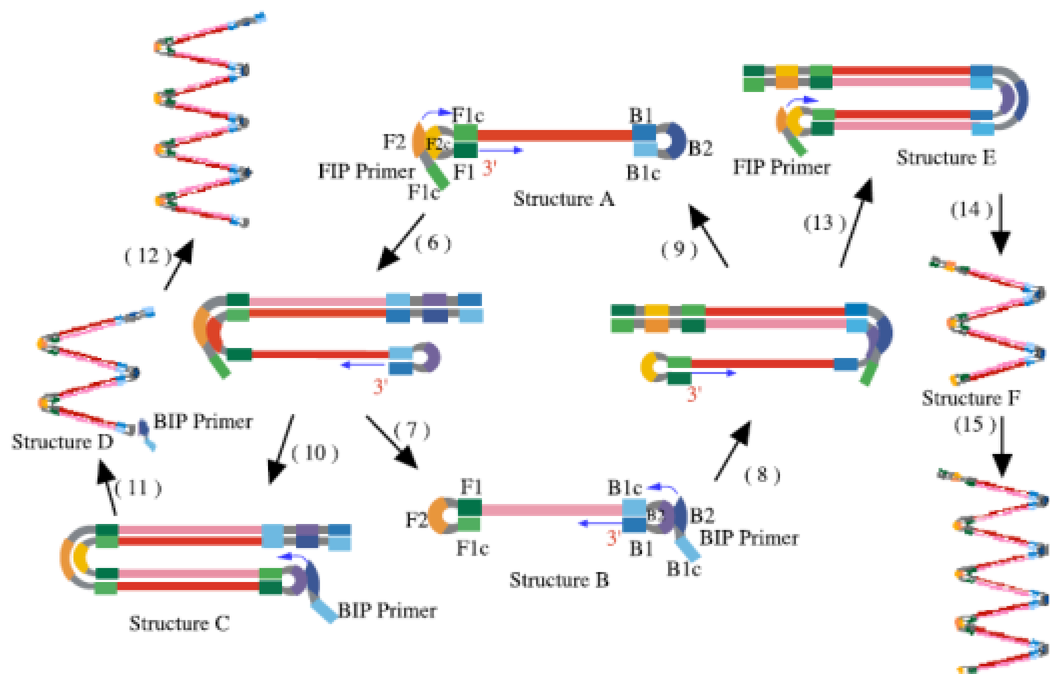

Figure 5.3. Cycling amplification step of LAMP reaction
(Source: Eiken genome site, 2007)

The cycling amplification step of LAMP reaction was illustrated in Figure 5.3. In this step, by the self-primed DNA synthesis, the dumbbell-like DNA structure is quickly turned into a stem-loop DNA. FIP hybridizes to the single stranded region in

the stem-loop DNA and starts strand displacement DNA synthesis. During this process, the single strand is released and forms a stem-loop structure at the 3' end because B1c and B1 are complementary regions. This structure starting from the 3' end of the B1 region is produced by self-primed DNA synthesis. DNA synthesis continues by displacement activity of the enzyme until the end of the reaction. Finally, different structures with various sizes are formed (Fang et al., 2008).

5.2. Comparison of qPCR and LAMP Methods

Quantitative PCR has many advantages such as quantification and specificity by the use of sequence specific probes over PCR. However, it requires an expensive instrument, skilled personnel and costly reagents.

The reaction in LAMP method is generally carried out within 60 min at isothermal conditions. LAMP method has other advantages such as simplicity, sensitivity and specificity. Because the thermal cycling is not required, this method can be carried out in a heating block or waterbath (Bista et al., 2007). The denaturation step, which enables the primers to reach the target site easily, is eliminated in LAMP method (Nagamine et al., 2001). In LAMP method, the quantification of template could be done by using a real time thermal cycler or real time turbidimeter (Mori et al., 2001; Aoi et al., 2006). The visual detection of the precipitate and visual detection after SYBR Green I, PicoGreen or ethidium bromide staining and detection with agarose gel electrophoresis can be done with this method (Mori et al., 2001).

However, in this method, four primers are designed targeted to six different regions on the template DNA so the primer design is more difficult and complex than qPCR method (Aoi et al., 2006). The lower sensitivity of LAMP method compared to PCR method was reported in a study that compared the sensitivities of these methods for the detection of Salmonella in the artificially inoculated milk samples (Deguo et al., 2008).

CHAPTER 6

MATERIALS AND METHODS

6.1. Materials

6.1.1. Cheese Samples

S. aureus strains were isolated from thirty four (n=34) white cheese samples. 34 cheese samples were purchased from İzmir, Manisa and Aydın cities located in Aegean Region of Turkey. All samples were stored at + 4 ºC until processed.

6.1.2. Reference Strains

The reference strain names and codes were given in Table 6.1. Reference strains were grown in nutrient broth at 37 ºC for 24 h. The DNA extraction procedure in Invitrogen Purelink^{TM} Genomic DNA kit was used.

Table 6.1. Reference strains used in the study

Strain Name	Strain Code
Staphylococcus aureus	RSKK 1009 ATCC 29213
Staphylococcus carnosus	NRRL-B14760
Staphylococcus epidermidis	ATCC 12228
Escherichia coli	NRRL B-3008
Escherichia coli O157:H7	ATCC 700728
Salmonella Typhimurium	CCM 5445

6.2. Methods

6.2.1. Isolation of Bacteria

For isolation of bacteria, 25 g of cheese sample was homogenized in 225 ml of sterile buffered peptone water. Serial dilutions up to 10^{-3} were prepared and 0.1 ml aliquots were streak plated on Baird-Parker agar supplemented with egg yolk tellurite. The agar plates were incubated at 37 ºC for 24–48 h to allow bacterial growth. The bacteria grown in the medium was enriched in the tryptic soy broth medium. The enriched bacteria were purified with streak plating. Purification was ended when the homogenous appearance of colonies was observed. Grey to black colonies were grown on this medium.

6.2.2. Storage of Isolates

For long term storage of isolates, 0.75 ml of active cultures grown in tryptic soy broth medium was mixed with 0.75 ml of glycerol solution. Glycerol solution was prepared with glycerol and tryptic soy broth at ratio of 40% (v/v) resulting in 20 % (v/v) glycerol. The isolate in this solution was stored at -80 °C.

6.2.3. Biochemical Identification of the Isolates

6.2.3.1. Gram Staining

For gram staining, the bacterial colonies grown on Baird Parker Agar supplemented with egg yolk overnight were used. A loop of bacteria was mixed with a few drops of water on the microscopic lam. They were dried and fixed by exposing to a flame. In the Gram staining procedure, the colonies were stained with crystal violet and Gram's iodine for 1 min.

More stain was removed by washing after each staining step. The colonies were fixed with 95% alcohol for 15 s and stained with safranine for 30 s. Excess stain was washed and dried gently with cotton towels.

When the colonies were observed under microscope, Gram positive bacteria seem blue-purple while Gram negative bacteria seem pink-red after Gram staining.

6.2.3.2. Catalase Test

Catalase test was used to differentiate catalase positive *S. aureus.* This enzyme breaks down hydrogen peroxide into oxygen and water molecules ($2H_2O_2 \rightarrow 2H_2O + O_2$). Oxygen production is observed by the formation of O_2 bubbles. For this test, 3 % H_2O_2 solution is dropped onto colonies grown on agar medium overnight.

6.2.3.3. DNase Activity

DNase Test Agar was performed for the determination of DNase activity and was prepared according to the manufacturer's description. The cultures were inoculated to the test agar and grown for a day at 37ºC. 37 % HCl solution was used onto the colonies for 5 minutes and removed. DNase activity of the colonies was determined by observing the clear zone around the colonies.

6.2.3.4. Coagulase Test

Coagulase test was performed in two ways to screen free and coagulase production of the isolates. For bound coagulase test of the bacteria, REMEL *Staphaurex* test kit was used following the manufacturer's description. Briefly an overnight culture grown on agar plates and a few saline drops were put onto test cards. The agglutination formed on the test card shows that the strain is *S. aureus*. For the determination of free coagulase production of the isolates, coagulase plasma (0.5 ml) in the clean test tube was mixed with the tested isolate. The test tube was incubated at 37 ºC and observed every 30 minutes for clotting by gently shaking the tube.

6.2.3.5. Anaerobic Utilization of Mannitol

The tested colony was streak plated onto mannitol salt agar medium and incubated at 37 ºC for 3 days. The fermentation pattern of the isolate was determined by observing the yellow color formation from phenol red of the agar medium by the effect of acidic byproduct. *S. aureus* can ferment mannitol.

6.2.4. Genomic DNA Isolation

Genomic DNA was isolated as described by Sudagidan, et al. (2008). The bacterial cultures stocked in glycerol (40% v/v) at -80 °C was inoculated into the 6 ml fresh tryptic soy broth in the tubes and allowed for growth at 37 ºC overnight. The tubes were vortexed. 200 µl of the cultures were taken into eppendorf tubes. They were centrifuged at 10000 rpm for five minutes and the supernatant was removed. The pellets were resuspended in 45µl dH_2O and 15 µl lysostaphin (100 µg/ ml). They were incubated at 37ºC for an hour in the water bath. After this step 15 µl proteinase K (100 µg/ ml) and 150 µl Tris HCl (pH=8.0) were added and incubated at 37ºC for an hour in the water bath. The samples were then boiled at 100ºC for five minutes. Genomic DNAs were stored at -20ºC. The concentration of isolated DNA was determined by using NanoDrop instrument.

6.2.5. Conventional PCR Amplification Conditions

PCR amplifications were conducted in a solution containing 10x PCR buffer (10mM Tris–HCl, pH 8.8; 1.5mM $MgCl_2$,50 mM KCl,0.1% Triton X-100), 2 mM of each dNTP,10 µM of each primer and 1.2U of Taq DNA polymerase and 2.5 µl of DNA template, in a final volume of 25 µl. Negative control of amplification was performed with 2.5 µl of water instead of DNA template. Oligonucleotide primers for amplification of staphylococcal genes were given in Table 6.2. Programs for the amplification of genes performed as described by Akineden et al. (2008) were shown in Table 6.3. Reactions were carried out in a BIO-RAD, C-1000 thermal cycler. PCR products (10 µL) were electrophoresed in 2.0 % agarose gel in TAE buffer (40mM Tris–acetate, pH 8.0; 1mM Na_2EDTA).

Table 6.2. Oligonucleotide primers for amplification of staphylococcal genes.

Target genes	**Oligonucleotide sequence (5´-3´)**	**Reference**
23S rDNA	F-ACGGAGTTACAAAGGACGAC R- AGCTCAGCCTTAACGAGTAC	Straub et al., 1999
16 S rDNA	F- AGAGTTTGATCCTGGCTCAG R- CCCACTGCTGCCTCCCGTAG	Riyaz Ul-Hassan et al., 2008
16S–23S rDNA	F-TCTTCAGAAGATGCGGAATA R-TAAGTCAAACGTTAACATACG	Forsman et al., 1997
ClfA	F-GGCTTCAGTGCTTGTAGG R-TTTTCAGGGTCAATATAAGC	Stephan et al., 2000
coa	F-ATAGAGATGCTGGTACAGG R-GCTTCCGATTGTTCGATGC	Hookey et al., 1998
nuc	F-GCGATTGATGGTGATACGGT R-AGCCAAGCCTTGACGAACTAAAGC	Brakstad et al., 1992
Spa X	F-CAAGCACCAAAAGAGGAA R-CACCAGGTTTAACGACAT	Frenay et al., 1996
Spa Igg	F-CACCTGCTGCAAATGCTGCG R-GGCTTGTTGTTGTCTTCCTC	Seki et al., 1998
femA	F-AAAAAAGCACATAACAAGCG R-GATAAAGAAGAAACCAGCAG	Mehrotra et al., 2000
Sau	F-GACGGCTTTGATGGCTAGTGG R-AGTTAATTCACGCCCTAGTG	Holochová et al., 2010

Table 6.3. PCR amplification conditions

Target gene	Amplification Program
23S rDNA	Pre-denaturation 5 min at 94 ºC, 37 cycles of denaturation at 94 ºC for 40 s, annealing at 64 ºC for 60 s, extension at 72 ºC for 75 s and a final extension of 3.5 min at 72 ºC.
16 S- 23 S rDNA	Pre-denaturation 5 min at 94 ºC, 30 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s, extension at 72 ºC for 30 s and a final extension of 3.5 min at 72 ºC.
Clf A	Pre-denaturation 5 min at 94 ºC, 35 cycles of denaturation at 94 ºC for 60 s, annealing at 57 ºC for 60 s, extension at 72 ºC for 60 s and a final extension of 3.5 min at 72 ºC.
Nuc	Pre-denaturation 5 min at 94 ºC, 37 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 30 s, extension at 72 ºC for 30 sec and a final extension of 3.5 min at 72 ºC.
Coa, *Spa Igg*	Pre-denaturation 5 min at 94 ºC, 30 cycles of denaturation at 94 ºC for 60 s, annealing at 58 ºC for 60 s, extension at 72 ºC for 60 s and a final extension of 3.5 min at 72 ºC.
Spa X	Pre-denaturation 5 min at 94 ºC, 30 cycles of denaturation at 94 ºC for 60 s, annealing at 60 ºC for 60 s, extension at 72 ºC for 60 s and a final extension of 3.5 min at 72 ºC.
femA	Pre-denaturation 5 min at 94 ºC, 35 cycles of denaturation at 94 ºC for 2 min, annealing at 57 ºC for 2 min, extension at 72 ºC for 60 s and a final extension of 7 min at 72 ºC.
Sau	Pre-denaturation 3 min at 94 ºC, 30 cycles of denaturation at 94 ºC for 45 s, annealing at 56 ºC for 60 s, extension at 72 ºC for 90 s and a final extension of 7 min at 72 ºC.

6.2.6. Sequence Analysis

The bacterial strains were identified by using the primers amplifying 350 bp fragment of 16S ribosomal DNA gene. The primers were:

Forward primer: 5´ AGAGTTTGATCCTGGCTCAG-3´

Reverse primer: 5´-CCCACTGCTGCCTCCCGTAG-3´

The PCR mixture was the same with the previously given in Section 6.2.5. The PCR conditions:

Step 1: 94 °C for 3 min
Step 2: 94 °C for 15 s (Denaturation)
Step 3: 50 °C for 15 s (Annealing) } 30 cycles
Step 4: 72 °C for 30 s (Extension)
Step 5: 72 °C for 5 min.

The amplified products were purified and sequenced with Genetic Analyzer 3130 XL (Applied Biosytems). One forward primer was used for sequencing. The sequences obtained were compared with the sequences in the NCBI database with BLAST Analysis. The sequences were aligned with ClustalW program adapted to Mega 5.2 program. Phylogenetic distance tree was constructed with Maximum Likelihood method with phylogency test of Bootstrap method with 1000 replications to investigate the similarity between different isolates.

6.2.7. qPCR Method

6.2.7.1. Preparation of the Quantification Standard

The standard strain was grown in 6 mL of tryptic soy broth (Merck Darmstadt, Germany) incubated at 37 °C for 18 h. The turbidity of bacterial suspension in tryptic soy broth was adjusted to 0.5 McFarland. Tenfold serial dilutions were prepared from this suspension. Colonies were enumerated by plating onto Baird Parker Agar supplemented with egg yolk tellurite emulsion. DNA extraction from the reference strain was performed according to the method described previously (Sudagidan et al., 2008).

6.2.7.2. Artifically Contaminated Cheese Samples

White cheese sample was purchased from a supermarket in İzmir. Tenfold serial dilutions of pure culture as described for constructing the quantification standard was

prepared. The cheese samples were artificially seeded using these dilutions. DNA extraction methods were performed directly by using these cheese samples.

6.2.7.2.1. DNA Isolation from Artificially Contaminated Cheese

DNA was extracted directly from artificially contaminated cheese with four different methods. The schematic figure of these methods was given in Figure 6.1.

6.2.7.2.1.1. DNA Extraction Using Food DNA Extraction Kit (Method 1)

A food DNA extraction kit based on silica-gel membrane technology for rapid and efficient purification of DNA without organic extraction and ethanol precipitation (Intron Biotechnology, Inc., Korea) was applied according to manufacturers' instructions. The steps in this procedure included lysis with lysis buffer, proteinase K and RNase A, precipitation, binding and washing steps with instant buffer solutions. After selective binding of DNA to the column, the final eluate was recovered with elution buffer.

6.2.7.2.1.2. DNA Extraction Using Beads (Method 2)

DNA extraction from artificially contaminated cheese samples was carried out using the procedure assessed by Bonaïti et al. (2006) with slight modifications. Briefly, 2 g of the cheese samples were suspended in 4 ml of 4 mol l^{-1} guanidine thiocyanate–0.1 mol l^{-1} Tris–HCl (pH 7.5) and 250 µl of 10% N-lauroylsarcosine. For the DNA isolation, the 2 ml eppendorf tubes were filled with 200 mg of glass beads (0.25-0.50 mm diameter) and with the alkaline solution which consist of 50 µl mixture of 20% sodium dodecyl sulfate (SDS) solution, 300 µl of 0.1 mol l^{-1} phosphate buffer pH 8.0, 300 µl of 50 mmol l^{-1} sodium acetate and 10 mmol l^{-1} EDTA (pH 5.5). Then 400 µl phenol–chloroform–isoamyl alcohol (25:24:1) at pH 8.0 was added. The tube was vortexed for 30 s. After cooling samples on ice for 1 min, vortexing was repeated. After centrifugation at 20,800 *g* for 30 min, the upper aqueous phase was decanted. Afterwards, two washing steps were performed. In the first with phenol–chloroform–

isoamyl alcohol (25:24:1; pH 8.0) and with chloroform–isoamyl alcohol (24:1) respectively. The bacterial DNA was precipitated by addition of 2 volumes of cold absolute ethanol to the aqueous phase. Following incubation at -20 °C for 2 h, the DNA was centrifuged at 20,800 *g* for 15 min. The pellet was washed twice with 80% ethanol, dried and resuspended in 100 µl of Tris–EDTA, 5 µl of the DNA solution was used for qPCR analysis.

6.2.7.2.1.3. DNA Extraction Using Trypsin (Method 3)

The procedure of Hein et al. (2001) with slight modifications was applied. In summary, 2 g of cheese samples were mixed with 45 ml of digestion buffer containing 1 mg trypsin per ml and subsequently homogenized in a stomacher for 1 min, and incubated at 40 °C for 3 h. After centrifugation at 5,700 *g* for 15 min at 4 °C, the fat layer and aqueous phases were discarded and the pellet was washed three times with TE buffer (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA at pH 7.5) and once with the PCR buffer (50 mmol l^{-1} KCl, 1.5 mmol l^{-1} M $MgCl_2$, 10 mmol l^{-1} M Tris-HCl at pH 8.4). Finally, the pellet was resolved in 250 µl of PCR buffer. Lysostaphin and proteinase K solutions were added to this solution, incubated for 1 h and 15 min at 60 and 95 °C, respectively. After centrifugation at 13,000 *g* for 5 min, 5 µl of supernatant was subjected to qPCR analysis.

6.2.7.2.1.4. DNA Extraction Using Combined Method of Trypsin and The Food DNA Extraction Kit (Method 4)

In this method, the food DNA extraction kit procedure was combined with the modified method of Hein et al. (2001). In this procedure, the process steps described in Method 2 was followed until the pellet resolved in 250 µl of PCR buffer and continued with the procedure described in Method 1. In summary, two grams of cheese samples were mixed with 45 ml of digestion buffer containing 1mg trypsin per ml and subsequently homogenized in a stomacher for 1 min, and incubated at 40 °C for 3 h. After centrifugation at 5,700 x *g* for 15 min at 4 °C, the fat layer and aqueous phases were discarded and the pellet was washed three times with TE buffer (10 mM Tris-HCl,

1 mM EDTA at pH 7.5) and once with the PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl at pH 8.4). Finally, the pellet was resolved in 250 µl of PCR buffer and 100 µl of this solution was subjected to the food DNA extraction kit as described in Method 1.

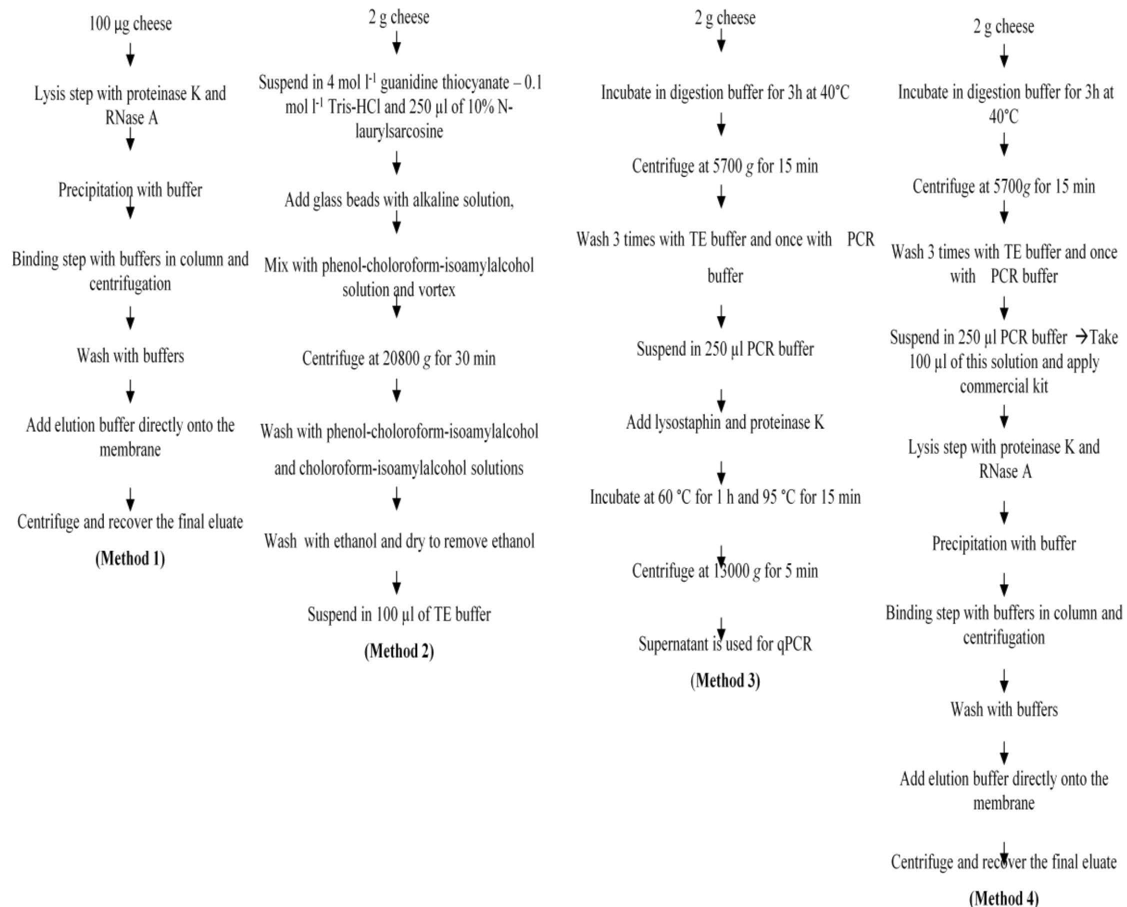

Figure 6.1. Methods used for DNA extraction from cheese samples.

6.2.7.3. Naturally Contaminated Cheese Samples

Cheese samples (n=7) were collected from different bazaars in İzmir and analysed immediately using traditional culture method and incubated at 37ºC for 24-48 h. The traditional plate count method includes homogenization of 25 g sample in 225 ml buffered peptone water and plating 0,1 ml aliquots in Baird Parker agar supplemented with egg yolk tellurite emulsion and incubation at 37 °C for 24–48 h. DNA extraction method was performed as decribed for artificially contaminated cheese samples.

6.2.7.4. TaqMan and LNA Probes Based qPCR Assay

The primers and probes were used in the qPCR analysis targeting *nuc* gene of *S. aureus* were given in Table 6.4. The size of the amplified product was expected to be 124 bp in length. TaqMan probe was labeled with 6-carboxy-fluorescein (FAM) and with 6-carboxy-tetramethyl-rhodamine (TAMRA) in 5′ and 3′ ends, respectively. The primes and probe were used as reported by Alarcón et al (2006). The LNA probe (was designed including 4 LNA monomers by using lna-tm.com program in exiqon web site (LNA probes, 2012).

Amplification assay of TaqMan and LNA probes based qPCR included in a total volume of 20 µl. This mixture composed of 10x probes master, 500 nM of each primer, 200 nM probe and 5 µl of template DNA. The thermal cycling programme started with 95°C for 10 min of incubation. 50 cycles of amplification included 95 °C for 15 s denaturation step, annealing at 60 °C for TaqMan and 57 °C for LNA probes. The reaction ended with extention step at 72 °C for 1 s. The data analyses were carried out using LightCycler® 480 Instrument software version 1.5 (Roche Diagnostics).

Table 6.4. The primers and the TaqMan probe used in qPCR analysis.

Primer	Size (bp)	Oligonucleotide sequence (5′→3′)
FP	32	CGCTACTAGTTGCTTAGTGTTAACTTTAGTTG
RP	28	TGCACTATATACTGTTGGATCTTCAGAA
TaqMan Probe	33	TGCATCACAAACAGATAACGGCGTAAATAGAAG
LNA Probe	20	AtCACaAACaGAtAACGGCG

6.2.8. LAMP Method

The primer sequences for LAMP method were given below targeting *nuc* gene of *S. aureus*.

F3 5´-GCATTTACGAAAAAAATGGTAGA-3´
B3 5´-TGTTCATGTGTATTGTTAGGTT-3´
FIP 5´-GCCACGTCCATATTTATCAGTTCTAAATGCAAAGAAAATTGAAGTCG-3´
BIP 5´-TATGCTGATGGAAAAATGGTAAACGTAAACATAAGCAACTTTAGCCAAG-3´

The LAMP reaction was carried out on a scale of 25.0 μl mixture containing 2.5 µl of Bsm buffer (20.0 mmol/L Tris- HCl (pH 8.8), 10.0 mmol/L KCl, 10.0 mmol/l (NH4)2SO4, 8.0 mmol/L MgSO4, 0.1% Tween 20), 1.0 mmol/L betaine, 1.6 μmol/l each primer FIP and BIP, 0.2 μmol/l each primer F3 and B3, 1.4 mmol/l dNTP, 8U *Bst* DNA polymerase, and 1.0 μl template DNA. For real time monitoring of LAMP reaction, the mixture in 96-well plates were incubated in a High-Throughput Real-Time PCR System (Roche Diagnostics, Mannheim, Germany).The negative control (sterilized ddH_2O instead of template DNA) was set in each LAMP reaction. The run set up was 60 cycles of 1 min at 63 °C, with fluorescence reading at the end of each of these cycles and melting curve analysis at 95 °C for 2 s with ramp rate of 4.4 °C/s and 2 s at 63°C. s with ramp rate of 2.2 °C/s. The temperature was then increased to 95 °C with continuous fluorescence monitoring.

CHAPTER 7

RESULTS AND DISCUSSION

In this study, the qPCR method was utilized for quantification of *S. aureus* cells with pure culture studies and artificially contaminated cheese samples. Firstly, TaqMan and LNA probe chemistries based qPCR methods were compared. The standard curves were constructed by using the *S. aureus* pure culture in two ways to compare the sensitivities of TaqMan and LNA probes. The first standard curve was constructed by using ten-fold dilutions of bacterial DNA of *S. aureus* pure culture. The second standard curve was constructed by using ten-fold dilutions of cell suspensions of *S. aureus* pure culture. TaqMan probes based qPCR method was found to be more sensitive in both of the standard curve methods. Then, the TaqMan probe based assay was used in artificially contaminated cheese samples. Four different DNA extraction methods were compared by carrying out the TaqMan probe based method to improve the sensitivity of the quantification range in artificially contaminated cheese samples. The most sensitive bacterial DNA extraction method was applied to quantitate *S. aureus* cells in naturally contaminated Turkish white cheese samples. LAMP method was performed for quantification of *S. aureus* cells with pure cultures and Turkish white cheese samples. The identification study was performed by isolating *S. aureus* from 34 Turkish white cheese collected from three different cities in Aegean Region of Turkey. The phenotypic and genotypic tests were compared to determine the standard method for quantification of *S. aureus*. The sequence analyses were performed to accurately identify the *S. aureus* isolates. The results of the phenotypic and genotypic tests were discussed in further sections.

7.1. Quantitative Real-Time PCR Method for Detection and Quantification of *S. aureus*

In this research, the qPCR method was utilized for direct quantitative detection of *S. aureus* by targeting *nuc* gene of *S. aureus* in Turkish white cheese. Samples having more than 10^5 CFU/g *S. aureus* have the potential of including staphylococcal

enterotoxins. That's why it is significant to develop an assay that accurately estimates *S. aureus* cell counts. Firstly, the detection limit and quantification range of the qPCR method was evaluated by using the *S. aureus* pure culture. The DNA extraction methods were evaluated with the more sensitive qPCR method for quantification of *S. aureus* in artificially and naturally contaminated white cheese samples. qPCR analyses were shown to quantify *S. aureus* cells accurately when combined with the proper DNA isolation method for milk samples (Aprodu et al., 2011).

7.1.1. Sensitivity of qPCR Assay with Pure Cultures

In the first part of the study, the qPCR method was optimized and the calibration curves were constructed using the *S. aureus* pure culture. Following this, four different bacterial DNA extraction methods were studies directly from white cheese samples. Although there are some studies on direct detection of *S. aureus* from milk and cheese samples, it is significant to optimize and adaptate these techniques to Turkish white cheese samples. After the development of DNA isolation method, qPCR analyses were performed for direct quantification of *S. aureus* cells in artificially and naturally contaminated white cheeses.

7.1.1.1. Determination of the Detection Limit and Quantification Range of qPCR Method

For the improvement of the capacity of qPCR analyses to quantify low levels of *S. aureus* cells, TaqMan and LNA probes based qPCR analyses were compared by targeting the *nuc* gene of *S. aureus* cells. The amplification of *nuc* gene as a target region was widely used for direct quantification of *S. aureus* in milk and cheese samples (Hein et al., 2005; Alarcón et al., 2006; Aprodu et al., 2011). The sensitivities of TaqMan and LNA probes based assays were compared by using *S. aureus* pure culture and artificially contaminated cheese samples. The standard curve studies were performed in two ways with *S. aureus* pure culture to find the most effective probe based assay for quantification. In the first method, standard curve was constructed by using ten-fold dilutions of DNA isolated from *S. aureus* pure culture. In the second way

standard curve was constructed using ten-fold dilution of cell suspensions of the pure culture. The most sensitive assay was used for quantification of *S. aureus* directly from cheese samples combined with qPCR.

7.1.1.1.1. Standard Curve Construction by Using Ten-fold Dilutions of DNA

Ten-fold dilutions of DNA isolated from *S. aureus* RSKK 1009 pure culture was prepared in a range between 10^0 and 10^7 CFU/ml. The qPCR analyses were performed with these dilutions. The averages of four C_t values were calculated for each dilution to construct the standard curve. Bacterial plate counts and C_t values for TaqMan and LNA probes were shown in Table 7.1. By plotting C_t values versus colony counts, the standard curves were generated separately for TaqMan and LNA probes based assays and illustrated in Figure 7.1 and Figure 7.2. The exact cell number was calculated as 5.88 CFU/ml detected by TaqMan probe based qPCR method. The coefficient of correlation (R^2) between log CFU/ml and C_t values was 0.989 and 0.998 for TaqMan and LNA probes, respectively. The slopes of the standard curves were -3.5968 and -3.7879 for TaqMan and LNA probes, respectively. The PCR efficiencies were calculated as 0.89 for TaqMan and 0.83 for LNA probes.

Table 7.1. Plate counts (CFU/ml) and C_t values obtained with the first standard curve method

CFU/ml	TaqMan C_t ave±STD	LNA C_t ave±STD
10^7	15.913±0.343	17.153±0.408
10^6	19.528±0.206	20.390±0.163
10^5	23.785±0.150	24.868±0.471
10^4	27.713±0.271	28.290±0.268
10^3	31.623±0.563	32.425±0.495
10^2	35.468±0.643	35.763±0.706
10^1	38.635±1.325	
10^0	39.860±1.370	

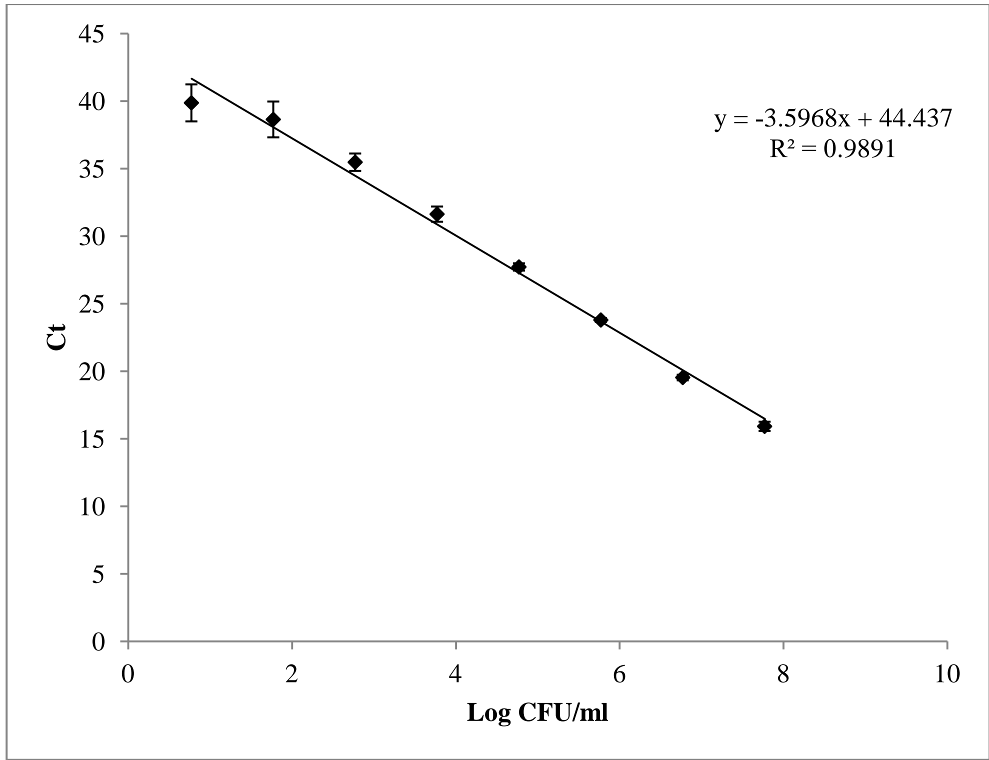

Figure 7.1. Standard curve generated with TaqMan probes based qPCR

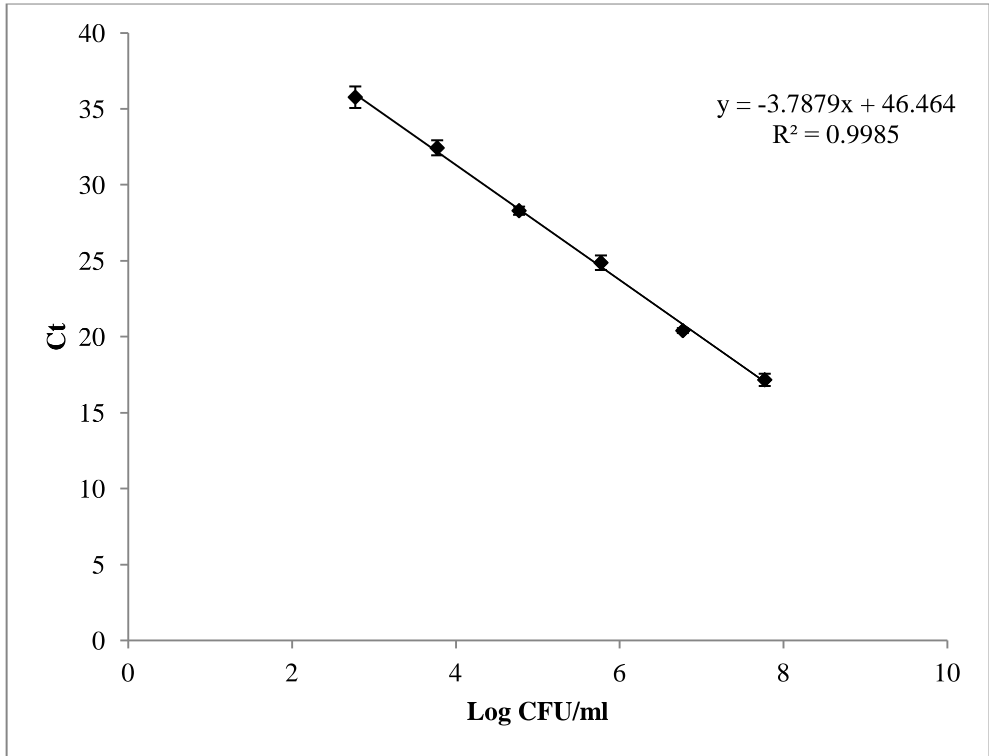

Figure 7.2. Standard curve generated with LNA probes based qPCR

7.1.1.1.2. Standard Curve Constructed Using Ten-fold Dilution of Cell Suspensions of the Pure Culture

In the second method of standard curve construction, ten-fold serial dilutions of pure culture were prepared. The bacterial DNA isolation was performed from each dilution. Representative amplification plot obtained using serial dilutions of DNA was shown in Figure 7.3. The C_t values obtained with qPCR assay for DNA of each dilution versus plate counts were plotted. Triplicate measurements were obtained for each dilution. By using the equation of each standard curve, efficiency of the PCR amplification was calculated as 0.81 by TaqMan and 0.76 by LNA probes. These standard curves were represented by Figures 7.4 and 7.5 respectively.

Table 7.2. Plate counts (CFU/ml) and Ct values obtained with the second standard curve method

Plate counts (CFU/ml)	TaqMan C_t ave±STD	LNA C_t ave±STD
10^7	19.119±0.440	18.766±0.664
10^6	25.412±0.091	24.438±0.238
10^5	27.269±0.574	26.909±0.548
10^4	32.246±0.220	31.413±0.342
10^3	36.318±0.525	35.337±0.139
10^2	40.233±0.363	39.748±0.150
10^1	42.257±0.347	

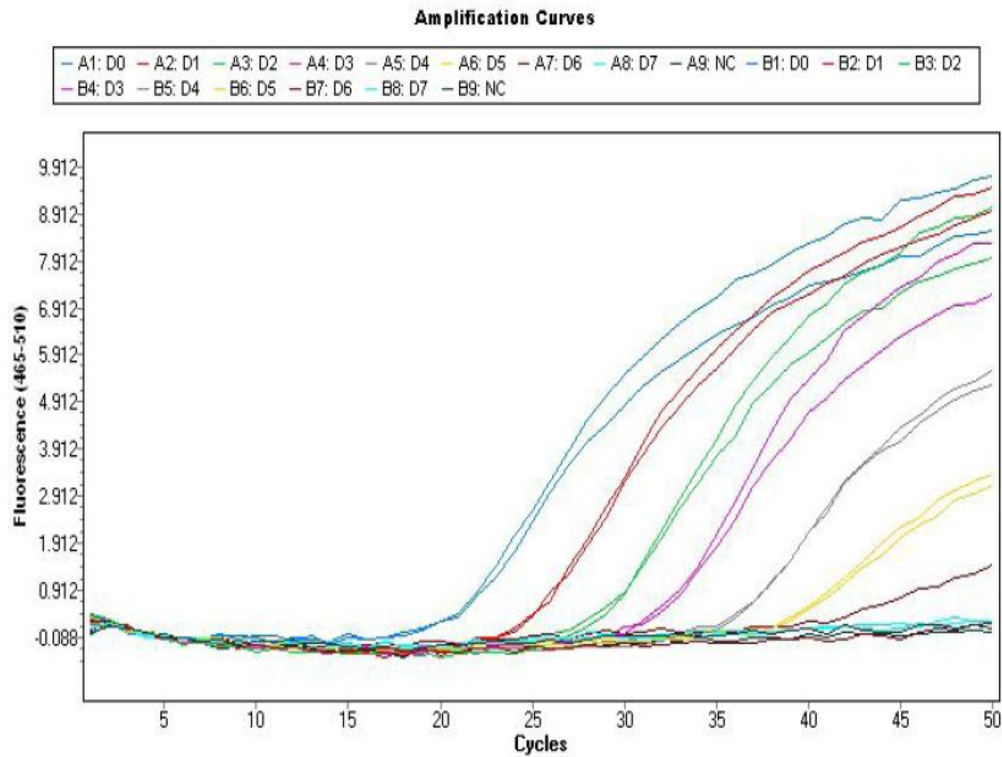

Figure 7.3. Amplification plot obtained using serial dilutions of DNA using TaqMan probe based qPCR method

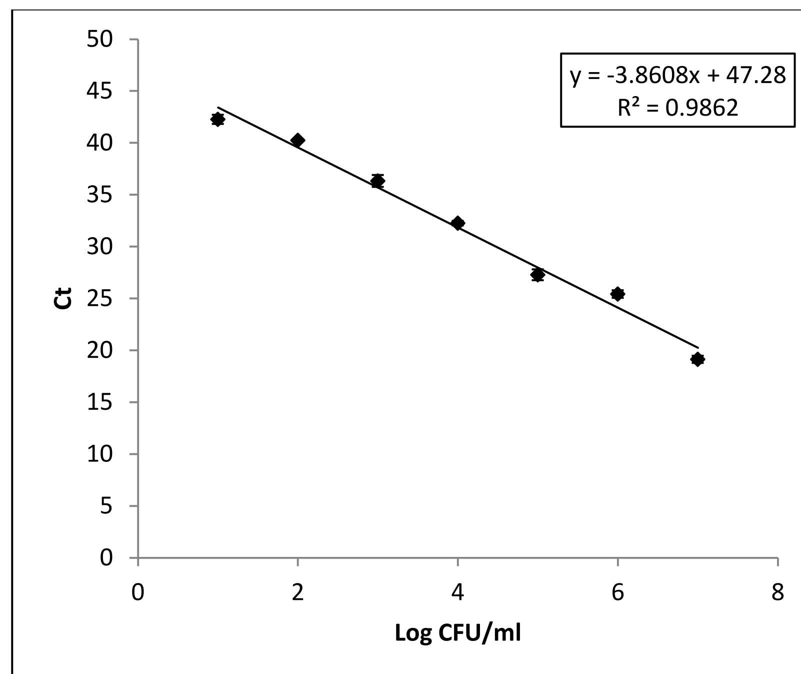

Figure 7.4. Standard curve generated with cell suspensions by TaqMan probes

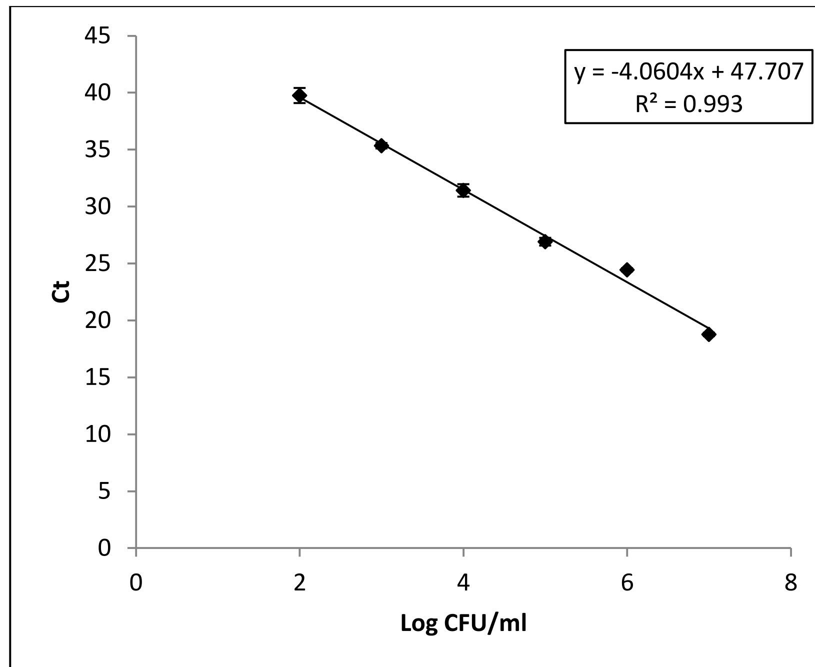

Figure 7.5. Standard curve generated with cell suspensions by LNA probes

Efficiency values of two qPCR based assays were evaluated according to the slope of the equation of the constructed line. TaqMan probe based qPCR based assay showed E of 0.89 and 0.81 for ten-fold dilutions of DNA and dilutions of calibrated cell suspensions. LNA probe based qPCR assay yielded E of 0.83 and 0.76 for ten-fold dilutions of DNA and calibrated cell suspensions. TaqMan probe based assay yielded higher efficiency values for ten-fold dilutions of DNA and calibrated cell suspensions. This result was opposed to the study which demonstrated that LNA probe based assay was more sensitive than TaqMan, Minor groove binder (MGB) and Scorpion probe chemistries for detection of thermotolerant *Campylobacter* (Josefsen et al., 2009). The similar result was obtained in another study that reported the comparison of LNA and MGB probes for detection of *S. aureus* enterotoxin genes (*sea* to *see*) (Letertre et al., 2003). However, in our study it is demonstared that LNA probe was not more effective than TaqMan probe for detection and quantification of *S. aureus*.

7.1.2. Specificity of qPCR Method

To test the specificity of qPCR method with TaqMan and LNA probes, negative controls such as *Staphylococcus carnosus*, *Staphylococcus epidermidis*, *Salmonella*

Typhimirium, *E. coli*, *E. coli* O157:H7 pure cultures were used. The qPCR method correctly classified cultures of negative controls. These isolates gave negative reaction to qPCR analyses. *S. aureus* RSKK1009, *S. aureus* ATCC 6538 and *S. aureus* ATCC 29213 were used as positive controls in qPCR analyses. Figure 7.6 shows the representative amplification curve of positive and negative controls.

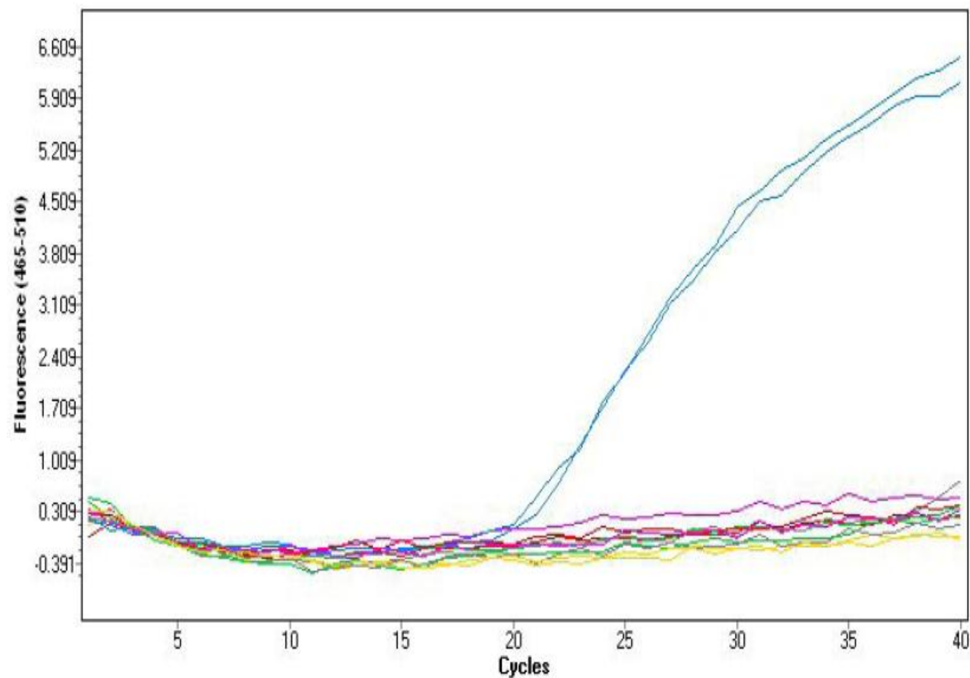

Figure 7.6. Amplification curve plot of positive control (*S. aureus* RSKK1009) and negative controls (*Staphylococcus carnosus*, *Staphylococcus epidermidis*, *Salmonella* Typhimirium, *E. coli*, *E. coli* O157:H7)

7.1.3. Comparison of Bacterial DNA Extraction Strategies for Detection of *S. aureus* in Turkish White Cheeses

The pure culture experiments demonstrated that TaqMan probes based qPCR method was more sensitive than LNA probe based assay. Therefore, the qPCR analyses were performed with TaqMan probes for quantification of *S. aureus* in white cheese samples. The detection limit of the TaqMan probes based qPCR assay was evaluated by using the artificially contaminated cheese samples to emphasize the real contamination case. For this purpose, the white cheese samples were artificially contaminated with serial dilutions of *S. aureus* pure culture at exponential phase. The ten-fold dilutions of

bacterial suspension that is equivalent to a 0.5 McFarland standard and determined as 7.00 log CFU/ml by plate count method were prepared. Each dilution was then inoculated to cheese sample. DNA isolation methods were compared to find the most sensitive method for quantification of *S. aureus* cells.

The capacity of detection level of qPCR is related to the preparation method used to isolate bacterial cells from food samples. Novel sample preparation method named flotation was developed with qPCR for quantitative detection of *Yersinia enterocolitica* in meat juice from pork quantifying to a level of $4.2x10^3$ cfu/ mL (Wollfs et al., 2004). In another study, a new two step filtration method with qPCR was used to quantify *Salmonella* in chicken rinse and spent irrigation water. This method allowed quantification to $7.5x10^2$ CFU/100 mL sample by distinguishing dead and viable bacterial cells (Wollfs et al., 2006).

The main disadvantage of qPCR has been considered on discriminating viable cells and DNA from dead cells (Wollfs et al., 2005). But in *S. aureus* studies performed with heat processed food products it must be established that although *S. aureus* cells are killed by the heat process, the enterotoxins remain because of their heat stability (Hein et al., 2001). Therefore in this study, qPCR method with the new DNA isolation method was used for determination of total contamination level of *S. aureus* without distinguishing viable and dead bacteria.

The proper DNA extraction method is useful and required for eliminating the PCR inhibitors such as fat and protein in the cheese samples. The fat and protein content of the cheese samples used in these methods were compared to the Turkish white cheese sample used in this study. It was found that they were similar with approximately 21.4% fat and 16% protein contents. The efficiency of qPCR analysis is another important parameter for actual quantification of *S. aureus*. For this purpose, four different DNA isolation methods were performed directly from cheese samples with TaqMan probe that is found as the more efficient qPCR assay in our study.

7.1.3.1. Bacterial DNA Extraction by Using The Food DNA Extraction Kit

In the first method DNA extraction from cheese samples was performed with a food DNA extraction kit. The complete information about the composition of the

ingredients was not described in detail by the manufacturer. The technical information indicated that the bacterial cells were lysed and enzymatically digested with RNase A and proteinase K according to manufacturers' instructions. After binding and precipitation steps, contaminants were removed by washing steps. Following buffering condition adjustment, DNA was selectively bound to a silica-gel membrane and finally DNA was eluted in elution buffer.

The binding mechanism in this silica column is based on the attraction between negatively charged DNA and positively charged silica particles. The major drawback of using these columns is that they can be for single use. The binding capacity is reduced by the DNA held by the silica matrix (Esser et al., 2006). The quality and quantity of DNA isolated is needed for efficient PCR analysis. DNA purity is found by calculating the ratio of absorbance at A_{260} to A_{280}. The pure DNA has A_{260}/A_{280} ratio of 1.7 to 2.0. Nanodrop results of DNA samples extracted by this method directly from cheese samples contaminated with *S. aureus* at the highest level (10^7 CFU/mL) were given in Table 7.3. The values indicate that there are some inhibitors such as proteins or contaminants in the solution that can inhibit PCR analyses.

In relation to these results, the qPCR analyses were performed and C_t values were calculated as slightly higher indicating lower sensitivity than the fourth DNA extraction method. Different extraction procedures such as commercial kits, boiling method and phenol-choloroform extraction were evaluated in the study for detection of *L. monocytogenes* from enriched pork raw sausage and mozzarella cheese samples. Commercial kits yielded lower amount of DNA as in our study (Amagliani et al., 2007).

7.1.3.2. Bacterial DNA Extraction by Using Beads

In the second method as modified from the method described in Bonaïti et al. (2006), the bacteria were treated with a chaotropic agent (guanidine), detergents (SDS, sodium lauryl sarcosinate), and chelating agent (EDTA). The lysates were mixed with glass beads to allow complete cell disruption. DNA bound to beads were washed, eluted in water and finally concentrated by ethanol precipitation. Nanodrop results of three DNA samples extracted by this method directly from cheese samples contaminated with *S. aureus* at the highest level (10^7 CFU/ml) were given in Table 6.3. This method gave amplification at higher C_t values than other DNA extraction methods. The disadvantage

of this method could be the contamination of DNA samples with glass beads or in several steps which cause cross contamination (Kim et al., 2001).

7.1.3.3. Bacterial DNA Extraction by Using Trypsin

The third method was the modified form of the method described by Hein et al. (2001). The cells were digested with digestion buffer and trypsin enzyme solution and incubated for about 3 hours for efficient digestion. This suspension was centrifuged and washed with buffer solutions. Lysostaphin and proteinase K were used for lysis and DNA solution was obtained. Proteinase K is an enzyme isolated from *Tritirachium album* and purified. This enzyme is used for hydrolyzing the proteins (Ebeling et al., 1974). Lysostaphin is an enzyme that is isolated from a bacterial culture of *Staphylococcus staphylolyticus.* The major component of lysostaphin is glycylglycine endopeptidase that lysis *Staphylococcus* species including *S. aureus* (Robinson et al., 1979). We could not obtain amplification with this method. DNA extraction from cheese samples can be inhibited by high level of lipid and protein found in the cheese composition. The reason of low isolation efficiency can be isolation of non-specific DNA target or these inhibiting substances in the cheese composition (Ercolini et al., 2004). Nanodrop results of this method revealed that although DNA yield was higher, the purity of extracted DNA was lower than other methods as given in Table 6.3. Another reason can be the washing steps that can cause loss of cells during extraction (Yang et al., 2007). The PCR analysis of the samples taken from some steps during DNA extraction showed that most of DNA of the gene of our interest was lost during washing step.

7.1.3.4. Bacterial DNA Extraction by the Combined Method Using Trypsin and The Food DNA Extraction Kit

In the fourth method that was the combined use of the modified method described by Hein et al. (2001) and the food DNA extraction kit, proteins are digested in the presence of detergent and trypsin. After buffering and washing steps, the food DNA extraction kit was used. In this new procedure, the advantage of the DNA extraction method by using trypsin with respect to high DNA amount was combined with the

procedure of food DNA extraction kit which provided higher DNA purity to improve the accuracy of quantification. The nanodrop results were represented by Table 7.3.

Table 7.3. Nanodrop results of DNA extraction methods

Bacterial DNA extracted by food DNA extraction kit		
Sample no	DNA amount ng/µl	A_{260}/A_{280}
1	17.12	3.37
2	12.11	3.11
3	10.01	1.81
Bacterial DNA extracted by using the beads		
Sample no	DNA amount ng/µl	A_{260}/A_{280}
1	15.11	1.18
2	54.23	0.93
3	389.17	1.24
Bacterial DNA extracted by using trypsin		
Sample no	DNA amount ng/µl	A_{260}/A_{280}
1	30.76	1.26
2	163.31	1.31
3	163.57	0.91
Bacterial DNA extracted by the using trypsin and the food DNA extraction kit		
Sample no	DNA amount ng/µl	A_{260}/A_{280}
1	37.30	1.97
2	26.17	1.92
3	20.43	2.06

7.1.3.5. Comparison of Bacterial DNA Extraction Methods

In an attempt to find the most sensitive qPCR method, these four procedures were compared according to their C_t values. The average C_t values were 27.8 ± 0.58 (M1), 34.2 ± 1.20 (M2) and 25.5 ± 0.24 (M4). The lowest C_t value corresponding to the highest sensitivity was obtained with the fourth method. This can be described by the long incubation period for 3 hours used for digestion and selective binding of DNA to silica gel membrane following binding and precipitation steps. Different C_t values of the target bacterial gene can be related to differences in bacterial DNA extraction

efficiency. Recovery of cells was estimated as the percentage of the ratio of bacterial cell counts calculated by qPCR to the number of cells counted by plate count method. As it is observed from Figure 7.7, the highest recovery result was found with the newly developed DNA extraction method (M4).

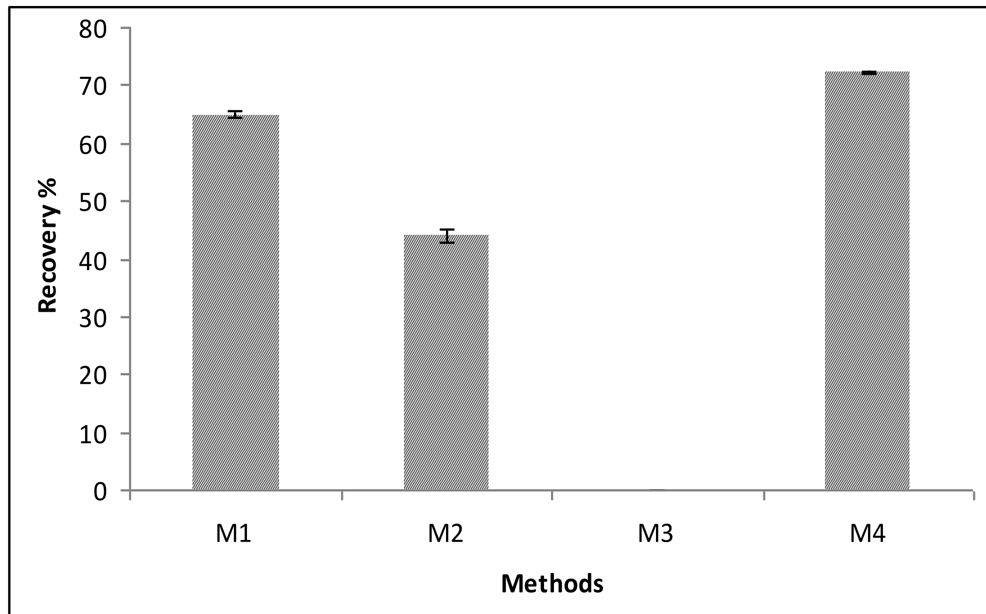

Figure 7.7. Comparison of four different methods based on qPCR results
M1: (Method 1), M2:(Method 2), M3:(Method 3), M4:(Method 4).

7.1.4. Comparison of TaqMan and LNA probes for Quantification of *S. aureus* Cells in Artificially Contaminated White Cheese Samples

The performances of TaqMan and LNA probes were evaluated with white cheese samples in qPCR system. Ten-fold serial dilutions of pure culture were added to each cheese sample. DNA extraction method were applied to white cheese samples. qPCR experiments were carried out by using the genomic DNAs isolated from cheese samples. Standard curves were constructed by using genomic DNA solutions for both of the qPCR systems. The E and R^2 values were calculated as 2.02 and 0.9522 for TaqMan probe. Figure 7.8 shows the standard curve constructed using DNA isolated from artificially inoculated cheese samples generated by TaqMan probes based qPCR assay. E of LNA probe was 2.32 and R^2 of the standard curve was 0.9523. Standard

curve constructed using DNA isolated from artificially inoculated cheese samples generated by LNA probes based qPCR assay is illustrated by Figure 7.9.

Efficiency of TaqMan probe was found to be higher than LNA probe because the efficiency reduces at values higher than 2.

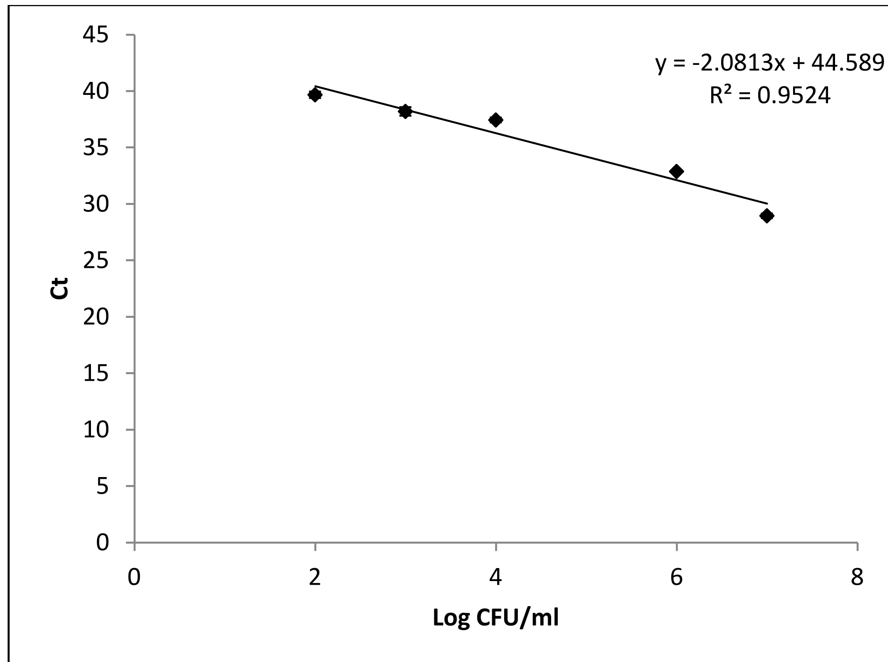

Figure 7.8. Standard curve constructed using bacterial DNA isolated from artificially inoculated cheese samples generated by TaqMan probes based qPCR assay.

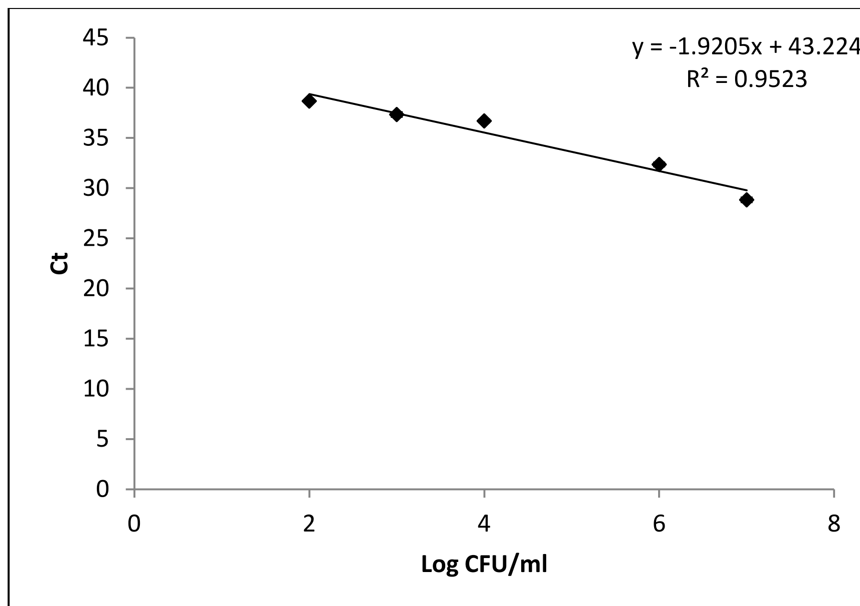

Figure 7.9. Standard curve constructed using bacterial DNA isolated from artificially inoculated cheese samples generated by LNA probes based qPCR assay.

7.1.5. Determination of Sensitivity of the Bacterial DNA Extraction Method Using Trypsin and the Food DNA Extraction Kit With TaqMan Probe Based qPCR Method

In order to evaluate the novel DNA extraction method for quantifying *S. aureus* in cheese samples, the experiments were performed with the fourth method due to its highest sensitivity behavior. 1 ml of bacterial suspension prepared by ten-fold dilutions of reference strain adjusted to McFarland standard 0.5 was added to the cheese samples. After DNA isolation with the fourth method, qPCR analyses were performed. The results are illustrated in Figure 7.10. For the two lowest concentrations, no quantitative data were obtained, since these concentrations fall outside the linear range of amplification. However, it was possible to confirm the presence of *S. aureus* cells. The reason of finding a lower R^2 value of the curve obtained from the direct DNA isolation method from the curve of the qPCR system obtained from serial dilutions of reference strain could be the presence of high quantity of proteins and fats present in the cheese that might adsorb or prevent detergents, chaotropic agent, chelating agents or beads from accessing to the cells (Bonaïti et al., 2006).

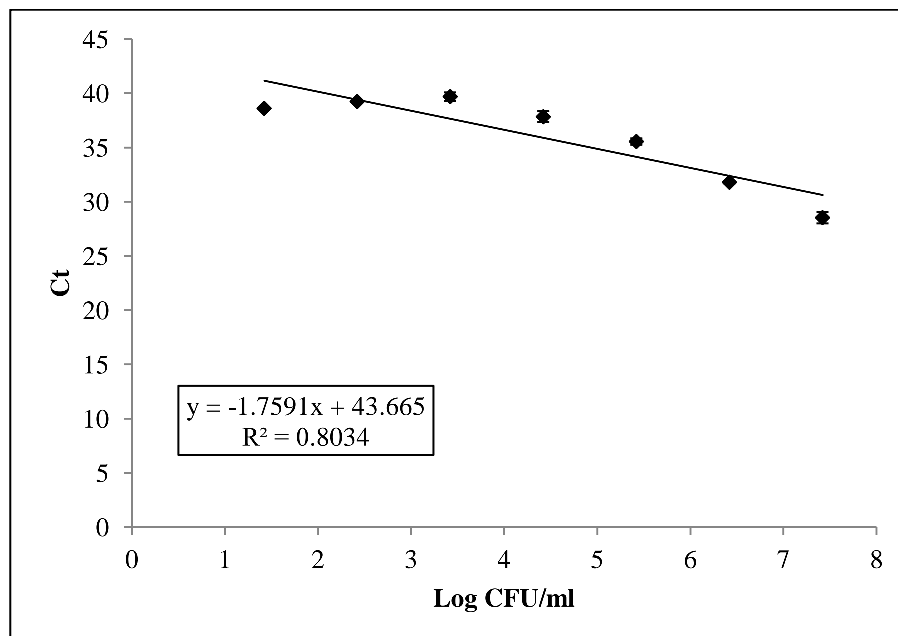

Figure 7.10. qPCR results of serial DNA suspensions obtained with the fourth isolation method with TaqMan probe based qPCR method

The qPCR results were represented by *S. aureus* copy number. They were compared to CFU numbers calculated by the plate count method. Figure 7.11 is the representation of correlation of plate count and qPCR results from cheese samples treated with the fourth method.

The coefficient of correlation between these values was 0.981. In this study, the qPCR results were not higher than the plate counts indicating the lower risk of false positives caused by the detection of DNA from dead cells. These results also showed that it was important to prepare standard curves for the cells in actual food samples because the curve could change with the actual samples. The levels of standard deviation were low enough to allow accurate quantification.

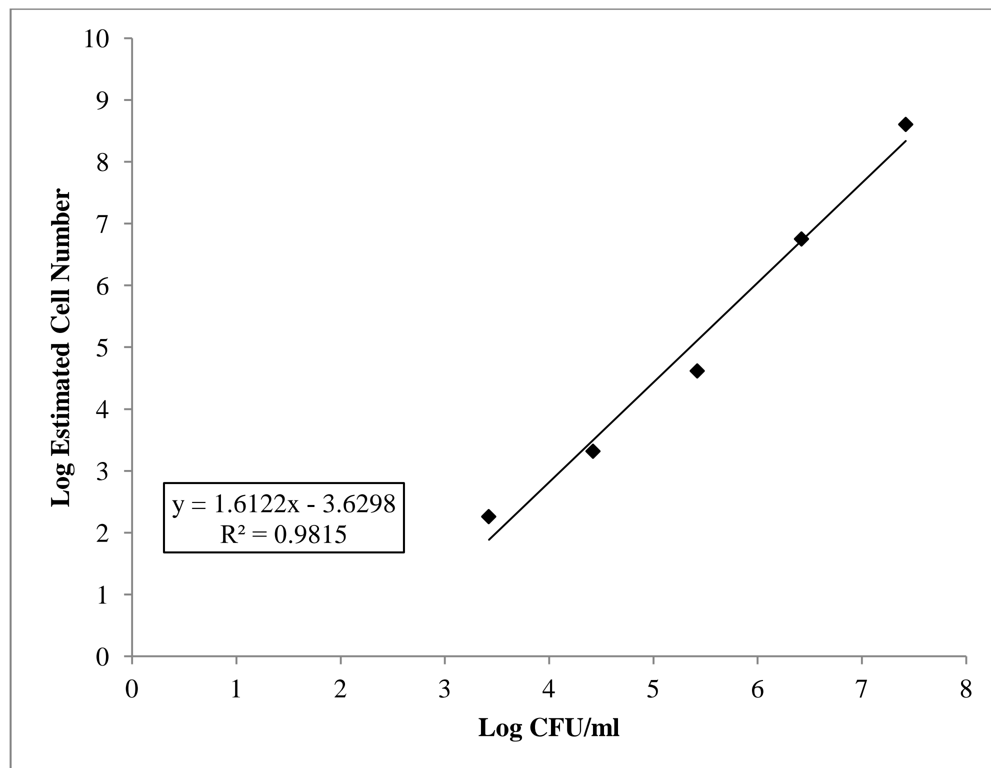

Figure 7.11. Correlation of plate count and qPCR results from cheese samples treated with Method 4.

qPCR is important for specificity and fast analysis time. It is increasingly used for detection and quantification of foodborne bacterial pathogens. Several studies have been performed for detection of *S. aureus* in food samples. In direct detection of pathogens applied to food samples, the efficiency of extraction can be reduced by inhibitory substances such as fats, proteinases and high concentration of Ca^{2+}

(Cremonesi et al., 2006). In this study, a novel DNA isolation method has been developed and when combined with qPCR, it was found to detect bacterial level of 13.2 CFU/g which was the lowest amount that amplification was observed. Poli et al. (2007) reported detection of *S. aureus* lower than 10^3 CFU/g in cheese samples by *nuc* targeted qPCR assay. In the study by Alarcón et al. (2006) it was reported that $4.9x10^3$ CFU/g had to be present to give positive reaction with TaqMan probe in actual food samples. In another study, qPCR was applied to quantify *S. aureus* cells in artificially contaminated cheeses of different types. It was demonstrated that $1.5x10^2$ to $6.4x10^3$ copies of the *nuc* gene/2 g cheese could be detected based on the cheese matrix (Hein et al., 2001). Detection sensitivity improved approximately one log order in our study.

Cheese is a difficult matrix for direct *S. aureus* detection and quantification by qPCR method due to its high fat content (Ercolini et al., 2004). There have been few studies on development of DNA extraction method directly from food samples for detection of *S. aureus* (Hein et al., 2001; Bonaïti et al., 2006). The main risk of *S. aureus* contamination frequently in milk and milk products is lack of proper hygienic conditions during food preparation (Akineden et al., 2008). For evaluating the safety of food production process, it is important to quantify the changes in microbial numbers (Schelin et al., 2011). Therefore, the detection of this bacterial pathogen is a significant issue for the safety and quality of traditionally produced cheeses (Cremonesi et al., 2007).

Traditional microbiological methods have several drawbacks such as long detection time, high cost and low sensitivity (Cremonesi et al., 2007). For that reason numerous PCR detection methods have been developed for the detection of *S. aureus* to replace these classical methods (Brakstad et al. 1992; Atanassova et al., 2001; Cremonesi et al., 2005; Yang et al., 2007; Zhang et al., 2009). Quantitative PCR method has been used in several studies for the detection of *S. aureus* in food samples (Hein et al., 2001; Alarcón et al., 2006; Poli et al., 2007; Fusco et al., 2011).

7.1.6. Comparison of TaqMan and LNA Probes for Quantification of *S. aureus* Cells in Naturally Contaminated White Cheese Samples

In this part of the study, standard curves generated by using artificially inoculated cheese samples was used to calculate the bacterial numbers in naturally contaminated cheese samples. Plate counts and qPCR enumeration in naturally

contaminated cheese samples were given in Table 7.4. The qPCR counts in two systems were found to be higher than plate counts. This could be related to complex nature of cheese that has high content of protein, fat and other substances such as calcium ions, plasmin that can inhibit PCR amplification (Rossen et al., 1992; Ercolini et al., 2004). The reason of higher detection limit in *S. aureus* detection than other bacterial species can be related to the presence of cell wall in Gram positive cells that can prevent cell lysis (Riyaz-Ul-Hassan et al., 2008).

Table 7.4. Plate counts and qPCR enumeration in naturally contaminated cheese samples.

Samples	Plating Log CFU/g	C_t TaqMan (mean)	TaqMan Log Estimated Cell Number/g	C_t LNA (mean)	LNA Log Estimated Cell Number/g
1	3.18	33.60	5.70	34.20	5.12
2	4.93	35.23	4.92	35.00	4.70
3	3.43	32.31	6.32	31.97	6.28
4	1.98	34.00	5.51	34.24	5.09
5	3.01	37.12	4.01	35.00	4.70
6	3.89	37.42	3.86	35.00	4.70
7	2.18	37.03	4.05	35.00	4.70

This study is one of the first studies that demonstrate the comparison of LNA and TaqMan probes in qPCR assay for quantification of *S. aureus* in white cheese samples. The importance of the study is the development of qPCR method for quantification of *S. aureus* without traditional culturing methods. The highest microbial counts of *S. aureus* in cheese samples are determined as 10^5 CFU/g for production of enterotoxins by the official regulations (Alomar et al., 2008). Although pasteurization of milk, fermentation and ripening steps retard *S. aureus* growth, it is important to detect and quantify this pathogenic bacterium in cheese (Akineden et al., 2008). By this qPCR method since species specific detection was performed, DNA of other bacteria was not detected by the developed method under the described conditions. The bacterial counts in qPCR method were calculated higher than the plate count method. The difference between qPCR and plate count results revealed that most of the bacteria could not be

counted by classical plate counting method. The reason of this can be related to a few numbers of clusters formed by the bacteria causing less number of plate count results. Another reason of this difference was described by detection of dead and viable cells together (Mäntynen et al., 1997). The dead cells could not be detected by the plate count method. The coefficient of correlation between the qPCR and plate count methods were high enough (0.9815) for the artificially contaminated cheese samples. Therefore, it is possible to calculate the plate counts by using the qPCR results.

7.2. Detection of *S. aureus* Using LAMP Method

LAMP is a novel method performed at isothermal conditions within an hour. In this study, this method was performed with serial dilutions of bacterial DNA isolated from each cell suspension in LightCycler480 instrument. There was a very few number of study on quantification of *S. aureus* by using LAMP method with this instrument.

The sensitivity and specificity of the method was determined by using serial DNA dilutions and negative controls. The standard curve was obtained by plotting the plate counts versus C_t numbers. The plot gave R^2 value of 0.73 with efficiency of 0.65 which was lower than qPCR analyses performed with pure culture. The experiments were repeated more than 2 times but the results were not consistent. Therefore the average of two values were taken and used to construct the standard curve (Figure 7.13). The study on detection of NS3 gene of Japanese B encephalitis virus (JEV) in swine samples by using reverse transcription LAMP assay was also performed with Lightcycler 480 High-Throughput Real-Time PCR System and successful results were obtained with this instrument as opposed to our findings (Tian et al., 2012).

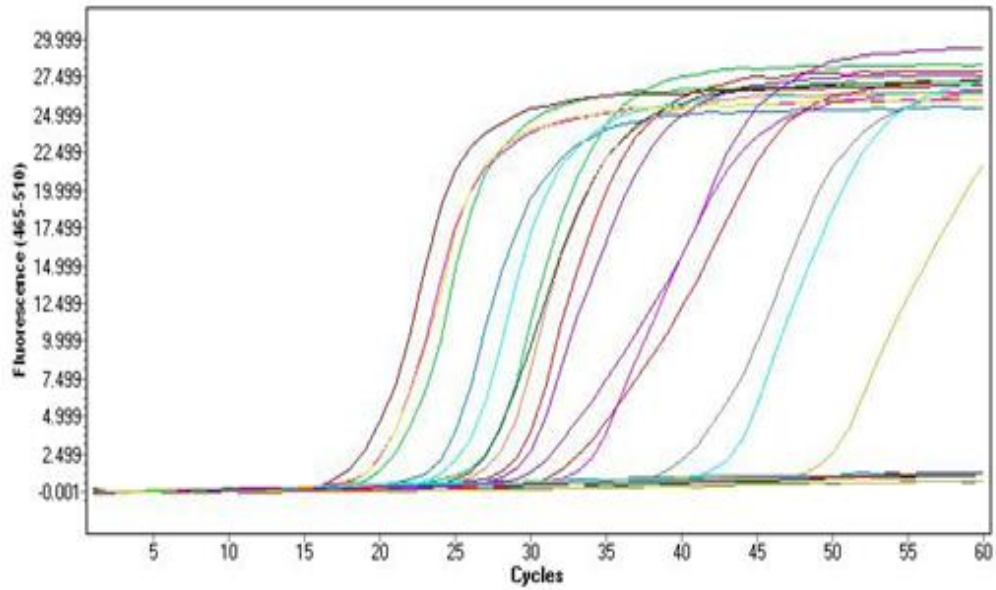

Figure 7.12. Amplification plot obtained using serial dilutions of bacterial DNA of cell suspensions of *S. aureus*, positive controls and negative controls (*S. epidermidis, S. carnosus, Ecoli O157:H7 and E. coli,* water) by using LAMP method

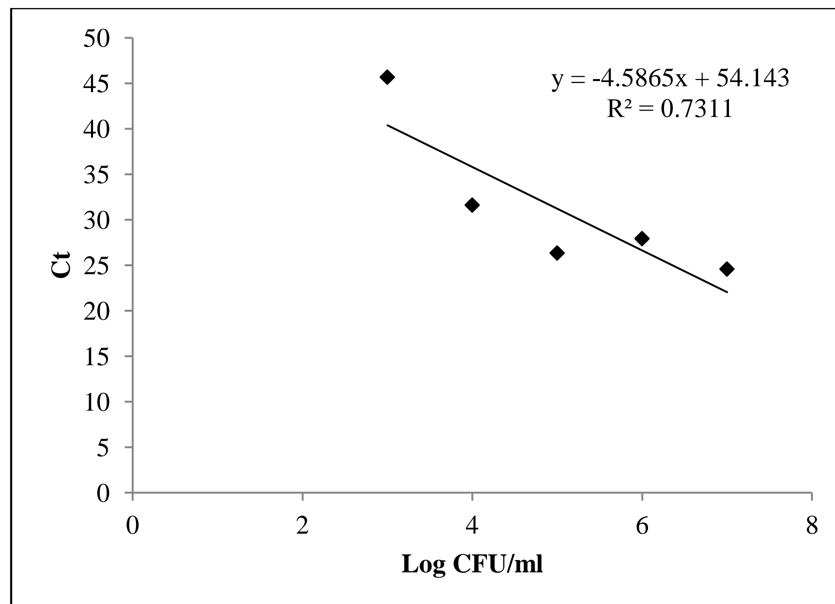

Figure 7.13. Standard curve constructed using bacterial DNA isolated from serial cell suspensions by LAMP method

7.3. Identification of *S. aureus* Isolates

7.3.1. Isolation of *Staphylococci*

S. aureus is an important pathogen causing community and hospital acquired diseases and food intoxication cases. White cheese is one of the most widely consumed food products in Turkey. Therefore it is necessary to characterize *S. aureus* strains isolated from white cheese samples and to investigate these strains phenotypically and genotypically. Thirty four (34) white cheese samples were investigated for the presence of *S. aureus* and 207 pure cultures were obtained as the result of subculturing experiments. Gram staining, catalase, slide coagulase, tube coagulase, mannitol fermentation and DNase activity tests were performed. The test results of 24 isolates were given in Table 7.5. 5 isolates gave positive results to Gram staining, catalase, coagulase, slide coagulase, tube coagulase and mannitol fermentation biochemical tests

7.3.2. Phenotypic Characterizations

7.3.2.1. Gram Reaction and Morphology

Gram staining test was applied as described in Materials and Methods section. The cells showing purple-violet color forming grape-like clusters were regarded as *Staphylococci* (Figure 7.14).

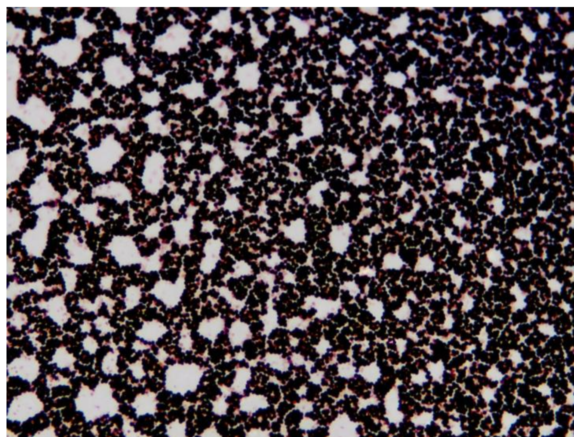

Figure 7.14. Gram staining image of *S. aureus* cells under light microscope

7.3.2.2. Biochemical Tests

Biochemical tests were carried out on 207 pure isolates. According to these biochemical tests, 114 isolates were Gram (+), 138 isolates were catalase (+), 104 isolates were Gram (+) and catalase (+), of the 93 isolates tested for tube coagulase reaction 5 isolates were coagulase (+), 105 isolates were tested by slide coagulase test and 54 isolates were coagulase (+), among 187 isolates 32 isolates showed DNase activity and 99 isolates fermented mannitol. 24 isolates were further investigated for the presence of *coa* and *nuc* genes by using PCR analyses. 3 isolates gave positive result to all biochemical tests performed. The phenotypic test results may lead to conclude inaccurately on identification of *S. aureus*. This could be related to the impact of environmental factors on gene expression. For this reason, a set of phenotypic tests should be used for bacterial identification (Gandra et al., 2005). Kateete et al. (2010) investigated the isolates that gave positive reaction to Gram staining and catalase tests. These isolates were tested for tube coagulase, mannitol and DNase reactions. These test results were combined with the PCR amplification of *nuc* gene which is regarded as the gold standard for *S. aureus* identification. Tiwari et al. (2008) compared slide coagulase test, tube coagulase test and Slidex Staph Plus test by regarding the *coa* gene amplification as the main criteria for identification of *S. aureus*. In this study, tube coagulase test was used as a routine test to differentiate *S. aureus* isolates.

DNase test was used as an additional test for screening *S. aureus* isolates. The production of DNase by *S. epidermidis* in small quantity can cause misidentification. Two isolates that gave negative reaction to DNase test gave positive reaction to other biochemical tests. Thermostable nuclease test was considered to confirm the other biochemical test results but *S. intermedius* and *S. hyicus* are the other thermostable nuclease positive *Staphylococcus* species associated with food poisoning (Gandra et al., 2005).

Table 7.5. Biochemical test results and PCR analyses of *coa* and *nuc* genes

Sample code	Gram staining	Catalase test	Slide coagulase test	DNase activity	Mannitol ferm.	Tube coagulase test	*coa*	*nuc*
PC	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+
4	+	+	+	-	+	+	+	+
5	+	+	+	-	+	+	+	+
6	+	+	+	-	-	-	-	-
7	+	+	-	-	+	-	-	-
8	+	+	+	-	-	-	-	-
9	+	+	+	-	-	-	-	-
10	+	+	+	-	-	-	-	-
11	+	+	+	+	-	-	-	-
12	+	+	+	+	-	-	-	-
13	+	+	+	-	+	-	-	-
14	+	+	+	-	-	-	-	-
15	+	+	+	-	+	-	-	+
16	+	+	+	-	-	-	+	-
17	+	+	+	+	+	-	-	-
18	+	+	+	-	+	-	-	-
19	+	+	+	-	+	-	-	-
20	+	+	+	-	+	-	+	+
21	+	+	+	-	-	-	+	-
22	+	+	+	-	+	-	-	-
23	+	+	+	-	+	-	-	-
24	+	+	+	+	+	-	-	-

7.3.3. Genotypic Characterizations

Genotyping of *S. aureus* is an important consideration for determining the strain origin, epidemiology of strain outbreaks and hospital outbreaks and molecular relatedness (Agius et al., 2007). For genotypic characterization of biochemically identified *S. aureus* cells, several target regions including the 23S rDNA, the spacer region between 16S-23S rDNA, *coa*, *clf*, *spaX*, *spa IgG* and *nuc* were amplified in the bacterial genome using PCR method. The results were summarized in Table 7.6.

Table 7.6. PCR analysis results of *S. aureus* isolates.

Sample code no	**23S rDNA**	**16S-23S**	***Coa***	***Clf***	***Spa X***	***Spa Igg***	***fem***	***Sau***
1	+	-	+	+	+	+	+	+
2	+	-	+	+	+	+	+	+
3	+	-	+	+	+	+	+	+
PC	+	+	+	+	+	+	+	+

PC: Positive control (*S. aureus* RSKK 1009)

The molecular characterization of *S. aureus* isolates yielded amplicon sizes of 1250 bp and 500 bp by the amplification of the genes encoding 23S rDNA and 16S-23S rDNA (Figure 7.15).

The coagulase production was used as an important phenotypic identification criteria among *S. aureus* isolates. Single banded *coa* PCR products were especially reported in *S. aureus* strains isolated from bovine milk. In this study single banded *coa* gene products were observed (Figure 7.16). The 600 bp products were generated. Regarding high polymorphic size amplicons generated by amplification of this gene, it does not allow identification of all *S. aureus* strains uniquitously (Palomares et al., 2003). *Coa* gene polymorphism has been utilized for typing of *S. aureus* strains previously (Goh et al., 1992; Shopsin et al., 2000).

In a study, genotypic screening of 128 *S. aureus* isolated from milk samples revealed the presence of *coa* gene in all isolates by PCR analysis. Besides the presence of *coa* gene and slide coagulase positive test result, two isolates were found to be tube coagulase negative (Motta et al., 2001). Another study demonstrated the importance of heterogeneity of *coa* gene products in the control of *S. aureus* mastitis (Sindhu et al., 2010). The study was performed to examine the genetic

32, relatedness of *S. aureus* isolates from 700 milk samples of cows with mastitis based on *coa* gene polymorphism by PCR restriction fragment length polymorphism (RFLP). This study concluded that the personnel in milking process was assigned in formation of double band by *coa* PCR (Karahan and Çetinkaya 2007). The clumping factor is the fibrinogen binding protein located on the surface of *S. aureus.* The reaction of bacteria and fibrinogen results in formation of clumping of bacterial cells (Eidhin et al., 1998). The amplification of the *clf* gene yielded an amplicon with 1000 bp (Figure 7.16).

Protein A is an important virulence factor of *S. aureus*. The immunoglobulin G (IgG)-binding protein A has the capacity to coat the *S. aureus* cell surface with incorrectly oriented IgG molecules (Stutz et al., 2011). The PCR amplification of the genes encoding IgG binding region of *S. aureus* yielded amplicon sizes of 1000 bp. Amplification of X region of protein A gave two different amplicon sizes of approximately 300 bp for positive control and 250 bp for the *S. aureus* isolates (Figure 7.17). Spa typing has been performed to investigate fourty-four *S. aureus* isolates from the milk of cows suffering from mastitis in Korea. Five clusters and two singletons based on 14 spa types were obtained (Hwang et al., 2010). The reproducibility of *spa* sequencing has been evaluated in a multicenter study and 100% intra- and interlaboratory reproducibility results were obtained (Aires-de-Sousa et al., 2006).

S. aureus strains produce an extracellular thermostable nuclease with species specific and unique sequence (Palomares et al., 2003). The presence of the gene encoding thermostable nuclease (*nuc*) in *S. aureus* strains isolated from cheese samples was analyzed in several studies (Hein et al., 2001; Borelli et al., 2011). The amplification of the *nuc* gene yielded a product size of 279 bp in all *S. aureus* isolates (Figure 7.18). *S. aureus* cells are not thermostable but their enterotoxins are resistant to heat. The detection of *nuc* gene by PCR method can be utilized for determining the presence of toxin producing bacteria regarding the heat stability of toxins (Hein et al.,

2001). Enterotoxin genes were also investigated by application of PCR targeting SEA, SEB, SEC, SED and SEE. However, all 3 strains were not found to be enterotoxigenic.

FemA gene is a target factor encoding methicillin resistance (Johnson et al., 1995). The *femA* gene presence is reported to be specific to all *S. aureus* isolates. Its 42 kDa protein is involved in cell wall methabolism and used as an internal positive control in reactions (Mehrotra et al., 2000). The amplification of this gene gave 129 bp size amplicons in all samples confirming the presence of *S. aureus* (Figure 7.18). Previously, the biochemical test results were confirmed by amplification of *femA* gene in PCR analysis (Günaydın et al., 2011). Another study focused on amplification of *femA* gene as a target region for identification of *S. aureus.* All the isolates tested for *femA* gene gave amplification band in PCR analysis (Riyaz Ul-Hassan et al., 2008). The amplification of the gene using sau1 and sau2 primers were used to generate 217-bp *S. aureus* species specific sequence derived from methyltransferase gene. This sequence is commonly found in *S. aureus* species and used as an internal control in previous studies (Pantůček et al., 2004; Holochova et al., 2010) (Figure 7.18).

The results indicate that except for 16S-23S region all the target regions tested positive to the isolated strains. Also, in another study, 5 of the 64 isolates which were confirmed as *S. aureus* tested negative for the 16S-23S rDNA intergenic spacer region (Akineden et al., 2008). In another study, it was aimed to investigate the appropriateness of 16S-23S rDNA intergenic spacer region for the identification of *Staphylococcus* strains. *S. aureus* was one of the very few species that showed heterogeneous patterns. This region did not allow identification at subspecies level (Mendoza et al., 1998).

Staphylococcus strains produce thermonuclease that degrades both DNA and RNA. The *nuc* gene encoding TNase protein has species-specific sequences (Brakstad et al., 1992). Detection of toxin genes does not necessarily indicate that the organism produces biologically active molecules or toxins. In a food system, PCR detection of toxin genes coupled with the specific detection of the producing species (*nuc*-PCR) represents the potential of toxin formation in food and hazardous food products due to the level of contamination (Ercolini et al., 2004).

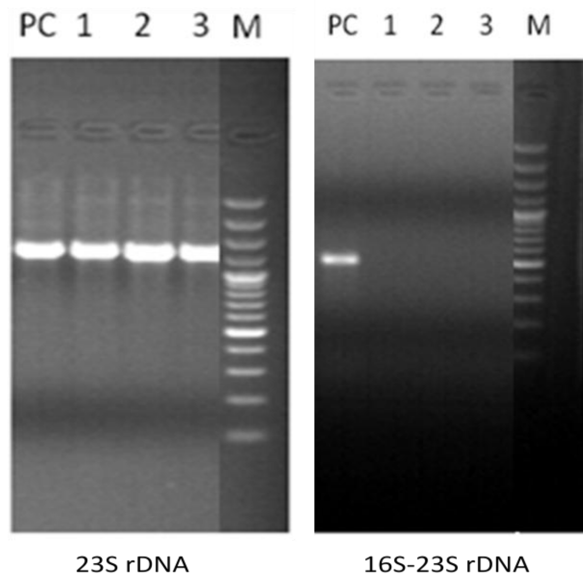

Figure 7.15. Amplification of the genes encoding 23S rDNA (1250 bp) and 16S-23S rDNA (500 bp). M, 100-bp ladder.

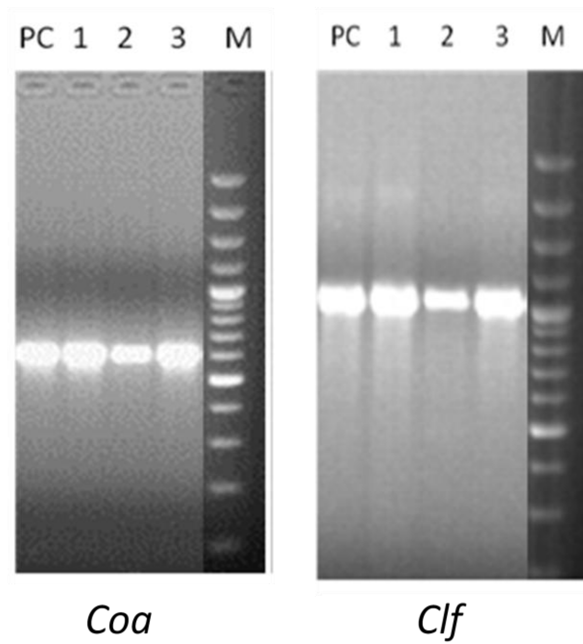

Figure 7.16. Amplification of the genes *Coa* (600bp) and *Clf* (1000 bp). M, 100-bp ladder

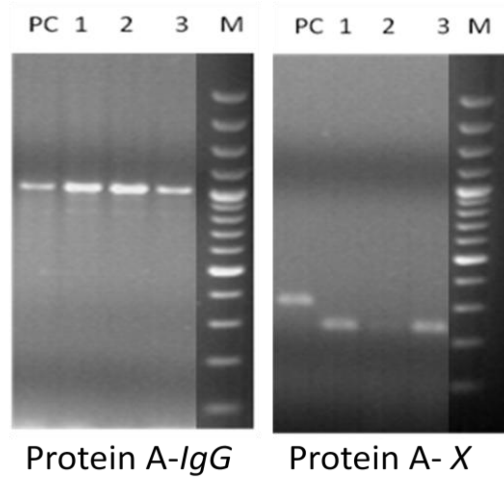

Figure 7.17. Amplification of the genes encoding the *IgG* binding region (1000 bp) of *X* region(PC, 300 bp, Lanes1-3, 250 bp) of protein A. M, 100-bp ladder

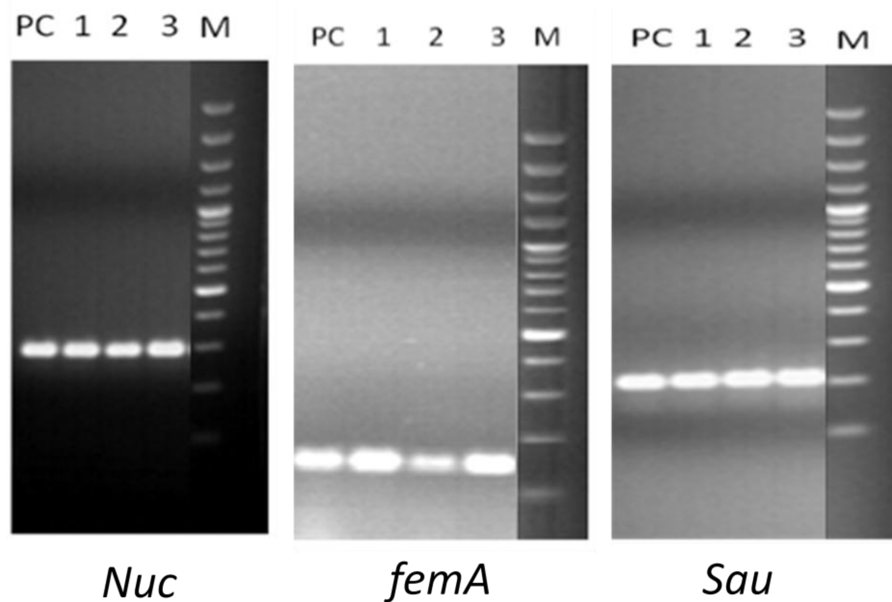

Figure 7.18. Amplification of the genes *Nuc* (279 bp), *femA* (129 bp) *and Sau* (217 bp) gene. M, 100-bp ladder

Biochemical tests are not enough for reliable identification of *S. aureus*. It was also reported that there is no single test that can definitely identify *S.aureus* (Kateete et al., 2010). Comparative analyses such as slide coagulase test, tube coagulase test, *coa* and *nuc* gene presence were examined to choose the gold standard method for identification of *S. aureus*. Tube coagulase test has been used for differentiation of *S.aureus* in most of the studies (Malathi et al., 2009; Akineden et al., 2011). In one of these studies; slide coagulase test, Slidex Staph plus test and tube coagulase test were compared and analysing the presence of *coa* gene by PCR was used as gold standard for

detection of *S. aureus* and tube coagulase test was recommended as routine test to correctly differentiate *S. aureus* from coagulase negative Staphylococci (Tiwari et al., 2008). However, it is important that coagulase negative strains of *S. aureus* have been reported. In these studies, the isolates gave negative reaction to tube coagulase test but they all carried *coa* gene when amplified with PCR (Vandenesch et al., 1994; Akineden et al., 2011). PCR amplification of *nuc* gene has been used as gold standard for the detection of *S.aureus* in many studies (Hein et al., 2001, Alarcón et al., 2006, Esan et al., 2009).

For confirmation of the biochemical and genotypic test results *S. aureus* isolates, 19 isolates were chosen among the isolates that gave negative results to some of the biochemical tests especially tube coagulase test. These isolates were tested for the presence of *coa* gene by PCR reaction and *nuc* gene by qPCR analysis.

In this study, the results showed that 3 isolates that tested negative in tube coagulase test were positive for the *coa* gene. Also 2 isolates that were tube coagulase negative had thermostable nuclease gene (*nuc*). These isolates gave positive reaction to slide coagulase test. In a previous study, 93 *S. aureus* isolates were investigated for the presence of *nuc* and *coa* genes with enterotoxin genes in comparison to coagulase and thermostable nuclease biochemical tests. Only one isolate showed lack of correlation between these tests and presence of these target genes. Although the coagulase and thermostable tests were negative, they express *coa* and *nuc* genes and they were not enterotoxigenic strains (Cremonesi et al., 2005). The tube coagulase test was regarded as the basic identification test for *S. aureus* but the test results are interpretable and positive results may turn to negative after overnight incubation.

7.4. Sequence Analyses of *S. aureus* Isolates

The sequence analyses were performed to clarify these biochemically and genotypically conflicting results for identification of *S. aureus*. 16S rRNA sequence typing was carried out with the total of 25 isolates. Phylogenetic analysis was performed by maximum likelihood method. Table 7.7. shows the sequence analysis results of the isolates

Table 7.7. Sequence analyses results of the *Staphylococcus* isolates

Sample code	Sequence Results	Sample code	Sequence Results
1	*S. aureus (94%)*	13	*S. aureus (83%)*
2	*S. aureus (98%)*	14	*S. carnosus (85%)*
3	*S. aureus (90%)*	15	*S. xylosus (88%)*
4	*S.pasteuri (85%)*	16	*Uncultured bacterium(91%)*
5	*S. saprophyticus (83%)*	17	*S. sciuri (83%)*
6	*Staphylococcus spp. (97%)*	18	*S. carnosus (81%)*
7	*S. epidermidis (99%)*	19	*S. saprophyticus (86%)*
8	*Macrococcus spp. (92%)*	20	*S. equorum (81%)*
9	*Staphylococcus spp. (87%)*	21	*S. carnosus (99%)*
10	*Staphylococcus spp. (93%)*	22	*S. xylosus (98%)*
11	*S. carnosus (86%)*	23	*S. saprohyticus (88%)*
12	*S. carnosus (99%)*	24	*Staphylococcus spp (84%).*
PC	*S. aureus (97%)*		

In Figure 7.19, the distance tree was shown with the relative position of some strains of *Staphylococcus* on the basis of 16S ribosomal gene sequence similarity. Strains were identified as *S. aureus* and other *Staphylococcus s*pp. The 16S ribosomal gene sequences of *S. hyicus* KC212040 and *S. intermedius* EU157209 strains were downloaded from NCBI website. These strains were grouped separately from our isolates.

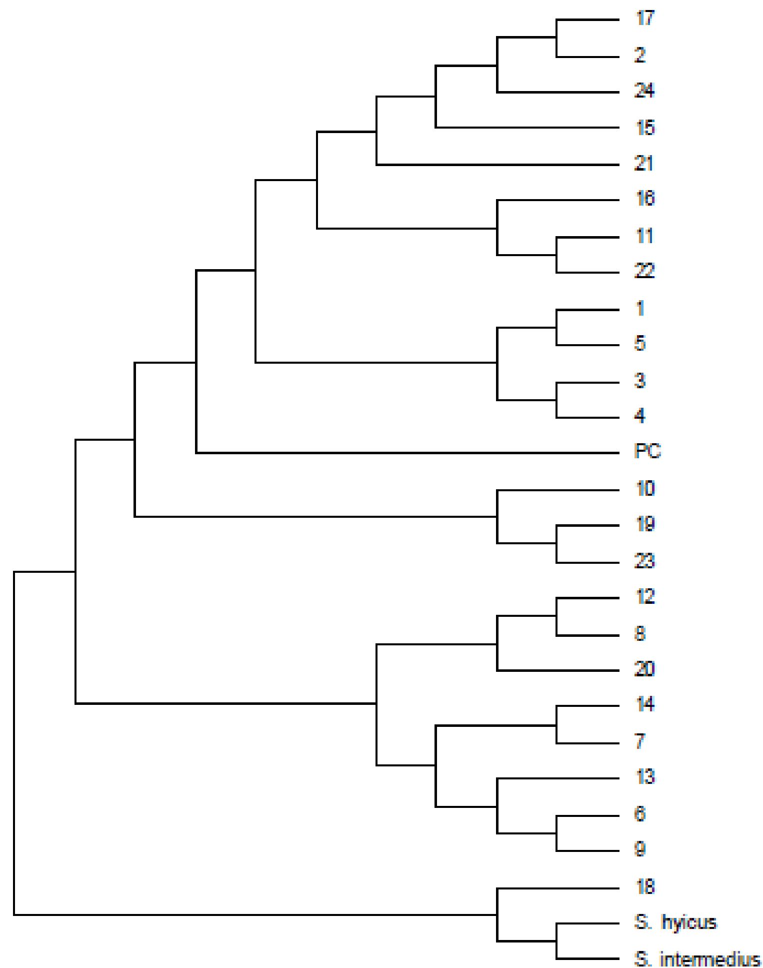

Figure 7.19. The distance tree showing the relative position of 24 isolates based on ribosomal gene sequence similarity.

Previous studies were reported implicating the importance of 16S rRNA sequencing for the identification of *S. aureus*. In one of these studies, 16S rRNA sequencing with *nuc* and *coa* gene amplification was used for identification of *S. aureus* genotypically among 219 *Staphylococcus* sp. isolated from food samples (Sudagidan and Aydin 2010). In another study, the PCR amplification of 16S rRNA with *nuc* gene was utilized to confirm the coagulase test results (Asfour and Darwish, 2011). Akineden et al. (2011) reported that 16S rRNA sequencing and amplification of *coa* gene are required for identification of *S. aureus* strains isolated from milk samples of a dairy cow with subclinical mastitis despite their negative reaction to coagulase test.

CHAPTER 8

CONCLUSIONS

In this study, qPCR method was utilized for direct quantitative detection of *S. aureus* by targeting the *nuc* gene of *S. aureus* in Turkish white cheese samples. The qPCR analyses were optimized by using *S. aureus* pure culture. TaqMan and LNA probe-based qPCR analyses were compared by targeting the *nuc* gene of *S. aureus* cells to improve the performance of qPCR analyses to quantify low levels of *S. aureus* cells.

TaqMan probe-based assay was found to quantify down to 5.88 *S. aureus* cell number. LNA probe-based assay was found to be 100 times less senssitive than TaqMan probe-based qPCR in pure culture studies. In parallel to pure culture studies, the sensitivity of TaqMan probe-based assay was found to be higher than LNA probe-based assay in artificially contaminated cheese samples performed to authenticate the real contamination case.

Four different methods were used to extract the bacterial DNA from white cheese. The most sensitive bacterial DNA extraction method was found to be the newly combined method of trypsin and the food DNA extraction kit for quantification of *S. aureus* cells. The detection range of *S. aureus* quantification was improved down to 13.2 CFU/g with the combined DNA extraction method of using trypsin and the food DNA extraction kit by using TaqMan probe based qPCR study in artificially contaminated cheese samples. In the naturally contaminated cheese samples, the bacterial numbers calculated by the qPCR method were higher than the plate counts which can be related to detection of live and dead cells together in qPCR analyses.

In the identification part of the study, 207 pure cultures were obtained and characterized phenotypically and genotypically. Out of the 207 isolated *S. aureus* colonies, 24 isolates were further studied for tube coagulase reaction and 3 isolates were tested positive. These 3 isolates were found to carry 23S rDNA, *coa*, *clf*, *spaX*, *spaIgG* and *nuc* target regions in PCR analyses. The 3 of the 5 isolates were identified to be *S. aureus* based on 16S rDNA sequencing. They were also tested positive for DNase reaction. Therefore, it is concluded that DNase test should be carried out in correlation with the presence of *nuc* gene for accurate identification of *S. aureus*.

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APPENDIX A

CHEMICALS USED IN THE EXPERIMENTS

Table A1 Chemicals Used in Experiments

No	Chemical	Code
1	Baird Parker Agar	BD-Difco 276840
2	Egg Yolk Tellurite	BBL 212357
3	Nutrient Broth	Merck 1.05443.0500
4	Tryptic Soy Broth	Merck 1.05459.0500
5	Crystal Violet	Sigma C3886
6	Potassium iodide	Sigma C6757
7	Safranine O	Fluka FL 84120
8	Hydrogen Peroxide	Merck 1.07209.1000
9	DNase Test Agar	BBL 211179
10	HCl	Riedel-deHaen 07102
11	Rapid Latex Test Kit	Staphaurex
12	Coagulase Plasma	BBL 240826
13	Mannitol Salt Agar	BD 211407
14	Lysostaphin	Sigma L7386
15	Proteinase K from Tritirachium album	Sigma-Aldrich P2308
16	Trizma Base	Sigma T6066
17	KCl	Amresco 0395
18	MgCl2	Amresco J364
19	dNTP	Fermentas R0182
20	Taq DNA polymease	Fermentas EP0402
21	Tris-Acetate	Amresco 0189
22	Na_2EDTA	Amresco 0105
23	Guanidine thiocyanate	Amresco 0380
24	N-Laurylsarcosine	Sigma-Aldrich L5777
25	SDS	Merck 8.17034

26	Sodium Phosphate Monobasic	Riedel-deHaen 04270
27	Sodium Phosphate Dibasic Dihydrate	Riedel-deHaen 04272
28	Sodium Acetate	Merck 1.06268
29	EDTA	Fluka 03685
30	Phenol-choloroform-isoamyl alcohol	Amresco K169
31	Choloroform	Applichem A3633
32	Isoamylalcohol	Applichem A2610
33	Ethanol	Sigma Aldrich 32221
34	Trypsin	Sigma Aldrich
35	10x Taq Buffer $(NH4)_2SO4$ (-$MgCl_2$)	Fermentas 00044197
36	Betaine	Sigma B2629
38	Bsm DNA Polymerase	Thermo Scientific EP0691
39	MgSO4	Amresco E541
40	Redsafe Dye	Chembio 21141

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ABSTRACTS, POSTERS, AND PRESENTATIONs (First Author)

- Kadiroğlu, P., F. Korel, Ç. Ceylan, TaqMan Probes based RT-PCR Method for the Quantitative Detection of Staphylococcus aureus in Traditional Cheese Samples of İzmir District, 23rd International ICFMH Symposium, 389, September 3-7, İstanbul, Turkey, 2012.
- Kadiroğlu, P., Korel, F., Quantitative detection of Staphylococcus aureus using real time-polymerase chain reaction method, 7. Food Engineering Congress, 196, November 24-26, Ankara, 2011.
- Kadiroğlu, P., Korel, F., Classification of Turkish extra virgin olive oils based on their volatile profiles using SPME-GC-MS in combination with chemometrics during storage, 5th International Symposium on Recent Advances in Food Analysis, 192, November 1-4, Prague, Czech Republic, 2011.
- Kadiroglu, P., Korel, F., Tokatlı, F. Discrimination of extra virgin olive oils using surface acoustic wave sensing electronic nose, 2nd International Congress on Food and Nutrition, October 24-26, Istanbul, Turkey, 2007.
- Kadiroğlu, P., Ocakoğlu, D., Gürdeniz, G., Özen, F.B., Tokatlı, F., Korel, F., Aroma fingerprints of Turkish extra virgin olive oils using surface acoustic wave sensing electronic nose, 4th Euro Fed Lipid Congress, 481, October 1-4, Madrid, Spain, 2006.