Global Journal of Biotechnology & Biochemistry 6 (4): 171-177, 2011
ISSN 2078-466X

Detection of *Staphylococcus aureus* in Bovine Milk and Its Product by Real Time PCR Assay

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Abstract: The SYBR Green RT-PCR assay is a useful diagnostic tool for quick, high throughput and reliable routine screening of *S. aureus* isolates. Moreover, the SYBR Green based quantitative detection of these pathogens in raw milk could remarkably contribute to clarify their actual role in Staphylococcal food poisoning and other clinical syndromes associated with the consumption of milk and milk-based products. In this work the incidences of *Staphylococcus aureus* in Kareish cheese, ice-cream and raw milk by using the traditional methods were 68, 50 and 40%, respectively and the lowest percentage was detected from yoghurt (14%). Real-time PCR assay was used to provide a rapid and sensitive method for the specific detection of *Staphylococcus aureus* in milk and its product. The melting point to *Staphylococcus aureus* was 83.4°C.

Key words: Real time PCR · Yoghurt · Kareish · Ice-cream · Raw milk · Melting point · SYBER Green

INTRODUCTION

Milk has long been referred to as the most perfect food for human from birth to senility it contains all the nutrients required for rapid growth and healthy development of the body. Kareish cheese and ice-cream are considered the most popular Egyptian dairy products. Their manufacture and handling techniques in Egyptian markets are still primitive and unhygienic [1, 2]. Many contaminants find their way to raw milk, from which they gain access to dairy products [3-7].

Milk and its products have been shown to be an ideal media for growth and multiplication of many microorganisms including staphylococci. They are common vehicles for staphylococcal food poisoning [8, 9]. *Staphylococcus aureus* infection is estimated to be present in up to 90% of dairy farms and is responsible for 35% of the economic loss in the dairy industry. *S. aureus* is a facultatively anaerobic Gram-positive bacterium. The majority of *S. aureus* strains are catalase-positive and coagulase-positive, which forms the basis of traditional identification methodology.

Staphylococcal food poisoning is a syndrome characterized by nausea, vomiting, diarrhea, general malaise and weakness. Such symptoms appear within 2-4 hours post -ingestion of contaminated food. Although the illness is seldom fatal, complications including dehydration and shock, can accompany severe attacks. *Staphylococcus aureus* is one of the most common agents in bacterial food poisoning outbreaks. It is also a major causative pathogen of clinical or subclinical mastitis of dairy domestic ruminants [10]. Rapid and reliable methods for detection of this microorganism in milk and other foods are needed. When primer set was used for the real-time PCR detection of *S. aureus* in milk samples without the pre enrichment step, samples with target cell numbers greater than 10(3)CFU/ml or CFU/g could be detected, indicating the potential quantitative ability of this real-time PCR assay. With a 10-h pre enrichment step, however, a detection limit of 1 CFU/ml or CFU/g could be obtained [11]. The real-time PCR assay does not provide false-positive results [12].

The introduction of real-time PCR provides the opportunity for the rapid detection of pathogens in food and clinical settings. Apart from saving time, real-time PCR is highly specific, sensitive and offers the potential for quantification [13]. The risk of cross-contamination is significantly reduced and high-throughput performance and automation are possible since no post-PCR manipulations are required. The aims of the present study

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Table 1: Types of the collected samples.

Samples	Number
Milk	50
Kareish cheese	50
Yoghurt	50
ice cream	50
Total	200

were to determine the incidence of *Staphylococcus aureus* in bovine milk and its products by using the traditional methods and to use real time PCR for confirmation of the traditional methods.

MATERIALS AND METHODS

Collection of Samples: As show in Table (1) two hundred samples of bovine milk and its product (Kareish cheese, Yoghurt and ice cream) were collected from different supermarkets at Cairo, Qaluopia and Port- said under aseptic conditions. The samples were immediately sent in an ice box to the laboratory.

Bacteriological Culture: Ten µl of each sample was streaked on to a 5% selective sheep blood agar containing 15mg/l of nalidixic acid and 10mg/l of colistin [14]. The plates were incubated for 48 hours at 37°C. After this time a smear was prepared from colonies and stained by Gram method. Colonies that had Gram- positive cocci were examined by catalase test to be processed for *S. aureus* diagnosis. These isolates were cultured on Baired Parker and mannitol salt agar media and the suspected colonies were identified according to [14].

Real -Time PCR

DNA Extraction: Extraction of DNA from milk samples Genomic DNA was extracted from milk according to the procedure described for blood [15], with some modifications. Forty ml of raw milk was centrifuged at 4°C for 30 min at 2,000 × g. The fat layer and the supernatant were discarded. The pellet was resuspended in 1 ml of PBS, pH 7.4 and centrifuged at 4°C for 10 min at 400 × g. The pellet was then resuspended in 50 ml of lysis buffer (0.32 *M* sucrose, 10 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$ and 1% Triton X-100). The nuclei pellet was centrifuged at 2,465 × g for 10 min at 4°C and washed twice with 10 ml of 0.075 *M* NaCl, 0.025 *M* EDTA. The pellet was then resuspended in 30 ml of 10 m*M* Tris-HCl, pH 8.0 and 2 m*M* EDTA; then, 100 *µl* of 10% SDS and 40 *µl* of proteinase K (10 mg/ml) were added. The resulting nuclear lysate was incubated at 65°C for 1 h. After incubation, 500 all of 5 *M* NaCl was added and the precipitated proteins were centrifuged at 1,811 × g for 10 min. The aqueous layer was recovered and DNA was precipitated after addition of 6 ml of isopropanol and centrifugation for 10 min at 1,811 × g. The resulting pellet was dried at room temperature and then redissolved in 500 *µl* of Tris-EDTA (10 m*M* Tris, 1 m*M* EDTA, pH 7.5). Total DNA yield ranged from 2.12 to 610.12 *µ*g per milk sample. The quality and quantity of DNA extracted were measured by spectro photometric absorption of UV light at 260 and 280 nm.

Extraction of DNA from Kareish Cheese: Five grams of Kareish cheese was added to 45 ml of digestion buffer diluted 1:10 and containing 12 U of pronase (Fluka Chemie, Buchs, Switzerland) per ml, homogenized in a Laboratory Blender Stomacher 400 (Seward, London, United Kingdom) for 1 min and subsequently incubated at 40°C for 3 h. After centrifugation at 5,700 × g for 15 min at 4°C, the fat layer and aqueous phase were discarded and the pellet was washed three times with TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]) and once with PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl [pH 8.4]). Finally, the pellet was resolved in 250 µl (Camembert) of PCR buffer and 143 U of lysostaphin (Sigma-Aldrich GmbH, Steinheim, Germany) per ml was added. This solution was incubated at room temperature for 15 min. After addition of 8 U of proteinase K (Roche Diagnostics GmbH) per ml, samples were held at 60°C for 1 h and afterwards incubated for 15 min at 95°C. Cell debris was removed by centrifugation at 13,000 × g for 5 min. Five micro liters of the supernatant was subjected to RT-PCR.

Extraction of DNA from Yoghurt Samples: Total DNA of yoghurt samples was extracted as carried by [mention author (s) [16] with modifications. Probiotic yogurt samples of 25 ml were diluted in 225 ml of sterile PBS (pH 7.4). One milliliter of 10-fold diluted probiotic yogurt samples was mixed with 100 µl 18% (w/v) sodium citrate and 50 µl 1 m-NaOH at room temperature, then the mixture was centrifuged at 6600×g for 10 min. The pellet was washed in PBS buffer. The washed pellet was resuspended in 100 µl MilliQ water and mixed with 100 µl 2% (w/v) Triton X-100 (Sigma). This mixture was heated at 100°C for 10 min, then immediately cooled in an ice-water and centrifuged at 6600×g for 10 min and the supernatant was stored at -20°C until its use.

Extraction of DNA from Ice Cream: The ice -cream samples were melted and DNA was extracted according to mention author (s)[15].

Real-Time PCR Amplification: Boiling was carried out to all positive and negative samples detected by traditional methods. Oligonucleotides primers were designed against a conserved region of the bacterial 16S gene as follows: the forward primer 5'-cga aag cgt ggg gat caa ac-3' and the reverse primer 5'-ccc agg cgg agt gct taa tg -3'. The expected amplicon size was 125 bp. Using Fermentas SYBR Green q PCR MM.ROX 1.25ML (K0251) program, The real-time PCR conditions consisted of an initial step of 95°C for 10 minutes followed by an amplification program for 40 cycles of 3 seconds at 95°C, 5 seconds at 61°C, 20 seconds at 72°C with fluorescence acquisition at the end of each extension. The amplification program was immediately followed by a melt program consisting of 60 seconds at 95°C, 60 seconds at 65°C and a gradual increase to 90°C at a rate of 0.2°C/sec with fluorescence acquisition at each temperature transition according to mention author (s) [17, 18]. The primers used for the SYBR green to detection of the strains were selected in to give optimal results when combined with the primers specific for the *S. aureus*. When SYBR Green is used, the increase in fluorescence emission is due to binding of the dye to dsDNA. The identification of the PCR product was performed by determining the melting temperature (T_m) of the amplicon after PCR.

RESULTS

Bacteriological Results: Using the traditional methods the highest incidence of *Staphylococcus aureus* was detected from kareish cheese while the lowest incidence was detected in yogurt (68 and 14%), respectively. In ice cream samples the incidence of *Staphylococcus aureus* reached to 50% while in raw milk the incidence of *Staphylococcus aureus* was 40% (Table 2).

Real Time PCR Results: Fig. 1 shows that The melting point for *S aureus* ranged from 82.4°C to 83.9°C for all samples. if we make imagine line from the highest peak to the button we can detected that the *Staphylococcus aureus* melting point at 83.4°C slightly range above or low.

Fig. 2 displays that the amplification plots appeared from the 18 to 24 cycles. The samples with amplification appeared at 18 cycles contained great amount of DNA while the samples with the amplification appeared at 24 cycles contained lower quantity of DNA so the amplification appeared later, samples were negative by traditional methods.

Table 2: Incidence of *Staphylococcus aureus* in examined samples by traditional methods

Type of samples	No. of examined samples	No. of + ve samples	% of + ve samples
Raw milk	50	20	40 %
Kareish cheese	50	34	68 %
Yoghurt	50	7	14 %
Ice-cream	50	25	50 %

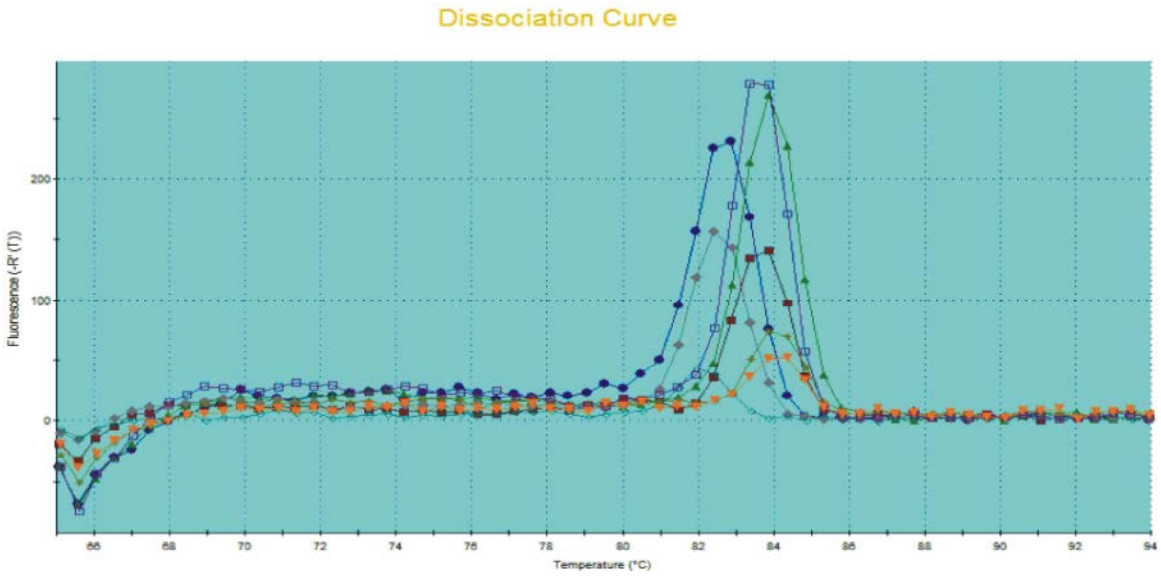

Fig. 1: Dissociation curve of all samples used after boiling

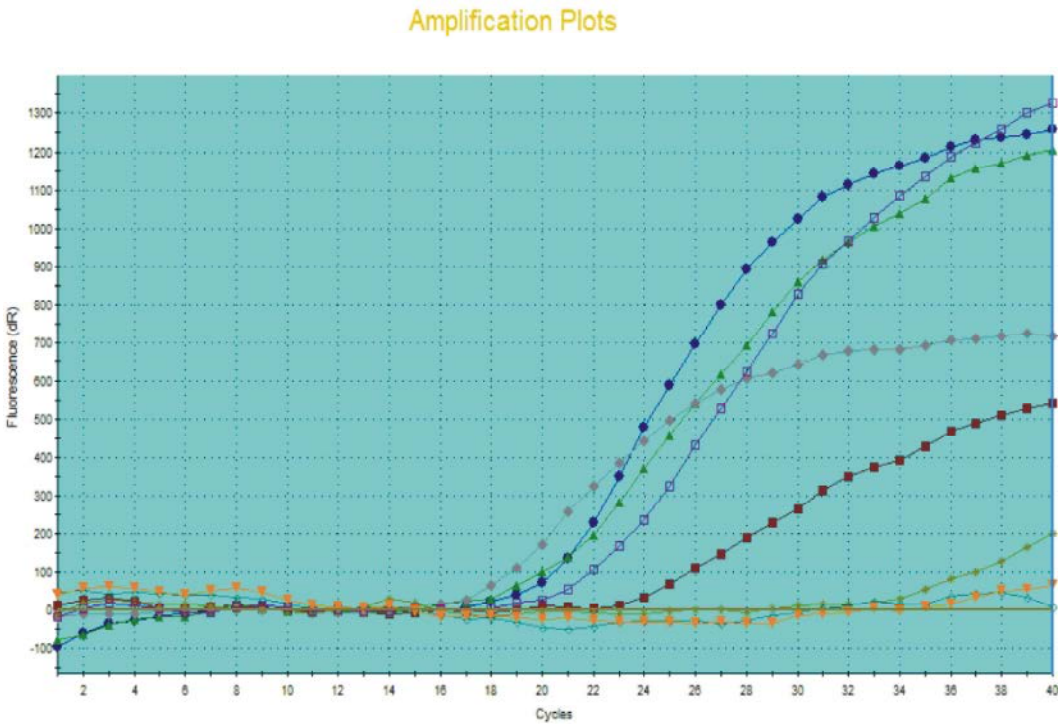

Fig. 2: The amplification plots for all samples used after boiling

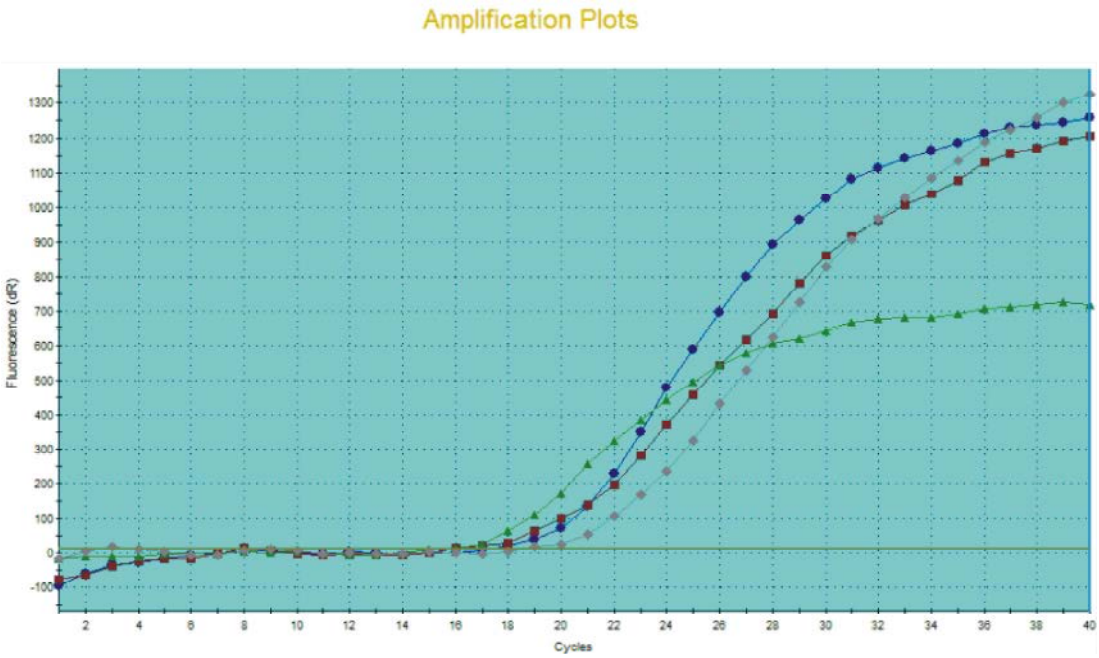

Fig. 3: The amplification plots of positive samples detected by the traditional methods appearing at 18 cycles.

----- ● ------ Kareish cheese ----- ■ ----- Raw milk
----- ◆ ------ Yoghurt ----- ▲ ----- Ice -cream

The amount of DNA in samples positive by traditional methods was great enough so that the amplification began in appearance at 18 cycles

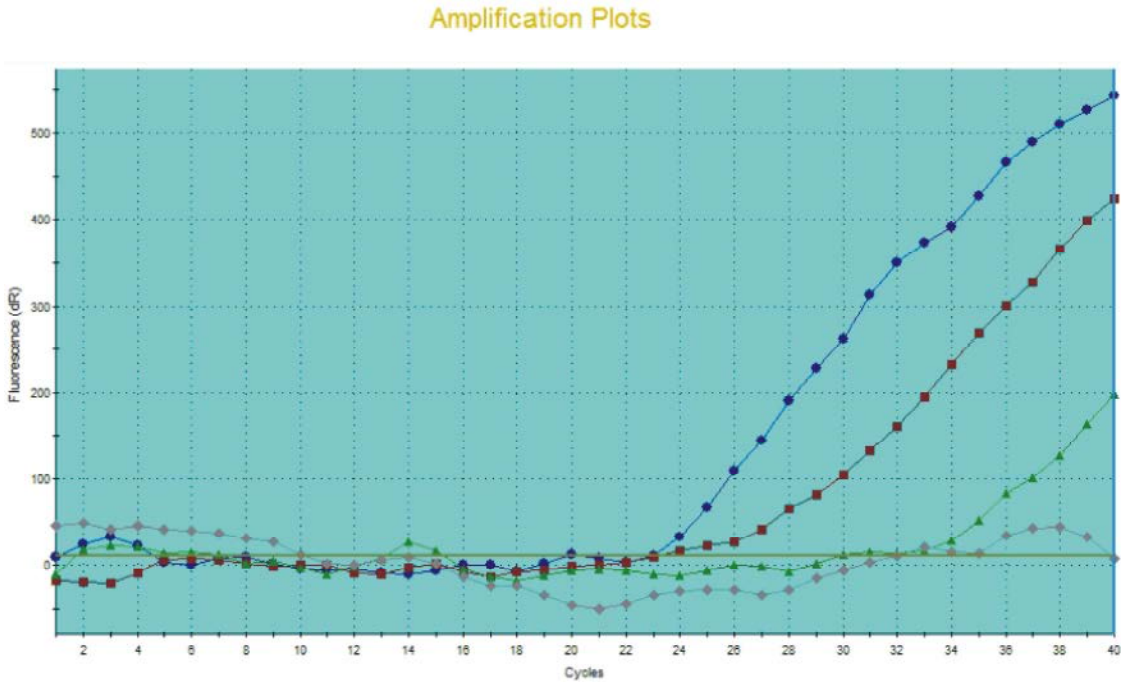

Fig. 4: The amplification plots of negative samples detected by the traditional methods

----- ● ------ Kareish cheese ----- ■ ----- Raw milk
----- ◆ ------ Yoghurt ----- ▲ ----- Ice -cream

From Fig. 4 we can see the appearance of amplification plots at 24 cycles as the amount of DNA was low so the amplification appeared at late cycles. i.e this samples contain *Staphylococcus aureus* but can not detected by the traditional methods because it low amount but it appear after using the real time PCR because it more sensitive than the traditional methods.

The amplification plots appear at cycles according to the amount of DNA which present in examined samples.

DISCUSSION

In the current study, *Staphylococcus aureus* was detected in 68% of examined Kareish cheese samples. This result is higher than that reported by mention author (s) [19, 20] who reported that the prevalence rate of *Staphylococcus aureus* was 10 % in fresh Kareish cheese samples. In addition, mention author (s) [21] reported that *Staphylococcus aureus* was present in 28% of the examined Kareish cheese samples. *Staphylococcus aureus* was detected in 50% of examined ice cream samples. Mention author(s) [20]. The high incidence of *Staphylococcus aureus* is indicative of poor hygienic measures during production, handling and distribution [4, 22]. The difference in the prevalence rates of *S. aureus* between the examined products may originate from the method of manufacture, storage and handling. Kareish cheese is made by farmers from raw milk that is not subjected to heat treatment. Street vendors put Kareish cheese in pans exposed to dust and flies. The lowest prevalence rate (14%) of *S. aureus* which was recorded in yogurt might be attributed to the effect of heating and then freezing during its manufacture which inhibits the multiplication of this microorganism and kills the microorganism.

The SYBR Green RT-PCR assay is a useful diagnostic tool for quick, high throughput and reliable routine screening of *S. aureus* isolates. Moreover, the SYBR Green based quantitative detection of these pathogens in raw milk and milk products could remarkably contribute to clarify their actual role in staphylococcal food poisoning and other clinical syndromes associated with the consumption of milk and milk-based products. [23], particularly with the development of a real-time PCR that detect more *S. aureus* than from the culture [24]. It is considered that *S. aureus* species specific real time PCR is useful for speeding up identification of *S. aureus* by replacing the current biochemical phenotypic schemes

which are time consuming. Additionally, if appropriate conditions are established, direct PCR identification of *S. aureus* from food and clinical specimens can be performed [25].

The results revealed out that Egyptian Kareish cheese and ice cream products sold in Cairo, Qaluobia and Port- Said city markets are contaminated with *S. aureus* which constitute public health hazards to consumers. Periodical examination of dairy products to ensure safety for consumers must be practiced. Adoption of reward and punishment policy may help to improve their hygienic standards. Overall, Good quality raw materials used in product processing, adoption of good manufactured practices and strict personal hygiene are recommended to ensure safety and high quality of the dairy products.

We have introduced a SYBR green real-time PCR assay for the fast (Isolate and detect *S. aureus* in under 4 hours) and conclusive identification of *Staphylococcus aureus*. The SYBR green represents an economically interesting alternative for the analysis of a large number of samples. And the melt curves do not overlap. Real-time PCR assays can be automated and are sensitive and rapid. From the samples directly and by using a recent technique we can detect the *Staphylococcus aureus* from milk and its products without using the traditional methods.

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