3.1 Introduction

Enterotoxin-producing *Staphylococcus aureus* is the most common cause of food-borne human illness throughout the world (Do Carmo *et al.* 2004; Le Loir *et al.* 2003). Staphylococcal enterotoxins (SEs) are the common agents and primary cause of food poisoning all over the world (Balaban and Rasooly 2000; Greenfield *et al.* 2002; Smyth *et al.* 2005). Enterotoxigenic strains of *S. aureus* have been reported to cause a number of diseases or food poisoning outbreaks because of the ingestion of contaminated dairy products or milk. Milk is a good substrate for *S. aureus* growth and enterotoxin production. In addition, enterotoxins retain their biological activity even after pasteurization (Asao *et al.* 2003). *Staphylococcus aureus* is able to grow in a wide range of temperatures 7 °C to 48.5 °C with an optimum of 30 °C to 37 °C (Schmitt *et al.* 1990), pH 4.2 to 9.3, with an optimum of 7 to 7.5 (Bergdoll 1989) and sodium chloride concentrations (up to 15%). These characteristics enable *S. aureus* to grow in a wide variety of foods. Many reports have also shown the prevalence of staphylococcus in other food stuffs (Ghosh *et al.* 2007; Warke *et al.* 2000; Soriano *et al.* 2002). The development of rapid, sensitive and infallible methods of detecting food-borne pathogens has received much impetus in the recent years owing to an increased public awareness of the health hazards associated with microbial contamination of food. Conventional methods of detecting bacteria in food comprises of propagation in selective enrichment media followed by microbiological and biochemical tests, which are cumbersome and time-consuming. The advent of nucleic-acid based assay systems like the polymerase chain reaction (PCR) has led to the emergence of improved, expedient and reliable methods of microbial identification and surveillance, capable of even detecting nonviable cells (Scheu *et al.* 1998; Josephson *et al.* 1993). The direct detection of pathogenic bacteria in food samples is a challenging task, hampered by the presence of PCR-inhibitory substances frequently associated with enrichment media, DNA isolation reagents and the food matrix itself and additionally compounded by the presence of high numbers of indigenous microflora (Rossen *et al.* 1992; Wilson 1997). PCR has been often experimented in milk for the direct detection of *S. aureus* (Ramesh *et al.* 2002; Cremonesi *et al.* 2006; Nakayama *et al.* 2006). Loop mediated isothermal amplification (LAMP) developed by Notomi *et al.* (2000) is simple, rapid, specific, sensitive and cost-effective. LAMP exhibits less sensitivity to inhibitory substances present in biological samples than PCR (Kaneko *et al.* 2007). Robustness of LAMP
against inhibitors can constitute a potentially valuable tool for rapid diagnosis of food-borne pathogens in food safety analysis. In the light of serious health related and economic implications caused by enterotoxigenic staphylococcus the objective of the present study was to optimize a simple, rapid and sensitive DNA extraction method directly from spiked milk and other food samples. PCR and LAMP methods were used to detect *S. aureus* directly from food samples obviating a pre-enrichment step; and sensitivity of both methods was compared. In addition in mixed culture systems (*S. aureus* and *Yersinia enterocolitica*) also sensitivity of both the methods were evaluated. Emerging pathogens like *Yersinia enterocolitica* are capable of surviving and growing in milk stored at low temperature (Stern *et al.* 1980) and has been associated with outbreaks of milk borne diseases (Black *et al.* 1978; Tacket *et al.* 1984). Studies have been made with many gram +ve and gram –ve bacteria. To cite an example with gram –ve bacteria *Yersinia enterocolitica* has been provided. In the present study, the use of Triton X - 100 for the extraction of DNA found to be cost-effective over other methods reported so far, wherein costlier reagents like guanidium thiocyanate (Cremonesi *et al.* 2006), paramagnetic nanoparticle (Amagliani *et al.* 2004), combination of organic solvents (diethyl ether), detergents (SDS), alkali (NaOH) (Ramesh *et al.* 2002) and petroleum ether (Nakayama *et al.* 2006) etc., were being used.

3.2 Materials

3.2.1. Molecular reagents and chemicals

All the glass wares used in the present investigation were sterilized by autoclaving at 121 °C for 20 min. Gamma-irradiated sterile disposable petriplates (HiMedia laboratories, Mumbai, India) were used in the present study. All the media used in this study were dehydrated culture media procured from HiMedia laboratories, Mumbai, India. The media were prepared according to the manufacturer’s instructions and sterilized by autoclaving at 121 °C for 20 min. The water used in the experimental trials was Mili-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA). Taq DNA polymerases, dNTP’s, 100 bp, 500 bp DNA marker, 6x loading buffer were purchased from Bangalore genei, Bangalore. Sybr Green I, Betaine was purchased, from Sigma Aldrich chemical company, Bangalore. Bst DNA polymerase was purchased from New England Biolabs. Triton X – 100 was purchased from HiMedia laboratories, Mumbai, India. Agarose, EDTA, Tris-HCl, Ethidium bromide
were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. Company, Bangalore.

3.2.2 Food samples

Food samples like raw milk, khoa, milk burfi, sugar cane juice and cooked rice (Twenty five) from five different sources were collected from the local markets. Samples were procured from local market in Mysore city, Karnataka, India and collected under sterile conditions and brought to the laboratory within 60 min of collection and subjected to analysis.

3.2.3 Reference cultures culture condition and preparation of cell suspension

The reference cultures included *Staphylococcus aureus* MTCC 96, *Yersinia enterocolitica* MTCC 859 were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. All the strains were maintained at 4 °C on BHI agar slants and sub cultured at regular intervals of 15 days. *S. aureus* and *Y. enterocolitica* were grown in 10 ml BHI broth at 37 °C and 32 °C respectively for 16 h at 150 rpm in an orbital shaker. The cells were harvested by centrifugation at 8000 x g for 20 min at 4 °C and resuspended in sterile saline prior to being used in the experiment.

3.2.4 Bacteriological media

Baird parker agar, BHI broth, BHI agar, PCA agar and were prepared as described under 1A.2.6.1 to 1A.2.6.4 and 2.2.6.1.

3.2.5 Diluent

Prepared as described under 1A.2.4.

3.2.6 Triton X -100 solutions

0.01, 0.1, 1, 2, 5 and 10% Triton X – 100 were prepared using sterile distilled water.

3.2.6.1 Lysis buffer

100 mM Tris – HCl (pH 8.5), 5 mM EDTA, 0.2% SDS and 200 mM NaCl. The buffer was autoclaved at 121 °C for 15 min and stored at 4 °C.
3.2.7 Primers

All the PCR and LAMP primers were synthesized from Sigma Aldrich, Pvt, Ltd, Bangalore, India.

3.2.8 \textit{entA}, \textit{entB}, \textit{entC} and \textit{entD} gene PCR primers

Oligonucleotide primers for the specific PCR amplification of \textit{entA}, \textit{entB}, \textit{entC} and \textit{entD} used in this study are listed in the Table 1A.2

3.2.9 \textit{entA}, \textit{entB}, \textit{entC} and \textit{entD} gene LAMP primers

Oligonucleotide primers for the specific LAMP amplification of \textit{entA}, \textit{entB}, \textit{entC} and \textit{entD} used in this study are listed in the Table 2.1 (2.2.8.3).

3.2.10 The reaction components for PCR amplification

The reaction components for PCR amplification was used as under 1A.2.7.17 and 1A.2.7.17a

3.2.11 LAMP reaction components

The reaction components for LAMP amplification was used as mentioned under Table 2.2 and Table 2.3 (2.2.10).

3.3 Methods

3.3.1 Sample preparation for milk, khoa, milk burfi, sugarcane juice and rice

Pasteurized steamed milk samples, khoa prepared by under constant stirring of milk in low flame, food samples (milk burfi, sugarcane juice and rice) from local market, Mysore, India were tested to ensure that there were no \textit{S. aureus} prior to inoculation.

3.3.2 Optimization of lysis of whole cells of \textit{S. aureus} for the isolation of genomic DNA from spiked milk sample.

3.3.2.1 Optimization of Triton X-100 concentration for the lysis of \textit{S. aureus} cells in spiked milk sample

Pasteurized milk samples were procured from the local retail outlets and steamed for 30 min prior to use. Saline suspensions (one ml) having known
concentration of *S. aureus* at $10^4$ cfu was spiked into 0.4 ml of milk samples in replicates. All the samples were vortexed and allowed to settle for 30 min at room temperature. Spiked milk samples were centrifuged at 2000 x g for two min at 4 °C; supernatant was recentrifuged at 8000 X g for 10 min at 4 °C; to the pellet different concentrations of 100 µl of Triton X-100 (0.01, 0.1, 1, 2, 5 and 10%) were added and boiled for 10 min.

### 3.3.2.2 Optimization of boiling time for the lysis of *S. aureus* cells in spiked milk sample

To another set of milk samples, spiking and centrifugation was carried out following above procedure. DNA isolation was carried out by lysing the pellet by adding 100 µl of 1% Triton X-100 and boiled at different time intervals (5, 10, 15, 30 and 45 min).

### 3.3.2.3 Effect of Triton X-100 method on recovery of DNA in spiked milk samples

Saline suspensions (1 ml) having known concentration of *S. aureus* in the range of $10^1$ - $10^{11}$ cfu were added individually to replicates of 0.4 ml of milk samples. Pellet was boiled by adding 100 µl of 1% Triton X-100 for 10 min. For each experiment control was maintained. After lysis, DNA was determined by spectrophotometric method.

### 3.3.3 Rapid lysis method (Dickinson *et al.* 1995) for the isolation of genomic DNA of *S. aureus* from milk sample

Pasteurized milk samples were procured from the local retail outlets and steamed for 30 min prior to use. 1 ml saline suspensions having known concentration of *S. aureus* in the range of $10^1$ - $10^{11}$ cfu were added individually to replicates of 0.4 ml of milk samples. All the samples were vortexed and allowed to stabilize for 30 min at room temperature. Spiked milk samples were centrifuged at 10,000 x g for 15 min at room temperature. The milk sample’s pellet was resuspended in 0.5 ml of lysis buffer (3.2.6.1). Milk sample lysates were incubated at 55 °C for 2 hr or until it solubilised and cooled on ice. For each experiment control was maintained. After lysis, DNA was determined by spectrophotometric method.
3.3.4 Optimization of lysis of whole cells of *S. aureus* for the isolation of genomic DNA from food (khoa, milk burfi, sugarcane juice and cooked rice) samples

3.3.4.1 Optimization of Triton X-100 concentration for the lysis of *S. aureus* cells in spiked food samples

Saline suspensions (1 ml) having known concentration of *S. aureus* at $10^4$ cfu was spiked into 0.4 ml / 0.4 gm of food samples in replicates. All the samples were vortexed and allowed to settle for 30 min at room temperature. Spiked food samples were centrifuged at 2000 x g for two min at 4 °C; supernatant was recentrifuged at 8000 X g for 10 min at 4 °C; to the pellet different concentrations of 100 µl of Triton X-100 (0.01, 0.1, 1, 2, 5 and 10%) was added and boiled for 10 min.

3.3.4.2 Optimization of boiling time for the lysis of *S. aureus* cells in spiked food sample

Food samples were spiked and centrifuged. DNA isolation was carried out by lysing the pellet with the addition of 100 µl of 1% Triton X -100 and boiled at different time intervals (5, 10, 15, 30 and 45 min).

3.3.4.3 Effect of Triton X-100 method on recovery of DNA in spiked food samples

Saline suspensions (1 ml) having known concentration of *S. aureus* in the range of $10^1$ - $10^{11}$ cfu were added individually to replicates of 0.4 ml /0.4 gm of food samples. Pellet was boiled by adding 100 µl of 1% Triton X-100 for 10 min. For each experiment control was maintained. After lysis, DNA was determined by spectrophotometric method.

3.3.5 Rapid lysis method for the isolation of genomic DNA of *S. aureus* from food sample

Saline suspensions (1 ml) having known concentration of *S. aureus* in the range of $10^1$ - $10^{11}$ cfu were added individually to replicates of 0.4 ml/0.4gm of food samples. All the samples were vortexed and allowed to stabilize for 30 min at room temperature. Spiked food samples were centrifuged at 10,000 x g for 15 min at room temperature. All the food sample pellets were resuspended in 0.5 ml of lysis buffer (3.2.6.1). Food samples lysates were incubated at 55 °C for 2 hr or until its solubilised and cooled on ice. For each experiment control was maintained. After lysis, DNA was determined by spectrophotometric method.
3.3.6 Spiking of food samples

Saline (1 ml) suspensions having known concentration of *S. aureus* in the range of $10^8$ - $10^1$ cfu were added individually to 0.4ml or 0.4gm of food samples. In another set of experiment where in the food samples (0.4 ml or 0.4 gm) were mixed with 0.5 ml saline suspensions of each of the bacterial strains (*S. aureus* and *Y. enterocolitica*) of known concentrations in the range of $10^6$ - $10^2$ cfu.

3.3.7 DNA extraction from food samples

To extract bacterial DNA from the spiked food samples, centrifuged sample pellet was boiled in required volume of 1% Triton X-100 for 10 min. This lysate was used for the PCR and LAMP reaction.

3.3.8 Analysis of food samples for the detection of enteroxigenic staphylococci

Twenty five food samples from five different sources were collected from the local market. For the enumeration of *S. aureus*, 1 ml or 1g aliquots of samples were serially diluted to 0.85% saline and appropriate dilutions were plated onto Baird-Parker agar (HiMedia, Mumbai) plates. The plates were incubated at 37 °C for 24 - 48 h and observed for characteristic colony morphology. For the enumeration of total bacterial count, 1ml / 1g aliquots of samples were serially diluted using 0.85% saline and appropriate dilutions were plated onto Plate Count Agar (PCA) (HiMedia, Mumbai) plates. The plates were incubated at 37 °C for 24 h. Aliquots of 1ml /1g of individual food samples were subjected to total DNA extraction using the method as described earlier in 3.3.7. The DNA preparations were used as templates in PCR and LAMP reaction.

3.3.9 PCR reaction and condition for the amplification *entA, entB, entC* and *entD* genes.

For the detection of enterotoxigenic staphylococci directly from food samples, PCR reaction and condition were as mentioned in 2.3.9.

3.3.9.1 Analysis of PCR products by agarose gel electrophoresis

The electrophoresis was performed as in 1A.3.5a
3.3.10 LAMP reaction and condition for the amplification of *entA*, *entB*, *entC* and *entD* genes.

For the detection of enterotoxigenic staphylococci directly from food samples, LAMP reaction and condition was used as mentioned in 2.3.12.

### 3.3.10.1 Analysis of LAMP products by agarose gel electrophoresis

The electrophoresis was performed as in 2.3.10 and 2.3.12.

3.4 Results

3.4.1 Effect of different concentrations of Triton X-100 on *S. aureus* cell lysis in spiked milk samples

To optimize the lysis of whole cells of *S. aureus* for the isolation of genomic DNA from milk sample, different concentrations of Triton X-100, 0.01, 0.1, 1, 2, 5 and 10% were studied separately (3.3.2.1). Among different concentrations of Triton X-100 used 1% Triton X-100 lysed cells effectively and showed the maximum recovery of genomic DNA (Figure 3.1).

**Fig. 3.1 Effect of Triton X-100 concentration on *S. aureus* cell lysis in spiked milk samples**

Different concentrations of Triton X-100 (0.01, 0.1, 1, 2, 5 and 10%) were used for lysis. The cell suspension in Triton X-100 was boiled and the DNA extracted was read spectrophotometrically against respective blanks.

3.4.2 Effect of different boiling periods on *S. aureus* cell lysis in spiked milk samples

Different boiling time intervals of 0, 1, 3, 5, 10, 15, 30 and 45 min were studied separately for the lysis of *S. aureus* cells from milk samples (3.3.2.2). The recovery of
DNA improved with boiling time and maximum recovery was obtained at boiling time of 10 min. The recovery did not improve further with continued boiling (Figure 3.2).

![Graph](image)

**Fig. 3.2** Effect of 1% Triton X-100 concentration at different boiling time intervals on bacterial cell lysis in spiked milk samples

### 3.4.3 Concentrations of *S. aureus* cells and recovery of DNA using Triton X-100 method in spiked milk samples

Different concentrations of *S. aureus* cells were spiked into milk sample for studying the efficacy of the above optimized method in the recovery of DNA from cell lysis (3.3.2.3). The *S. aureus* cells were used at $10^1$-$10^{11}$ cfu. The optimized condition, 1% Triton X-100 and a boiling time of 10 min was used. The recovery of DNA increased with increase in the cell count (Figure 3.3).

![Graph](image)

**Fig. 3.3** Effect of 1% Triton X-100 concentration at the boiling time of 10 min on bacterial cell lysis in spiked milk sample

Saline suspensions having *S. aureus* in the range of $10^{11}$ – $10^1$ cfu were spiked separately to milk samples. DNA extracted was read spectrophotometrically against respective blanks.
3.4.4 Comparison of Triton X-100 method and rapid lysis method on bacterial cell lysis in spiked milk samples

Different concentrations of *S. aureus* cells were spiked into milk sample for studying the efficacy of the above optimized Triton X – 100 method (3.3.2.3) and Rapid lysis method (3.3.3) in the recovery of DNA from cell lysis. The *S. aureus* cells were used at the $10^1$-$10^{11}$ cfu. The recovery of DNA increased with increase in the cell count in both methods. Compared to rapid lysis method, Triton X - 100 methods was found to more effective in maximum recovery of DNA in spiked milk samples (Figure 3.4).

![Figure 3.4 Comparison of Triton X-100 method and Rapid lysis method on bacterial cell lysis in spiked milk samples](image)

Saline suspensions having *S. aureus* in the range of $10^{11}$ – $10^1$ cfu were spiked separately to milk samples. DNA extracted was read spectrophotometrically against respective blanks.

3.4.5 Effect of different concentrations of Triton X-100 on *S. aureus* cell lysis in spiked food samples (khoa, milk burfi, sugarcane juice and cooked rice)

To optimize the lysis of whole cells of *S. aureus* for the isolation of genomic DNA from spiked food sample, different concentrations of Triton X-100, 0.01, 0.1, 1, 2, 5 and 10% were studied separately (3.3.4.1). Among different concentrations of Triton X- 100 used, 1% Triton X-100 lysed cells effectively and showed the maximum recovery of genomic DNA (Figure 3.5).
Different concentrations of Triton X-100 (0.01, 0.1, 1, 2, 5 and 10%) were used for lysis. The cell suspension in Triton X-100 was boiled and the DNA extracted was read spectrophotometrically against respective blanks.

3.4.6 Effect of different boiling periods on S. aureus cell lysis in spiked food samples

Different boiling time intervals of 0, 1, 3, 5, 10, 15, 30 and 45 min were studied separately for the lysis of S. aureus cells from food samples (3.3.4.2). The recovery of DNA improved with boiling time and maximum recovery was obtained at boiling time of 10 min. The recovery did not improve further with continued boiling (Figure 3.6).

![Fig. 3.5 Effect of Triton X-100 concentration on S. aureus cell lysis in spiked food samples](image)

![Fig. 3.6 Effect of 1% Triton X-100 concentration at different boiling time intervals on bacterial cell lysis in spiked food sample](image)
3.4.7 Concentration of *S. aureus* cells and recovery of DNA using Triton X-100 method in spiked food samples

Different concentrations of *S. aureus* cells were spiked into food sample for studying the efficacy of the above optimized method in the recovery of DNA from cell lysis (3.3.4.3). The *S. aureus* cells were used at the $10^1-10^{11}$ cfu. The optimized condition, 1% Triton X-100 and a boiling time of 10 min was used. The recovery of DNA increased with increase in the cell count (Figure 3.7).

![Fig. 3.7 Effect of 1% Triton X-100 concentration at the boiling time of 10 min on bacterial cell lysis in spiked food sample](image)

Saline suspensions having *S. aureus* in the range of $10^{11} – 10^1$ cfu were spiked separately to food samples. DNA extracted was read spectrophotometrically against respective blanks.

3.4.8 Comparison of Triton X-100 method and rapid lysis method on bacterial cell lysis in spiked food samples

Different concentrations of *S. aureus* cells were spiked into food samples for studying the efficacy of the above optimized Triton X – 100 method (3.3.4.3) and rapid lysis method (3.3.5) in the recovery of DNA from cell lysis. The *S. aureus* cells were used at the $10^1-10^{11}$ cfu. The recovery of DNA increased with increase in the cell count in both methods. Compared to Rapid lysis method, Triton X – 100 method was found to more effective in maximum recovery of DNA in spiked milk samples (Figure 3.8).
Saline suspensions having *S. aureus* in the range of $10^{11} - 10^1$ cfu were spiked separately to food samples. DNA extracted was read spectrophotometrically against respective blanks.

### 3.4.9 Sensitivity of PCR and LAMP assays

PCR and LAMP assays using *ent*A, *ent*B, *ent*C and *ent*D primers in conjunction with DNA extracts (3.3.7) of milk, khoa, milk burfi, rice and sugarcane juice having known concentration of *S. aureus* (3.3.6) were carried out. The detection limit of PCR was $10^4$ CFU/g, $10^4$ cfu/ml and LAMP was $10^2$ cfu/g, $10^2$ cfu/ml (Table 3.1). PCR amplification of *ent*C gene using DNA extracts of staphylococcus spiked in milk, khoa and milk burfi is shown in Figure 3.9. LAMP amplification of *ent*C gene using DNA extracts of staphylococcus spiked in milk, khoa, milk burfi, cooked rice and sugarcane juice is shown in Figure 3.10 and Figure 3.11.

### Table 3.1 Comparision of sensitivities of LAMP and PCR reactions for detection of *S.aureus* (*ent*A, *ent*B, *ent*C and *ent*D genes) in spiked food samples

<table>
<thead>
<tr>
<th>Food samples</th>
<th>PCR sensitivity (entA entB entC and entD genes) (cfu g$^{-1}$/cfu ml$^{-1}$)</th>
<th>LAMP sensitivity (entA entB entC and entD genes) (cfu g$^{-1}$/cfu ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^1$</td>
<td>$10^2$</td>
</tr>
<tr>
<td>Milk</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Khoa</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>milkburfi</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cooked rice</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sugarcane juice</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 3.9 PCR based direct detection of *S. aureus* in spiked food samples (milk burfi, sugarcane juice and boiled rice)

Agarose gel electrophoresis of *entC* gene PCR product. Lane M: Molecular size marker; Lanes: 1- $10^3$ cfu g$^{-1}$, 2- $10^5$ cfu g$^{-1}$ (milk burfi); 3- $10^3$ cfu ml$^{-1}$, 4- $10^5$ cfu (sugarcane juice); 5- $10^3$ cfu g$^{-1}$, 6- $10^4$ cfu g$^{-1}$ (boiled rice); 7- control.

Fig. 3.10 LAMP based direct detection of *S. aureus* in spiked food samples (milk burfi, sugarcane juice and boiled rice)

Agarose gel electrophoresis of *Ent C* gene LAMP product. Lanes: M, 100 bp DNA marker, 1- $10^2$ cfu g$^{-1}$, 2- $10^5$ cfu g$^{-1}$ (milk burfi); 3- $10^3$ cfu ml$^{-1}$, 4- $10^5$ cfu ml$^{-1}$ (sugarcane juice); 5- $10^2$ cfu g$^{-1}$, 6- $10^3$ cfu g$^{-1}$ (boiled rice); 7- control
1000 bp

100 bp

Fig. 3.11 LAMP based direct detection of *S. aureus* in spiked milk and khoa samples

Agarose gel electrophoresis of *entC* gene LAMP product. Lane M: Molecular size marker, 100 bp DNA marker; Lanes 1-3: 10^2-10^4 cfu/ml (milk); Lanes 4-6: 10^2-10^4 cfu/g (khoa); Lane 7- control.

3.4.10 Impact of background flora on PCR and LAMP sensitivity

The sensitivity of PCR and LAMP for enumerating a specific bacterial pathogen in presence of co-existing microbes (*Y. enterocolitica*) was investigated by extracting DNA from food samples spiked (3.3.6) with equal number of *S. aureus* and *Y. enterocolitica* in the range of 10^6-10^1 cfu/g and cfu/ml. Despite the presence of *Y. enterocolitica*, the sensitivity of PCR (Figure 3.12) and LAMP (Figure 3.13 and 3.14) to *S. aureus* remained same. However, for PCR reaction detection limit was 10^4 cfu/ml and 10^4 cfu/g, whereas for LAMP reaction it was 10^2 cfu/ml and 10^2 cfu/g for detection of *S. aureus* (Table 3.2). Thus, LAMP has been proven to be more sensitive than PCR.

Table 3.2 Comparision of sensitivities of LAMP and PCR reactions for detection of *S. aureus* (*entA, entB, entC* and *entD* genes) in presence of *Y. enterocolitica* in spiked food samples

<table>
<thead>
<tr>
<th>Food samples</th>
<th>PCR sensitivity (<em>entA, entB, entC</em> and <em>entD</em> genes) (cfu g^{-1}/cfu ml^{-1})</th>
<th>LAMP sensitivity (<em>entA, entB, entC</em> and <em>entD</em> genes) (cfu g^{-1}/cfu ml^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^2 10^3 10^4 10^5 10^6</td>
<td>10^2 10^3 10^4 10^5 10^6</td>
</tr>
<tr>
<td>Milk</td>
<td>-    -    +    +    +</td>
<td>+    +    +    +    +    +</td>
</tr>
<tr>
<td>Khoa</td>
<td>-    -    -    +    +</td>
<td>-    -    +    +    +    +</td>
</tr>
<tr>
<td>Milk burfi</td>
<td>-    -    -    +    +</td>
<td>-    -    -    +    +    +</td>
</tr>
<tr>
<td>Cooked rice</td>
<td>-    -    -    +    +</td>
<td>-    -    -    +    +    +</td>
</tr>
<tr>
<td>Sugar cane juice</td>
<td>-    -    -    +    +</td>
<td>-    -    -    +    +    +</td>
</tr>
</tbody>
</table>
Fig. 3.12 PCR based direct detection of *S. aureus* in presence of *Y. enterocolitica* in spiked food samples (milk burfi, sugarcane juice and boiled rice)

Agarose gel electrophoresis of *entC* gene PCR product. Lane M: Molecular size marker; Lane 1: $10^3$; Lane 2: $10^4$ cfu/g (milk burfi); Lane 3: $10^3$; Lane 4: $10^4$ cfu/ml (sugarcane juice); Lane 5: $10^3$; Lane 6: $10^4$ cfu/g (boiled rice); Lane 7 – control.

Fig. 3.13 LAMP based direct detection of *S. aureus* in presence of *Y. enterocolitica* in spiked food samples (milk burfi, sugarcane juice and boiled rice)

Agarose gel electrophoresis of *entC* gene LAMP product. Lane M: 100bp DNA marker; Lane 1-2: $10^3-10^4$ cfu/g (milk burfi); Lane 3-4: $10^2-10^3$ cfu/ml (sugarcane juice); Lane 5-6: $10^2-10^3$ cfu/g (boiled rice).
Fig. 3.14 LAMP based direct detection of *S. aureus* in presence of *Y. enterocolitica* in spiked milk and khoa samples.

Agarose gel electrophoresis of *entC* gene LAMP product. Lane M: Molecular size marker, 100 bp DNA marker; Lanes 1-3: $10^2-10^4$ cfu/ml (milk); Lanes 4-6: $10^2-10^4$ cfu/g (khoa); Lane 7- control

3.4.11 Evaluation of market food samples for direct detection of enterotoxigenic *S. aureus*

The viable counts of mesophilic aerobes and *S. aureus* in food samples are presented in Table 3.3. The total count in the food samples varied from 2.0-7.5 $\log_{10}$ cfu/ml or cfu/g, without any enrichment, almost all the food samples displayed the presence of *S. aureus* with a highest count of 6.1 $\log_{10}$ cfu/ml for raw milk (sample number two), with the exception of raw milk (sample number three); khoa (sample number seven); milk burfi (sample number 14), cooked rice (sample number 19 and sugarcane juice sample number 23) which showed no *S. aureus* growth. DNA extracted from the food samples as described earlier was subjected to PCR and LAMP reaction. Among the food samples raw milk and milk burfi (sample number one and 12 respectively) the DNA extract gave positive for *entC* and *entD* in both PCR and LAMP amplification. DNA extracts of khoa (sample number six) shows positive for *entC* in LAMP and PCR was negative as the *S. aureus* count was very low i.e. $10^3$ cfu/g. In sugarcane juice (sample number 21) DNA extract shows positive for *entC* and *entD* genes in both PCR and LAMP amplifications. Cooked rice sample DNA extract showed negative to *entA*, *entB*, *entC* and *entD* in both PCR and LAMP amplifications (Table 3.3).
Table 3.3 Analysis of market food samples for enterotoxigenic *S.aureus* by conventional plating, PCR and LAMP methods

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Food product</th>
<th>Total bacterial count (\text{log}<em>{10}\text{CFU g}^{-1}/\text{log}</em>{10}\text{CFU ml}^{-1})</th>
<th><em>S. aureus</em> count (\text{log}<em>{10}\text{CFU g}^{-1}/\text{log}</em>{10}\text{CFU ml}^{-1})</th>
<th>PCR</th>
<th>LAMP</th>
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3.5 Discussion

Foodborne diseases are a widespread and a growing public health problem, both in developed and developing countries. According to World Health Organization (2007) report, in 2005 alone, over 1.8 million people worldwide, most of whom are children, succumbed to death due to microbial contaminated food and water. The poor handling practices, lack of sanitation and unhygienic conditions contribute to the prevalence of *S. aureus* in food stuff (Ankita 2010; Ramesh and Nageswara Rao 1987; Singh 1985; Sudershan et al. 2009). The U. S. Food and Drug Administration (FDA) reported an outbreak having many typical elements of staphylococcal food poisoning; food involved, means of contamination, inadequate food handling measures and symptoms all pointed to *S. aureus*. In the National Health and Nutrition Examination Survey conducted in 2001-2002 in the United States, it was estimated that nearly one third (32.4%) of the non-institutionalized population including children and adults were nasal carriers. In the United States of America, Olsen et al. (2000) reported that *S. aureus* was involved in food poisoning. Except for France and the USA no data are available for the staphylococcal food borne intoxication and hospitalization rate. This rate is 15% and 18% in reported *S. aureus* cases for France (Haeghebaert et al. 2002) and the USA (Mead et al. 1999) respectively. The hospitalization rate for all cases has been estimated to be 1% in the USA. However, there is an under reported numbers of cases as only 1% were the estimated cases in the USA. Forty two documented outbreaks of foodborne intoxication, with 1,413 notified cases and one death occurring from 1993 to 1997 has been reported. *S. aureus* has been estimated to cause approximately 1,85,000 illnesses, 1,750 hospitalizations, two deaths per year, all from consumption of contaminated foods (Mead et al. 1999).

In developing countries like India, it forms a serious health hazard, with its implication on reduced socio-economic status of the rural populace. Many reports have also shown the staphylococcal food-poisoning outbreak in Indian subcontinent (Warke et al. 2000; Ghosh et al. 2007; Nema et al. 2007; Das et al. 2010). There are reports of food borne illness associated with the consumption of fruit juices at several places in India and elsewhere (Bhaskar et al. 2004; Chumber et al. 2007; Ghosh et al. 2007). Staphylococcal food poisoning out breaks has been well documented from dairy products (Tamarapu et al. 2001). Milk and milk products are often stored under refrigeration and the ability of pathogenic serotypes of *S. aureus* to survive and grow at low temperature makes it a public health concern for dairy industries. According to
Anderson et al., (1996), staphylococcal enterotoxins (SEs) are very resistant to heat; staphylococcal enterotoxin A (SEA), for example, retains some biological activity after 28 min at 121 °C. The recent identification of new SEs has considerably increased the perceived frequency of enterotoxigenic staphylococci isolates, indicating that the pathogenic potential of S. aureus may be greater than previously visualized (Rall et al. 2008).

Food safety has emerged as an important global issue with international trade and public health implications. The detection of pathogenic bacteria is a fundamental objective of food microbiology ensuring food quality. The emergence of nucleic acid based technique for detection of pathogenic organisms in food sample has received much impetus in recent past. The PCR has been widely accepted as the method of choice for rapid and reliable detection of microbes in food. This technique can be extremely useful for pure microbial cultures, but when applied directly to food samples its efficiency can be markedly reduced. The crux of this problem lies in the inhibitory substances in food samples. In an attempt to overcome this, a variety of treatments have been examined, e.g. lysozyme, lysostaphin, proteinase K, detergents, boiling, centrifugation, filtration, DNA affinity purification columns and magnetic beads coated with specific antibodies or lectins (Kroll 1993). This study focuses on another alternative, the extraction of bacterial nucleic acids from foods. Essentially, the DNA extraction method proposed in this study does not require any enrichment step; it is a sensitive, rapid, cost effective and simple method. This method was found to be more effective than Rapid lysis method (Dickinson et al. 1995) in all the spiked food samples. It reduces sample loss by encompassing only three basic steps. Food sample filtrates are centrifuged and pellet was boiled in water bath for 10 min using 1% Triton X-100. The DNA isolated using this procedure can be used for direct PCR and LAMP amplification assays without any purification or precipitation steps. In the anticipated method PCR was inhibited by food matrix and sensitivity reduced. LAMP was not affected by inhibitors and sensitivity increased more than 100 times than PCR. Ramesh et al. (2002) reported that, the presence of coexisting bacteria in the sample can attenuate the specific detection of the target bacterial species. Remarkably, in the present study, despite the presence of coexisting bacteria like Y. enterocolitica the level of sensitivity of both LAMP and PCR for specific detection of target bacteria remains unaltered and food matrix effect does not interfere in LAMP. The level of sensitivity for PCR was found to be $10^4$ cfu/g (4 log$_{10}$ cfu/g) and for
LAMP it was $10^3 \text{ cfu/g (3 log}_{10} \text{ cfu/g)}$. In food sample where *S. aureus* count was $10^3 \text{ cfu/g (3 log}_{10} \text{ cfu/g)}$ or less, only LAMP shows positive and PCR fails to detect. Thus, LAMP has been proven to be a powerful tool, which is useful for the detection and reliable identification of enterotoxin genes of pathogen from diverse food source, especially for resource-limited laboratories in developing countries. For routine use in food analysis, detection methods need to be simple, specific, robust and reliable. In the present study the replica experiments with food samples yielded the same results indicating the reproducibility of the method developed and uninoculated samples failed to give any amplification. The implications of the present study are promising and the DNA extraction method in combination with judicious choice of LAMP can be extended to detect pathogens present in food samples reducing the ill effects of food contamination resulted by length of the time taken for their identification.