Visual detection of *Escherichia coli* contamination in milk and fruit juice using loop-mediated isothermal amplification

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Abstract We developed a dye-based visual detection technique for *Escherichia coli*, a common contaminant in raw milk and fruit juice. We used hydroxylnapthol blue (HNB) dye directly to *E. coli*-contaminated milk and juice samples, prior to loop-mediated isothermal amplification (LAMP) of *tuf* gene. The samples detected using HNB dye was further validated by running them in gel electrophoresis. LAMP was used for amplification of *tuf* gene as it is more specific and sensitive over conventional PCR. The detection limit of LAMP was estimated to be of $10^{1 \text{ to } 4}$ cfu ml^{-1} of *E. coli*, as against $10^{2 \text{ to } 5}$ cfu ml^{-1} in PCR. However, the detection limit of *E. coli* in milk (10^1 cfu ml^{-1}) was lower compared to fruit juice (10^4 cfu ml^{-1}) suggesting that the presence of fruit pulps in juice sample interfered with the detection. The developed HNB-based visual detection is superior over other dye based detection methods as HNB is added prior to LAMP reaction, and therefore the samples need not be opened for mixing dyes; thereby reducing the chances of cross contamination of the samples. The present study established a specific, easy-to-use, efficient, rapid and naked-eye based detection technique for *E. coli* contamination in milk and pulpy fruit juice.

Keywords HNB dye · LAMP · *Escherichia coli* · Detection

Introduction

Raw or processed milk and fruit juices are well-known source that supports the microbial growth leading to spoilage of the product as well as infection /intoxication of consumers (Murinda et al. 2004; Oliver et al. 2005). Among the common

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microbial contaminants, *Escherichia coli* (*E. coli*) is the most predominant as indicated by the microbes in fecal pollution (Bonacorsi and Bingen 2005). Generally the non-pathogenic population of *E. coli* comprises main inhabitants of the intestinal tract of most mammalian species including humans (Marrs et al. 2005; Ochman and Selander 1984). However, very often the pathogenic population causes severe intestinal and extra intestinal diseases (such as the urinary tract, meninges, and the bloodstream) in humans (Johnson 2003; Klein et al. 2006).

The conventional culture-based diagnosis of *E. coli* infections requires bacterial culture to be obtained after 1–2 days of incubation in suitable growth media and subsequent confirmatory testing. The culture-based detection is thus not only laborious but time-consuming, requiring selective enrichment on different agar media followed by presumptive biochemical, serological or molecular confirmation (Romero et al. 1995). Although the DNA based diagnosis using PCR can provide fast results, its use is restricted partly because it requires considerable skill and expensive equipment. PCR based technique is quick and it remains effective for detection of pure microbial cultures. Thus, application of PCR for the direct detection of microbial contaminants in food has been limited due to the complex nature of the starting materials, including certain inhibitors of PCR amplification (Rossen et al. 1992; Wilson 1997). Moreover the sensitivity of PCR amplification of *E. coli* from contaminated milk samples was found to be low compared to that of bacterial cultures. Besides some false-negative PCR results were also reported (Romero et al. 1995). The variety of inhibitors including organic and inorganic substances (such as antibiotics, detergents, enzymes, fats, phenolics, polysaccharides, proteins and salts) are reported to inhibit the PCR amplification efficiency resulting in reduced sensitivity or false positive reactions (Rossen et al. 1992; Wilson 1997). This necessitates the development of a rapid and sensitive method especially for the detection of microbial contaminants in sources like milk and fruit juices.

Loop mediated isothermal amplification (LAMP) is a novel nucleic acid based amplification technique which can amplify the target DNA with high specificity, efficiency, and rapidity under isothermal condition (Nagamine et al. 2002; Notomi et al. 2000). Besides, LAMP does not involve sophisticated instruments like thermal cycler, gel electrophoresis apparatus. A water bath or heating block is sufficient to provide a constant temperature as the amplification proceeds under isothermal conditions and positive LAMP reactions can be visualized with naked eye (Mori et al. 2001). Unlike PCR, LAMP does not require a denatured template as it employs *Bst* DNA polymerase with strand displacement activity (Notomi et al. 2000). The LAMP reaction is more rapid generating large amounts of the target DNA in a short time. Another important advantage of the LAMP reaction is the ability to overcome the interference during amplification as caused by some inhibitory materials such as a culture medium and other biological substances that commonly affect the efficiency of the PCR (Kaneko et al. 2007). In this direction, the present study aimed to develop a dye-based quick and rapid LAMP-mediated detection of *E. coli*, a common bacterial contaminant in fruit juices and milk, targeting *tuf* gene.

Material and methods

Bacterial strains and culture conditions

Bacterial strains used in this study include *Escherichia coli* strain JM109, *E. coli* strain DH5α, *Salmonella typhimurium*, and *S. enterica*. The strains were cultured overnight in LB medium at 37 °C, at 180 rpm. Bacterial genomic DNA was prepared by lysis of bacterial pellet in 100 μl of lysis buffer (20 mM Tris–HCl, pH 8.0 and 1.2 % Triton X-100) that was boiled for 10 min.

Primers for PCR reaction

tuf gene sequences available from public databases were analyzed with GCG programs (version 8.0; Accelrys, Madison, WI, USA). Based on multiple sequence alignment and the oligo primer analysis software (Version 5.0; National Biosciences, Plymouth, MN, USA) PCR primers were designed from *tuf* gene, highly conserved regions of the *E. coli*. The chosen *E. coli*-specific PCR primers were TEcoI553 (5′-TGGGAGCGAAAATCCTG-3′) and TEcoI754 (5′-CAGTACSGGTAGACTTCTG-3′).

Primers for LAMP reaction

In order to design the species-specific primers for LAMP reaction, we first searched the nucleic acid sequence of *E. coli* deposited in the GenBank database, and used BLAST program to choose the species-specific gene sequences. The accession number of the *tuf* gene was J01609. The sequence was further analyzed by the Primer Explorer V1 software program (http://venus.netlaboratory.com/partner/lamp/pevl.html) to have LAMP primers: F3 (Forward outer primer), B3 (Backward outer primer), FIP (Forward Inner primer), and BIP (Backward inner primer) (Table 1). In addition, each internal primer that recognizes both sense and anti-sense strands of the target DNA was connected by a TTTT spacer. Lamp designed LAMP primers were outsourced for synthesis.

PCR amplification conditions

Colonies of *E. coli* strains were resuspended in 25 ml sterile nuclease free distilled water (SNW) and denatured at 95 °C for 10 min. The samples were centrifuged at 8000 rpm for 5 min to pellet down the debris. The supernatant was used as a template. The PCR was carried out as per standard protocol. Amplification conditions were, initial denaturation at 94 °C, 5 min; denaturation at 94 °C, 30s; primer annealing at 56 °C, 30s and extension at 72 °C, 1 min. This step was repeated for 35 cycles in an automated thermal cycler. Final extension was done at 72 °C for 5 min. The PCR amplicons were run on 1.5 % agarose gel and gel stained with ethidium bromide where visualized under the UV transilluminator.

Artificial inoculation of samples

For contamination of milk and fruit juice, common *E. coli* strains (DH5α, JM109), *Salmonella typhimurium*, and *S. enterica* were employed. Pasteurized whole milk (Mother dairy Co.) and fruit juice (Mango Maza) was artificially inoculated with each of the bacterial strains @10^7 cfu ml^{-1} (and hereafter referred as stock samples). The inoculated stock sample (10^7 cfu ml^{-1}) was serially diluted up to 7 folds (adding 1 ml of the stock to 9 ml of the sample). Thus varied levels of bacterial contamination (like 10^7, 10^6, 10^5, 10^4, 10^3,

Table 1 Primer sequences of LAMP reaction targeting *E. coli tuf* gene

Primers	Sequence (5′-3′)	Product (bp)
F3	CGTTGAATACGACACCCCG	229
B3	CGTCATCAACCATGTCGCA	
FIP	CTGAGCAGCACCGGTGATCATGTT TTCACTACGCACACGTAGACTG	82
BIP	GATGCCGCAGACTCGTGAGCTTTT ATGATGTACGGAACGCCTAC	
LF	TTTAACATAGTCGGCGTGCCC	125
LB	ATCCTGCTGGGTCGTCAG	

F3 forward outer primer, *B3* backward outer primer, *FIP* forward inner primer, *BIP* backward inner primer, *LF* forward loop primer, *LB* backward loop primer

10^2, 10^1, 10^0) in the samples were prepared. The bacterial number in the culture was subsequently estimated by standard plate counts. Bacterial cells in the samples were recovered by centrifugation at 6000 g for 20 min at 4 °C and the pellets were then treated at 95 °C for 10 min.

Sample preparation for direct visualization of *E. coli*

Milk

Milk samples were centrifuged at 10,000 rpm for 10 min and supernatant containing milk fat was removed. Milk protein like casein and whey were removed by washing with 0.5 M EDTA at 10,000 rpm for 7 min. A wash with mild non-ionic detergent (0.1 %) was used to remove remaining fats and hydrophobic proteins. Two subsequent sterile nuclease free water (SNW) washes (8000 rpm for 5 min) were performed to get rid of any trace EDTA or Triton X-100. Bacterial pellet was denatured in 25 ml of SNW at 95 °C for 5 min, centrifuged at 8000 rpm for 5 min. The DNA present in supernatant was used as a template for PCR and LAMP.

Mango juice

The artificially inoculated mango juice was centrifuged at 1500 rpm for 10 min to selectively pellet down the pulp. 1 ml of the supernatant obtained was centrifuged further at 7000 rpm at 10 min. The pellet was washed thrice with SNW. The pellet was dissolved in 25 ml of SNW and kept for incubation at 95 °C for 5 min. After spinning at 8000 rpm, the supernatant obtained was used as template for the PCR and LAMP.

Optimization of LAMP conditions

The LAMP reactions were optimized with respect to $MgSO_4$, deoxynucleotide triphosphate (dNTPs), betaine and temperature. Five concentrations of $MgSO_4$ (10 mM, 20 mM, 30 mM, 40 mM, 50 mM), six concentrations of dNTPs (2 mM, 4 mM, 4 mM, 6 mM, 8 mM, 10 mM), four concentrations of betaine (5 M, 4 M, 3 M, 2 M), and five ranges of temperature (55 °C, 57 °C, 59 °C, 61 °C, 63 °C) were used to optimize the LAMP reaction. These concentrations were chosen based on previous reports. LAMP reactions were put with each variable to optimize the best amplification conditions using a standard reaction mixture. A 25 μl of the standard reaction mixture contained 0.5 μl FIP (100 μM), 0.5 μl BIP (100 μM), 0.5 μl F3 (10 μM), 0.5 μl B3 (10 μM), 1 μl *Bst* DNA polymerase (8U) (large fragment; New England Biolabs Inc., USA), 2.5 μl thermopol buffer (10X), 4 μl $MgSO_4$ (20 mM or variable tested), 3.5 μl dNTP mix (10 mM or variable tested), 5 μl betaine (5 M or variable tested), 1 μl template

DNA (100 ng), and 6.0 μl water. LAMP reaction was carried out at 61 °C for 1 h.

Visual detection of LAMP products

One μl of 120 μM hydroxynapthol blue (HNB) dye was added to each tube before LAMP amplification. The non-amplified template (along with LAMP mixture) was used as control. The change of dark violet colour (control) to light blue colour indicated the positive for amplification.

Results and discussion

Microbial contaminants in raw milk and fruit juice are the major concern for health related hazards in human being. The microbe-contaminated food materials lead to infection / intoxications to the consumers (Murinda et al. 2004; Oliver et al. 2005). *Escherichia coli* is the most common contaminants in fruit juice and milk (Bonacorsi and Bingen 2005; Johnson 2003). The early detection of microbial contaminants is essential to reduce the toxic-food related health hazards. The conventional culture-based detection of microbial contaminants and their subsequent identification take long time and also tedious. DNA-based detection through polymerase chain reaction (PCR) is effective but issues like false positives, sensitivities are very common (Romero et al. 1995; Rossen et al. 1992; Wilson 1997) and it also requires highly expensive equipments including thermal cycler. The diagnostic technique based on loop mediated isothermal amplification (LAMP) is becoming popular as it overcomes these problems. The LAMP reaction is more sensitive as could rapidly generate large amounts of the target DNA in a short time amplifying three different regions of the target DNA (Notomi et al. 2000). Besides, the LAMP could overcome the interference caused by inhibitory materials such as a culture medium and other biological substances that commonly affect the efficiency of the PCR (Nagamine et al. 2002). Therefore, in the present study, we developed a dye-based quick and rapid detection method for *E. coli* in contaminated fruit juices and milk. For this purpose we used LAMP-amplified *tuf* gene of *E. coli*. Pasteurized whole milk and fruit juice was artificially contaminated with varied doses of bacterial culture. The HNB dye was preferred over the other dyes as it can be mixed directly in the sample prior to amplification and thereby the reaction tubes were not required to be opened reducing the risk of cross-contamination.

Optimization of LAMP assay condition for *E. coli* detection

We initially optimized and standardized the LAMP assay for *E. coli* detection by using two outer, two inner primers and loop primers from *tuf* gene (Table 1) and DNA template from

the *E. coli* strain. The *tuf* gene was chosen because it is highly conserved in *E. coli* (Maheux et al. 2009). The desired LAMP products were further confirmed through restriction digestion with *Sma* 1. The restricted profile of LAMP product resulted two distinct fragments, at 48 bp and 181 bp, confirming the single site-specific digestion of the desired amplicon of 209 bp (Fig. 1). The appearance of 9 visible bands starting at 209 bp in a ladder-like fashion on agarose gel confirmed the specific amplification (Fig. 2).

Effect of Mg++ concentration

Concentration of free Mg++ affects primer annealing and DNA polymerase activity (Saiki et al. 1998). In this study, the effect of Mg++ concentrations on LAMP reaction was determined. The Mg++ concentration at 30 mM gave the optimal amplification (Fig. 2a). Though the concentration ranging from 20 to 40 mM gave amplification but quality (as revealed by distinct ladder formation was observed at 30 mM. The concentration of Mg++ are reported to be varied ranging from 0 to 150 mM. The concentration determined in this study is lower than reported concentration as used in other studies (Wang et al. 2012).

Effect of deoxynucleotide triphosphate concentration

The deoxynucleotide triphosphate (dNTP) concentration largely affects the specificity of DNA polymerase amplification (Innis et al. 1988). The LAMP reaction in presence of various concentrations of dNTP was tested. The dNTP concentrations ranging from 0 to 10 mM amplified the target
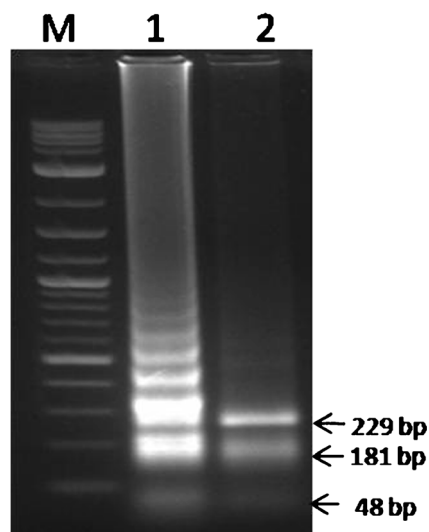
Fig. 1 Restriction analysis of LAMP products using *sma1* M=1 kb ladder; 1=Undigested LAMP- amplified product from *E. coli*, 2=*sma*1 restricted LAMP product showing two distinct fragments, at 48 bp and 181 bp. Also showing some undigested product resulted from partial digestion

DNA, but 4 mM gave the maximal reaction product (Fig. 2b). The concentration of dNTP was found to be lower compared to the other reports (Wang et al. 2012).

Effect of betaine concentration

Betaine plays important role in improving the DNA amplification (Frackman et al. 1998). Thus LAMP reaction in presence of different concentrations of betaine was tested (Fig. 2c). We observed that with the increase in betaine concentrations the LAMP reaction efficiency also improved. The concentration of 5 M betaine resulted best amplification. This concentration falls within the range of other studies (Nagamine et al. 2002; Notomi et al. 2000). Contrary, there are few reports, wherein lower concentration of betaine (0.8 M) was found to be optimal for LAMP reaction (Yeh et al. 2005). The differences in the concentration of betaine are most likely due to the differences in the target DNA sequences (Henke et al. 1997). Though the exact role of betaine in improving the LAMP reaction is not fully understood, but the suggested mechanisms explain that betaine makes DNA templates accessible for DNA polymerase (Henke et al. 1997) and it destabilizes GC-rich DNA sequences (Rees et al. 1993).

Effect of temperature

Although the *Bst* DNA polymerase has the optimal activity at 65 °C, several reports showed this enzyme can amplify DNA templates at lower temperatures in the LAMP reaction (Iwamoto et al. 2003; Parida et al. 2004; Yoshikawa et al. 2004). The effect of temperature on the LAMP reaction was determined. In our study, LAMP reaction temperature at 61 °C generated ladder-like pattern products, but no such typical pattern product was detected at 55 °C and 57 °C (Fig. 2d).

Specificity of LAMP-based detection of *E. coli* targeting *tuf* gene

To determine the specificity of the primers to the *E. coli*, *tuf* gene from 3 species of bacteria, namely *S. typhimurium*, *S. enterica*, *E. coli* strain DH5α (used as positive control), that are closely associated with *E. coli* were tested. Each bacterial species was cultured overnight and harvested by centrifugation. The DNA templates were prepared by lysing bacterial pellets in the lysis buffer. We observed that primers only amplified the *tuf* gene (Fig. 3). This result suggests that these primers are specific for detection of *E. coli* and is consistent with other studies of specificities of LAMP assays in bacterial detection (Enosawa et al. 2003; Iwamoto et al. 2003; Maruyama et al. 2003).

Fig. 2 Optimization of the LAMP reaction for detection of *E. coli*. The optimized parameters are marked by underlined gel wells in the gel panel as well as its corresponding values in the legend. **a**. Effect of MgSO₄ on the LAMP: M=1 kb ladder, 1=−ve control (*E. coli*), 2=10 mM, 3= 20 mM, 4=30 mM, 5=40 mM, 6=50 mM. **b**. Effect of deoxynucleotide triphosphate on the LAMP: M=1 kb ladder, 1=+ ve control (*E. coli*), 2=−ve control, 3=2 mM, 4=4 mM, 5= 4 mM, 6=6 mM, 7=8 mM, 3= 10 mM. **c**. Effect of betaine on the LAMP: M=1 kb ladder, 1=5 M 2=4 M, 3=3 M, 4=2 M. D. Effect of temperature on LAMP: M= 1 kb ladder, 1=55 °C, 2=57 °C, 3=59 °C, 4=61 °C, 5=63 °C
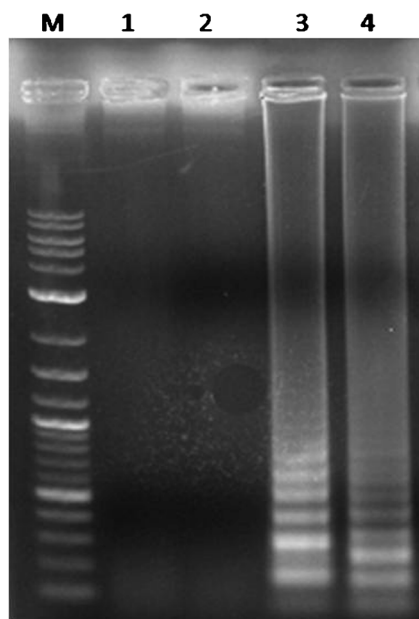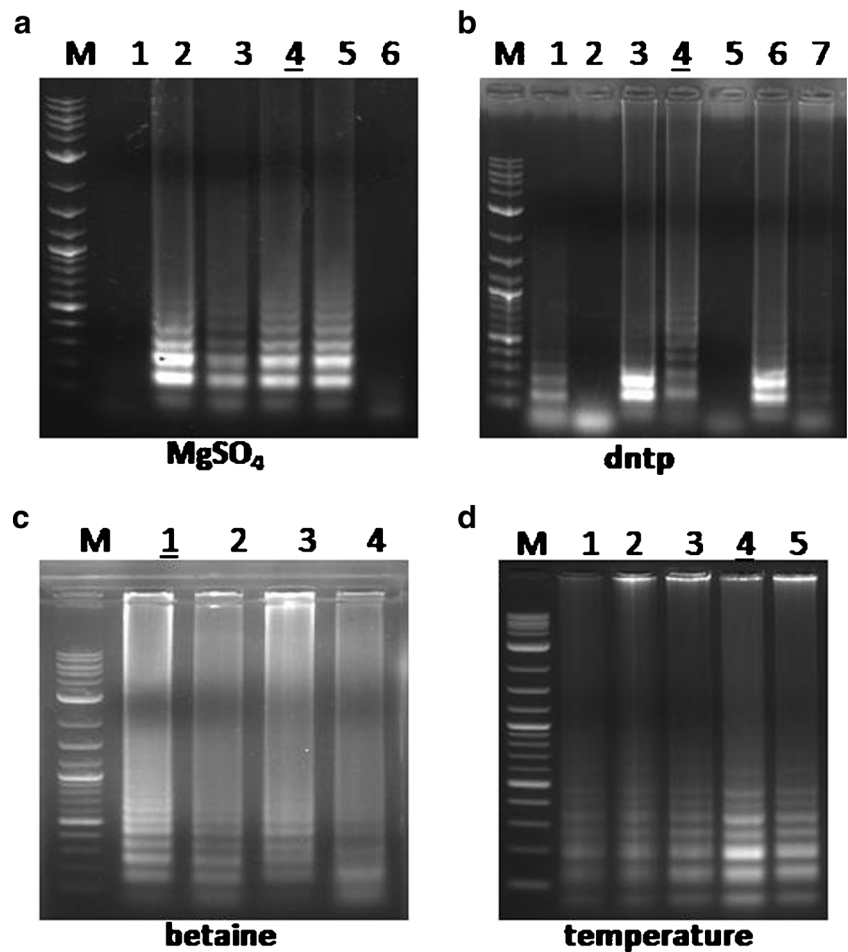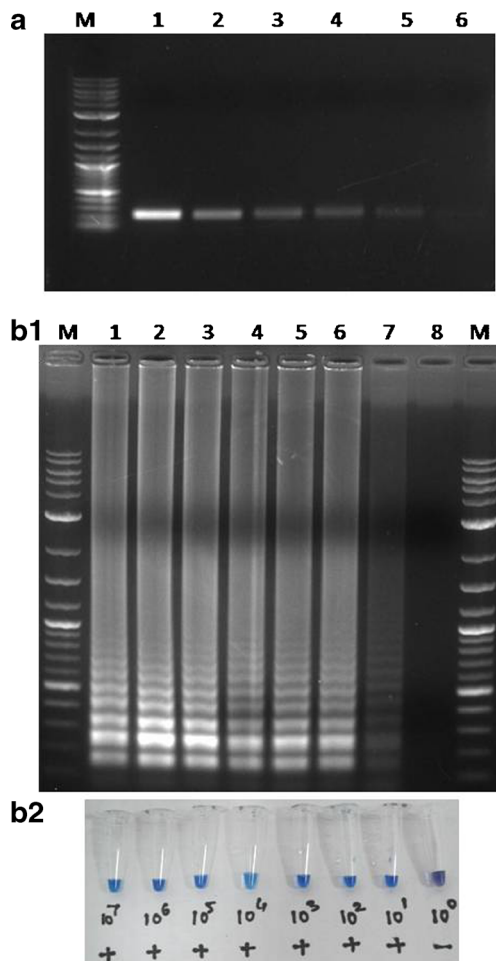

Fig. 3 Specificity of the *tuf* gene of *E. coli* used as LAMP-based detection marker M=1 kb ladder, 1=*S. typhimurium*, 2=*S. enterica*, 3=*E. coli* (DH5α), 4=*E. coli* (JM109)

Comparative sensitivity of PCR and LAMP assay for the detection of *E. coli* in milk

Milk, one of the most consumed dairy products, is highly susceptible to bacterial contamination. Macromolecules like proteins and fats present in milk might interfere with PCR assay. Thus, removal of theses macromolecules are required to prevent PCR inhibition. Most of the casein proteins (around 80 % of total milk protein) are incorporated in the micelles, together with a high proportion of the available calcium and inorganic phosphate (Ward et al. 1997). We added chelating agent like EDTA to sequester calcium ions and to disrupt the micelles. EDTA has shown to be used for protein denaturation and thereby increases the proportion of casein and whey protein in supernatant (Ward et al. 1997). We used triton X-100 that disperses fat flocculants to remove any residual lipids (Bringe et al. 1996). The pellet was washed with SNW to remove the left over detergent, if any, and used as template for PCR. To determine the comparative sensitivity of the developed LAMP assay with PCR detection, raw milk sample was artificially contaminated with different cell density of *E. coli*. The PCR technique used was able to detect as few
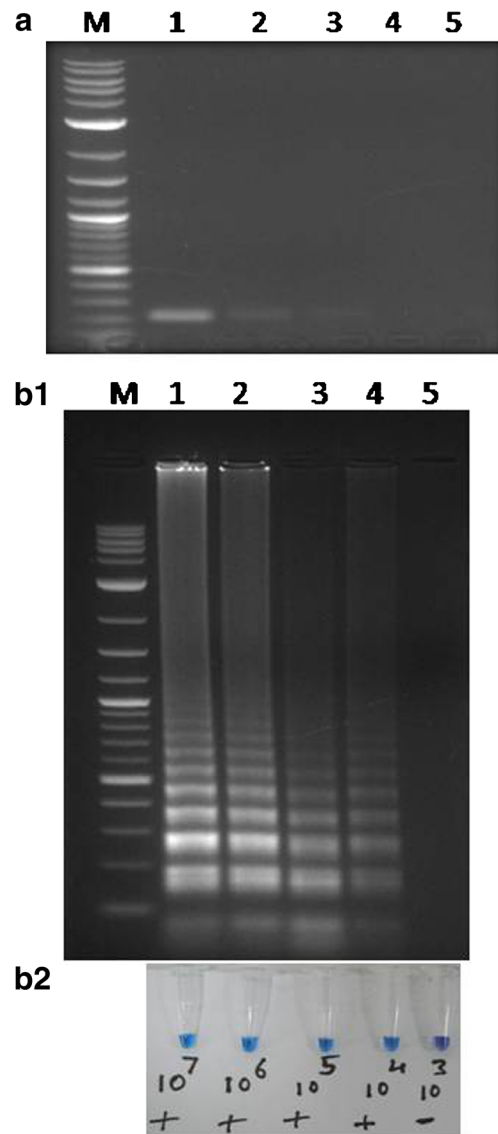
as 10^3 cfu ml^{-1}. Contrary the developed LAMP assay was found to be highly sensitive with the detection limit as low as 10^1 cfu ml^{-1} (Fig. 4). The higher sensitivity of the LAMP assay compared to the PCR was documented in several studies with various bacterial strains including *E. coli*, *Edwardsiella ictaluri*, *Flavobacterium columnare*, and *Salmonella* spp. (Hara-Kudo et al. 2008; Song et al. 2005; Teh et al. 2014; Wang et al. 2008, 2009; Yano et al. 2007; Yeh et al. 2005, 2007). However, most of these studies, particularly with reference to *E. coli*, were designed to detect virulence loci or markers of specific strains, such as verotoxin (Hara-Kudo et al. 2008; Song et al. 2005; Yano et al. 2007). In the present study we target *tuf* gene, a highly conserved gene across the *E. coli* strains (Maheux et al. 2009) and thus the developed LAMP method would not be limited to a particular strain rather would be more useful for detecting contamination of *E. coli*, irrespective of the strains.

Comparative sensitivity of PCR and LAMP assay for the detection of *E. coli* in fruit juice

Fruit juices are good source of microbial contamination. The efficiency of the designed PCR technique was tested with mango juice sample (Mango maza brand). The spiked fruit juice was neutralized with NaOH to remove the acids present which might inhibit PCR. The pulp was eliminated by low-speed centrifugation which is sufficient to pellet down bacteria. Washes with SNW can remove proteins that pellet along with the bacteria. To compare the sensitivity of LAMP with conventional PCR detection, fruit juice was contaminated

Fig. 4 Validation of dye-based detection with gel-based detection of *E. coli* in milk. **a**. PCR panel: M=1 kb ladder, 1 to 6=10^7, 10^6, 10^5, 10^4, 10^3, 10^2 cells of *E. coli* ml^{-1}. **b1**. LAMP panel: M=1 kb ladder, 1 to 8= 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 cells of *E. coli* ml^{-1}. **b2**. Dye panel: The presence of *E. coli* (as labeled in **b**1) were visualized directly after adding hydroxy napthol blue (1 μl of 120 μM) in the tube prior to LAMP reaction. "+" indicates amplification and "-" indicates no amplification

Fig. 5 Validation of dye-based detection with gel-based detection of *E. coli* in fruit juice. **a**. *PCR panel*: M=1 kb ladder, 1 to 5=10^7, 10^6, 10^5, 10^4, 10^3 cells of *E. coli* ml^{-1}. **b1**. *LAMP panel*: M=1 kb ladder, 1 to 5=10^7, 10^6,10^5, 10^4, 10^3 cells of *E. coli* ml^{-1}. **b2**. *Dye panel*: The presence of *E. coli* (as labeled in B1) were visualized directly after adding hydroxy napthol blue (1 μl of 120 μM) in the tube prior to LAMP reaction. "+" indicates amplification and "-" indicates no amplification

with *E. coli* at varied cell density. The result indicated that PCR was able to detect only 10^5 cfu ml^{-1} while the detection limit for LAMP assay was around 10^4 cfu ml^{-1} (Fig. 5). The lower detection limit of LAMP as well as PCR with fruit juice sample compared to milk samples is thought to be due inhibition of amplification reaction by the fruit pulps (Rossen et al. 1992).

Dye based visualization of LAMP products

The amplified LAMP products were directly visualized using 1 µl of 120 µM hydroxynapthol blue dye (HNB) (Figs. 4b2, 5b2). We observed the colour change in positive LAMP reaction from violet to light blue. The result indicated the simplicity of the developed LAMP technique where the amplification can be directly visualized without any aid of gel electrophoresis apparatus. Further we validated the HNB-based visualization with gel electrophoresis-based visualization. HNB has been used for visualization of LAMP reaction with λ DNA (Goto et al. 2009) and recently with human metapneumovirus (Wang et al. 2012). In this line, we demonstrate the utility of HNB for naked-eye visualization of bacterial contamination.

In conclusion, the present study established a specific, easy-to-use, efficient, rapid and naked-eye based detection technique for *E. coli* contamination in milk and pulpy fruit juice. The technique utilizes HNB dye-based visualization of LAMP-amplified *tuf*-gene. We target *tuf*-gene, a much conserved domain, in order to detect the *E. coli* irrespective of the strains contamination. We used LAMP-amplification technique as it is highly sensitive compared to conventional PCR. Thus, the developed technique would be of immense use in detecting the *E. coli* contamination as it overcomes the cumbersome process of gel-electrophoresis. Further, the technique is superior over other dye based detection methods as HNB is mixed with the sample prior to LAMP reaction and thereby reduces the chances of cross contamination of the samples during the process of opening of reaction tubes for mixing of dyes as needed in other cases.

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