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# Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid Detection of *Bacillus cereus* Toxin Genes

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**A B S T R A C T**

In this communication we describe a Loop mediated isothermal amplification (LAMP) based method for quick and effective detection of specific *Bacillus cereus* enterotoxin genes. Primers for the enterotoxin genes (hemolysin-A (*hblA*), Enterotoxin-T (*bceT*), Enterotoxin-FM (*entFM/cwpFM*) and Cytotoxin-K (*CytK*) of *Bacillus cereus* were designed using Primer explorer 4. Hydroxynaphthol blue (HNB), SYBR Green I and Calcein were used to enable visual detection. An attempt was made to quantify the DNA using Varioskan Flash microplate reader. The primers showed negligible cross reactivity with other food borne pathogens studied. The lowest concentration of standard DNA detected corresponded to $10^2$, $10^2$ and $10^3$copies of DNA respectively with Calcein, SYBR Green I and HNB dyes. The DNA isolated directly from the food matrix was used to validate the applicability of the developed LAMP assay. Negligible food matrix interference was observed in most cases. The method is quick, accurate and economical for onsite analysis. HNB was suitable for visual detection of *B. cereus* in some spiked food samples. Calcein proved to be a better dye for detection of spiked food samples when read in a Varioskan flash reader. This is one of the first reports on LAMP based detection of *Bacillus cereus*. The method is suitable for directly testing of food samples without any enrichment techniques and therefore could find its application in food safety analysis onsite. Such a detection system will ensure better food safety and can also be extended to clinical diagnosis of pathogenic organisms.

## Introduction

*Bacillus cereus* is a facultative anaerobic, spore-forming, motile microorganism that can be commonly isolated from food. It is known to produce four toxins, one emetic toxin and three potential diarrheal toxins (Ehling-schulz, *et al.*, 2004; Samapundo, *et al.*, 2011). The emetic syndrome is mainly characterized by vomiting 0.5–6 h after ingestion of the contaminated food. In the diarrheal syndrome, symptoms appear 8–16 h after ingestion and include abdominal pain and diarrhea. In general, both types of

food-borne illness are relatively mild and usually do last not more than 24 h (Dierick *et al.,* 2005). However, it is difficult to accurately determine the incidence of *Bacillus cereus* infection as most of the cases go unreported or misdiagnosed (Granum 2005). *Bacillus cereus* spores usually survive high temperature cooking and later multiply to cause the disease symptoms (Rajkovic *et al.,* 2013). Detection of these toxin producing bacteria in food can check widespread contamination.

Presently PCR based methods (Gracias and McKillip 2011) and existing kits used for detection of *B. cereus* have been shown toyield equivocal results (Burgess and Horwood 2006). PCR based nucleic acid detection though sensitive; requires highly pure form of DNA which is difficult to obtain from food samples. Also, Taq polymerase used in PCR reactions is often inhibited by the presence of food isolates(Schrader *et al.,* 2012).

Loop mediated isothermal amplification, first reported by Natomi *et al.,* 2000 (Notomi *et al.,* 2000a), is an alternative to the present PCR based nucleic acid detections. LAMP uses *Bst* polymerase, having strand displacement activity and therefore amplifies at a single temperature. Four to six primers, which identify different regions of the target gene, make the assay specific. LAMP based detection of pathogens is a good alternative and can complement PCR because of its simplicity, speed and accuracy(Parida *et al.,* 2006). The primary characteristics of the LAMP assay are its ability to amplify nucleic acid under isothermal conditions at 65°C with *Bst* DNA polymerase; as a result it allows the use of simple and cost effective reaction equipment. The second characteristic is that LAMP has high specificity and high amplification efficiency, with DNA being amplified $10^9$–$10^{10}$ times in 15–60 min. The high amplification efficiency of LAMP is attributed to no time loss of thermal change (as in PCR) because of its isothermal nature(Wang 2013).

In this communication we describe a LAMP assay to detect four toxin genes (*hblA, bceT, entFM / cwpFM* and *cyt K*) of *Bacillus cereus.* This is one of the first reports on detection of *B. cereus* diarrheal toxins using LAMP method. The applicability of the developed method was tested with spiked food samples.

## Materials and Methods

### Test Cultures and Media

*Bacillus cereus* MTCC 1272 (reference culture) maintained on brain Heart infusion (BHI) agar supplemented with 0.1% glucose (HiMedia, Mumbai, India) and other test cultures (Table 1) used in the cross reactivity studies were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

### Isolation of Bacillus Cereus Strains

A total of 25 different types of food matrices including meat and meat products, milk and milk based products, cakes and pastries, fried snacks, egg and egg products, fast foods, raw vegetables, fermented foods, etc., were used in this study (Table 2). The choice of food sample for isolation and spiking studies was done in accordance to the FDA methods validation guidelines for microbial pathogens(Service 2011).

A homogeneous sample was prepared in 0.85% sterile saline. The isolation of the cultures was done as described by (Samapundo *et al.,* 2011). Aliquots of 0.1 mL from appropriate dilutions were surface plated on *B.cereus* specific Polymyxin B sulphate pyruvate egg yolk mannitol

bromothymol blue agar (HiMedia Laboratories, Mumbai, India) plates. The inoculated plates were incubated at 37°C for 24 - 48 h. Presumptive colonies of *B. cereus* were selected based on characteristic colony features (peacock blue colored colonies with a surrounding zone of egg yolk precipitation). Further they were tested for β-hemolytic activity on blood agar plates (HiMedia Laboratories, Mumbai, India) containing 5% defribinated sheep blood. The isolated colonies were maintained on Brain Heart Infusion (HiMedia Laboratories, Mumbai, India) agar containing 0.1% glucose. The DNA isolated from these isolates was used as the template for LAMP reactions.

## Isolation of DNA

DNA from bacterial cultures was isolated by phenol: chloroform method as described by (Sambrook *et al.*, 1989). The DNA was checked for its purity and was quantified by measuring the absorbance ratio at 260/280 nm using a Biospectrometer-Basic, Eppendorf India Pvt Ltd.

## LAMP Primers

A total of six sets of primers for each toxin producing genes of target pathogenic *B. cereus* genome were used in this study. They were designed using Primer Explorer-version 4 (http://primerexplorer.jp/ elamp4.0.0/index.htmL) software. Four sets of LAMP primers designed for the *hblA*, *cytK*, *bceT* and *entFM/cwpFM* gene are given in Table 3. All oligonucleotide primers used in this study were synthesized by Sigma–Aldrich chemical Company, Bangalore, India.

## LAMP Assay

LAMP assay was carried out as described by (Tomita *et al.*, 2008a) with slight modifications, wherein a 25 µL reaction mixture containing, 8 U of *Bst* polymerase large fragment (New England Bio Labs, Beverly, MA, USA), 5 µL of template DNA ($10^1$ - $10^8$ copies, preheated at 95° C for 5 min, chilled on ice) along with 1.6 µM each of inner primer (FIP and BIP), 0.2 µM each of outer primer (F3 and B3 primer). 0.8 µM of each loop primer (LF and LB), 1.4 mM of deoxynucleotide triphosphate, 0.5 M of Betaine (Sigma, St Louis, MO, USA), 20 mM of Tris-HCl (pH 8.8), 10 mM of KCl, 10 mM of $(NH_4)_2SO_4$, 8 mM $MgSO_4$ and 0.2% Tween-20. The reaction mixture was incubated at 65 ºC for 60 min in a water bath and then heated at 80 ºC for 2 min to terminate the reaction. All the reactions were performed in triplicates.

LAMP products were subjected to electrophoresis on a 1.5% agarose gel, visualized under UV light after ethidium bromide staining and documented in a gel documentation system (Cleaver scientific Ltd, microDoc unit, UK).

## Specificity of the LAMP Primers

Specificity of LAMP primers designed was checked with genomic DNA isolated from other food- borne pathogens given in Table 1. The LAMP assay conditions used were the same as mentioned earlier.

## Sensitivity of LAMP Reaction

Total DNA was isolated from overnight reference *B.cereus* culture. The DNA obtained was diluted in the concentration range of $10^8$ to $10^1$ copies and used to study the sensitivity of LAMP assay as given above. The copy number was calculated using the online portal http://cels.uri.edu/gsc/cndna.htmL.

## Analysis of LAMP Products

Performance of three dyes HNB, Calcein

and SYBR Green I used in the study were compared. The Quantitative estimation was carried out by measuring the colour change of Hydroxynaphthol blue (HNB) (SRL, Mumbai, India) at 120 µM concentration, 1 µL SYBR Green I (Sigma Aldrich, Bangalore, India) at 1X concentration and Calcein (SRL, Mumbai, India) at 2 µM concentration were used in analysis of the LAMP products. The readings were recorded at 15 min intervals, using a Varioskan Flash microplate reader (Thermo Fisher, Finland).

## Spiking of Food Samples to Study Matrix Interference

A total of 25 different types of food samples mentioned in Table 2, spiked with $10^2$ to $10^8$cells $g^{-1}$ or mL food were used to study matrix interference. Aliquots of 1g or 1mL food sample were used for the study.

## Preparation of Spiked Food Samples for LAMP Testing

The food matrices spiked with $10^2$ to $10^8$cells $g^{-1}$ or mL (aliquots of 1 g or 1 mL) food after equilibration for 1h were suspended in isotonic saline and centrifuged at 2000 g for 10 min to remove the food matrix. The supernatant was boiled for 15 min with lysis solution consisting of 20 mM Tris-HCl (pH 8), 2 mM EDTA (pH 8) and 1.2% Triton X-100 (Sowmya *et al.,* 2012). The boiled sample was centrifuged for 10 min at 3000 g and supernatant was used directly for detection by LAMP.

## Results and Discussion

## Primer Design for *B. cereus* Toxin Genes and LAMP Assay

LAMP primers generated from Primer explorer-V4, for the toxin genes (*hblA, bceT, cytK, entFM/cwpFM*) of *B. cereus* were checked for the 5' and 3' stability and the best primer set was chosen for our study (Table 3).

The LAMP assay was performed according to (Tomita *et al.,* 2008a). The time dependence of the reaction for the *hblA* gene ($10^4$ copies of DNA) at 15 min intervals was checked on an agarose gel (1.5 %). A distinct ladder like pattern was observed (Fig. 1).

## Evaluation of *B.cereus* Food Isolates

DNA extracted from all the 25 food sample isolates was subjected to LAMP analysis to check for the presence of *B.cereus* diarrheal toxin genes. All the food sample isolates mentioned in Table 3 showed positive results for *hbl*A toxin gene. 16 isolates of the 25 isolates studied showed postitive amplification for the *bceT* gene and *entFM/cwpFM*. 14 of the 25 isolates showed positive results for *cytK* gene (Table 2).

## Sensitivity and Competence of the Dyes

Hydroxynapthol Blue, Calcein and SYBR Green I were compared in detecting reference *B.cereus* (MTCC 1272)diarrheal toxin genes.

Hydroxynapthol Blue**:** The colour change of HNB was in agreement with the pattern obtained in agarose gel (Fig. 1). The colour change from violet to blue (measured by absorbance at 650 nm) was less at 15 min interval and was maximum at the end of 1 h which corresponded with the readings obtained in a Varioskan Flash microplate reader. DNA concentrations as low as $10^3$ copies of DNA could be read with HNB dye (Fig. 2). The colour change could be visually differentiated at the DNA concentrations studied (Fig 2E).

**Table.1** List of Test Cultures

| Bacterial Species | MTCC[1] No |
|---|---|
| *Bacillus cereus* | 1272 |
| *Vibrio cholerae* | 3906 |
| *Salmonella parathypi* | 735 |
| *Listeria monocytogenes* | 1143 |
| *Streptococcus pneumoniae* | 655 |
| *Staphylococcus aureus* | 96 |
| *Yersinia enterocolitica* | 859 |
| *Escherichia coli* | 729 |

Microbial type cell culture collection, IMTECH, Chandigarh

**Table.2** Analysis of Food Isolates for the Presence of *B. Cereus* Toxin

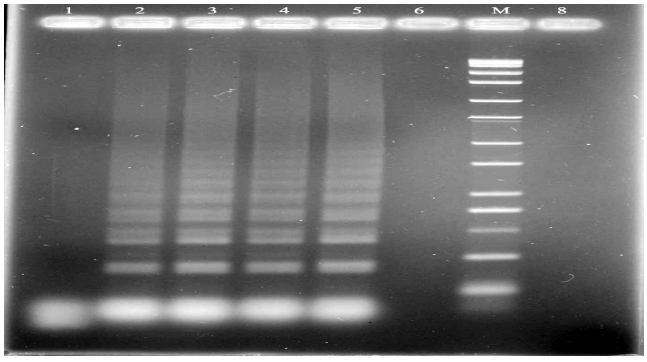
| S.No | Food samples | Isolate Number | *hblA* | *bceT* | *entFM* | *cytK* |
|---|---|---|---|---|---|---|
| 1 | Condensed Milk | HkmBC1 | +[2] | + | + | -[3] |
| 2 | Milk | HkmBC2 | + | + | – | + |
| 3 | Butter | HkmBC3 | + | - | + | + |
| 4 | Curds | HkmBC4 | + | + | + | - |
| 5 | Paneer | HkmBC5 | + | + | – | - |
| 6 | Cheese | HkmBC6 | + | + | – | + |
| 7 | Milk burfee | HkmBC7 | + | + | + | + |
| 8 | Colored Rice | HkmBC8 | + | + | + | + |
| 9 | White Rice | HkmBC9 | + | + | + | + |
| 10 | Apple pie | HkmBC10 | + | - | + | + |
| 11 | Chapatti | HkkmBC11 | + | - | + | + |
| 12 | Noodles | HkmBC12 | + | + | + | - |
| 13 | Chicken sausage | HkmBC13 | + | - | + | + |
| 14 | Meat (Pork) | HkmBC14 | + | + | – | + |
| 15 | Carrot | HkmBC15 | + | + | + | - |
| 16 | Boiled potato | HkmBC16 | + | + | + | + |
| 17 | Beetroot | HkmBC17 | + | + | + | - |
| 18 | Tomato | HkmBC18 | + | - | - | - |
| 19 | Orange juice | HkmBC19 | + | - | - | - |
| 20 | Minced Chicken | HkmBC20 | + | + | + | + |
| 21 | Samosa | HkmBC21 | + | + | + | + |
| 22 | Panipuri | HkmBC22 | + | - | - | - |
| 23 | Bhel puri | HkmBC23 | + | + | - | + |
| 24 | White sauce | HkmBC24 | + | - | + | - |
| 25 | Tomato ketchup | HkmBC25 | + | - | - | - |

[1]Microbial type cell culture collection, IMTECH, Chandigarh
[2] Positive amplification +
[3] Negative amplification -

**Table.3** List of LAMP Primers

| Genes | Primer | Sequence |
|---|---|---|
| *hbl*A gene (L20441.1)[4] | hbl F3 | TGCTATTTTGGGTCTACCAAT |
| | hbl B3 | GGACATATAAGTAAGAGCGTTAA |
| | hbl FIP | ACGTAATTCTGCTAATAAAGGCTCTTTGGCGGTATTATAGTGGGA |
| | hbl BIP | AACCTTAAATCGTGTAGTTGGAGTTATCATCAAGCGCCTTGTC |
| | hbl LF | TGCTATTTTGGGTCTACCAAT |
| cytK gene (DQ019311.1) | cytkF3 | CTAGCGTATCTTATCAACTTGG |
| | cytkB3 | CCGTTAAAGAATACGTTCCAT |
| | cytkFIP | GACCAGTTGCACCAGCTTCATGGCTCTGTTAAAGCTTCTG |
| | cytkBIP | AAGTCACTTGGTCTGACTCTGTTTTACGTTTTTGTTCGTTTGG |
| | cytk LB | CGCTAGGGCCATTAGGCGT |
| *bceT* gene (D17312.1) | bceT F3 | GAAGTAATAAGCGTACCATCTG |
| | bceT B3 | GAAGTAATAAGCGTACCATCTG |
| | bceT FIP | ACGGTCTAGCTAACGCTTCAATTTAACTGGATGCTGTTATTGACT |
| | bceT BIP | GGTTACCACCACTTCCAGAAAG-TTCTTTTCTTTCGCCCATG |
| | bceT LB | GAAGTAATAAGCGTACCATCTG |
| *ent*FM gene (AY789084.1) | entfmF3 | GGTTATGTAAGTGCAGACTTC |
| | entfmB3 | TCAAAACCAGCAGGTGTT |
| | entfmFIP | CGTCTTTACCTGGTTGTTGAACGGTTAAGTTTGTAAAAGGCGGA |
| | entfmBIP | CCAACAACAGGTGGAGATACATCGCAGTTCTGTATGGTGAAC |
| | entfmLF | TCGCTGGATTCGCTAGATCTTT |
| | entfmLB | GGTTATGTAAGTGCAGACTTC |

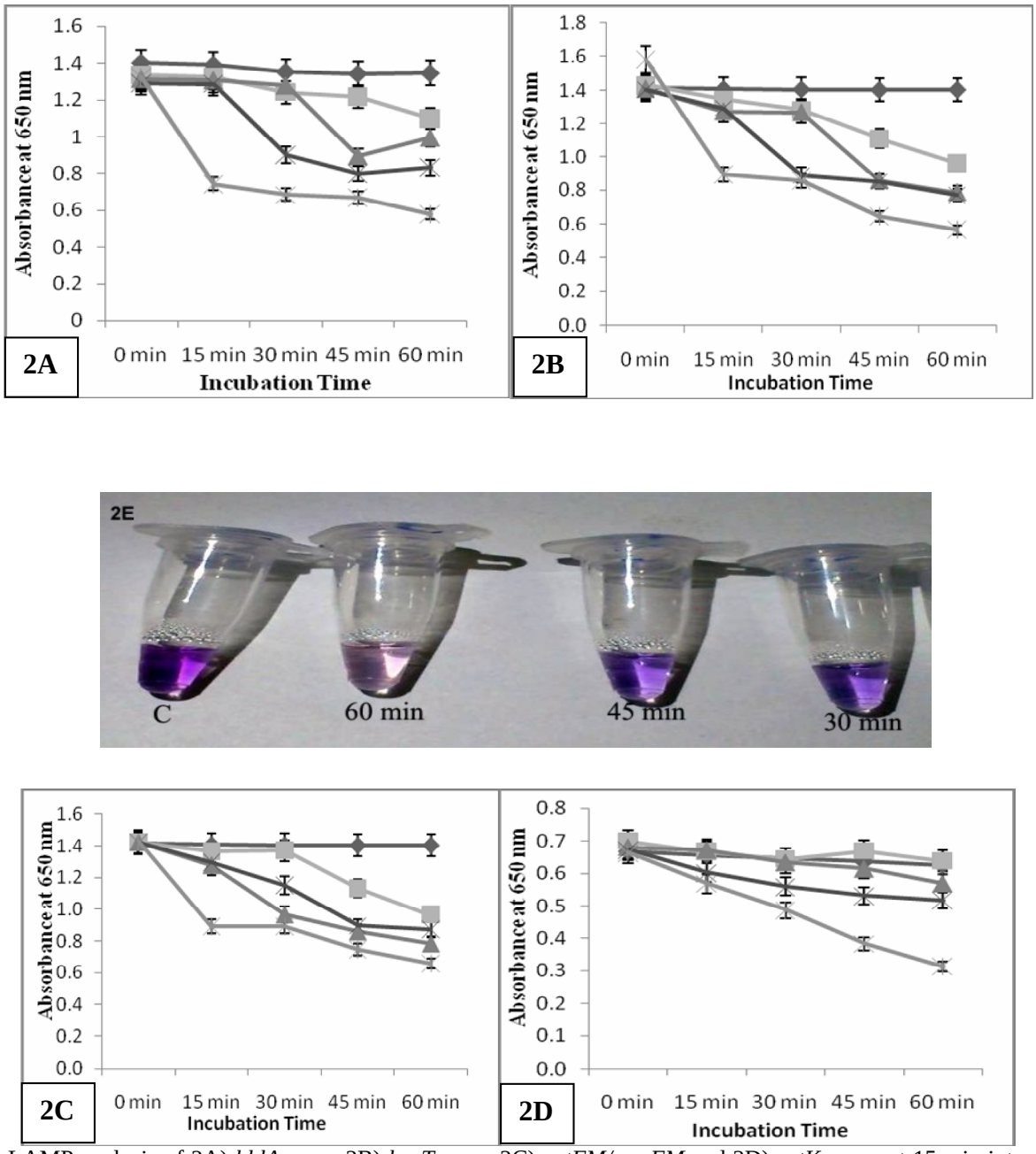
**Figure 1. Agarose Gel Showing Positive LAMP Amplification for *HBL*A Gene at Different Intervals of Time**




Lane 1: 0 min; Lane 2: 15 min; Lane 3: 30 min; Lane 4: 45 min; Lane 5: 60 min; Lane 6: Control and Lane M: 100 kb Marker

[4]GenBank Acession Number

**Figure.2** LAMP Analysis of Toxin Genes of *B. Cereus* with HNB

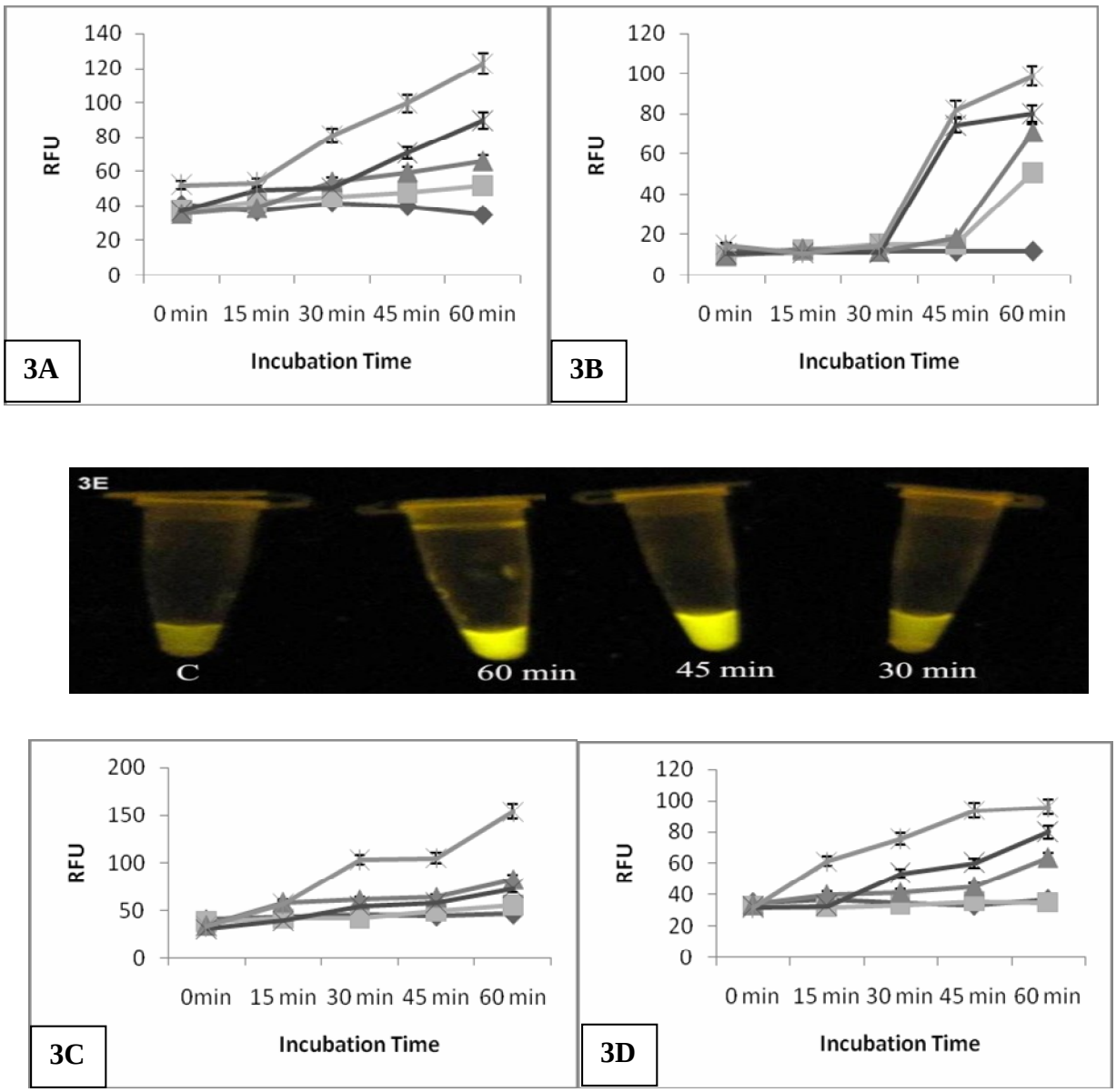



LAMP analysis of 2A) *hblA* gene; 2B) *bceT* gene; 2C) *entFM/cwpFM* and 2D) *cytK* genes at 15 min intervals with varying concentrations of standard *Bacillus cereus* DNA.

2E) color change in tubes containing $10^4$ copies of DNA under UV light at varying incubation times:Tube C-control, Tube 1: 60 min, Tube 2: 45 min, Tube 3: 30 min.

(◊) Control, (■) $10^1$ copies, (▲) $10^4$ copies, (x) $10^7$ copies, (*) $10^8$ copies

**Figure.3** LAMP Analysis of Toxin Genes of *B. Cereus* with Calcein

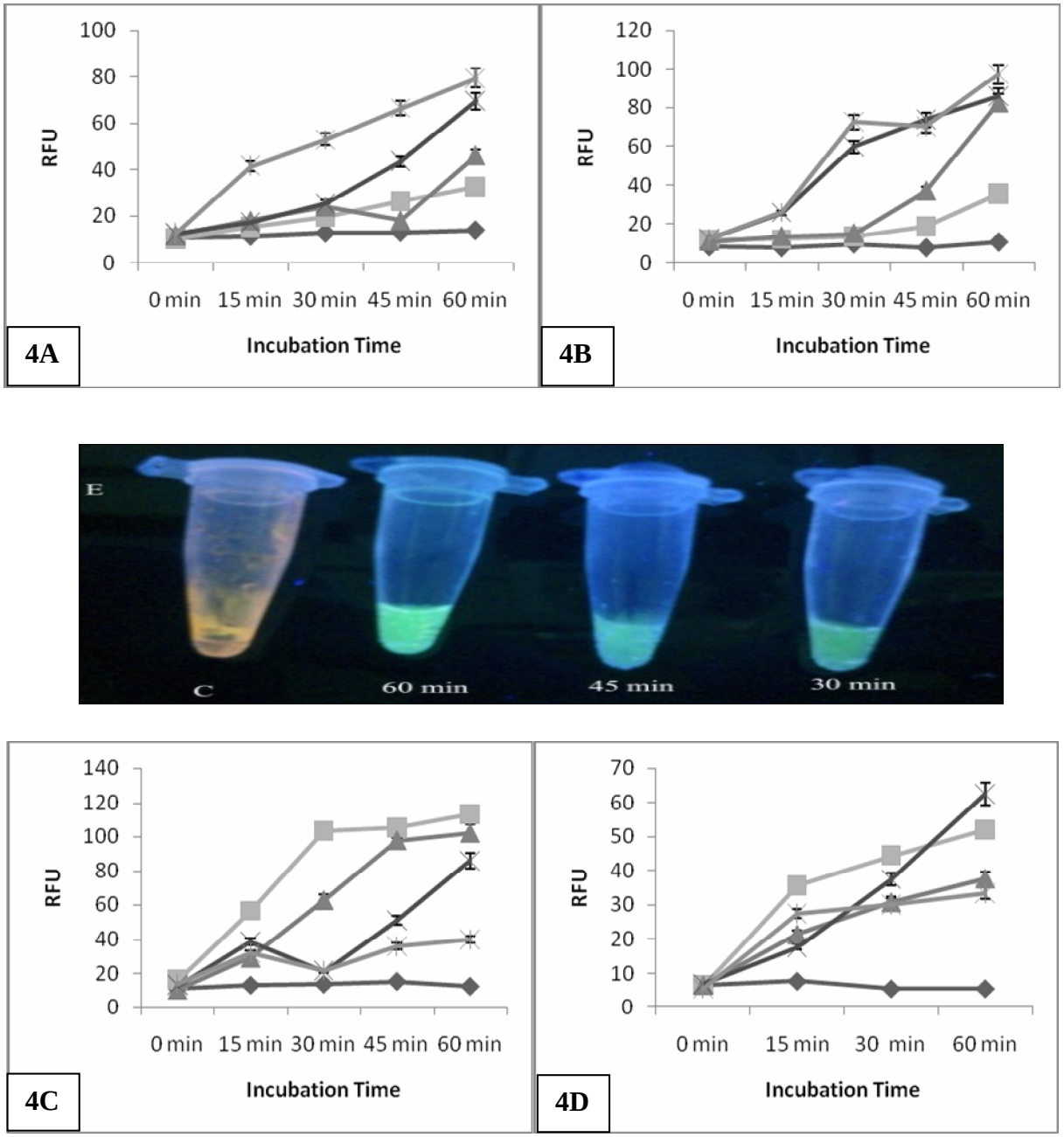



LAMP analysis of 3A) *hblA* gene; 3B) *bceT* gene; 3C) *entFM/ cwpFM* and 3D) *cytK* genes at 15 min intervals with varying concentrations of standard*Bacillus cereus* DNA.

3E) color change in tubes containing $10^4$ copies of DNA under UV light at varying incubation times: Tube C: control, Tube 1: 60 min, Tube 2: 45 min, Tube 3: 30 min.

(◊) Control, (■) $10^1$ copies, (▲) $10^4$ copies, (x) $10^7$ copies, (*) $10^8$ copies

**Figure.4** LAMP Analysis of Toxin Genes of *B. Cereus* with SYBR Green I




LAMP analysis of 4A) *hblA* gene; 4B) *bceT* gene; 4C) *entFM/ cwpFM* and 4D) *cytK* genes at 15 min intervals with varying concentrations of standard *Bacillus cereus* DNA.

4E) color change in tubes containing $10^4$ copies of DNA under UV light at varying incubation times: Tube C: control, Tube 1: 60 min, Tube 2: 45 min, Tube 3: 30 min.

(◊) Control, (■) $10^1$ copies, (▲) $10^4$ copies, (x) $10^7$ copies, (*) $10^8$ copies

Calcein**:** The colour change from orange (control) to bright yellow (amplified product) was in agreement with the agarose gel results (Fig.1). The colour change was difficult to be differentiated visually (Fig. 3E). However, it could be read using a

Varioskan Flash reader (absorption/emission spectra 495/517) and DNA concentrations as low as $10^2$ copies of DNA could be detected with Calcein dye (Fig. 3).

SYBR Green I: SYBR green I, a DNA intercalating dye, commonly used for LAMP analysis gave us good results and was sensitive upto $10^2$ copies of DNA (Fig. 4). The colour change from orange to green could be visually differentiated (Fig. 4E) and also could be quantified at 497/520 nm (absorption/emission spectra). On triplicate analysis, the instances of false positive results were observed thus hampered the reproducibility of the assay.

## Specificity of the LAMP Assay

In order to evaluate and verify the specificity of the primers used in this study, each primer set was tested on DNA templates from 7 different bacterial test cultures (Table 1). The analysis indicated that all primers were specific for *B. cereus* diarrhealtoxin genes and there was no amplification due to cross reactivity with other target organisms (data not shown).

## Matrix Interference in Spiked Food Samples

The effect of food matrix on the detection efficiency of LAMP assay was carried out with three dyes. The method developed involved spiking of food samples with reference *B.cereus* (MTCC 1272). The DNA was isolated by boiling in presence of triton X-100 (1.2%) which resulted in good recovery of DNA from food samples. HNB dye showed positive colour change from violet to blue with all food matrices tested except milk and beetroot samples (Table 2, Sample No. 2 and 17). However, the same food matrices did not interfere when Calcein was used. Even though colour change in Calcein from orange to yellow was not easy to differentiate with naked eyes, there was a marked increase in the absorption/emission spectra (495/ 517) of the spiked food samples when read using a Varioskan flash microplate reader. The colour change produced by SYBR Green I from orange to green (497/520) was equivocal as it produced false positive results in some control food matrices as well. Starchy food samples (Table 2, sample No: 8, 9, 16 and 21) showed positive amplification in case of all four toxigenic genes studied with negligible matrix interference. However, a few food samples such as raw beetroot, orange juice, panipuri, tomato and tomato sauce (Table 2, sample No. 17, 22, 18 and 25) interfered with the LAMP assay.

LAMP assay is highly specific as LAMP primers bind to six different regions on the target DNA. The assay can be carried out with both denatured and non-denatured DNA (Nagamine *et al.,* 2001). However, denatured DNA template gave better amplification.

SYBR Green I fluoresces when it binds to the major grove of DNA. The SYBR Green dye, more often than not, binds to the primers and the template DNA present in the reaction mixture resulting in false positive results (Papin *et al.,* 2010).

HNB and Calcein being metal chelator dyes detect the $Mg^{2+}$ ion generated on amplification (Goto *et al.,* 2009; Notomi *et al.,* 2000b). In our studies we observed that the accuracy of HNB was dependent mainly on the presence of $Mg^{2+}$ and the pH of the reaction mixture. The pH between 8.6 – 8.9 was found to be optimum. HNB was found to be more acceptable for visual detection. The colour change was easily distinguishable with naked eyes. The food matrices that imparted colour or have an

acidic pH, however, interfered with the reaction sensitivity. This was especially true with beetroot and milk samples used in our study.

Calcein resulted in sensitive quantitative detection upon reading the fluorescence intensity in a Varioskan flash microplate reader. The colour change from orange to yellow although difficult to detect visually (Wastling *et al.,* 2010) could be easily quantified in the microplate reader due to increase in the fluorescence as a result of the $Mg^{2+}$ formation, a by-product of amplification reaction. Visual detection was however possible when a UV lamp was used to visualize the colour change.

LAMP reactions are generally performed using DNA isolates and have seldom been validated on direct spiked food samples. To validate the performance of LAMP reaction obtained using standard DNA, analysis was carried out with spiked food samples.

Contrary to the findings of Wastling *et al.*, 2010, Calcein was found to be less prone to food matrix interference. Calcein equilibrated with $MnCl_2$ only fluoresces when its $Mn^{2+}$ ions are replaced with $Mg^{2+}$ ions produced during amplification (Tomita *et al.,* 2008b). Since the colour imparted by food matrix does not interfere with fluorescence of Calcein, it produced consistent results on analysis of spiked food samples. SYBR Green I showed false positive results with non- *B. cereus* spiked food sample as well. The dye intercalates with the DNA obtained from direct boiling method and primer dimers present in the reaction mixture and therefore produced a non-specific colour change. Also, Since SYBR Green I dye is added post reaction, the chances on contaminating the product is high (Chen *et al.,* 2011), which is not the case with HNB and Calcein.

In conclusion, we observed HNB to be suitable for visual detection of *B. cereus* spiked food samples barring some food matrices that imparted colour. Calcein proved to be a better dye for detection of spiked food samples as the food matrix did not interfere with the detection sensitivity. The presence of *Hbl* toxin gene could be detected in all the food isolates tested, probably this is due to the predominance of Hbl toxin compared to other toxin genes as seen in our previous studies on the immunodetection of *B. cereus* Hbl toxin in food matrices (Machaiah and Krishnan 2014). To our knowledge, this is one of the first reports on LAMP based detection of *Bacillus cereus*. Work on development of an onsite detection kit for detection of *Bacillus cereus* in food and environmental samples are underway. Such a detection system will ensure better food safety and can also be extended to clinical diagnosis of pathogenic organisms.

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