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Most Probable Number - Loop Mediated Isothermal Amplification (MPN-LAMP) for Quantifying Waterborne Pathogens in Less Than 25 Minutes

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Abstract

We are reporting a most probable number approach integrated to loop mediated isothermal technique (MPN-LAMP) focusing on Gram-negative *Escherichia coli* and Gram-positive *Enterococcus faecalis* bacterial cells without nucleic acids extraction. LAMP assays for *uidA* from *E. coli* and *gelE* from *E. faecalis* were successfully performed directly on cells up to single digit concentration using a commercial real time PCR instrument. Threshold time values of LAMP assays of bacterial cells, heat treated bacterial cells (95 °C for 5 min), and their purified genomic DNA templates were similar, implying that amplification could be achieved directly from bacterial cells at 63 °C. Viability of bacterial cells was confirmed by using propidium monoazide in a LAMP assay with *E. faecalis*. To check its functionality on a microfluidic platform, MPN-LAMP assays targeting < 10 CFU of bacteria were also translated onto polymeric microchips and monitored by a low-cost fluorescence imaging system. The overall system provided signal-to-noise (SNR) ratios up to 800, analytical sensitivity of < 10 CFU, and time to positivity of about 20 min. MPN-LAMP assays were performed for cell concentrations in the range of 10⁵ CFU to < 10 CFU. MPN values from LAMP assays confirmed that the amplifications were from < 10 CFU. The method described here, applicable directly on cells at 63 °C, eliminates the requirement of complex nucleic acids extraction steps, facilitating the development of sensitive, rapid, low-cost, and field-deployable systems. This rapid MPN-LAMP approach has the potential to replace conventional MPN method for waterborne pathogens.

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Keywords

loop-mediated isothermal amplification; most probable number; propidium monoazide; detection limit; point-of-care diagnostics

1. Introduction

Sample preparation is a key aspect in the implementation of sample-in-answer-out gene analysis systems. Sample preparation methods to recover nucleic acids require several steps depending on the sample being analyzed, but often includes cell enrichment, cell lysis and nucleic acid purification (Mariella, 2008). Enrichment/concentration of target cells becomes important in the sensitive diagnosis of infectious disease agents due to their low concentrations in the samples (e.g., 200 human immunodeficiency virus particles in 1 ml of blood containing about 5×10^9 red blood cells). Sample preparation techniques such as filtration (Mach and Di Carlo, 2010), immuno-separation (Dharmasiri et al., 2010), and di-electrophoresis (Hu et al., 2005) for cell enrichment, and biochemical, thermal, electrical, and mechanical methods for cell lysis (Brown and Audet, 2008), and silica-based solid-phase methods for nucleic acid separation and purification have been translated on microfluidic chips. Such chips are being integrated, for sample preparation, with nucleic acid amplification and detection modules for gene analysis systems with sample-in-answer-out capabilities (Le Roux et al., 2014). However, microfluidic-based sample preparation modules are limited by their nucleic acid extraction efficiency and cumbersome sample processing, affecting the sensitivity and total detection time of gene analysis systems (Myers et al., 2013). As such, development of rapid gene analysis systems require either the elimination of sample preparatory steps or improvements in their protocols.

Microfluidic polymerase chain reaction (PCR) systems are known to carry out DNA amplification directly from bacterial cells (Atrazhev et al., 2010; Manage et al., 2011) by using polymerases which are less prone to inhibition (Kermekchiev et al., 2009; Matheson et al., 2010; Zhang et al., 2010). *Taq* polymerase, the enzyme commonly used to amplify nucleic acids in PCR is inhibited by a variety of compounds such as hemoglobin, humic acid and cellular debris (Abu Al-Soud and Radstrom, 1998). Therefore, microfluidic PCR systems are often coupled with dedicated cell lysis and nucleic acid purification steps to remove cell debris and compounds that could inhibit DNA amplification (Witek et al., 2006; Park et al., 2011). Another promising approach in this regard could be the use of intracellular enzymes to perform amplification (Qian et al., 2014) or translocation of the enzymes inside the cells (Patel et al., 2014; Wang et al., 2014).

Loop-mediated isothermal amplification (LAMP), initially reported in 2000, is a robust molecular method that has been extensively applied for the DNA/RNA-based amplification of a variety of pathogens (Notomi et al., 2000; Seyrig et al., 2011; Tourlousse et al., 2012). LAMP is a highly sensitive, specific, and rapid technique for nucleic acid amplification (Zhang et al., 2014). Most importantly, it is not affected by the presence of cellular debris and other compounds which typically inhibit PCR (Poon et al., 2005). LAMP with bacterial cells in conventional PCR vials has been previously performed (Craw and Balachandran,

2012) but it required separate heating of bacterial cells and mixing of cell lysate in the reaction solution. Notably, direct heating of the reaction solution above 80 °C deactivates *Bst* polymerase (Matovu et al., 2010). *In situ* LAMP is a modification of LAMP for DNA amplification directly from bacterial cells (Ye et al., 2011). *In situ* LAMP requires prior fixation and permeabilization of bacterial cells on a solid surface followed by a LAMP reaction at 63 °C. Nucleic acids are amplified inside the cells by the inclusion of the LAMP reaction components in the permeabilized cells. Due to the steps involved in sample processing, *in situ* LAMP is performed in about 3-4 h. Digital polymerase chain reaction (Pohl and Shih, 2004) and digital recombinase polymerase amplification (Shen et al., 2011a) are performed for quantifying low concentrations of nucleic acids. MPN-LAMP technique applied directly to bacterial cells would be more effective in target qualification, due to the elimination of upstream sample processing and the associated loss of nucleic acids.

Direct cell-based microfluidic LAMP would reduce the instrument complexity as compared to microfluidic PCR due to the isothermal nature of the LAMP reaction. Direct cell-based LAMP without any sample processing has yet to be translated on a microfluidic platform. Here, we performed the genetic amplification, without any sample processing, from Gram-negative and Gram-positive bacteria at an isothermal temperature on a commercial real time PCR system. We have also applied this concept to amplify the genetic sequences from bacterial cells on a microchip with most probable number technique, resulting in MPN-LAMP – a technique that could be used for many waterborne and other pathogens where analytical sensitivity, quantification, and time-to-result are key factors.

2. Materials and methods

2.1 Bacterial cultures and genomic DNA preparation

Enterococcus faecalis was obtained from American Type Culture Collection (ATCC, 19433, Manassas, VA). *Escherichia coli* K12 strain C3000 was graciously provided by Professor Joan B. Rose (Water Quality, Environmental, and Molecular Microbiology Laboratory, Michigan State University). Frozen stocks of *E. coli* and *E. faecalis* were re-suspended in sterile test tubes containing Trypticase Soy Broth (TSB, 211768, BD, Sparks, MD). To revive the cells, these tubes were incubated at 37 °C in a shaker-incubator (New Brunswick Scientific, Edison, NJ) at 150 rpm for 12 h. Next, cultures were inoculated into 25 ml of TSB and incubated at 37 °C for about 8-12 h to achieve mid-exponential phase. Bacterial cultures were placed overnight in the refrigerator at 4 °C. Cultures were serially diluted in TSB and colony forming units of *E. coli* and *E. faecalis* were enumerated by plating 0.1 ml of culture dilutions on Trypticase Soy Agar (TSA, 211043, BD, Sparks, MD) plates, which were incubated at 37 °C for 12 h. Cell concentrations were about 1×10^8 CFU/ml for both the bacterial species. Genomic DNA of *E. coli* strain K-12 (700926D) and *E. faecalis* strain V583 (700802D) were purchased from ATCC. Before use, dried genomic DNA was re-suspended in nuclease-free sterile water (Fischer Scientific, Pittsburgh, PA).

2.2 LAMP primer design

LAMP primers were designed for β -D glucuronidase (*uidA*) and glutamate decarboxylase (*gadA*) genes of *E. coli* K-12 strain C3000, and gelatinase (*gelE*) gene of *E. faecalis* strain

V583. Primer sequences used are listed in Table S1 (supplementary material). A set of six specific LAMP primers (F3, B3, FIP, BIP, LF and LB) was designed for each target gene by using Primer Explorer V4 (Eiken Chemicals Co., Tokyo, Japan) and was synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

2.3 Microchip fabrication

Microchips with circular wells were fabricated in 127 μm thick polyester film (8567K52, McMaster-Carr, Los Angeles, CA) using a 10 cm $\times$ 12 cm perforated steel sheet with multiple 1 mm holes, 0.5 mm center to center (Diamond Manufacturing Company, Wyoming, PA), and a piece of silicon rubber (70 A, McMaster-Carr, Los Angeles, CA). The sandwiched system was preheated to 220 $^{\circ}\text{C}$ in a press (Carver press model 4386; Carver, Wabash, IN) with an embossing pressure of 1000 lb, applied for 3 min. The system was subsequently cooled to 105 $^{\circ}\text{C}$ while maintaining the embossing pressure. The microchip was then extracted from the perforated sheet, rinsed with distilled water, and air-dried at 65 $^{\circ}\text{C}$ in an oven. The microchips consisted of multiple circular wells each with 1 mm diameter and 2 μl volume per well (Fig. S1).

2.4 Experimental setup

The gene amplification and detection system was built by integrating a light source, optical filters, thin film heater, and a charge-coupled device (CCD) camera. Details of the fluorescence imaging system were reported previously (Ahmad et al., 2011). This system was slightly modified to obtain homogenous light for high throughput imaging. In the modified version, a light emitting diode (LED) (05027-PM12, LED Supply) was replaced by a 525 nm LED array (LIU002; Thorlabs, Inc., Newton, New Jersey). The LED array consists of 20 individual LEDs on a printed circuit board, providing homogeneously distributed light on larger area. Other components such as the excitation filter (FF01-534/20-25, Semrock Inc., Rochester, NY), emission filter (FF01-572/28-25, Semrock), CCD camera with AutoStar Envisage imaging software (MEADE DSI Pro, Irvine, CA), lens (16 mm, 15774, Deal Extreme), thin film heater (HK5165R52.3L12, Minco, Minneapolis, MN), thermocouple (5SRTC-TT-T-40-36, Omega Engineering Inc., Stamford, CT), pulse width modulator IC (DRV102T, Texas Instruments, Dallas, TX), USB DAQs (USB-DAQ 6009, National Instruments, Austin, TX), and LabVIEW VI (developed in-house) were same as the previously reported setup.

2.5 LAMP assay on Chromo4™

LAMP reactions on the commercial real time PCR instrument (Chromo4™, Bio-Rad Laboratories, Hercules, CA) were performed in triplicate in a volume of 25 μl consisting of 1.6 μM each of FIP and BIP primer, 0.2 μM each of F3 and B3 primer, 0.8 μM each of LF and LB primer, 0.8 M betaine (Sigma, St Louis, MO), 1.4 mM of each dNTP (Invitrogen Corporation, Carlsbad, CA), 20 mM Tris-HCl (pH 8.8), 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 8 mM MgSO_4 , 8 mM Triton X-100, 0.64 units/ μl of large fragment of *Bst* DNA polymerase (New England Biolabs Inc., Ipswich, MA), 0.2% Pluronic® F-68 (Invitrogen, Carlsbad, CA), 1 mg/ml bovine serum albumin (New England BioLabs), and 2 μM of SYTO-82 (Invitrogen Corporation, Carlsbad, CA). Depending on the type of LAMP assay, DNA or cell-based, the LAMP reaction solution was mixed with 1 μl of DNA solution (10^5 genomic copies as DNA

template/ μl) or bacterial solution (10^5 CFU/ μl) without any prior treatment. To examine the effect of heating on cell lysis, cell solutions were separately pre-heated at 95°C for 5 min before mixing in the LAMP reaction solution. Additionally, to examine the effect of *Bst* polymerase heating on the performance of LAMP, reaction solutions mixed with bacterial cells were preheated at 95°C for 5 min. To establish standard curves for quantitative analysis of *E. coli* and *E. faecalis*, LAMP assays with 10-fold serial dilutions of bacterial cells (10^5 CFU to < 10 CFU) were prepared. Sample replicates of 1×10^{-8} dilution were expected to contain less than 10 bacterial cells and was verified by plating the dilution on Trypticase Soy Agar. LAMP assays without any DNA template or bacteria were also prepared to serve as negative controls. Sample replicates and negative LAMP controls were loaded in 0.2 ml PCR tubes (VWR International, West Chester, PA) and incubated at 63°C for about 30 to 40 min on Chromo4™. Fluorescence signals of the LAMP assays were measured every minute in channel 2 (excitation wavelength range from 500-535 nm and emission wavelength range from 560-580 nm). LAMP assays for all the dilutions were performed in triplicates (except for < 10 CFU dilution, for which 6 replicates were used).

To detect viable *E. faecalis* in the presence of dead cells and extracellular DNA templates in the cell solution, LAMP assays were performed with DNA-modifying propidium monoazide (PMA) dye. PMA stock solution (20 mM, Biotium Hayward, CA) was diluted to $500\ \mu\text{M}$ in dimethyl sulfoxide. A volume of $9\ \mu\text{l}$ of cell solutions (10^5 CFU/ μl) were placed in three PCR tubes (0.2 ml). The solution of the second tube was preheated at 95°C for 5 min to lyse the cells. The solutions in the first and third tubes were not treated with heat. A volume of $1\ \mu\text{l}$ of $500\ \mu\text{M}$ PMA solution was mixed in the solutions of first and second PCR tubes to achieve PMA concentration of $50\ \mu\text{M}$. These two tubes were incubated in the dark for 20 min followed by exposure with blue light (450 nm LED, LXML-PR01-0500, Ontario, Canada) for 15 min. The solution in the third tube was neither mixed with PMA nor exposed to the light. For LAMP assays, $1\ \mu\text{l}$ of each cell solution was mixed with $24\ \mu\text{l}$ LAMP reaction solutions and incubated at 63°C for 30 min on Chromo4™. Solutions from these three tubes were also streaked on TSA plates and incubated for 12 h at 37°C .

2.6 MPN-LAMP on microchip

For < 10 CFU-based MPN-LAMP assays on microchip, $1\ \mu\text{l}$ of *E. coli* and *E. faecalis* solution (< 10 CFU/ μl) was dispensed in each well ($n=10$) followed by $1\ \mu\text{l}$ of LAMP solution. Wells were covered with mineral oil (Sigma, St. Louis, MO) during the reaction to avoid solution evaporation. The microchip was placed on the heating stage for incubation at 63°C for 30 min and real-time CCD imaging was used to monitor the fluorescence.

To confirm the amplification from single digit CFU of bacteria and to calculate their respective most probable number (MPN) concentrations, MPN-LAMP assays of *E. coli* (*gadA* gene) and *E. faecalis* (*gelE* gene) with serial dilutions from 1×10^{-1} to 1×10^{-10} were performed in an endpoint format. Each of the 40 wells was dispensed with $1\ \mu\text{l}$ of *E. coli* or *E. faecalis* dilution solution followed by $1\ \mu\text{l}$ of LAMP solution. Five replicates of digital assays for each dilution were performed to match with the 5-tube MPN analysis. The microchip was placed on the heating stage for incubation at 63°C for 40 min and fluorescence was checked by endpoint CCD imaging.

2.7 Fluorescence signal analysis

Opticon™ software (Bio-Rad Laboratories, Hercules, CA) was used to extract the raw fluorescence signals of LAMP assays on the commercial PCR instrument. For MPN-LAMP assays in real-time, CCD imaged the microchips in time-lapse mode at every 30 s at an exposure time of 5 s. Imaging was stopped when signals from the positive wells plateaued. For endpoint MPN-LAMP assays, the microchip was imaged after 40 min. A gain of 100 and an offset of 60 were used for CCD imaging. Images were saved in flexible image transport system format. CCD images were opened in Image J (National Institutes of Health, Bethesda, MD) and a microarray profile plugin (<http://www.optinav.com/imagej.html>) was applied to obtain fluorescence signals from the images. A microarray grid (circular wells with diameter of 1 mm) was used to extract the mean of signals from the same pixel area of all the time lapse images. To calculate SNR values of LAMP and MPN-LAMP assays on the commercial real-time PCR instrument and on the microchip, respectively, raw fluorescence signals were baseline corrected by subtracting the average fluorescence signals obtained in the first 3 min and dividing by their standard deviation. The average of the SNR from the triplicates was plotted with respect to amplification time. It was then defined as the time at which the SNR reached an arbitrary cut-off of 10 or higher. It values were calculated for individual LAMP reaction of triplicates and an average value of It is reported here. The error bars in the plots represent the standard deviations of the mean from three replicate experiments unless otherwise indicated. Data analysis and plotting was performed with Microsoft Excel (Microsoft, Redmond, WA). Contrast of the images of MPN-LAMP assays were modified for visual clarity, which would not affect the signal values.

2.8 Quantification of Most Probable Number (MPN)

MPN concentrations of LAMP dilutions of < 10 CFU were quantified as per the statistical method of result rejection developed by Hurley and Roscoe, and Taylor (Taylor 1962; Hurley and Roscoe 1983). 5 replicates were made for each dilution to calculate the MPN concentrations of the assays. The formula shown below, described by Hurley and Roscoe (Hurley and Roscoe, 1983), was used for analysis.

$$\sum_{i=1}^k \frac{d_i p_i v_i}{1 - \exp(-d_i x_i v_i)} = \sum_{i=1}^m d_i n_i v_i$$

Here, k is the levels of dilution, n_i is the number of subsamples, p_i is the number of positive subsamples, d_i is the dilution factor, v_i is the volume of each subsample, and x is the estimated density of the microorganism. The applied assumptions were that bacteria were distributed in the sample without any clustering and wells containing even single digit viable bacteria would produce the growth (or in our case amplification). We have assumed that the MPN values at the microscale volume would be consistent with the milliliter volume as used in the developed method. The result rejection method is based on that if the dilutions provide all the positive results, the calculation included only the most diluted samples. If the dilutions provide all the negative results, calculation included only the most concentrated samples.

3. Results and discussion

3.1 LAMP assays from genomic DNA templates and bacteria

LAMP assays with 10^5 genomic DNA copies and 10^5 CFU of *E. coli* (*uidA* gene) and *E. faecalis* (*gelE* gene) were performed at 63 °C on a commercial PCR instrument. To ensure bacterial cell lysis during the reaction, LAMP assays were also performed with both type of bacteria, separately preheated at 95 °C for 5 min. LAMP assays were also performed in which LAMP reaction solution containing bacterial cells were preheated at 95 °C for 5 min to examine the effect of heating on *Bst* polymerase. LAMP assays with genomic DNA templates and bacterial cells of *E. coli* and *E. faecalis* showed the DNA amplification at (Fig. 1a). DNA amplification was also achieved for the LAMP assays of bacterial cells, in which the bacterial cells were separately pretreated with heat (data not shown). No DNA amplification was achieved for the LAMP assays of bacterial cells, in which the LAMP reaction solutions containing bacterial cells were pretreated with heat (data not shown). Negative controls did not show any increase in the SNR value during the reaction.

Gram-positive and Gram-negative bacteria contain peptidoglycan layer, which is cross-linked with peptide bonds to provide the rigidity to the cell walls. The peptidoglycan layer in Gram-positive bacteria is thicker (15 nm to 80 nm) and more cross-linked than in Gram-negative bacteria (~10 nm), resulting in more robust cell walls (Huang et al., 2008). LAMP assays have been reported for gene amplification directly from prokaryotic and eukaryotic cells (Iwasaki et al., 2003; Bosward et al., 2016). However, prior heating of samples containing bacterial cells at 95 °C (3-10 min) for cell lysis and centrifugation to separate bigger cell debris were required before LAMP (Poon et al., 2006; Chen and Ge 2010). To our knowledge, this is the first demonstration of DNA amplification at 63 °C directly from bacterial cells without any processing of cellular samples.

Average SNR of bacterial cell assays were higher than DNA-based assays. Also, the average SNR from the cell assay of *E. faecalis* was higher than the cell assay of *E. coli*. For example, the highest average SNR from *E. coli* cell assay and DNA-based assay were 725 and 352, respectively. The highest average SNR from *E. faecalis* cell assay and DNA-based assay were 1,893 and 1,275, respectively. The highest average SNR from *E. faecalis* cell assay was 2.6-fold higher than *E. coli* cell assay. Although SYTO-82 is a DNA intercalating dye, higher SNR from bacterial cell assays than DNA-based assays might be due to some interaction of SYTO-82 dye with the cell membranes, in addition to the interaction with genomic DNA. In another experiment, *E. coli* growth was monitored in the presence of 2 µM SYTO-82. SYTO-82 was not appropriate for cell growth monitoring, but it showed a 2-fold higher fluorescence signal in the presence of *E. coli* cells than in water. Therefore, the interaction of SYTO-82 dye with the cell membrane cannot be completely ruled out. Also, higher SNR from *E. faecalis* cell assay than *E. coli* cell assay supports this hypothesis due to the thicker peptidoglycan layer in Gram-positive *E. faecalis* than Gram-negative *E. coli*. Higher standard deviations (SNR) in the cell assays than DNA-based assays were observed, which might be due to non-specific interactions of SYTO-82 with cell membranes.

Average Tt values of DNA-based assays were slightly lower than bacterial cell assays. For example, the average Tt values of *E. coli* DNA-based and cell assay were 6 min (SNR:

19.97) and 7 min (SNR: 13.89), respectively. The average Tt values of *E. faecalis* DNA-based and cell-based assays were 6 min (SNR: 161.1) and 7 min (SNR: 49.87), respectively. Also, the average Tt values of the bacterial cell-based LAMP assays, in which the cells were preheated at 95 °C for 5 min were exactly the same as the cells without any preheating (data not shown). LAMP assays containing bacterial cells, which were directly heated at 95 °C for 5 min did not show any amplification (data not shown). This was due to the fact that direct heating of LAMP reaction mixture at 95 °C deactivated *Bst* polymerase (Matovu et al., 2010).

3.2 LAMP assays for quantitative analysis of *E. coli* and *E. faecalis*

To establish standard curves for quantitative analysis, LAMP assays targeting *uidA* gene and *gelE* gene with 10-fold serial dilutions of *E. coli* and *E. faecalis*, respectively from 1×10^{-1} to 1×10^{-10} were performed at 63 °C on a commercial PCR instrument. Out of 6 samples of single digit cell assay (1×10^0 X dilution) of *E. coli* and *E. faecalis*, only 3 and 4 assays, respectively, showed the positive amplification. LAMP assays of *E. coli* and *E. faecalis* showed a consistent increase in the SNR values reaching to a plateau in 15 min for 10^5 CFU and between 20-30 min for higher dilutions. Tt values showed a consistent increment with respect to decreasing cell concentrations (Fig. S2). Negative controls did not show any increase in the SNR throughout the reaction. For both assays, standard deviations in the SNR values showed an increase with respect to decreasing cell concentrations. Additionally, standard deviations in the SNR values of the *E. faecalis* cell assays were higher than the *E. coli* cell assays. This could be attributed to the higher non-specific interactions of SYTO-82 with higher amount of cell membranes of *E. faecalis*.

The standard curves for the LAMP assays of both bacteria were established by plotting the log of CFU of bacteria versus the Tt values (Fig. 1b). The cell-based LAMP assays on the commercial PCR instrument were quantitative and sensitive to the level of single digit CFU. Higher standard deviations in the average Tt values were observed for *E. faecalis* assay than *E. coli* assay. Correlation coefficients (R^2) of the fitted line were 0.98 and 0.97 for *E. coli* and *E. faecalis* assays, respectively. Tt values of LAMP assay from 10^5 CFU to < 10 CFU of *E. coli* were from 8 min to 18.7 min, respectively. Tt values of the LAMP assay from 10^5 CFU to < 10 CFU of *E. faecalis* were from 6.7 min to 13.3 min, respectively. Faster amplification of the *E. faecalis* assay was due to rapidly increasing SNR values as compared to *E. coli* assay (Figs. 1a, b). Therefore, no preheating or processing was needed for cell-based LAMP assays i.e., LAMP can be performed by incubating the bacterial cells in the LAMP reagent mixture presumably because enough of the cells are permeabilized, providing the access of genetic contents to the LAMP reaction solution.

3.3 Optimization of LAMP assay with PMA for the detection of viable *E. faecalis* cells

LAMP assays were performed with 10^5 CFU of *E. faecalis* (*gelE* gene) treated with PMA to confirm that the DNA amplification was only from viable bacteria and not from extracellular DNA in the solution (Fig. 2). The differentiation of live and dead cells is a challenge in DNA-based molecular detection techniques (Girones et al., 2010). Prior treatment of pathogens with ethidium monoazide (EMA) (Nogva et al., 2003) and PMA (Nocker et al., 2009) is being applied to test cellular viability by using DNA-based molecular detection

techniques. EMA and PMA are photo-reactive dyes with high affinities for DNA. These dyes intercalate into double stranded DNA and form covalent linkage upon exposure to blue light, resulting in chemically modified DNA, which cannot be amplified by PCR or LAMP reactions. These dyes are designed to be cell-impermeable thus targeting only compromised or dead cells and extracellular DNA, without affecting the viable cells. Comparative investigations of membrane permeability characteristics of EMA and PMA have shown that EMA could penetrate the membranes of viable cells leading to cell loss (Pan and Breidt 2007). However, due to higher molecular weight and higher positive charge of PMA than EMA, it remains membrane impermeable (Yáñez et al., 2011). Compared to EMA, PMA has been shown to have greater success and non-lethal effects in identifying the viability of viruses (Parshionikar et al., 2010), bacteria (Nocker et al., 2009; Kralik et al., 2010; Chen et al., 2011; Liang et al., 2011), and protozoa (Brescia et al., 2009) coupled with PCR, real-time PCR, and LAMP assays. Therefore, LAMP with PMA was selected to test the viability of *E. faecalis*. An optimum concentration 50 μ M PMA was selected for this study as a range of concentrations from 30 to 50 μ M was found to be effective in other studies (Pan and Breidt, 2007; Vesper et al., 2008). *E. faecalis* treated with PMA was exposed to a blue LED for 15 min for the PMA-DNA complex formation. A longer exposure time (15 min) with a less intense LED was selected over a shorter exposure with brighter halogen lamp as heat generated by the halogen lamp has been reported to kill viable bacteria (Wang and Levin, 2006; Vesper et al., 2008; Delgado-Viscogliosi et al., 2009).

LAMP assays of *E. faecalis* (non-lysed) with and without PMA treatment showed DNA amplification with the same Tt value of 7 min. SNR value (70.1) at the Tt of non-PMA treated cells was higher than the SNR value (50) at the Tt of PMA treated cells. Tt and the corresponding SNR of PMA treated lysed cells were 12 min and 14.5 min, respectively. Cell growth was only observed from the non-lysed cells solutions (with and without PMA treatment) (Fig. S3). No cell growth was observed from the heat treated cell solution. LAMP assays showed a drastic shift in the Tt value of PMA-treated lysed cells than PMA-treated non-lysed cells. A difference in Tt value of 5 min was observed, which corresponded to 4-log fold reduction in the cell concentration due to PMA treatment. No effect of PMA treatment was observed in LAMP assays with non lysed cells (with and without PMA treatment). However, a slight lower SNR value of PMA treated cells than non-PMA treated cells might be due to death of few viable cells by the heat generated by LED light. Additionally, heat treatment (95 °C for 5 min) killed all the viable cells as observed by the lack of any cell growth on TSA plate (Fig. S3). These results confirmed that the DNA amplification (Fig. 1 a, b) was directly from viable bacteria rather than extracellular DNA in solution.

3.4 MPN-LAMP assays of single digit bacterial CFU

MPN-LAMP assays from < 10 CFU of *E. coli* and *E. faecalis* were performed on polyester microchips and imaged with a CCD imaging system developed by our group (Ahmad et al., 2011) (Fig. 3). Polyester material is compatible with LAMP reaction, as recently reported by our group (Stedtfeld et al., 2012). Also, polyester is comparatively cheaper than cyclic olefin copolymer and no surface treatment of polyester is needed during/after microfluidic chip fabrication for performing LAMP, thus a cost effective and easy-to-use option. MPN-LAMP

assays with single digit CFU of *E. coli* (*uidA* gene) and *E. faecalis* (*gelE* gene) were performed without any processing (Fig. 3).

MPN-LAMP assay of *E. coli* provided a maximum average SNR of 438 and an average Tt of 21 min, respectively. And MPN-LAMP assay of *E. faecalis* provided a maximum average SNR of 738 and an average Tt of 17.5 min, respectively. Due to statistical variations in the dilution at < 10 CFU level (1×10^{-8} dilution), only limited number of positive replicates showed DNA amplification. For example 5 and 8 positive amplifications were achieved out of 10 sample replicates in *E. coli* and *E. faecalis* assays, respectively. Negative controls did not show any increase in the SNR throughout the reaction. Higher SNR from *E. faecalis* assay than *E. coli* assay was consistent with the results from commercial PCR instrument. Additionally, Tt values for *E. coli* and *E. faecalis* assays on the microchip (5 s CCD exposure) was higher by 2.3 min and 4.2 min, respectively, than the assays on commercial PCR instrument. We had earlier demonstrated that increasing the CCD exposure time reduces the Tt value of microchip-based real-time fluorescence LAMP assays (Ahmad et al., 2011). However, in this case, reaction wells were covered with mineral oil to avoid evaporation. Due to the higher refractive index of mineral oil (1.5) than air (1.0), a portion of emitted photons from reaction wells might have been refracted from the oil-air interface, reducing the number of emission signals reaching to the CCD and increasing the Tt of MPN-LAMP assays. Also in this case, the working distance between the CCD and the microchip was increased for image focusing, further reducing the incoming emission signals to the CCD. Tt could have been further reduced by increasing the CCD exposure time from 5 s. However, increasing the CCD exposure time than 5 s would result into poor image contrast, reducing image clarity.

3.5 Quantification of most probable number by MPN-LAMP

MPN-LAMP assays were performed by targeting *gadA* and *gelE* with 10-fold serial dilutions (1×10^{-1} to 1×10^{-10}) up to 10^5 CFU of *E. coli* and *E. faecalis*, respectively (Fig 4). For each dilution, 5 replicates were performed to calculate the MPN concentrations of the assays by using the concept of 10-fold dilution of 5-tube MPN analysis. LAMP primers for *gadA* of *E. coli* were not tested earlier. Therefore, LAMP assay from *E. coli* cells was performed on a commercial PCR instrument at 63 °C to confirm the single digit CFU-level sensitivity of the assay. The designed LAMP assay for *E. coli* (*gadA* gene) was sensitive to < 10 CFU (Fig. S4). This LAMP assay showed slower amplification (Tt: 27 min) than the < 10 CFU assay designed for *uidA* of *E. coli*. Therefore, for the MPN-based LAMP assays, the microchips were incubated for 40 min before imaging.

MPN-LAMP assay of *gadA* of *E. coli* showed amplification from 10^5 CFU to < 10 CFU (Fig. 4a). Only 2 out of 5 sample wells containing single digit CFU showed amplification. No amplification was observed with the highest dilutions of 1×10^{-9} and 1×10^{-10} . Based on the 5-well MPN analysis [5 2 0], average MPN concentration of 1×10^{-8} dilution was 0.49 ($p = 0.05$) per 2 μ l of reaction volume. Similarly, MPN-LAMP assay of *gelE* of *E. faecalis* showed amplification from 10^5 CFU to < 10 CFU (Fig. 4b). However, only 3 out of 5 sample wells containing single digit CFU showed amplification. No amplification was observed with the highest dilutions of 1×10^{-9} and 1×10^{-10} . Based on the 5-well MPN

analysis [5 3 0], average MPN of 1×10^{-8} dilution was 0.79 ($p = 0.05$) per 2 μl of reaction volume. For both the cases, no amplification was observed in all 5 replicates with highest dilutions of 1×10^{-9} and 1×10^{-10} . In 1×10^{-8} dilution there is an average of 1 CFU/ μl but due to bacterial distribution at such small volumes some of the wells might contain 0, 1, 2 or even 3 cells. The results also implied that the MPN-LAMP had a large dynamic range and single digit CFU sensitivity. This approach of MPN-LAMP would therefore be valuable for the detection of low concentrations of target cells in higher reaction volumes. Digital PCR and digital recombinase polymerase amplification assays based on DNA amplification have already been reported, which rely on the readout of low-volume and high throughput wells/ compartments (positive/negative) at the end of reaction (Pohl and Shih, 2004; Heyries et al., 2011; Shen et al., 2011a; Shen et al., 2011b). MPN analysis is generally performed using culture-based approaches, e.g., broth/agar dilution, which takes about 10-12 h for the results (Rompré et al., 2002; Kinzelman et al., 2003). MPN-LAMP could be used for the rapid bacterial enumeration by temporal gene amplification from bacteria, and thus has the potential to replace the conventional MPN method.

3.6 Potential mechanism(s) of MPN-LAMP

The effect of temperature shock (from 50 °C to 70 °C) on the membrane permeability of a Gram-negative (*E. coli*) and a Gram-positive (*Deinococcus radiodurans*) bacteria has already been reported (Baatout et al., 2005). A significant increase in the cell permeability of both the bacteria was observed at temperatures higher than 45 °C. Therefore, in our case, the incubation of bacterial cells at 63 °C might have increased the cell permeability leading to the inclusion of LAMP reaction components in the cells and DNA amplification may have occurred inside the cells, as reported in case of *in situ* LAMP (Ye et al., 2011). The results illustrated that gene amplification was possible either by lysis or permeabilization of Gram-negative and Gram-positive bacterial cells at 63 °C. DNA-based assays were faster than cell assays by 1 min. In the DNA-based assays, DNA templates were easily accessible to the LAMP reaction components. However, in cell assays, diffusion of LAMP reaction components inside the cells or their diffusion through cell debris might have slightly increased the Tt values.

4. Conclusions

LAMP assays for rapid and sensitive gene amplification directly from *E. coli* and *E. faecalis* bacterial cells (without any sample processing) were performed. PMA-LAMP was performed to test the viability of *E. faecalis*, which confirmed that DNA amplification was only from live bacterial cells. MPN-LAMP assays of 1×10^{-8} dilution were also translated on microchips. DNA amplification from MPN-LAMP assays of single digit CFU-level was confirmed by MPN analysis. Success of the LAMP method directly from bacterial cells at a temperature of 63 °C might due to increased cell permeability at this temperature leading to DNA amplification. Direct DNA amplification from bacterial cells at this moderate temperature would eliminate any sample processing steps and reduce the complexity of gene analysis instruments. In addition, MPN-LAMP has the potential to replace the conventional MPN method. This work would be valuable for use with simple, sensitive, and rapid integrated gene analysis systems with sample-in-answer-out capability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Most probable number based loop mediated isothermal amplification approach is proposed.
- It is advantageous because of very low detection limit < 10 CFU.
- Live cell DNA amplification is confirmed by using propidium monoazide PMA.
- Amplification can be achieved at moderate temperature without processing the cells.
- The approach has also been translated on microfluidic platform.

Fig. 1.

(a) LAMP assays of 10^5 genomic DNA copies and 10^5 cells of *E. coli* (*uidA* gene) and *E. faecalis* (*gelE* gene) on a commercial PCR instrument

Fig. 1.

(b) Standard curves of RT-LAMP assays of *E. coli* (*uidA* gene) and *E. faecalis* (*gelE* gene) cells on a commercial PCR instrument.

Fig. 2.
LAMP assays of 10^5 *E. faecalis* non-lysed cells (with and without PMA treatment) and PMA treated lysed cells on a commercial PCR instrument.

Fig. 3. MPN-LAMP assays of single digit CFU of *E. coli* (*uidA* gene) and *E. faecalis* (*gelE* gene). Inset showed the CCD time lapse images of microchip with 10 sample replicates and 10 negative controls. The error bars represented the standard deviations of the mean from 5 and 8 positive replicates (amplified) and 10 negative replicates for *E. coli* and *E. faecalis*, respectively.

Fig. 4. MPN-LAMP image for (a) *E. coli* (*gadA* gene) and (b) *E. faecalis* (*gelE* gene). Cell number per well (left) and number of positive reaction (right) were shown in the image.