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Most probable number - loop mediated isothermal amplification (MPN-LAMP) for quantifying waterborne pathogens in <25 min

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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 10^5 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.

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 Rådström, 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

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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., 2006). 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 × 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 °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 °C while maintaining the embossing pressure. The microchip was then extracted from the perforated sheet, rinsed with distilled water, and air-dried at 65 °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 $(NH_4)_2SO_4$, 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.