Biosensors and Bioelectronics 30 (2011) 255–260

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

# Handheld device for real-time, quantitative, LAMP-based detection of *Salmonella enterica* using assimilating probes

Daniel M. Jenkins[a,*], Ryo Kubota[a], Jin Dong[b], Yong Li[b], Dennis Higashiguchi[c]

[a] Department of Molecular Biosciences and Bioengineering, University of Hawai'i, Mānoa, USA
[b] Department of Human Nutrition, Food, and Animal Sciences, University of Hawai'i, Mānoa, USA
[c] Department of Microbiology, University of Hawai'i, Mānoa, USA

ARTICLE INFO

*Article history:*
Received 12 July 2011
Received in revised form
14 September 2011
Accepted 17 September 2011
Available online 22 September 2011

*Keywords:*
Isothermal amplification
Gastroenteritis
Agricultural diagnostics
Point-of-care instrument

ABSTRACT

A simple handheld instrument was designed to enable real-time detection of the LAMP reaction in a standard PCR tube using newly described assimilating probes as sequence-specific reporter molecules. The system was validated using DNA isolated from *Salmonella enterica*, demonstrating accurate temperature control with little power and little overshoot of setpoint temperatures, with rapid and accurate detection often in less than 30 min and within 20 min for reactions with high ($>10^5$) genome copy numbers. The system could be used for quantitative determination of pathogen DNA, with a limit of detection of about 15 genome copies in purified DNA or 25 cells in DNA extracts from chicken rinsate – comparable to values obtained when running the same reaction on a commercial benchtop real-time PCR instrument. Positive classification of standards nominally containing a single genome equivalent was demonstrated, and no false positives were reported. Detection of *S. enterica* in rinsate from a contaminated chicken sample required 48 h enrichment prior to the LAMP reaction or plating on semi-selective media. The new system demonstrates a major compelling advantage of the LAMP reaction, in that it may be enabled in simple, low-power, handheld devices without sophisticated custom miniaturized disposables. This new diagnostic system is especially promising for on-site diagnostics in the food and agricultural industries where laboratory space is often primitive if it is available at all.



## 1. Introduction

*Salmonella enterica* is a gram negative bacterium that commonly results in acute gastroenteritis, and globally accounts for almost 50% of all outbreaks of food-borne illness (Greig and Ravel, 2009). Certain populations of the pathogen are causal agents of the deadly typhoid fever (Brooks et al., 2007), and are especially prevalent in developing countries. In 2010, it is estimated that infection by non-typhoidal *Salmonella* in the United States alone resulted in over 19,000 hospitalizations and 400 deaths (United States Centers for Disease Control and Prevention, 2011). The pathogen is often associated with poultry and egg products as illustrated by *Salmonella* outbreaks traced back to Iowa egg producers in 2010 (Layton, 2010), but it is becoming more common to encounter the pathogen on fresh vegetables (Sant'ana et al., 2011) and other processed foods (Weise, 2009). It is estimated that in 2010 *Salmonella* infection in the US cost $2.7 billion in medical bills, lost wages, and premature death (United States Department of Agriculture, Economic Research Service, 2011), exclusive of costs associated with product recalls, disease containment and control measures, and incalculable losses to the reputations of implicated agricultural products and producers regardless of the true source of contamination.

Current regulations in the United States require that all poultry operations operate a HACCP (Hazard Analysis and Critical Control Points) plan to mitigate risk of food-borne illness (United States Food and Drug Administration, 2009). These plans include mandatory screening for Salmonella, usually requiring that samples be shipped to state-run diagnostic laboratories running time consuming culture-based methods or capital intensive benchtop instruments. Delays in transit, analysis, and reporting make it difficult to respond in a timely manner to control and contain disease organisms when they occur. The main objective of this research is to develop simple, rapid, inexpensive tools to enable typical producers and processors to implement their own diagnostic programs on-site, where access to dedicated laboratory facilities is often lacking.

For the greatest speed, flexibility, and adaptability, our work is focused primarily on gene-based diagnostics which can readily be adapted to emerging pathogens using advances in sequencing

* Corresponding author at: 1955 East West Rd., Rm. 218, Honolulu, HI 96822, USA. Tel.: +1 808 956 6069; fax: +1 808 956 3542.
*E-mail address:* danielje@hawaii.edu (D.M. Jenkins).

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doi:10.1016/j.bios.2011.09.020

and bioinformatics (Kubota et al., 2011c), and which can more reliably be designed to rapidly discriminate closely related pathogen populations which may have different virulence or other risk factors to agriculture and human health. Examples include identifying cold tolerant populations of the bacterial wilt pathogen *Ralstonia solanacearum* which pose great risks to agricultural production in temperate regions of North America, or discriminating lethal strains of *Escherichia coli* (Dempsey and Neuman, 2011) from less harmful and more ubiquitous strains.

To facilitate accelerated detection on a simple platform, most biosensor development has focused on direct analyte interaction with a biorecognition element resulting in physico-chemical changes that can be detected by optical, electrochemical, or seismic measurements (Lazcka et al., 2007). However, it has been challenging to develop such systems that have the levels of sensitivity and selectivity required for reliable diagnostics of disease organisms (Lazcka et al., 2007). A variety of relatively inexpensive and disposable point-of care gene-based diagnostic systems have been demonstrated that may be transferable to the agricultural setting (Weigl et al., 2008; Yager et al., 2008), including microfluidic devices where DNA is extracted and amplified by PCR (Chen et al., 2010; Lui et al., 2009). Most development is focused on PCR based diagnostics, where for speed, portability and low-power thermal cycling systems must necessarily be miniaturized. Alternative approaches to thermal cycling include moving PCR reactions through a system with spatial temperature gradients (Cao et al., 2011; You et al., 2011), including systems designed for natural convective flow through various temperature zones (Muddu et al., 2011; Zhang and Xing, 2010). To circumvent the need for rapid thermal cycling and the incumbent design complexities, we have focused our work on developing functional molecular assays using isothermal amplification reactions. While a variety of isothermal nucleic acid amplification mechanisms are available, in our experience the most suitable is Loop mediated AMPlification (LAMP; Notomi et al., 2000) as it requires only a single enzyme (strand displacing polymerase) and does not require preliminary manipulations to build a molecular motif capable of continuous self-replication. We have successfully demonstrated the use of LAMP for detection of various pathogens (Kubota et al., 2011b, 2011c), including in soil drainage from infected plants (Kubota et al., 2008). We have also developed a new FRET based probe to allow sequence specific real-time monitoring of the LAMP reaction (Kubota et al., 2011a).

Instruments for real time monitoring of the LAMP reaction based on turbidimetric measurements of magnesium pyrophosphate precipitated as a byproduct of the polymerization reaction are commercially available (LA-200 LoopAMP real time turbidimeter, Eiken Chemical Co., Japan), and portable versions are reported in the literature (Lee et al., 2008). Others have reported analogous fluorescence based real-time instruments for isothermal amplification reactions, using non-specific intercalating dyes for reporting positive reactions. These include a handheld instrument for NASBA based detection (Smith et al., 2007) and another for LAMP (Liu et al., 2011). Both of these instruments use custom designed miniaturized disposable elements. We have attempted a similar approach in earlier prototypes to economize on power, controlling temperature in a custom disposable by laser irradiation, with feedback control enabled by a PCB-mounted infrared pyrometer. While temperature control was effective in these designs, material incompatibilities with the disposable made reactions inconsistent, and we took a new approach to design a system around industry standard disposables which can be prepared and used simply by personnel in the field, analogous to the rationale for Qiu et al. (2010) to design a point of care thermal cycler for relatively large volume (~10–100 μl) PCR reactions. In this manuscript, we report on a prototype handheld device to enable real-time, sequence specific detection of the LAMP reaction in standard PCR tubes using assimilating probes. To illustrate the functionality of the device, we have demonstrated its use for detecting *S. enterica* in rinsate from dressed chicken samples.

## 2. Materials and methods

### 2.1. Preparation of DNA standards

*S. enterica* subsp. *enterica* ser. Typhimurium (ATCC #14028) was grown on Brain Heart Infusion (BHI) agar (Catalog No. 221610, Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 24 h at 35 °C. DNA were purified from these cells with the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. DNA concentrations were quantified photometrically (absorbance measurements at 260 nm and 280 nm with Nanodrop® ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., Rockland, DE, USA). The copy number of template genomic DNA was estimated on a mass basis assuming a genome size of approximately 4.95 Mb with 53% GC content (McClelland et al., 2001), resulting in a genome mass of about 5.1 fg.

### 2.2. Preparation and culture-based analysis of chicken rinsate

Chicken was purchased from a local grocery store. Two drumsticks with skin-on, weighing approximately 180 g, were covered in 180 ml of buffered peptone water (Catalog No. 212367, Beckton Dickinson) and agitated mildly for 2 min. The rinsate was then collected and distributed into 9 ml aliquots in sterile, 40 ml glass tubes with plastic screw tops. One tube was set aside for general microbial characterization, while all other tubes were stored at −20 °C until their use for preparation of live *S. enterica* standards. Total culturable microbial counts were estimated by plating the chicken rinsate directly on non-selective Tryptic Soy Agar (TSA; Catalog No. 236950, Becton Dickinson) and incubating for 24 h at 35 °C. Enumeration of *S. enterica* in rinsate was done by direct plating on semi-selective Xyline-Lysine-Desoxycholate (XLD) agar (Catalog No. 221284, Becton Dickinson), incubating for 24 h at 35 °C, and counting characteristic black colonies. For more reliable culture based detection of *S. enterica* contamination in the original sample, rinsate was first pre-enriched by incubating for 24 h at 35 °C. One ml of the pre-enriched rinsate was subsequently diluted into 9 ml of RV broth (Difco™ Rappaport-Vassiliadis R10 from Becton Dickinson) and enriched further by incubation for another 24 h at 42 °C. *S. enterica* growing in the enriched RV broth were detected by plating onto XLD agar as described above. Presence of *S. enterica* in enriched RV broth was also confirmed by PCR (Fey et al., 2004).

### 2.3. Preparation of S. enterica standards in chicken rinsate

Cultures of *S. enterica* (ATCC 14028) were prepared by inoculating 10 ml of sterile Tryptic Soy Broth (TSB; Catalog No. 286210, Becton Dickinson) with 0.1 ml of a previously frozen (−80 °C) culture. After 12 h at 35 °C, 0.1 ml of the resulting culture was inoculated into 10 ml of fresh TSB which was then grown out for another 12 h at 35 °C. The resulting stock cultures generally contain approximately $10^9$ CFU/ml *S. enterica*, but for quantitative studies viable cells in the pure culture were enumerated by plating out a $10^7$ dilution onto TSA. Serial dilutions (1 in $10^2$ to 1 in $10^8$) of the stock culture were made in chicken rinsate prepared as described above. *S. enterica* load in these preparations was estimated on the basis of the cell counts observed in the inoculum from pure culture, and colony counts of the original chicken rinsates plated out on semi-selective XLD agar. Six aliquots of 1 ml from each dilution were removed for analysis by LAMP following DNA extraction. DNA from three aliquots was extracted using a commercial kit (PrepMan™ Sample Preparation Reagent, Applied Biosystems, Foster City, CA,