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6200 Aurora Ave., Suite 200W, Des Moines, IA 50322-2864

Lena Diaz,[1] Yong Li,[2] Ryo Kubota[1] and Daniel M. Jenkins[1]*

[1]Dept. of Molecular Biosciences and Bioengineering and [2]Dept. of Food Science and Human Nutrition, University of Hawaii at Manoa, Honolulu, HI 96822, USA

Characterization of a Portable, Non-instrumented Incubator for Enrichment of *Escherichia coli* O157:H7 and *Salmonella* Serovar Typhimurium and Detection by Loop Mediated Isothermal Amplification (LAMP)

ABSTRACT

We developed a handheld, non-instrumented incubator (NI-incubator) to enable direct enrichment of pathogenic bacteria in the field. The incubator contains a custom cartridge, enclosed in a thermos, filled with a phase-change material that can store latent energy at the desired temperature (37°C) after addition of approximately 300 mL of boiling water. Temperature profiles recorded in four NI-incubators over 12 hours (n = 3) illustrated that the devices maintained temperatures above 35°C for over nine hours. To quantify bacterial growth rates in NI-incubators, cultures of *Salmonella* Typhimurium (ATCC 14028), *Escherichia coli* O157:H7 (C7927), *Escherichia coli* K12, and generic *E. coli* (environmental isolate) were grown in buffered peptone water and subjected to a temperature profile representative of those observed in NI-incubators. The empirical growth characteristics were used to predict the minimum enrichment periods required to achieve reliable detection (> 95% positive) of a given quantity of inoculum by loop mediated isothermal amplification (LAMP). Predictions were validated by inoculation of 5 g of spinach with 2.33 ± 0.58 CFU of *E. coli* O157:H7 and of 5 g ground chicken inoculated with 4.5 ± 0.5 CFU *Salmonella* Typhimurium, which could be reliably detected by LAMP after 6 and 8 hours (h), respectively (n = 3 each), of enrichment in NI-incubators.

INTRODUCTION

New molecular techniques and advances in automated instrumentation that make possible the rapid, convenient and sensitive detection of foodborne pathogens are becoming commercially available for point-of-care diagnostics and on-site testing. However, constraints on sample size and composition typically mean that these systems are unlikely to detect pathogens at concentrations below 10^3 or even 10^4 CFU/mL, levels well above regulatory thresholds posing significant health risks of many bacteria. For example, a nucleic acid-based PCR assay targeting *Escherichia coli* O157:H7 in ground beef homogenate demonstrated a 10^3 CFU/mL limit of detection (LOD) *(21)*, while the infectious dose of *E. coli* O157:H7 is 10^1–10^3 CFU *(10)*. In another instance, a nucleic acid-based loop mediated amplification

*Author for correspondence: Phone: +1 808.781.1343; Fax: +1 808.956.3542; Email: danielje@hawaii.edu

(LAMP) assay demonstrated a 10^3–10^4 CFU/mL LOD of *Salmonella* Typhimurium (*S.* Typhimurium) in cantaloupe, spinach and tomato *(7)*, while the infectious dose of *S.* Typhimurium is 10^1–10^3 CFU *(13)*. Thus, the sensitivities of rapid methods (i.e., methods with biosensors based on nucleic acid amplification, hybridization, or other biological interactions) are generally insufficient for direct detection of pathogens and require culture enrichment prior to analysis. This is especially true when the LOD of low or zero tolerance pathogens by rapid methods is greater than the infectious dose (ID) and regulatory thresholds *(24)*. Therefore, conventional culture enrichment methods, requiring times of 16–24 h, are often necessary to achieve cell densities appropriate to detecting small quantities (10^0–10^2 CFU/mL) of pathogens by rapid methods. While culture enrichment increases the time to detection, it allows time for damaged or stressed cells to repair and dilutes inhibitors present in sample food matrix, so that nucleic acid-based assays such as LAMP can directly detect pathogens with little sample processing and DNA extraction.

LAMP is an isothermal method of amplifying template strands of nucleic acids that makes it attractive for portable low power diagnostic applications. LAMP has been demonstrated to be 10–100 times more sensitive than PCR in detecting *Listeria ivanovii (25)* on spiked stool samples, vero toxin producing *E. coli* on beef *(12)* and *S.* Typhimurium on chicken *(20)*. Effective means of enriching and/or concentrating target pathogens still require transport of samples to laboratory facilities with benchtop instruments, qualified technicians, and good sanitary conditions and practices. In this study, we demonstrate the use of a portable non-instrumented incubator (NI-incubator) to enrich the target cells to the detection level of specific LAMP assays, enabling POC enrichment and rapid detection of trace pathogens from food samples.

MATERIALS AND METHODS

Bacterial strains

Salmonella Typhimurium (ATCC 14028), *Escherichia coli* O157:H7 (C7927), and *Escherichia coli* K12 (capsular antigen K12) were stored at -80°C prior to experimentation. After removal from storage, strains were propagated overnight at 37°C on MacConkey sorbitol agar and xylose lysine deoxycholate (XLD) agar for *E. coli* O157:H7 and *S.* Typhimurium, respectively. Generic *Escherichia coli* was isolated from the environment (environmental isolate, Ala Wai Canal, Honolulu, HI, USA) using selective media plates CHROMagar™ ECC (CHROMagar, Paris, France). We chose to use this generic *Escherichia coli* isolated from the environment because our lab has previously conducted studies with this isolate. Specifically, we developed a LAMP assay to detect generic *E. coli (8)*. Details of bacteria propagation prior to inoculation for each experimental condition are outlined in the respective sections to follow.

Appropriate serially diluted volumes of inoculated cultures were used in tested enrichment conditions for ground chicken and spinach inoculation.

Non-instrumented incubator

A commercially available insulated food thermos of about 11.5 cm outer diameter (850 mL Thermal Container, Life Without Plastic, Wakefield, Quebec, Canada) was used to enclose a custom fabricated cartridge designed to accommodate 50 mL bags of liquid media for enrichment *(Fig. 1A)*. The 4 inch (~10 cm) outer diameter cartridge is composed of a section of 3 inch (~7.5 cm) inner diameter thin-walled aluminum tubing, with polycarbonate caps and outer walls enclosing a phase change material (PureTemp 37°C, Entropy Solutions, LLC., Plymouth, MN, USA) for latent energy storage *(Fig. 1B)*. Phase change material stores thermal energy as latent heat at the material's melting temperature and can therefore maintain a specific temperature, i.e., 37°C, for extended periods of time. The phase change material was melted so it could be poured into the walls of the cartridge, leaving approximately 1 cm headspace within the cartridge walls to allow thermal expansion/contraction before the top cap of the assembly was secured. To prevent leakage of the phase change material, all joints in the cartridge were permanently sealed with a marine grade sealant (Marine Adhesive Sealant 5200, 3M, St. Paul, MN).

Each cartridge contained approximately 160 g polycarbonate, 67 g aluminum, and 190 g phase change material, representing a total sensible heat capacity of 680 J K^{-1} and latent heat capacity of 42180 J *(Table 1)*. By recording the first order temperature decay of boiling water enclosed in the thermos, we determined that its total resistance to heat transfer was approximately 0.1 min K J^{-1}, a somewhat conservative value given that convective heat transfer from water inside the thermos should be faster than transfer from the cartridge to the thermos through an air gap. Based on these estimates, the NI-incubator theoretically could maintain the design temperature for at least six hours in a 25°C ambient environment.

NI-incubator heating

To heat the NI-incubator for sample enrichment, the removable cartridge was first filled to the rim with boiling water (~300 mL) and then sealed in the thermos for 15 minutes. To prevent scalding injuries, water was poured into the cartridge already positioned in the thermos, and then the lid was fastened onto the thermos. After a 15-minute equilibration period during which the entire contents of the thermos approached 37°C, the cartridge was removed from the thermos and then returned to it with the sample/media, after the water had been discarded.

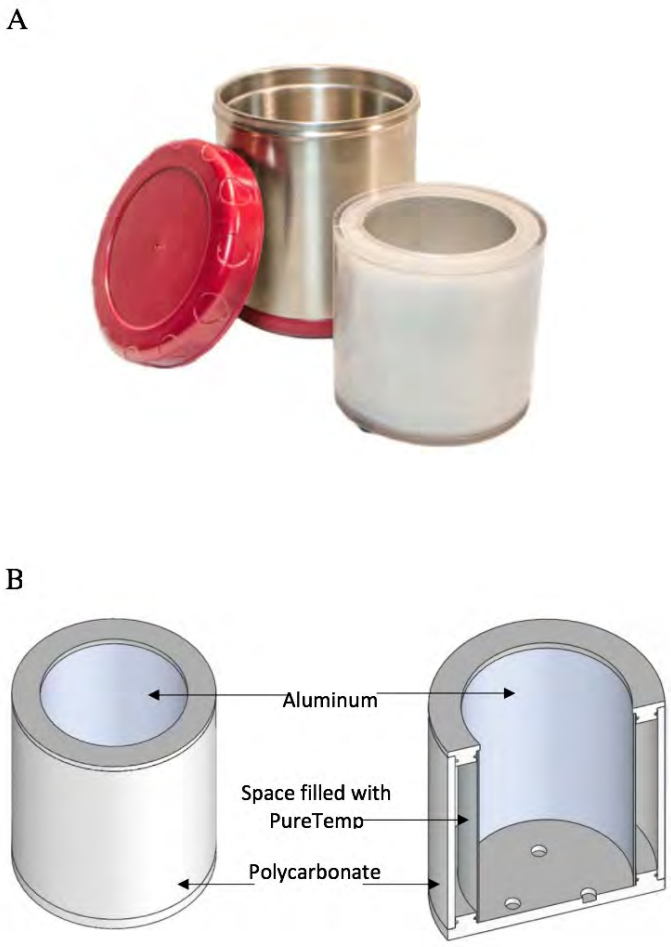

Figure 1. Image of non-instrumented incubator thermos and cartridge (A) Solid Works CAD model of custom designed NI-incubator cartridge, with cutaway view (B) showing the space between the cylinder walls where PureTemp 37°C phase change material is filled. The WhirlPak™ sample bag sits inside the cartridge during incubation.

TABLE 1. Material composition and thermal characteristics of incubator cartridges

	mass (g)	C_p* (J g^{-1} K^{-1})	Lf† (J g^{-1})	SC‡ (J K^{-1})	LC⁋ (J)
Aluminum	67	0.902•	n/a	60	-
Polycarbonate	160	1.25••	n/a	200	-
PureTemp37°C•••	190	2.21	222	420	42180
Total	**417**	-	-	**680**	**42180**

* Specific heat capacity. • *(6)*

† Latent heat of fusion. •• *(4)*

‡ Sensible heat capacity. ••• *(2)*

⁋ Latent heat capacity.

NI-incubator temperature profile

A representative NI-incubator temperature profile was determined by averaging the recorded temperature profiles observed in four different NI-incubators over a period of 12 hours (n = 3 for each NI-incubator), with 50 mL of tap water in a closed media bag. The temperature was recorded every minute of the incubation periods, using USB based recorders (Track-It™Temperature Data Loggers With Display, Monarch Instrument, Amherst, NH), each of which was calibrated against a single type-K thermocouple, which itself had previously been calibrated using standards of an ice bath with distilled water, boiling distilled water, and references in between using temperatures indicated on a multimeter with temperature measurement capability (Fluke model 179 multimeter, Fluke Corp. Everett, WA). Calibration of the USB-based recorders was conducted by recording steady state temperature values reported by each of these devices, along with the calibrated thermocouple reading. In this calibration setup, the calibrated thermocouple itself was used in a custom circuit to override the feedback control of the temperature on the rack of a commercially available laboratory incubator (Model 637D, Fisher Scientific, Dubuque, IA), and temperature was programmed to increase each hour in 1°C increments over the range from 25°C to 40°C. It was assumed that data immediately before each step was at "steady state."

NI-incubator enrichment growth characteristics

Growth characteristics of four selected bacterial strains, *Salmonella* Typhimurium (ATCC 14028), *Escherichia coli* O157:H7 (C7927), *Escherichia coli* K12, and generic *Escherichia coli* (environmental isolate, Ala Wai Canal, Honolulu, HI, USA), were determined by incubating inoculated media for 12 hours in the incubator setup already described, with temperature programmed to replicate the representative NI-incubator temperature profile recorded in prior experiments (i.e., average temperature values of the 12 recorded profiles at each point). Initial liquid cultures were prepared by picking single colonies of each bacterial strain on a selective media plate and inoculating each into 100 mL of autoclaved buffered peptone water (BPW). The cultures was allowed to grow overnight at 37°C. Then, 100 L of serially diluted initial liquid culture was used to start the simulated NI-incubation by inoculating to three different 100 mL WhirlPak bags (A, B, C) containing 50 mL of BPW. Initial liquid culture concentrations (CFU/mL) were determined by plating serial dilutions individually onto plate count agar (PCA) media plates. Every two hours during the incubation, 100 L samples were taken from each bag and bacteria enumerated by plating following appropriate dilution (n = 3 for each count). Prior to each sampling, the bags were gently inverted several times to homogenize the culture.

Artificial contamination of ground chicken and spinach samples

Ground chicken and fresh spinach were purchased from a local supermarket and kept at 4°C until inoculation. Prior to each experiment, single bacterial colonies were picked from MacConkey Sorbitol agar (*E. coli* O157:H7) or xylose lysine deoxycholate (XLD) agar (*S.* Typhimurium) media and propagated twice overnight at 37°C in 10 mL of TSB to achieve a target stock concentration of approximately 10^9 CFU/mL. Approximately 5 g portions of ground chicken were weighed aseptically, placed into sterile WhirlPak bags, and inoculated using a pipette with approximately 100 L of 10^1 CFU/mL dilution of *S.* Typhimurium culture to achieve approximately 1–10 CFU total in 5 grams of ground chicken. Samples were left undisturbed for 20 minutes to allow the bacteria to bind to the meat surface *(23)*.

Spinach leaf samples were washed 3 times with deionized (DI) water and dried in a biosafety cabinet for 30 minutes at room temperature. Spinach leaves were then inoculated by the spot method recommended by the National Advisory Committee on Microbiological Criteria for Food and used with spinach and green onion matrices by Durak et al. *(9)*. One hundred L of *E. coli* O157:H7 serially diluted culture was distributed equally between spinach leaf surfaces to achieve a total inoculation of 1–10 CFU in 5 grams of spinach, and the samples were then dried in a biosafety cabinet for 1 hour at ambient temperature.

At the same time, control samples of 9 g of ground chicken or spinach were homogenized in individual 81 mL volumes of primary enrichment BPW medium (1:10 dilution) and aseptically distributed into nine subsamples (~1 g of homogenized sample/ 9 mL BPW) in sterile 15 mL conical centrifuge tubes (Falcon™, Thermo Fisher Scientific, Waltham, MA, USA). Homogenization was achieved by hand massaging the WhirlPak bag contents for 2–5 minutes until large chunks of sample were mostly dispersed and well mixed. Spinach and ground chicken homogenate subsamples were then inoculated with *S.* Typhimurium and *E. coli* O157:H7, respectively, to achieve equivalent concentrations of 10^{-1} to 10^7 CFU/g food (~10^{-2} –10^6 CFU/mL final dilutions in media). These samples were used to determine the direct detection limits of two unique LAMP assays targeting *E. coli* O157:H7 and *S.* Typhimurium without prior enrichment.

Pathogen enrichment by NI-incubation

For enrichment of artificially inoculated food in NI-incubators, samples were aseptically transferred to 45 mL of sterilized BPW to obtain a 1:10 dilution and a total volume of approximately 50 mL in a WhirlPak™ bag. Once sealed in WhirlPak™ bags, the samples were massaged by hand for 2 minutes before being placed into a pre-heated NI-incubator cartridge enclosed in a thermos. The incubation periods were 6 h for inoculated spinach and 8 h for inoculated ground

chicken to enrich *E. coli* O157:H7 and *S.* Typhimurium, respectively. One un-inoculated sample was used as a negative control for each spinach and ground chicken sample to ensure that samples were not already contaminated. All experiments were conducted in triplicate.

Sampling and DNA template extraction

Nucleic acid based loop mediated isothermal amplification (LAMP) assays *(17)* were performed using template DNA extracted directly from different media/samples. Contents of WhirlPak bags were homogenized by gentle inversion a few times before 1 mL samples were transferred into sterile centrifuge tubes, which were then boiled at 100°C for 10 minutes to lyse cells and release cellular DNA *(22)*. Following boiling, food particles were removed by low-speed centrifugation at 1,000 g for 2 minutes (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany); 5 µl of crude cell lysates were then pipetted from the liquid fraction and placed directly into individual PCR tubes for assay by LAMP.

Pathogen detection (LAMP assay)

A previously published LAMP primer set targeting the genes *VT2, VT2vha,* and *VT2vhb* of Vero-toxin-producing *E. coli* was used to detect *E. coli* O157:H7 (*E. coli* primers *(12)*). A separate LAMP primer set (Se primers; *(14)*) was used to detect *S.* Typhimurium specifically. All LAMP reactions have 6 primers and were performed in 25 L (total volume) of sample containing 40 pmol of each backward inner primer (BIP) and forward inner primer (FIP), 5 pmol of each outer primer (B3 and F3), and 20 pmol of each loop primer (LB and LF). Reactions were prepared by adding 5 L of a stock primer solution and 5 L of sample (containing target template DNA) to 15 L of commercially available Isothermal Mastermix with dye (Catalog No. ISO001, Optigene, Inc., Horsham, UK). All primers had been synthesized commercially (Integrated DNA Technologies, Coralville, IA, USA). Assays for bacteria in inoculated food before and after NI-incubator enrichment were performed in 0.2 mL reaction tubes (TempAssure, Optical Caps, USA Scientific Inc., Orlando, FL, USA) in an 8-well isothermal amplifier Platform (BioRanger™, Diagenetix Inc., Honolulu, HI, USA) and incubated at 65°C for 31 minutes. LAMP reactions to determine detection limits by use of control samples without enrichment were performed in 0.1 mL TempPlate semi-skirt PCR 96 well plates (Catalog No. 1402-9100, USA Scientific, Inc. Ocala, FL, USA) in a real-time PCR machine (Applied Biosciences StepOnePlus™), also at 65°C for 31 minutes. Fluorescence values corresponding to fluorescein were recorded every minute during the 31-minute reactions in the StepOnePlus™ PCR machine, and every 30 seconds in the BioRanger™ isothermal amplifier. For the BioRanger, threshold times (t_T) of positively classified reactions are reported as the time at which the maximum rate of fluorescence increase occurs, to control for variations in reaction intensity and fluorescence sensitivity between channels. For the StepOnePlus™ PCR machine, the t_T was estimated as the time required for the fluorescence value to exceed a threshold value equivalent to the pooled average plus three standard deviations of the fluorescence values observed throughout the reactions of triplicate negative control reactions *(15)*. To compare relative amplification efficiencies for different reaction conditions and primers, we used the reciprocal of the negative slope of standard curves relating threshold time to initial template concentration. These values are inversely related to the amplicon doubling time *(15)*, and are completely analogous to standard methods of estimating PCR amplification efficiency *(26)*. Reactions were conducted in triplicate for each sample type (chicken, spinach) and treatment (before and after enrichment in NI-incubator), for un-inoculated control samples and non-template controls, and for each bacterial concentration in experiments to determine detection limits prior to enrichment. LAMP detection rates are defined as the ratio of number of positive assays to total number of assays performed, for samples known to contain target DNA. Detection rates were calculated based on the percentage of positive samples classified by observed $t_T < 29$ minutes.

RESULTS

NI-incubator temperature profile

The mean observed temperature in four NI-incubator assemblies (total n = 12) remained above 35°C for 9.3 hours and above 33°C for 12 hours *(Fig. 2)*. While the PureTemp phase change material was designed to be maintained at 37°C, the recorded temperature from T = 0 h to T = 9 h was approximately 2°C lower, which can likely be attributed to temperature gradients between the cartridge/phase change material and the other space within the thermos, and potentially to the effect of impurities in the PureTemp material on the uniformity of the melting temperature.

NI-incubator growth characteristics

Growth rates of four selected bacterial strains at the representative NI-incubator temperature profile were determined by sampling 100 µL from each culture every two hours and plating the samples for enumeration. The observed temperatures during these growth experiments aligned closely with the representative temperature profile observed in non-instrumented incubators, allowing for short disturbances coinciding with the two-hour intervals when the incubator was opened to sample cultures for plating of each Ala Wai isolate *(Fig. 3A)*, *S.* Typhimurium *(Fig. 3B)*, *E. coli* KI2 *(Fig. 3C)*, and *E. coli* O157:H7 *(Fig. 3D)*. Normalized growth curves *(Fig. 4)* indicated that all of the bacterial strains grew at similar rates under the temperature conditions of the non-instrumented incubators, with doubling times of 24.13 min (*S.* Typhimurium), 24.97 min (*E. coli* K12), 21.86 min (*E. coli* Ala Wai isolate), and 19.19

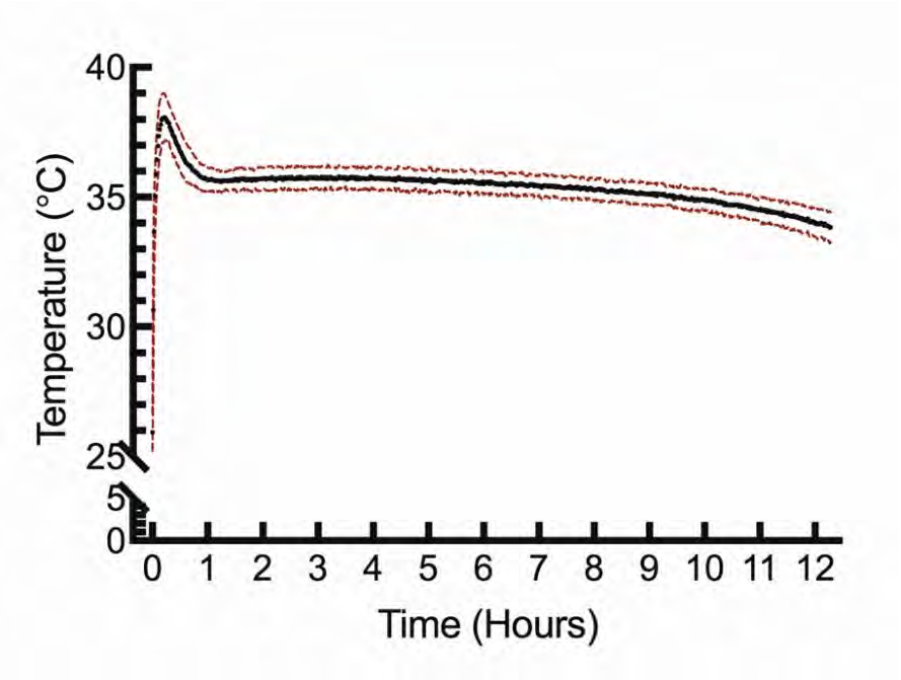

Figure 2. Non-instrumented incubator temperature profiles recorded every minute over 12 hours. The solid black line represents the average, while dotted lines represent ± standard deviation of temperature (°C) from 4 different NI-incubator assemblies, each replicated in triplicate (total n = 12).

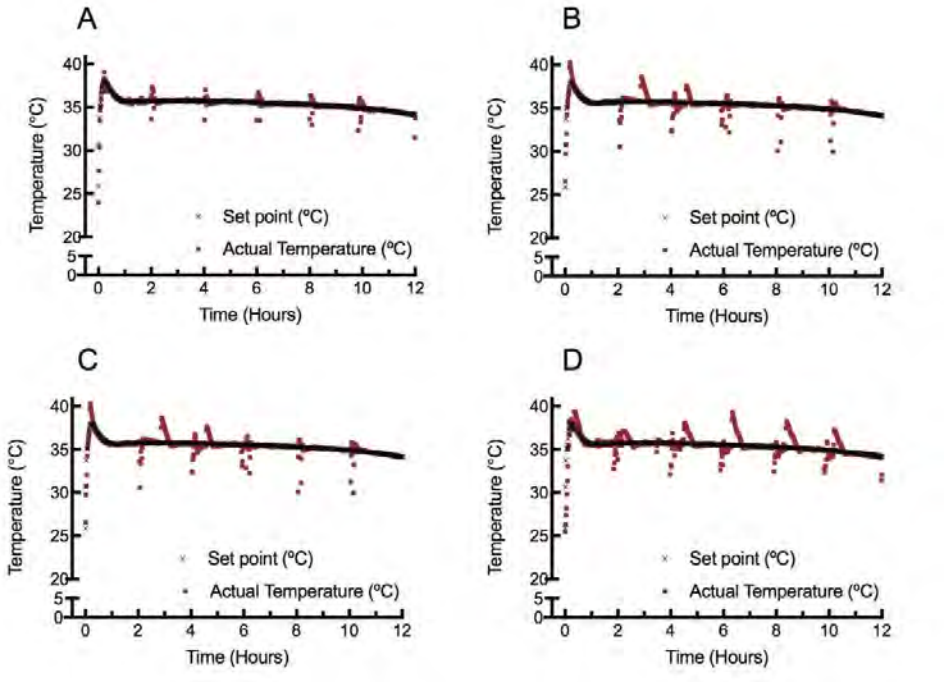

Figure 3. Set point (black line) and actual (red square) temperatures (°C) observed in laboratory incubator programmed to replicate observed NI-incubator temperature profiles, in growth experiments for each of the experimental bacterial strains used in this research. Brief deviations of the actual temperature from the set point occur at two hour intervals coinciding with opening the incubator to sample cultures for bacterial enumeration. Individual panels represent temperatures during the growth of a generic isolate of *Escherichia coli* (A), *Salmonella* Typhimurium (ATCC 14028) (B), *Escherichia coli* K12 (C), and *Escherichia coli* O157:H7 (C7927) (D).

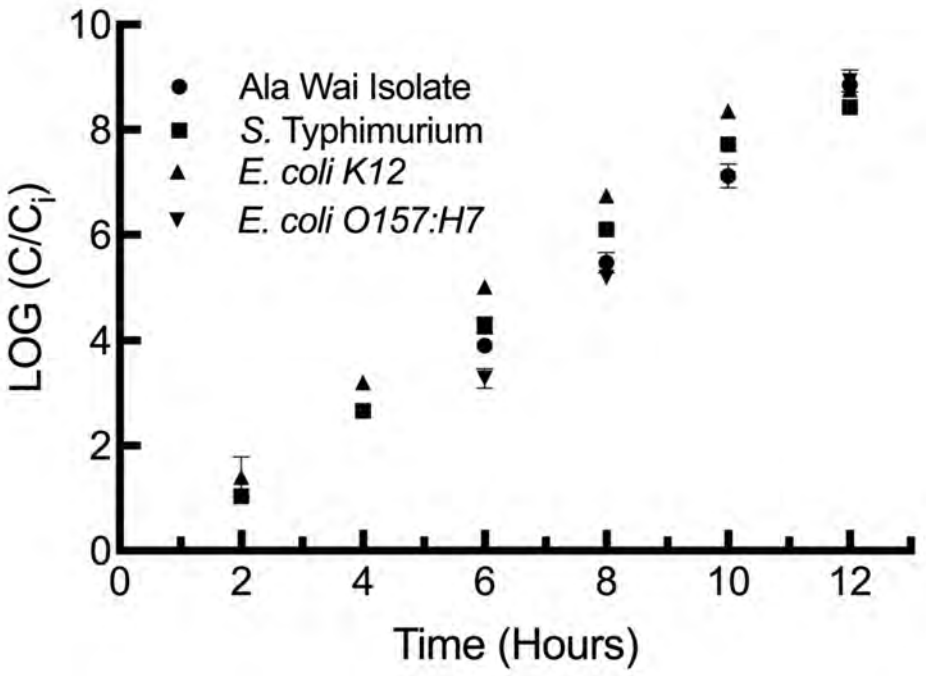

Figure 4. Normalized growth characteristics of *Salmonella* Typhimurium (ATCC 14028) (C_i = 1.82 CFU/mL), *Escherichia coli* O157:H7 (C7927) (C_i = 0.062 CFU/mL), *Escherichia coli* K12 (C_i = 0.96 CFU/mL), and generic *Escherichia coli* (Ala Wai isolate) (C_i = 0.027 CFU/mL) in 50 mL BPW and enriched in a laboratory incubator for 12 hours with temperature programmed to replicate previously observed NI-incubator temperature profiles. Observed plate counts (C) were normalized to the initial concentrations (C_i) for each independent replicate Error bars show standard deviations of the means (n = 3).

min (*E. coli* O157:H7). Doubling times (t_D) were determined by estimating the rate constant 'a' for exponential growth of cell population (c) during the linear phase:

$$c_{(t)} = c_{(t=0)}\, e^{at} \qquad \text{eq. 1}$$

from the negative slope of the natural log of c vs time. The time for the population to double (t_D) can easily be demonstrated to be the natural log of 2 divided by 'a'. These doubling times are consistent with expectations of growth of *S.* Typhimurium and *E. coli* in non-selective enrichment media. For example, a study evaluating growth media for *E. coli* O157:H7 for beef processing testing procedures reported the doubling time of *E. coli* O157:H7 in ground beef enriched in BPW (42°C) as 16 to 18 minutes *(11)*. In another study, on the growth of *Salmonella* in enteral feeds, *S.* Typhimurium doubling times ranged from 21 to 34.8 minutes at 25°C *(19)*.

Sensitivity of LAMP using samples without enrichment

The lowest concentration reliably detected by LAMP targeting *S.* Typhimurium on ground chicken without enrichment was 10^5 CFU/mL *(Fig. 5A)*, with t_T = 26.67 ± 1.15 *(Fig. 5C)*. No positive detection (0/3 assays positively detected) occurred in samples containing 10^{-2} CFU/mL–10^4 CFU/mL *S.* Typhimurium. The lowest concentration reliably detected by LAMP targeting *Escherichia coli* O157:H7 on spinach without enrichment was 10^4 CFU/mL (3/3 assays positively detected, *Fig. 5B*) with mean t_T =15.33 ± 0.57 *(Fig. 5C)*. No positive detection occurred in samples containing 10^{-2} CFU/mL–10^3 CFU/mL *E. coli* O157:H7 concentrations. Although spinach and ground chicken samples were processed by the same methods, differences in sensitivity were observed between the LAMP assays targeting *E. coli* O157:H7 and those targeting *S.* Typhimurium on these inoculated foods. Similarly, the amplification efficiency, as indicated by the negative reciprocal of the slope of the standard curve *(Fig. 5C)*, was greater in assaying *E. coli* on spinach than in assaying *S.* Typhimurium on ground chicken. Unremoved inhibitors present in the ground chicken homogenate may have caused greater inhibition of LAMP than the inhibitors present in the spinach homogenate. During sample homogenization, components in ground chicken were more readily dissolved into the BPW media than were the components in spinach. In contrast, spinach leaves were harder to homogenize into solution and larger portions of the sample remained intact.

Sensitivity of LAMP using NI-incubator enriched samples

After an 8-hour NI-incubator enrichment of three ground chicken (5 g) samples inoculated with 4.5 ± 0.5 CFU *S.* Typhimurium (~ 0.9 CFU/g), target *S.* Typhimurium

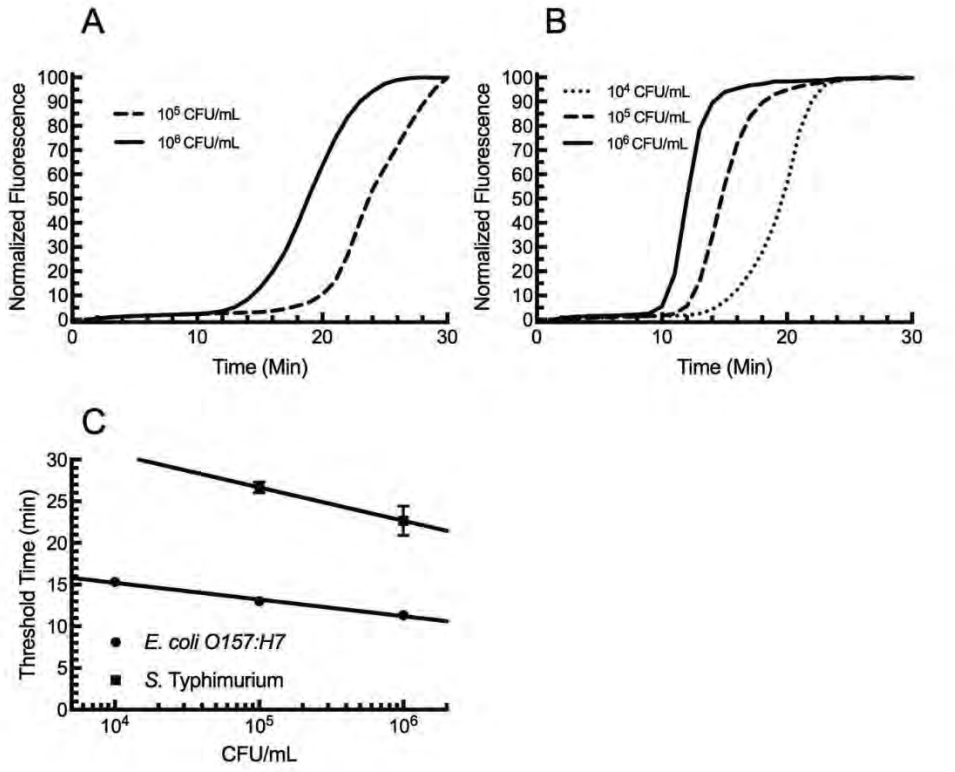

FIGURE 5. Representative LAMP amplification curves for detection of *Salmonella* Typhimurium on ground chicken (A) and *Escherichia coli* O157:H7 on spinach (B) without enrichment. Mean observed threshold times for (■) *Salmonella* Typhimurium (•) *Escherichia coli* O157:H7 (C). Error bars are standard errors of the means from 3 replicate assays. Only concentrations resulting in amplification in each of three replicates are shown.

was enriched to quantities equal to or greater than the detection level of LAMP without enrichment (10^6 CFU/g) and positively detected by LAMP at a rate of 100% in 3 experimental replicates. Mean LAMP t_T values for the detection of *S.* Typhimurium for each experimental replicate are listed in *Table 2*. Prior to enrichment (T = 0 h), *S.* Typhimurium was not detected in any of the samples *(Table 2)*.

After a 6-hour enrichment period of spinach (5 g) samples inoculated with a mean 2.7± 0.6 CFU *E. coli* O157:H7 (~0.5 CFU/g), target *E. coli* O157:H7 was enriched to quantities equal to or greater than the detection level of LAMP without enrichment (10^5 CFU/g) and positively detected by LAMP at a rate of 100% in 3 experimental replicates. Mean LAMP t_T values for the detection of *E. coli* O157:H7 for each experimental replicate are listed in *Table 3*. Without enrichment (T = 0 h) *E. coli* O157:H7 was not detected in any experimental replicate *(Table 3)*. Ground chicken samples required longer enrichment than spinach samples for *S.* Typhimurium and were enriched 2 hours longer to compensate for the observed assay inhibition caused by chicken components.

DISCUSSION

Subjecting samples to short (≤ 8 hours) enrichment periods using the NI-incubators described in this study achieved cell densities equal to or above the common LOD (10^3–10^4 CFU/mL) of nucleic acid-based assays (i.e., LAMP, PCR) directly testing food sample homogenates.

In general, smaller representative samples from a larger quantity of food product are sampled and tested to make interpretations on the demonstrated bacterial quantities, a form of traditional testing called lot-batch testing. The number of units that comprise representative samples varies according to the sampling category to which the food type is assigned and must be statistically significant. According to the United States Food and Drug Administration (FDA) Food Safety and Inspection Service (FSIS) laboratories sampling plans for *Salmonella* species, the standard analytical unit (25 g) is enriched at a 1:9 sample-to-broth ratio *(1)*. For category II foods (foods that are not subject to a process lethal to *Salmonella*), 30 analytical units are required for testing. These 30 units (25 g each) may be composited into two 375 g samples and enriched at a 1:9 sample-to-broth ratio (375 g sample/3375 mL enrichment broth) *(1)*. This

TABLE 2. LAMP detection frequency and threshold times (t_T) of *S.* Typhimurium in ground chicken

		Pre-enrichment		Post 8-h enrichment	
Incubator	Inoculation CFU	% Positive	t_T (min)[a]	% Positive	t_T (min)
A	4	0	n/a	100	24.5 ± 1.5
B	5	0	n/a	100	19.83 ± 1.15
C	4.5	0	n/a	100	25.5 ± 3.12
Control	0	0	n/a	0	n/a

[a]Mean ± standard deviation of threshold times (t_T) from 3 independent assays

TABLE 3. LAMP detection frequency and threshold times (t_T) of *E. coli* O157:H7 in spinach

		Pre-enrichment		Post 6-h enrichment	
Incubator	Inoculation CFU	% Positive	t_T (min)[a]	% Positive	t_T (min)[a]
A	2	0	n/a	100	17 ± 0.5
B	3	0	n/a	100	21 ± 3.12
C	2	0	n/a	100	19 ± 1.16
Control	0	0	n/a	0	n/a

[a]Mean ± standard deviation of threshold times (t_T) from 3 independent assays

study was performed using analytical units of 5 g. While 5 g is less than the FDA recommended analytical unit size of 25 g, multiple 5 gram samples using multiple incubators can be enriched to achieve a composite 25 g analytical unit size. Additionally, the NI-incubators may be redesigned and scaled in size to meet sample size requirements to meet the validation criteria of an alternate FDA method, i.e., 25 g sample/225 mL enrichment broth.

Microbiological analysis of a large number of samples acquired on a random basis during lot-batch testing is often cumbersome and typically employed on products for which no information is available. In a well-designed food production scheme, where hazards affecting food manufacturing and processing have been properly evaluated and preventative controls to prevent microbial contamination have been implemented, lot-batch testing by design should not be necessary. The Food Safety Modernization Act (FSMA) currently requires food facilities to devise and implement an analysis of hazards and preventative controls to minimize or prevent the identified hazards but leaves the development of such protocols to the growers' or processors' discretion. Microbiological testing for verification relies on results from pathogen testing to assess whether their Hazard Analysis Critical Control Point (HACCP) systems are operating correctly to ensure safe and unadulterated products (*5*). For example, thermal inactivation uses precise times and temperatures required to yield < 1 CFU/100 g of *Salmonella* in a certain food product. In this case, thermal inactivation is a critical control point (CCP) during production and needs periodic examination to ensure proper

functionality. Microbial verification could consist of sampling the final product for key indicator microorganisms or for *Salmonella (5)*. In this design, food safety is ensured through performance monitoring of the implemented CCPs and not by direct microbiological testing.

The methods presented in this study are being evaluated as a screening tool rather than as a reference or alternative method and are not intended to replace current standardized testing methods by regulatory agencies (i.e., FDA). Instead, we potentiate the application of commercially available portable microbiological diagnostic test kits and platforms (i.e., NI-incubators and molecular diagnostics) to support real-time routine pathogen-oriented testing or periodic microbiological verification testing (i.e., indicator tests) to control the presence of microorganisms in the product along the entire distribution chain.

For the isolation and identification of *Salmonella* from poultry, the United States Department of Agriculture (USDA) recommends enriching 25 g ± 2.5 g raw poultry product in 225 ± 4.5 mL buffered peptone water at 35 ± 2°C for 20–24 hours followed by the BAX® PCR assay *(18)*. Similarly, the FDA recommends incubation of enrichment broth mixtures for 24 hours at 42°C for screening of *E. coli* O157:H7 in sprouts by real-time PCR *(3)*. Although LAMP is generally considered to be more sensitive and specific than PCR, it has not yet been approved by regulatory agencies as a validated assay for screening for pathogens in food or water. The amplified template DNA product produced during LAMP reactions are in the form of fragments and cannot be enumerated or cloned, making it challenging to quantify the number of pathogens present in the original sample. In this study, we inferred detection limits and estimated sample concentrations (CFU/mL) by generating a standard curve according to the t_T values of artificially spiked samples with different target bacterial concentrations *(16)*. Although considerable research is required to establish LAMP as a quantitative method, LAMP shows great potential as a prescreening method to identify presumptively positive samples, especially when coupled with shortened enrichment procedures. For example, when following the Food Safety and Inspection Service USDA procedures to isolate and identify *Salmonella*, PCR screening tests are performed approximately 48 hours after receiving the sample in a lab and after primary and secondary enrichments. Enrichment using NI-incubators and detection by handheld molecular diagnostics platforms supporting LAMP technology could not only reduce the initial screening time to a 9-hour-or-less workday but also allow all procedures to be done on site or in the field. Analysis of leafy greens to detect 1 CFU/g requires less enrichment time (approximately 6 hours) and could easily be completed in an 8-hour shift. All procedures used in this research, including enrichment (NI-incubator), DNA extraction (boiling method) and molecular testing (LAMP via BioRanger™) can be completed during POC testing following simple procedures.

The methods in this study were intentionally rudimentary to demonstrate baseline performance of the NI-incubators and simplicity of use. Target pathogens in frozen samples or in samples containing injured but viable cells may be more difficult to detect and screening for them may require longer enrichment times. For these sample types, longer enrichment periods could be achieved at 35°C if the cartridge is re-filled with boiling water a second time after the first 12-hour enrichment period. Many portable options can easily be used to boil water, including small electric kettles or camping stoves. A can of sterno can be used where electric power is not available. According to the FDA, primary enrichment of *E. coli* O157:H7 should be conducted at a temperature of 42°C. Enrichment temperatures can be varied by manufacturing cartridges with different PureTemp phase change materials to accommodate enrichment requirements (i.e., varying temperatures) for different pathogens.

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