Journal of
Bioengineering & Biomedical Science

Birmpa et al., J Bioengineer & Biomedical Sci 2015, 5:3
DOI: 10.4172/2155-9538.1000164

Research Article Open Access

A Loop-mediated Isothermal Amplification Platform for the Detection of Foodborne Pathogens

Birmpa A[1], Kouroupis G[2], Kalogeropoulos K[1], Kokkinos P[1], Kritsonis P[2] and Vantarakis A[1]*

[1]*Environmental Microbiology Unit, Department of Public Health, Medical School, University of Patras, 26504, Greece*
[2]*Metricon Digital Systems, Athinon 65, Rio, Patras, 26441, Greece*

Abstract

Salmonella spp., *Listeria monocytogenes* and viruses constitute major concern for public health. It is known that RTE foods are consumed raw, thus they are often associated with outbreaks of food poisoning. In the present study, the effectiveness of a LAMP fully automated platform able to detect foodborne pathogens in less than an hour, was evaluated. Food samples were inoculated with known pathogens, such as *S. enteritidis*, *L. monocytogenes* and hAdV 40/41. With LAMP assays pathogens can be detected shortly and without the need of sophisticated equipment. The results of the present study were then compared to those of the LAMP assays detected by a Light Cycler Roche platform which is a real-time PCR instrument and were in concordance. The developed LAMP platform that is presented in this study could become a valuable, robust, innovative, powerful, cheap and fast monitoring tool which can be extensively used for routine analysis and screening of contaminated foods by the food industry and the Public Health Authorities.

Keywords: LAMP; Food borne pathogens; Automated platform

Introduction

New devices have been developed for specific nucleic acid detection in many areas such as clinical diagnostics, environmental monitoring and food-quality control [1-3]. Molecular techniques like polymerase chain reaction (PCR) and isothermal assays (e.g. loop mediated isothermal amplification, LAMP) are being translated on micro/microfluidic chips with a goal of developing a sample in answer-out gene analysis system for diagnostics [4]. When a new diagnostic tool is developed, specific criteria should be fulfilled for the detection of the nucleic acids. The new device or method mustbesensitive, selective and have high-throughput applicability. Apart from these diminishing the cost of the whole detection assay is another major objective for novel method or tool development [5].

Loop-mediated isothermal amplification (LAMP) is an accurate, fast, and cost-effective alternative isothermal amplification technique characterized by high sensitivity and high specificity [6]. It can amplify a few copies of DNA to 10^9 copies in less than an hour under isothermal conditions (60-69°C) without thermo cycling [7]. Considering these facts, simple, cost-effective equipment is required which can be used to a disposable integrated micro-reactor [8]. Bacterial, viral, fungal and parasitic pathogens have been detected through the LAMP assays [9].

Ready-to-eat (RTE) fruits and vegetables are considered important components of a healthy and balanced diet and recognized as an important source of nutrients for humans [10]. In the majority of contamination cases, fresh produce becomes contaminated on the farm during growing or harvesting [11]. Recent foodborne outbreaks in Europe have been caused by Noroviruses present in lettuce [12] or HAV in semidried tomatoes [13]. *S. enteric* (serovar Enteritidis) is one of the most common Salmonella strains which are associated with salmonellosis outbreaks [14]. It is well known that a "zero tolerance" policy for *L. monocytogenes* in RTE foods exists [15].

Instruments applying the turbidimetric measurements through magnesium pyrophosphate byproduct generation are available in order to monitor LAMP reactions. Literature evidence is available for such techniques [16]. For LAMP methods, instruments which are usingfluorescence probes along with isothermal amplification protocols have been reported [17].

Many miniaturized isothermal systems which are based on the strand-displacement activity of a DNA polymerase (NASBA, LAMP, HDA, RCA, MDA, and RPA) to cyclically amplify a target in short time (less than an hour).

Strand displacement phenomena of DNA polymerase (NASBA, LAMP, HDA, RCA, MDA, and RPA) have been used by different isothermal systems. This method is used to amplify a particular target in a cyclic manner, in less than an hour. The afore mentioned methods are nucleic acid sequence-based amplification (NASBA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), multiple displacement amplification (MDA), helicase-dependent amplification (HDA) and recombinase polymerase amplification (RPA) [18].

Different microfluidic-coupled PCR amplification approaches have been developed. The amplified products can be visualized by different methods such as electrophoretic, turbidimetric and electrochemical or by simple visual evaluation of the solution color change resulting from the SYBR green stain [18].

Lee et al. [16] studied the development of an integrated isothermal device to amplify and detect hepatitis B virus (HBV) DNA using LAMP amplification method [16]. The device was made of a disposable polymethyl methacrylate (PMMA) based micro reactor which also contains an optical detection system, sensitive to temperature alteration. The device can detect the alteration in turbidity owing to the magnesium pyrophosphate precipitation in real-time monitoring. An on-chip LAMP device was presented by Zhang et al. [19] containing

***Corresponding author:** Vantarakis A, Associate Professor, Environmental Microbiology Unit, Department of Public Health, School of Medicine, University of Patras, Greece, Tel/Fax: +302610969875; E-mail: avanta@upatras.gr\

Received: July 28, 2015; **Accepted:** August 21, 2015; **Published**: September 04, 2015

Citation: Birmpa A, Kouroupis G, Kalogeropoulos K, Kokkinos P, Kritsonis P, et al. (2015) A Loop-mediated Isothermal Amplification Platform for the Detection of Foodborne Pathogens. J Bioengineer & Biomedical Sci 5: 164. doi:10.4172/2155-9538.1000164

a total reaction volume of 10 µL [19]. A microLAMP (µLAMP) was developed with an eight-channel chip containing an optic sensor capable to detect specific targeted nucleic acids. A comparatively low volume of 5 µl was used in this LAMP based approach [20].

The detection of pathogens using molecular techniques (i.e Real-Time PCR) needs precise protocols and highly skilled personnel. Moreover, the sample preparation and nucleic acid extraction are extremely demanding. For these reasons, there is an immense demand for a compact device-platform that can accomplish all the processes quickly. This has led to the advance of microfluidics, which has been widely applied in the molecular diagnostics and gene amplification [9]. The advantage of microfluidics is that it reduces the consumption of the costly reagents [9].

The aim of the present study was the development and evaluation of a simple, cost-effective platform using specific loop-mediated isothermal amplification (LAMP) assays for the detection of *Salmonella, Listeria* and Adenoviruses, in artificially inoculated RTE matrices (vegetables and soft fruits). This novel platform is advantageous since it has high specificity, sensitivity, low cost and it can be used as an easy diagnostic tool without the need of sophisticated equipment, by the food industry and the Public health authorities. It is the first platform for the detection of foodborne pathogens including hAdV 40/41 in real RTE fruits and vegetables.

Materials and Methods

Preparation of food samples for LAMP assays

Bacterial strains used were *S. enteritidis* NCTC 6676 and *L. monocytogenes* NCTC 11994 (HPA, Colingdale, UK). Lenticules with the microorganisms were rehydrated in 9 mL of peptone saline (0.1%) (Oxoid, UK), and after 20 min, working cultures were streaked onto Tryptic Soy Agar (TSA; Oxoid, UK), incubated at 37°C for 24 h, and stored at 4°C. Each bacterial type was cultured in 20 mL Tryptone Soya Broth (TSB; Merck, UK) at 37°C for 17 h, harvested by centrifugation at 4000X g for 20 min at 4°C and washed three times with buffered peptone water (BPW; Oxoid). The final pellets were resuspended in BPW, corresponding to approximately 10^8-10^9 CFU/mL. HAdV 40/41 strains were kindly donated by Dr. Annika Allard from the Department of Clinical Virology of the Umeå University Hospital (Sweden).

Fresh RTE products purchased from a local supermarket (Patras, Greece) the day of the experiment, were romaine lettuce (*Lactuca sativa L. var. longifolia*), strawberries (*Fragaria x ananassa*), cherry tomatoes (*Solanumly copersicum var. cerasiforme*), green onions (*Allium spp*), and sour berries (*Prunus cerasus*), and were inoculated with microorganisms such as *Salmonella, Listeria*and hAdV40/41. They were used for the specificity and evaluation tests of the developed LAMP assay.

All food samples were rinsed with sterile water to remove some of the natural flora before treatment. For the inoculation of the samples, a spot-inoculation method was applied to inoculate the bacteria or viruses on their surface. Briefly, 100 µL of *S. enteritidis*, *L. monocytogenes* and hAdV 40/41 were spotted separately with a micropipette on 10 different areas of the surface of each food sample weighing 10 g for *Salmonella* and *Listeria* and 25 g for hAdV 40/41, in order to simulate real conditions. After spiking, the samples were dried, for 1 hour at 22 ± 2°C, to allow bacterial attachment. All processes were performed in a class II biosafety cabinet (Cytair 155, FluFrance).

Buffered Peptone Water (90 ml) and Half Fraser Broth (90 ml) were added to 10 g of each food sample artificially inoculated with *S. enteritidis* and *L. monocytogenes*, respectively. The samples were homogenized for *Salmonella* and *Listeria* testing, respectively. The hAdV 40/41 spiked food samples were diluted in 40 ml TGBE (Tris Glycine 1% Beef Extract Buffer) (Sigma-Aldrich, USA) solution and then the pH was adjusted to 7.2. Then nucleic acid extraction followed using a NucliSENSminiMAG kit (bioMerieux, France), according to previous published protocols [10] for hAdV 40/41 detection.

Nucleic acid extraction

Nucleic acids (NA) from viral concentrates of fresh products were extracted using a NucliSENSminiMAG kit (bioMerieux, France), according to previous published protocols (Kokkinos). A negative control was included in all the nucleic acid extraction procedures. Finally, the NA eluates (100 µl) were stored at −70°C, until used. The food samples at this stage were characterized as extracted samples and were used for the subsequent LAMP assay. *L. monocytogenes* nucleic acids were extracted using the Genomic DNA from tissue (Nucleospin tissue, Macherey-Nagel, Germany), according to the manufacturer's instructions.

Lamp assay for pathogens detection

Six primers (two inner primers, two outer primers and two loop primers), targeting *Salmonella* enterica invasion protein (invA) gene were used for the *S. enteritidis* LAMP reactions [21]. The reaction was carried out in a total of 25 µl and contained 16 µl of Tin Isothermal Mastermix, 25 µMinvASalm FIP, 25 µMinvASalm BIP, 5 µMinvASalmF3, 5 µMinvASalmB3, 12,5 µMinvASalmF-Loop, 12,5µ MinvASalmB-Loop and 3 µl of template DNA [22]. The thermal profile of the reaction used was according to that described [22].

For *Listeria monocytogenes* detection six primers (two inner primers, two outer primers and two loop primers) targeting hlyA gene of *L. monocytogenes* were used. The obtained primers were purified using HPLC(high-performance liquid chromatography). Positive and negative controls were used in each run. The LAMP reaction was carried out in a total volume of 25 µl. The optimal conditions as well as the thermal profile were based on the assay [23].

For hAdV 40/41 detection the LAMP assay was conducted in a total volume of 20 µl consisting of isothermal Master Mix (ISO-001tin Isothermal Mastermix, OptiGene, UK) (12 µl), the set of six primers (outer, inner, and loop primers), and target DNA (2 µl). The sequences of the oligonucleotide primers, the thermal profile and the optimal conditions of the LAMP assay were previously described by Ziros et al. [24].

All the above samples were analyzed using a Light Cycler Nano Instrument (Roche). Positive and negative controls were included in each run. Aliquots of 10 µl of LAMP products were electrophoresed on 2% agarose gels and were visualized by ethidium bromide (Sigma) staining. In each reaction tube 1 µl of 1,000 X SYBR green dyes was added to aid in the detection of the amplified products.Fifteen minutes were needed for incubation. Positive reaction was detected through a yellowish green color formation and reddish orange denoted the negative reaction.

Specificity and sensitivity of the LAMP assays

The specificity and sensitivity of the LAMP assays for Salmonella, Listeria and hAdV 40/41 detection have been previously evaluated [21,23-25].

Results

LAMP platform

The developed LAMP platform (Figure 1) holds eight tube series so as to simultaneously detect pathogens. The temperature ranges between 20-70°C. The temperature accuracy is the predetermined temperature for each pathogen ±1°C. The duration to maintain the preferred temperature can be manually set and then the lid after the predetermined time was opened to expose the tubes either to electrophoresis (Figure 2) or to SYBR Green dye (Figure 3). The option of SYBR Green dye was accomplished automatically by inserting SYBR Green dye with prefilled syringe tubes. Finally, it was observed if any of the tubes fluoresces under UV light illumination and the qualitative diagnostic result is automatically given on the screen of the platform by means of specialized LED indications (Figure 1). The result is expressed in terms of green LED indication (positive reaction), red LED indication (negative reaction), and yellow LED indication (ambitious reaction) (Figure 1).

1: Tube holder, 2: Prefilled syringe tube holder, 3: Lid made of copper for better temperature control, 4: UV light lamp, 5: Photoresistors, 6: Copper tubes holders, 7: PID temperature controller, 8: Time controller, 9: LED indicators

In order to rise the temperature up to 60-69°C and maintain it steady for the predetermined time sample tubes are placed into pure copper holders. The temperature was selected according to each protocol designed for each specific target pathogen (65°C for *Salmonella*, 63°C for *Listeria* and 69°C for hAdV 40/41). The copper is connected with resistors to the power supply to rise its temperature and consequently to uniformly spread its temperature to the tubes. During initialization the copper automatically rises to include the tubes. The rest of the circuitry is not in contact with the copper so the overheating is avoided. During the steady temperature phase the lids of the tubes are in a higher temperature in order to avoid evaporation of the testing material. By pressing a button in the machine, the lid is lifted and without access of the user, the copper is bottomed and the tubes are exposed to the UV light. After 10 minutes, the photoresistors are activated and the result is automatically given. The temperature setting is performed by means of a PID temperature controller. The timer is implemented using custom electronics. The servomotors for the automatic lifting of the copper as well as the LED indicators are controlled by the electronic systems.

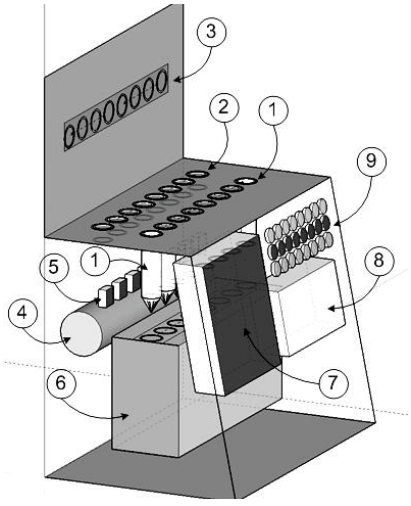

Figure 1: The developed LAMP platform.

Experimental results with the developed LAMP platform

The developed LAMP platform was used each time for separate pathogen detection. For every pathogen, 5 food samples were tested, including 1 positive and 2 negative controls (one without the template DNA and one inoculated with another pathogen-not the target pathogen-). The samples were tested in triplicate. Both options of pathogen detection (agarose gel electrophoresis and visual detection by LED indicator) were used and the results were verified.

Experimental results with customized light cycler nano instrument (Roche)

The specific isothermal amplification of the DNA of *Salmonella*, *Listeria* and hAdV 40/41 strains on food samples generated ladder-like pattern bands on agarose gel. No amplification was observed in LAMP reactions without template DNA (negative control) and in the control reactions with non-*Salmonella*/non-*Listeria*/non-hAdV 40/41 DNA. LAMP assay successfully detected the aforementioned targets in food samples within 60 min. Moreover, there was no difference between

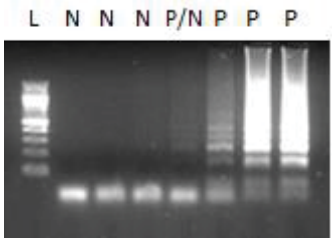

Figure 2: Electrophoresis of Ladder (L), Negative (N), Positive/Negative (P/N), Positive (P) Food samples (i.e romaine lettuce).

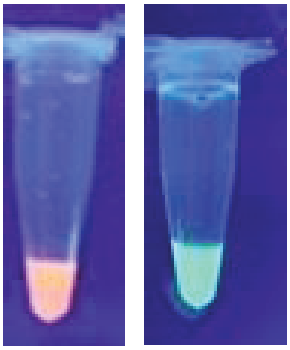

Figure 3: Negative (N) (reddish orange sample) and Positive (P) (yellowish green sample) lettuce samples after LAMP amplification.

the LAMP results detected by agarose gel electrophoresis of LAMP products or visual detection of LAMP products after SYBR Green addition and observation under UV light (Figures 2 and 3).

Finally, all the food samples were tested with both our developed LAMP platform and the Light Cycler Nano Instrument (Roche). The samples were tested in triplicate and at least two tubes were taken per sample. The results were verified by both methods tested.

Conclusions

Our study underlined the usefulness of the developed LAMP platform for the bacteriological and virological analysis of fresh RTE foods. The developed LAMP platform is expected to provide a very robust, innovative, powerful, cheap and fast molecular diagnostic tool, for the food industry and the public food authorities, without the need for sophisticated and demanding education of the personnel. However, the modest power requirements required for this device can be a barrier so as to have a truly portable (handheld) battery-operated system that is suitable for field settings or for diagnostics on an everyday basis in food companies.

Acknowledgements

This study was financially supported by The Greek State Scholarships Foundation-IKY Iky Fellowships of Excellence for Postgraduate Studies in Greece-Siemens Program.

References

1. D'Agata R, Corradini R, Ferretti C, Zanoli L, Gatti M, et al. (2010) Ultrasensitive detection of non-amplified genomic DNA by nanoparticle-enhanced Surface-Plasmon Resonance Imaging Biosens Bioelectron 25: 2095-2100.
2. D'Agata R, Breveglieri G, Zanoli LM, Borgatti M, Spoto G, et al. (2011) Direct detection of point mutations in nonamplified human genomic DNA Anal Chem 83: 8711-8717.
3. Marchelli R, Tedeschi T, TonelliA (2012) DNA analyses in food safety and quality: Current status and expectations. In: Spoto G, Corradini R (Eds.). Detection of Non-Amplified Genomic DNA. Springer Dordrecht Heidelberg Netherland.
4. Farhan A, Hashsham Syed A (2012) Miniaturized nucleic acid amplification systems for rapid and point-of-care diagnostics: A review. AnalyticaChimicaActa 733: 1-15.
5. Shi L, Perkins RG, Fang H, Tong W (2008) Reproducible and reliable microarray results through quality control: Good laboratory proficiency and appropriate data analysis practices are essential. Curr Opin Biotechnol 19: 10-18.
6. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, et al. Loop-mediated isothermal amplification (LAMP).
7. Parida M, Sannarangaiah S, Kumar Dash P, Rao PVL, Morita K (2009) Rev Med Virol 18: 407-421.
8. Szu-Yuan L, Chun-Nan L, Holl M, Meldrumc, Deirdre R, et al. (2007) Efficient, specific, compact hepatitis B diagnostic device: Optical detection of the hepatitis B virus by isothermal amplification. Sensors and Actuators B 127: 598-605.
9. Mohammadali S, Uddin AM, Mona T, Mohammed Z (2011) Microfluidic electrochemical assay for rapid detection and quantification of Escherichia coli. Biosensors and Bioelectronics 31: 523-528.
10. Nuanualsuwan S, Cliver DO (2003) Capsid functions of inactivated human picornaviruses and feline calicivirus. Applied and Environmental Microbiology 69: 350-357.
11. Kokkinos P, Kozyra I, Lazic S, Bouwknegt M, Rutjes S, et al. (2012) Harmonised investigation of the occurrence of human enteric viruses in the leafy green vegetable supply chain in three European Countries. Food and Environmental Virology 4: 179-191.
12. Feng H, Barbosa-Canovas G, Weiss J (2011) Ultrasound Technologies for Food and Bioprocessing. Springer New York.
13. Gogate PR (2007) Application of cavitational reactors for water disinfection: Current status and path forward. J Environ Manage 85: 801-815.
14. Techathuvanan C, D'Souza DH (2012) Reverse-transcriptase loopmediated isothermal amplification as a rapid screening/monitoring tool for Salmonella enterica detection in liquid whole eggs. J Food Sci 77: M200-M205.
15. Gombas DE, Chen Y, Clavero RS, Scott VN (2003) Survey of Listeria monocytogenes in ready-to-eat foods. J Food Prot 66: 559-569.
16. Lee SY, Huang JG, Chuang TL, Sheu JC, Chuang YK, et al. (2008). Compact optical diagnostic device for isothermal nucleic acids amplification. Sens Actuator B 133: 493-501.
17. Kokkinos PA, Ziros PG, Bellou M, Vantarakis A (2014) Loop-mediated Isothermal amplification (LAMP) for the Detection of Salmonella in Food. Food Anal Methods 7: 512-526.
18. Maria ZL, Giuseppe S (2013) Isothermal Amplification Methods for the Detection of Nucleic Acids in Microfluidic Devices. Biosensors 3: 18-43.
19. Zhang C, Xing D (2010) Single-molecule DNA amplification and analysis using microfluidics. Chem Rev 110: 4910-4947.
20. Fang X, Liu Y, Kong J, Jiang X (2010) Loop-mediated isothermal amplification integrated on microfluidic chips for point-of-care quantitative detection of pathogens. Anal Chem 82: 3002-3006.
21. Ziros P, Kokkinos P, Papanotas K, Vantarakis A (2012) Loop-mediated isothermal amplification (LAMP) for the detection of Salmonella spp. isolated from different food types. JMBFS 2: 152-161.
22. D'Agostino M, Diez-Valcarce M, Susana R, Losilla-Garcia B, Nigel C (2015) A Loop-mediated amplification-based method for analyzing animal feed for the presence of Salmonella. Food Analytical Methods.
23. Li W, Yue L, Jin C, Zhenbo X, Qingpin Z (2012) Development and application of a simple loop-mediated isothermal amplification method on rapid detection of Listeria monocytogenes strains. MolBiol Rep 39: 445-449.
24. Ziros PG, Kokkinos PA, Allard A, Vantarakis A (2015) Development and Evaluation of a Loop-Mediated Isothermal Amplification Assay for the Detection of Adenovirus 40 and 41. Food Environ Virol 7: 276-285.
25. Hara-KudoY, Yoshino M, Kojima T, Ikedo M (2005) Loop-mediated isothermal amplification for the rapid detection of Salmonella. FEMS Microbiol Lett 253: 155-161.

Citation: Birmpa A, Kouroupis G, Kalogeropoulos K, Kokkinos P, Kritsonis P, et al. (2015) A Loop-mediated Isothermal Amplification Platform for the Detection of Foodborne Pathogens. J Bioengineer & Biomedical Sci 5: 164. doi:10.4172/2155- 9538.1000164