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

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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 must be sensitive, 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 1010 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 using fluorescence 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...
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 Nuclisens miniMAG 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 Nuclisens miniMAG 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 Tn 
Isothermal Mastermix, 25 μM MinvASalm FIP, 25 μM MinvASalm BIP, 5 μM MinvASalmF3, 5 μM MinvASalmB3, 12.5 μM MinvASalmF-Loop, 12.5 μM 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].

### Materials and Methods

#### Preparation of food samples for LAMP assays

Bacterial strains used were *S. enteritidis* NCTC 6676 and *L. 
monocytogenes* NCTC 11994 (HPA, Colindale, 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. longfolia), strawberries (*Fragaria x ananassa*), cherry tomatoes 
(*Solanum lycopersicum var. cerasiforme*), and green onions (*Allium spp*.
(*Lactuca sativa* L. var. longfolia), strawberries (*Fragaria x ananassa*), 
cherry tomatoes (*Solanum lycopersicum var. cerasiforme*), green onions (*Allium spp*.), 
and sour berries (*Prunus cerasus*), and were inoculated with microorganisms 
such as *Salmonella*, *Listeria* and hADV 40/41. 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)
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).

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.

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
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.

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