Journal of Microbiology, Biotechnology and Food Sciences

Ziros et al. 2012 : 2 (1) 152-161

SHORT COMUNICATION

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR THE DETECTION OF *SALMONELLA SPP.* ISOLATED FROM DIFFERENT FOOD TYPES

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ABSTRACT

The objective of this study was the application and evaluation of a loop-mediated isothermal amplification (LAMP) method for the detection of *Salmonella spp.* strains isolated from food samples. Salmonella specific *invA* gene sequences (50 strains, 15 serotypes) were amplified at 65^{o}C in 60 min. All of the strains of *Salmonella* subsp. Enterica were shown to be positive using the LAMP reaction assay, whereas, all other bacteria, virus and yeasts tested in this study were negative. LAMP products could be visually detected under day light or ultraviolet light, while the specific amplification of the DNA of *Salmonella* strains generated ladder-like pattern bands on agarose gel. LAMP is suitable for the sensitive, rapid, and inexpensive detection of *Salmonella* spp. in food analytical laboratories.

Keywords: Loop-Mediated Isothermal Amplification, *Salmonella*, Detection, Food Matrices

INTRODUCTION

Foodborne diseases are a serious threat to public health (**Yang *et al.*, 2010**). Salmonellosis, a major foodborne infectious disease worldwide, is caused by 2500 serovars, and is most often attributed to the consumption of contaminated foods such as poultry, beef, pork, eggs, milk, seafood, nut products, and fresh produce (**Techathuvanan *et al.*, 2010; Yang *et al.*, 2010; Li *et al.*, 2009; Wang *et al.*, 2008; Ohtsuka *et al.*, 2005**). The traditional *Salmonella* detection includes pre-enrichment, selective enrichments and plating on selective agar media followed by biochemical and serological tests which requires 5 to 7 d for completion and is time consuming, labour intensive and costly to meet food safety control in routine food analytical laboratories (**Techathuvanan *et al.*, 2010; Okamura *et al.*, 2009; Wang *et al.*, 2008**). To satisfy the desired rapidity, polymerase chain reaction (PCR) and real-time PCR assays have been applied but still require long time periods and expensive equipment, such as a thermal cycler (**Okamura *et al.*, 2008; Wang *et al.*, 2008; Pochop *et al.*, 2011**). Novel rapid *Salmonella* detection assays without the need for sophisticated equipment or labour remain in high demand (**Techathuvanan *et al.*, 2011**).

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification assay that is rapid, specific, and simple, and has provided a new alternative for molecular diagnosis (**Notomi *et al.*, 2000**). The LAMP assay has been applied for the detection of *Salmonella*, and its higher sensitivity has been demonstrated. (**Okamura *et al.*, 2008; Hara-Kudo *et al.*, 2005; Ohtsuka *et al.*, 2005**).

The objective of our study was the application and further evaluation of a diagnostic LAMP-based assay for the detection of *Salmonella* in foods, and its potential use in routine food analytical laboratories.

MATERIAL AND METHODS

Bacterial and non bacterial control strains

Fifty strains of 15 different serotypes of *Salmonella* subsp. Enterica, and 10 strains of 3 bacterial species other than *Salmonella*, 2 virus species, and 2 yeast species (Table 1) were used to further evaluate the specificity of the LAMP assay. *Salmonella* strains were cultured according to ISO method 6579:2002. Non-Salmonella bacterial species were cultured on Tryptic Soy Agar (TSA), and yeast species on Malt Extract Agar (MEA). Virus aliquots of

human adenovirus (hAdV type 35) and human Norovirus GI and GII (NoV) were used. Appert SA and the Environmental Microbiology Unit are both accredited (E.SY.D., Hellenic Accreditation System S.A) for the Detection of *Salmonella* spp, (ISO 6579:2002).

Nucleic acids extraction

Bacterial, viral and yeast nucleic acids were extracted using the Blood and Tissue kit (Qiagen), QIAamp Viral RNA mini kit (Qiagen), and DNeasy Plant Mini Kit (Qiagen), respectively, in line with the manufacturer's instructions, using QIAcube (Qiagen, Hilden, Germany) fully automated platform.

LAMP assay

In this study, six primers, two inner primers, two outer primers and two loop primers, targeting *Salmonella* enterica invasion protein (*invA*) gene were used for the LAMP reactions. (**Hara Kudo *et al.*, 2005**). The reaction components were mixed in a tube incubated at 65 ^{o}C for 60 min using a thermal cycler (MJ Mini™ Personal Thermal Cycler, BIO-RAD, USA) and then heated to 80 $^{\circ}$C for 2 min to terminate the reaction. Aliquots of 10 µl of LAMP products were electrophoresed on 2% agarose gels and were visualized by ethidium bromide (Sigma) staining.

LAMP specificity

Salmonella and non *Salmonella* strains (Table 1) were used to determine LAMP specificity. Aliquots (2 µl) of each DNA template as prepared above were subjected to LAMP amplifications. Specificity tests were repeated twice.

RESULTS AND DISCUSSION

The diagnostic accuracy of the assay was shown to be 100%. The specific amplification of the DNA of *Salmonella* strains generated ladder-like pattern bands on agarose gel (Figure 1). No amplification was observed in LAMP reactions without template DNA (negative control) and in the control reactions with non *Salmonella* strains. All of the 50 strains of 15 serotypes of *Salmonella* subsp. Enterica were shown to be positive using the

LAMP reaction assay, whereas, all other bacteria, virus and yeasts tested in this study were negative. LAMP assay detected *Salmonella* within 60 min.

Sensitive and rapid detection methods for *Salmonella* in food are required. While a variety of isothermal nucleic acid amplification mechanisms are available, the most suitable is LAMP 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 (**Jenkins *et al.*, 2011**). LAMP is a novel nucleic acid amplification assay that is rapid, specific, and simple. This assay has been used for the *in vitro* specific detection of *Salmonella* in pure culture (**Techathuvanan *et al.*, 2010; Okamura *et al.*, 2008; Wang *et al.*, 2008; Hara-Kudo *et al.*, 2005**). The detection sensitivity of the LAMP assay for *Salmonella* using pure culture has been determined to be > 2.2 cfu/test tube using nine serotypes (**Hara-Kudo *et al.*, 2005**), 10^1 CFU/mL for pure overnight culture *S.* Typhimurium (**Techathuvanan *et al.*, 2010**), 100 fg DNA/tube (**Wang *et al.*, 2008**), 10^3 CFU/ml broth (**Okamura *et al.*, 2008**). To confirm the specificity of LAMP, various *Salmonella* serotypes and non *Salmonella* strains were tested in this study. The assay inclusivity has been increased by other *Salmonella* Enterica subspecies than those previously tested (*Salmonella* Deversoir, *Salmonella* Hermannswerder, *Salmonella* London, *Salmonella* Meleagridis, *Salmonella* Putten). The detection of various *Salmonella* serotypes was slightly different among serotypes, but the difference seemed to be due to the concentration of the *Salmonella* cells. The fifty strains of 15 serotypes of *Salmonella* subsp. Enterica were amplified, but not 10 strains of 3 bacterial species other than *Salmonella*, 2 virus species, and 2 yeast species. This complies with the studies of **Hara-Kudo et al. (2005)** and **Techathuvanan et al. (2010)** which reported that *inv*A primers did not cross-react with other foodborne pathogens (**Techathuvanan *et al.*, 2010; Hara-Kudo *et al.*, 2005**). The LAMP assay does not require well-equipped laboratories to be performed, and the procedure may be easily standardized among different laboratories (**Okamura *et al.*, 2008**). However, some current limitations of the used assay need to be considered, such as the inability to distinguish between the various *Salmonella* serovars as the assay is based on the detection of the *inv*A gene (**Techathuvanan *et al.*, 2012**). Further development of other LAMP assays specific for the different serotypes will improve the ability for the identification and discrimination of *Salmonella* serovars (**Okamura *et al.*, 2008**). Analytical *Salmonella* detection protocols consisting of a pre-enrichment overnight step, followed by a nucleic acids extraction-purification step, and the final Salmonella specific LAMP assay, could improve detection speed to <1 d for the total assay. LAMP is expected to provide a platform for convenient and feasible testing for regular screening purposes in

diagnostic laboratories or for deployment in field-testing (**Okamura *et al.*, 2009**). This LAMP assay has the potential to become a standardized method for the rapid detection of *Salmonella* in diagnostic food laboratories (**Techathuvanan *et al.* 2012; Francois *et al.*, 2011; Li *et al.*, 2009**).

Fifty strains of 15 different serotypes of *Salmonella* subsp. *enterica*, and 10 strains of 3 bacterial species other than *Salmonella*, 2 virus species, and 2 yeast species were used to further evaluate the specificity of the LAMP assay (Tab 1).

Table 1 *Salmonella* and non *Salmonella* control strains tested in this study

No.	Salmonella strain	Strain ID	No. of strains	Typing and confirmation method	Food matrix	Amplification of invA
						LAMP
1	Blockley	Δ38462	2	Serotyping by Slide Agglutination, White – Kauffmann (accredited method)	Raw poultry meat Greece	+
2	Bredeney	Δ39625	3	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
3	Deversoir	Δ40661	5	Serotyping by Slide Agglutination	Taboulen salad Greece	+
4	Hermannswerder	Δ42266	5	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
5	Montevideo	Δ42271	3	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
6	Typhimurium	Δ42474	2	Serotyping by Slide Agglutination	Raw pig meat Greece	+
7	Snftenberg	Δ430	3	Serotyping by	Food toping	+

		94		Slide Agglutination		
8	Putten	Δ43147	5	Serotyping by Slide Agglutination	Sesame India	+
9	Virchow	Δ43473	2	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
10	London	Δ44560	4	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
11	Hadar	Δ47737	2	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
12	Oraniemburg	Δ52876	2	Serotyping by Slide Agglutination	Pie with greens Greece	+
13	Enteritidis	Δ54686	3	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
14	Thompson	Δ54688	3	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
15	Meleagridis	Δ55050	6	Serotyping by Slide Agglutination	Raw poultry meat Greece	+
16	*Legionella pneumophila* NCTC 12821		2			-
17	*Enterococcus faecalis* NCTC 775		1			-
18	*Escherichia coli* NCTC 9001		2			-
19	*Saccharomyces*	A420	1	Nested PCR -	Concentrate	-

	cerevisiae	4		Sequencing	d orange juice	
20	*Zygosaccharomyces bailii*	A4196	1	Nested PCR - Sequencing	Soft drink	-
21	Human Adenovirus type 35		1			-
22	Human Norovirus GI		1			-
23	Human Norovirus GII		1			-

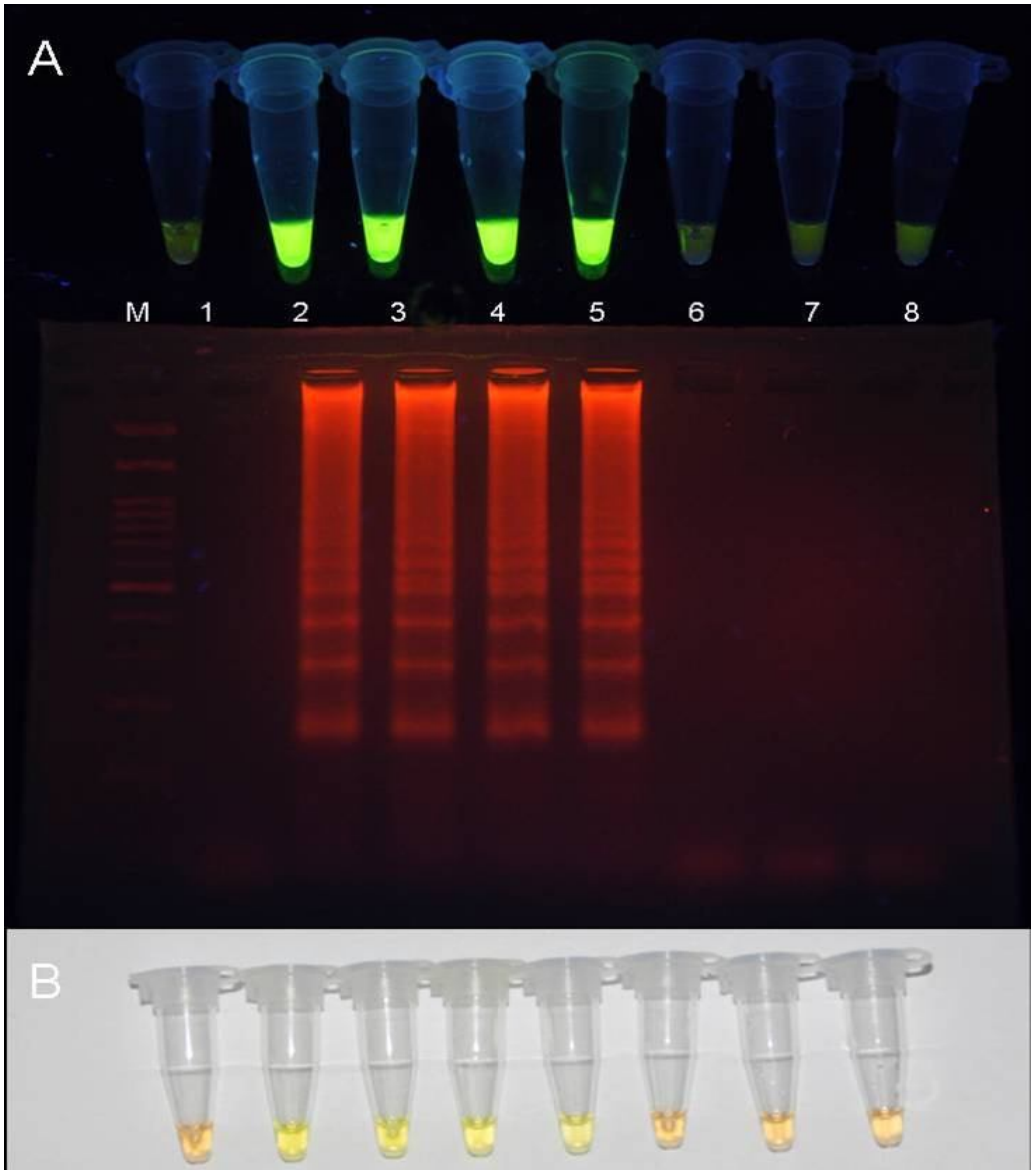

Figure 1 A. Visual detection of LAMP products under UV light (upper part). Representative agarose gel electrophoresis of LAMP products from nucleic acid extracts of pure cultures of

selected Salmonella serotypes. M: 100 bp DNA ladder; Lane 1: Negative water control; Line 2: *Salmonella* Meleagridis, Line 3: *Salmonella* Hermannswerder; Line 4: *Salmonella* Deversoir; Line 5: *Salmonella* London; Line 6: *Escherichia coli*; Line 7: *Legionella* spp; Line 8: human Adenovirus (lower part). **B.** Visual detection of LAMP products under day light.

CONCLUSION

Salmonella specific *invA* gene sequences were successfully amplified at 65°C in 60 min, by a loop-mediated isothermal amplification (LAMP) method, while the bacterial, viral and yeast control strains tested were negative. The study aimed to evaluate the inclusivity of a developed LAMP method for the detection of *Salmonella spp.* strains isolated from different food matrices, proved to be a sensitive, rapid and inexpensive detection method, which could be of interest for screening purposes in food analytical laboratories.

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