Food Control 88 (2018) 190–197

Contents lists available at ScienceDirect

Food Control

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

Development of a novel loop-mediated isothermal amplification (LAMP) assay for the detection of *Salmonella* ser. Enteritidis from egg products☆

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ARTICLE INFO

Article history:
Available online 2 February 2018

Keywords:
LAMP
Salmonella Enteritidis
Egg
*prot*6E
Detection

ABSTRACT

Salmonella ser. Enteritidis is a major public health concern worldwide. Loop-mediated isothermal amplification (LAMP) is a novel simple, easy-to-operate detection technology that amplifies DNA with high speed, efficiency, and specificity under isothermal conditions. The objective of this study was to develop a new LAMP assay for the detection of *Salmonella* ser. Enteritidis. The *Prot*6E gene located on a virulence plasmid of *Salmonella* ser. Enteritidis, encoding fimbrial biosynthesis protein, was the target for detection. The primer set was designed by using Primer Explorer V4 software and evaluated for its effectiveness in detecting *Salmonella* ser. Enteritidis strains SE12 (PT14b), 18579 (PT4), and CDC_2010K_1441 (PT8) using isothermal master mix under an approximately 35 min reaction by Genie III instrument (OptiGene, UK). Ratio of outer and inner primers, amount of DNA template, reaction temperature and time were optimized. Inclusivity test using 114 *Salmonella* ser. Enteritidis strains showed 97.4% positive for *prot*6E gene. For exclusivity testing, 34 non-*Salmonella* ser. Enteritidis *Salmonella* strains (27 serotypes) and 35 non-*Salmonella* strains (14 species) were tested and they were 100% negative. Results from the LAMP assay on 200 samples from egg products inoculated with 1–5 CFU/25 g completely matched with that from culture method and real-time PCR, and less than 1.2–12 CFU per reaction *Salmonella* ser. Enteritidis could be detected. This LAMP method can be of high value to the food industry due to its various advantages such as speed, specificity, sensitivity, cost- and labor-efficiency.

1. Introduction

Salmonella is a major foodborne pathogen that causes human gastrointestinal illness, which can be transmitted by contaminated food products such as eggs, milk, poultry, and vegetables. *Salmonella* is estimated to cause more than 1.2 million illnesses each year in the United States, with more than 23,000 hospitalizations and 450 deaths, according to the Centers for Disease Control and Prevention (CDC) (CDC, 2011). *Salmonella* ser. Enteritidis is considered the most frequent *Salmonella* serotype causing salmonellosis outbreaks associated with poultry, eggs, and egg products, due to its unique ability to contaminate the egg contents through vertical transmission (Guard-Petter, 2001; Martelli & Davies, 2012). The American Egg Board (AEB) reported that egg consumption was estimated to be 274.6 eggs per capita in June, 2017, in the U.S. (AEB, 2017). From 2009 to 2015, *Salmonella* ser. Enteritidis was always on the top of the laboratory-confirmed human *Salmonella* infections reported to CDC, and the incidence has increased from 2.6 in 2009 to 2.83 per 100,000 persons in 2015 (CDC, 2017a, 2017b). Moreover, *Salmonella* ser. Enteritidis is the predominant serovar associated with *Salmonella* outbreaks in the European Union, representing

☆ Mention of trade names or commercial products in the paper is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the U. S. Food and Drug Administration.

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https://doi.org/10.1016/j.foodcont.2018.01.006

41.3% of 82,409 confirmed cases of human salmonellosis; egg and egg products were the most important source of foodborne *Salmonella* outbreaks, accounting for 62.6% of *Salmonella* ser. Enteritidis outbreaks in 2012 (EFSA & ECDC, 2014). Therefore, effective testing of egg products for the presence of *Salmonella* ser. Enteritidis is important.

Conventional microbiological methods for detection of *Salmonella* from eggs and egg products take about five days (FDA, 2017; Zhang, Thau, Brown, & Hammack, 2013), which is time-consuming, labor intensive and costly. Although numerous quick molecular methods, such as immunoassay, DNA microarrays, conventional PCR, and real-time PCR, have been developed and used for detecting *Salmonella* ser. Enteritidis in shell eggs and egg products (Almeida, Cerqueira, Azevedo, & Vieira, 2013; Kim & Lee, 2016; Pasquali, De Cesare, Valero, Olsen, & Manfreda, 2014; Wang, Huang, Ma, Shi, & Cai, 2014), a quicker and less expensive technology is always most preferred. The loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method, which is based on the principle of a strand displacement reaction and the stem-loop structure that amplifies the target gene fragment under isothermal conditions (Tomita, Mori, Kanda, & Notomi, 2008). It has been widely studied in recent years for nucleic acid analysis due to its simplicity, rapidity, high efficiency, and outstanding specificity. Most LAMP *Salmonella* methods are aimed at the genus *Salmonella* using the *Salmonella* invasion gene (*inv*A) as a target. While these methods can detect *Salmonella* ser. Enteritidis, but they cannot differentiate *Salmonella* ser. Enteritidis from other *Salmonella* serotypes. In this study, we developed a LAMP method targeting *prot*6E gene to detect *Salmonella* ser. Enteritidis from egg products in support of the Food and Drug Administration (FDA) Egg Rule which specifically focuses on *Salmonella* ser. Enteritidis (FDA, 2009). The *prot*6E gene is located on a highly conserved, low copy number, 60-kb virulence plasmid (Clavijo, Loui, Andersen, Riley, & Lu, 2006). The gene was used successfully to detect *Salmonella* ser. Enteritidis by PCR, although the role of *prot*6E is not clear. It was believed that *prot*6E may provide unique surface fimbriae to bacterial cells and alter their interaction with egg albumen components, and therefore contributing to the colonization of deeper tissues among other functions (González-Escalona, Zhang, & Brown, 2012; Malorny, Bunge, & Helmuth, 2007). The objective of this study was 1) to develop a new LAMP method for the detection of *Salmonella* ser. Enteritidis; 2) evaluate the method with inoculated eggs products; and, 3) compare it with a culture method and a PCR assay for *Salmonella* ser. Enteritidis.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All strains were obtained from the Center for Food Safety and Applied Nutrition (CFSAN), Food and Drug Administration microbiological culture collection. And the details about these strains were listed in Tables 2–4. All strains were cultured overnight at 37 ± 2 °C in trypticase soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ).

2.2. Sample preparation

Twenty different kinds of egg products (including egg nog, liquid egg whites, liquid egg yolk, etc.) were ordered online (http://www.amazon.com/), with the exception of the deviled egg halves and egg salad, which were purchased from a local grocery store in Maryland. A total of 200 food samples were analyzed in this study. The details of the samples and sample preparation were reported in our previous publication (Hu et al., 2016). Briefly, egg products were inoculated at 1–5 CFU/25 g.Inoculated wet products were stored 2–3 days at 4 °C before microbiological analysis, while dry inoculated products were stored at room temperature for 2 weeks before use. Half of the samples were processed and culturally confirmed using the FDA *Bacteriological Analytical Manual* (BAM) Chapter 5, *Salmonella* method (FDA, 2017); the other half were processed according to the 3M Molecular Detection System (MDS) and the Neogen Amplified Nucleic Single Temperature Reaction (ANSR) Pathogen Detection System (PDS) requirement, using buffered peptone water (BPW) as pre-enrichment broth (the rest of the procedure followed FDA BAM). Pre-enriched samples were withdrawn for DNA template preparation. The 3M MDS and Neogen ANSR PDS results were confirmed culturally per the BAM *Salmonella* culture method.

2.3. DNA extraction

DNA templates used in this study were prepared using InstaGene Matrix kit (Bio-Rad, Hercules, CA). Briefly, 50 μl of samples (or 1–3 colonies) were added into 200 μl of InstaGene matrix, the suspension tubes were incubated at 56 °C for 25 min, and then vortexed at high speed for 10 s. After incubation at 100 °C for 8 min, the tubes were vortexed and spun at 12,000 rpm for 3 min. The supernatant was transferred to a new microcentrifuge, and stored at −20 °C before use.

2.4. Design of LAMP primer

Based on the *prot*6E gene sequence of *Salmonella* ser. Enteritidis (GenBank: U66901.1), 8 primer sets were selected from more than 100 sets designed using PrimerExplorer V4 software (http://primerexplorer.jp/e/v4_manual/index.html). And each primer set consisted of four primers targeting six regions on specific gene, that is, a forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3). All primers were assessed for specificity before use in LAMP assays by doing a BLAST search with sequences in GenBank (https://www.ncbi.nlm.nih.gov/guide/howto/design-pcr-primers/).

2.5. Development of LAMP assay

The LAMP reaction was performed using an Isothermal Master Mix (OptiGene, Horsham, UK) in a 25 μl volume, which contained 20 pmol each of FIP and BIP, 5 pmol each of F3 and B3 primers, 5.0 μl of DNA extraction, and 15.0 μl Isothermal Master Mix. The LAMP assay was conducted under isothermal condition at 65 °C for 30 min followed by a heating and cooling step from 98 °C to 80 °C (ramping at 0.05 °C per sec) in Genie III (OptiGene, Horsham, UK). The amplicons were run on 2.0% E-Gel® EX Agarose Gels by using E-Gel® iBase™ Power System and Safe Imager™ Real-Time Transilluminator (Invitrogen, Carlsbad, CA).

2.6. Optimization of the LAMP reaction

Ratio of outer and inner primers, amount of DNA template per reaction, reaction temperature and time were compared to optimize the LAMP condition. In the optimization study, 20 μl amplified DNA fragments were analyzed by electrophoresis on 2.0% agarose gel. For determining the best reaction temperature, the reaction mixture was incubated for 30 min at 59 °C, 61 °C, 63 °C, 65 °C, 67 °C, 69 °C, and 71 °C, respectively, and then terminated at a range from 98 to 80 °C (0.05 °C per sec). The ratios of outer and inner primers from 1:1 to 1:7 were tested, and the amount of template DNA from 1.0 μl to 8.0 μl at 1.0 μl interval were compared. Moreover, to investigate the minimum reaction time required in a LAMP run, the