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Salmonella detection in powdered dairy products using a novel molecular tool

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ABSTRACT

In this study, we developed a rapid, specific, and sensitive loop-mediated isothermal amplification technique combined with a lateral flow dipstick (LAMP-LFD) method to detect *Salmonella* targeting the *siiA* gene in powdered infant formula (PIF). The specificity of the detection method (LAMP-LFD) approached 100% using 21 *Salmonella* and 31 non-*Salmonella* bacterial strains. This detection method exhibited high sensitivity limits for pure cultures at 3.7 cfu/mL and in PIF at 2.2 cfu/g without enrichment. To evaluate the applicability of the LAMP-LFD method, we detected 60 positive PIF samples and 20 negative PIF samples. The results showed that the method of LAMP-LFD had a high diagnostic specificity of 100% for detection of *Salmonella* in PIF. To reduce incidence of LAMP contamination, we applied propidium monoazide (PMA) to eliminate carryover contamination of LAMP. At the same time, we found that PMA does not affect observation of LFD for measurement of LAMP signal. The results verified that the method of LAMP-LFD targeting the *siiA* gene is rapid, accurate, and sensitive for *Salmonella* detection in PIF, and that PMA shows great potential to be widely used to eliminate the amplicon contamination risk generated by the highly sensitive LAMP reaction in the detection process.

Key words: loop-mediated isothermal amplification, lateral flow dipstick, *Salmonella*, powdered infant formula

INTRODUCTION

Salmonella is one of the most common foodborne pathogenic bacteria worldwide, infecting humans and a variety of other animals via their respective habitats, feeding activities, or reservoirs (Zhao et al., 2003; Alves et al., 2015). Thus, *Salmonella* can infect humans by improper cooking and processing of foods of animal origin such as raw milk, meat, and eggs (Moussa et al., 2010). If ingested, *Salmonella* may cause diseases such as typhoid fever and salmonellosis (Salzman et al., 2003; Zhang et al., 2003). Previous studies have reported that powdered infant formula (**PIF**) is a newly identified source of *Salmonella* infection in infants, leaving the dairy industry with a high priority to develop innovative means to detect *Salmonella* in PIF (Cahill et al., 2008).

Conventional culture-based methods for detection of *Salmonella* require 2 to 3 d (Salam et al., 2013) and are not suitable for rapid detection of *Salmonella*. In recent years, many methods have been developed to rapidly detect nucleic acid targets of *Salmonella*, including PCR, real-time PCR, and loop-mediated isothermal amplification (**LAMP**; Zhao et al., 2010; Maciel et al., 2011). The methods of PCR and real-time PCR have many disadvantages, including a relatively high cost for equipment and consumables, and the need for trained personnel. However, LAMP is based on a nucleic acid amplification approach developed by Notomi et al. (2000). It may generate up to 10^9 copies of the target DNA within 1 h using a specific stem-loop structure based on auto-cycling strand displacement and isothermal conditions (60 to 65°C; Notomi et al., 2000). Therefore, LAMP is a fast, isothermal process (requiring only a heat block) and is robust, offering greater sensitivity than other comparable methods (Mori and Notomi, 2009). Thus, it is a method with great promise for food safety applications, or clinical diagnostics, especially for resource-poor laboratories (Dhama et al., 2014; Kokkinos et al., 2014). Many different monitoring methods are available for results of LAMP, including agarose gel electrophoresis (**AGE**), real-time turbidity measurement (Wang et al., 2012), electrochemical methods (Safavieh et al., 2012), lateral flow dipstick (**LFD**; Jung et al., 2015), or ELISA-based quantification (Ravan and Yazdanparast, 2012). Previous studies have reported that the visual detection method of LFD have many advantages such as rapid speed, long-term

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stability, accuracy, user-friendly format, and not requiring expensive laboratory equipment (Peng et al., 2014). Thus, LFD is the preferred method and has attracted significant attention (Rigano et al., 2014).

Many target genes have been used to detect *Salmonella* by different methods. Some, such as *iroB*, *hilA*, *rfb*, *opmC*, and *invA* are occasionally detected in some non-*Salmonella* bacteria (Luk et al., 1993; Bäumler et al., 1997; Ziemer and Steadham, 2003; De Clercq et al., 2007; Chen et al., 2011). Conversely, genes such as *fimA*, *iroB, pipA*, and *stn* may not be detected in all *Salmonella* strains (Bäumler et al., 1997; Khoo et al., 2009; Smith et al., 2010; Wang et al., 2013). It is worth mentioning here that *invA* encodes a *Salmonella* invasion protein and is thus considered a virulence gene located on *Salmonella* pathogenicity island (**SPI**) 1, so it is frequently used to detect *Salmonella* (Chen et al., 2011). However, several studies have shown that some *Salmonella* strains including the important strain of *Salmonella* Kentucky lack this gene (Turki et al., 2012). Therefore, a more specific and conserved gene is needed as an ideal target for accurate detection of *Salmonella* in food. The *Salmonella* SPI4 has an important role in intra-macrophage survival and cooperation with the SPI for invasion (Gerlach et al., 2008). The SPI4 region includes genes from *siiA* to *siiF* that are important for adhesion to polarized epithelial cells (Kiss et al., 2007). Wille et al. (2014) reported that *siiA* and *siiB* encode novel type I secretion system subunits that have important roles in controlling the SPI4-mediated adhesion of *Salmonella enterica*. Also, the *siiA*-regulatory protein is essential for SPI4-encoded type I secretion system function and can affect the expression of *Salmonella* virulence genes (Gerlach et al., 2007; Wille et al., 2014). Therefore, *siiA* has an extremely important role in the field of *Salmonella* pathogenesis. Hassena et al. (2015) used the novel gene of *siiA* to detect *Salmonella* by the conventional method of real-time PCR (Hassena et al., 2015). To the best of our knowledge, this is the first report using the LAMP and LAMP-LFD methods to specifically target the *siiA* gene for detection of *Salmonella* in a dairy food safety application.

It is well known that after repeated experiments, spurious aerosolized LAMP amplicons of various lengths may be present in pipettes, equipment, reaction reagents, the working environment, and even experimenters' skin (He and Xu, 2011; Kil et al., 2015). Amplicon amounts less than 10 ag have been successfully used to simulate the level of aerosol droplets, confirming contamination (Hsieh et al., 2014). Therefore, the high sensitivity of LAMP might also become its largest potential disadvantage (Ou et al., 2014). Also, it is of great significance to eliminate any contamination from previous LAMP reactions. Until now, some methods to eliminate carryover contamination have been established, such as the use of separate rooms for each step of LAMP, 5% sodium hypochlorite solution to wipe the surface of the entire experimental environment, UV light (Ou et al., 2014), uracil-DNA-glycosylase (Hsieh et al., 2014), a closed tube with an agar dye capsule (Karthik et al., 2014), and overlaying the top of the LAMP reaction mixture with sterile mineral oil (Wu et al., 2014). Although the results of these methods are credible and effective, it could be time-consuming, labor-intensive, occasionally inhibitory to LAMP amplification, and unable to eliminate the contaminants from the environment. It is well known that propidium monoazide (**PMA**) has been applied in selective detection of viable bacteria and removal of exogenous DNA signal in quantitative PCR experiments (Elizaquível et al., 2012; Schnetzinger et al., 2013). Therefore, PMA appears to be useful for the elimination of LAMP carryover contamination.

In this study, we used the *siiA* gene to develop a rapid, specific, and sensitive LAMP-LFD method for the detection of *Salmonella* in PIF and applied PMA to eliminate the LAMP carryover contamination. Thus, the aim of this study was to provide an accurate, fast, sensitive, and convenient detection system for the detection of *Salmonella* in the dairy industry.

MATERIALS AND METHODS

Bacterial Strains and DNA Extraction

A total of 50 bacterial strains including 21 *Salmonella* strains and 31 non-*Salmonella* strains were used to evaluate the specificity of the LAMP method (Tables 1 and 2). All *Salmonella* strains were grown in 20 mL of NB medium at 37°C shaking at 5 × *g* for 8 h to achieve late-log phase (10^8 cfu/mL). Non-*Salmonella* strains were cultured in 20 mL of TSB medium under similar conditions.

In this study, *Salmonella* Typhimurium ATCC 14028 was used as the positive control and the template DNA was extracted from 1 mL of bacterial culture by the Gen Elute Bacterial Genomic DNA Kit (TIANGE, Beijing, China) following the manufacturer's instructions (http://www.tiangen.com/?productShow/t1/1/id/18.html) and suspended in 100 μL of Tris-EDTA buffer. Templates were stored at −20°C until further use.

LAMP Target Sequence and Primer Design

The LAMP primers were designed based on the conserved sequence between the *Salmonella siiA* gene (STM4257, GenBank accession number: NC_003197) and the 7 different strains of *Salmonella* Pathogenicity