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Can a visual loop-mediated isothermal amplification assay stand out in different detection methods when monitoring *Campylobacter jejuni* from diverse sources of samples?

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ABSTRACT

Consumption and exposure to *Campylobacter*-contaminated food are important causes of bacterial diarrhea. This paper reports the development and evaluation of a visual loop-mediated isothermal amplification (LAMP) assay targeting the highly conserved *cjaA* gene of *Campylobacter jejuni* (*C. jejuni*). This assay could be used for specialized detection and primary screening of retail food samples in China. When applied to 475 different samples, PCR (14.9%) and bacterial culture (12.7%) showed lower detection rates than the LAMP, which demonstrated 51 positive (16.6%) with a 100% diagnostic sensitivity to bacterial culture with no targets undetected. When tested with samples that had different cleanliness, the specificity (0.955), accuracy (0.961), positive likelihood ratio (22.417), kappa coefficient (0.844) for retail food samples tested by LAMP were higher than for fecal and environmental samples. Moreover, when tested with food samples that had different *C. jejuni* carry rates, LAMP was more sensitive in monitoring raw pork meat samples-the specificity (0.958), accuracy (0.958), and positive likelihood ratio (23.600) were higher than for chicken samples compared with a bacterial culture. The LAMP test enables the cost-effective detection of *C. jejuni* in food samples in 40 min, and it can also function as a primary screening approach or a complementary method in large-scale and on-site assays of food.

1. Introduction

Campylobacter jejuni is one of the most common foodborne pathogens that causes gastroenteritis worldwide (Wei et al., 2016), which is characterized by diarrhea, abdominal cramping, and fever (Acheson & Allos, 2001; Du et al., 2016). The consumption of *Campylobacter*-contaminated food (such as poultry, meat, raw milk, seafood, fruits and vegetables) is the principal cause of human campylobacteriosis and also poses a potential cross-contamination risk. However, to date, domestic information on contaminated foods in retail is still inadequate, and frequently used *C. jejuni* detection methods are unsuitable for on-site and large-scale food inspection. Moreover, one unspecialized detection method has often been used to test various samples. All of these factors have been identified as a hindrance for the effective implementation of food safety control and intervention measures (Cook, Odumeru, Lee, & Pollari, 2012; FAO/WHO, 2009; Huang, Zong, Zhao, Zhu, & Jiao, 2016). To meet these challenges, a rapid and cost-effective assay must be developed.

Bacterial culture is the current "gold standard" for *C. jejuni* detection, but the method only detects viable bacteria and is not suitable for large-scale surveys. Alternative detection methods for *C. jejuni* have recently been developed, including antibody-based detection, PCR, and DNA microarrays (Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010). However, most of these procedures are still time-consuming and require specialized skills or facilities (Romero et al., 2016). For example, certain methods of PCR-based testing of food samples require complicated sample preparation because certain food ingredients could inhibit the reaction (Rossen, Norskov, Holmstrom, & Rasmussen, 1992). Without these drawbacks, the loop mediated isothermal amplification (LAMP) assay is cost-effective and is especially suitable for on-site and large-scale inspections (Venkatesan, Balamurugan, Bhanuprakash, Singh, & Pandey, 2016). LAMP was developed by Notomi et al. (2000) and has a higher efficiency because it uses Bst DNA polymerase (Li et al., 2016), which is unusual in that more than

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500 mg/ml DNA could be synthesized (Wu, Chen, & Levin, 2015). Moreover, the robust nature of the Bst polymerase can simplify sample preparation (Francois et al., 2011). LAMP has currently emerged as a promising candidate for reliable identification of foodborne bacterial pathogens (Niessen, Luo, Denschlag, & Vogel, 2013).

In the present study, we developed a visual LAMP assay to detect *C. jejuni* in food samples. This assay's effectiveness was compared with bacterial culture and PCR for 475 samples (food, fecal and environmental samples). Samples with different cleanliness and *C. jejuni* carry rates were also tested to determine if the assay could be used in specialized applications such that the optimized LAMP assay could play a more effective role in the large-scale testing of food samples.

2. Materials and methods

2.1. Bacterial strains and samples

A *C. jejuni* (NCTC 11168) standard strain was used to optimize the LAMP assay and provide a positive control for the three assays tested. A total of 101 *C. jejuni* (NCTC 11168, ATCC 33560, 99 *C. jejuni* isolates from dogs, geese and chickens), 56 *Campylobacter coli* isolates from pigs and chickens and 10 non-*C. jejuni* bacteria were used to test the analytical sensitivity and evaluate the specificity of the assay.

A total of 308 fresh retail food samples were collected at random from several markets weekly from April to July 2016, including 184 slaughtered poultry meat samples (119 pork meats and 65 chicken samples), 53 seafood, 51 vegetable and edible fungi and 20 fruit samples. These markets are the major sources of staple foods in Yangzhou, a city in China.

To assess efficiency of the LAMP assay, two other types of representative samples with different cleanliness were also collected from April to July 2016, including 94 fecal samples (i.e., 20 duck and 62 pig fecal samples were collected from private farms and 12 fecal samples from patients with diarrhea were collected from the Yangzhou People's Hospital), and 73 environmental samples from the previously mentioned poultry (i.e., 18 feedstuff samples, 23 ground wipe samples and 32 water samples) were included. After collection, the samples were tightly sealed in sterile plastic wrap and directly transported to the laboratory on ice in an insulated container.

2.2. Template preparation

Genomic DNA used for the optimization and detection limit assays was prepared with a TIANamp Bacterial DNA Kit (TIANGEN BIOTECH, Beijing, China) according to the manufacturer's instructions. A boiling method was used for genomic DNA extraction for the other tests in this study. The suspended bacterial cultures were boiled for 10 min, cooled to room temperature, and centrifuged at 12000 g for 4 min. The resultant supernatant was used as the template DNA. Moreover, the DNA template was verified by agarose gel electrophoresis and subsequently adjusted to a final concentration of 100 ng/μL and stored at −80 °C for further experiments.

2.3. LAMP assay

2.3.1. LAMP reaction

LAMP primers (Fig. 1) were designed to target the *C. jejuni* NCTC 11168 conserved *cjaA* region (GenBank, Gene ID: 905273) with the PrimerExplorer Version 4 online tool (http://primerexplorer.jp/e/). The specificity of the LAMP primers were also confirmed with the BLAST online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Primer premier Version 5. The primers (Table 1) were the commercially synthesized by Genscript, China.

The LAMP reaction was carried out in a total volume of 25 μl. The optimized reaction mixture contained 0.8 mM of dNTP (TaKaRa, Dalian, China), 0.2 μM each of F3 and B3, 0.8 μM each of LF and LB, 1.6 μM each of FIP and BIP, 4 mM of $MgCl_2$ (TaKaRa, Dalian, China), 0.8 M Betaine (Sigma, USA), 8U of Bst DNA Polymerase Large Fragment (Vazyme, Nanjing, China), 2.5 μl of 10 × ThermoPol Buffer (Vazyme, Nanjing, China), 2 μl of template DNA, and RNase-free Water (TaKaRa, Dalian, China) was added to a final volume of 25 μl. RNase-free Water was also used as a negative control. The LAMP assay was incubated at 65 °C for 30 min, and then heated at 80 °C for 3 min to terminate the reaction. To monitor the real time amplification of the target DNA, a real time LAMP assay was developed (Arimatsu, Kaewkes, Laha, Hong, & Sripa, 2012). In the optimized LAMP reaction mixture, 1:3000 diluted SYBR green I (Invitrogen, CA) was added for real time detection with a melting curve analysis on the FAM channel step using an ABI 7500 Real Time PCR System (Life Tech, USA), according to the manufacturers instruction.

2.3.2. Observation of the LAMP results

To confirm that LAMP assay correctly amplified the target, the products were visualized by electrophoresis in a 1.0% agarose gel and stained with ethidium bromide. A DL2000 DNA ladder (TaKaRa, Dalian, China) was used as a molecular weight marker. Magnesium pyrophosphate was observed as a by-product of the amplification reaction. Dyes were employed in order to observe the products with the naked eye. Adding 120 μM metal ion indicator dye hydroxy naphthol blue (HNB) to the reaction mixture changed the color of the solution from violet to sky blue in the presence of the LAMP amplicon (Wongratanacheewin, Pumidonming, Sermswan, & Maleewong, 2001). The LAMP amplification results could also be visually inspected under a light box or via UV irradiation by adding 1 μl of a 1:100 dilution of the fluorescent dye SYBR Green I (Invitrogen, Carlsbad, CA) to the products. The color of the reaction solution changed from orange to green, indicating a positive reaction.

2.3.3. Sensitivity and specificity analysis

In LAMP sensitivity analysis assays, genomic DNA was diluted with TE buffer (Tris-EDTA Buffer, pH 8.0) to a final concentration of 10 ng/μl. Subsequently, a 10-fold serial dilution was made from 1×10^{1} to 1×10^{-7} ng/μl, and each of the diluted DNA samples was used as a template for LAMP and conventional PCR. The conventional PCR assay was optimized at an annealing temperature of 55 °C for 30 s to amplify a 213 bp amplicon with two outer primers (F3 and B3).

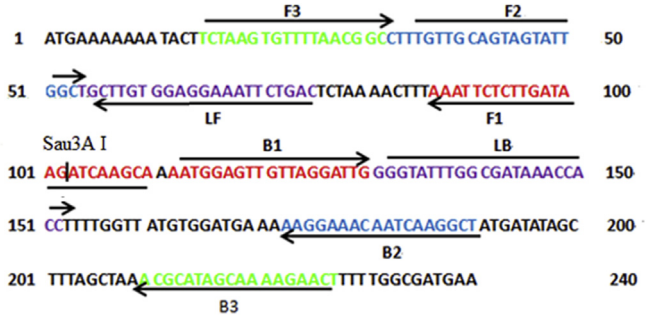

Fig. 1. Oligonucleotide primers were used to obtain the *cjaA* sequence of *Campylobacter jejuni*. Arrows and different colours indicated the position of the primers in the target sequences and the site of the restriction enzyme *Sau*3A I, respectively.