**Development of a multiplex real-time recombinase polymerase amplification (RPA)
assay for rapid quantitative detection of *Campylobacter coli* and *jejuni* from eggs and
chicken products**

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ABSTRACT

Chicken products are known as main vehicles for human infections and foodborne illness with *campylobacteriosis*. This study describes a rapid and sensitive method for the detection of *C. coli* and *C. jejuni* from eggs, chicken meat, and chicken broth using the multiplex real-time recombinase polymerase amplification (Rti-RPA) and its comparison to a culture dependent method. The optimal procedure for the multiplex Rti-RPA assay was set in isothermal condition at 45°C for 20 min and the detection sensitivity was at 1 CFU/reaction in pure culture. In the case of food applications, the detection limit of *C. coli* and *C. jejuni* using the RPA assay was 1 CFU/mL from chicken broth, 10³ CFU/g from egg and chicken meat samples without a pre-enrichment procedure, and 1 CFU/g from 24 h enriched egg and chicken meat samples. Quantifications of *Campylobacter* spp. in spiked food samples by the RPA assay were not significantly different with the results by the culture method. This multiplex Rti-RPA assay is considerably faster than other DNA amplification methods and exhibits no losses in detection sensitivity and specificity. This multiplex Rti-RPA is a simple, rapid detection and quantification assay that is completed within a 20 min runtime. The assay is applicable in the surveillance of *C. coli* and *C. jejuni* contamination in eggs and chicken products.

Key words: *Campylobacter*, RPA, Rapid method, Food Safety, Poultry products.

1. INTRODUCTION

Campylobacter is recognized as one of the most common causes of diarrheal illness in the United States (CDC, 2014) and campylobacteriosis is the most frequent foodborne illness in Europe (EFSA, 2015a; EFSA, 2015b). In 2012, the incidence of *Campylobacter* infection increased by 14% compared with those of 2006-2008 and among the species-confirmed cases of *Campylobacter* infections, *C. jejuni* (90%) and *C. coli* (8%) were identified as dominant species in the US. (CDC, 2014) The *Campylobacter* infections are mainly associated with contaminated foods. The EFSA reported that chicken meats directly account for 20-30% of infection cases and poultry meats, milk, and mixed products are believed to be sources of the outbreaks (EFSA, 2015a; EFSA, 2015b). In 2012, outbreaks of *C. jejuni* infection associated with undercooked chicken livers were reported in the US (CDC, 2013).

Isolation and identification of *Campylobacter* spp. require methodological expertise due to their particular growth requirements, feasible mutations, and the phenotypic similarities between the species. Over the last decade, nucleic acid based techniques have been established as alternatives to the traditional culture methods, such as the polymerase chain reaction (PCR), real-time PCR, and isothermal amplification assays (Abu-Halaweh, Bates, & Patel, 2005; Dong, Cho, Hahn, & Cho, 2014; Mateo, Cárcamo, Urquijo, Perales, & Fernández-Astorga, 2005; Nogva, Bergh, Holck, & Rudi, 2000; Yamazaki, Taguchi, Kawai, Kawatsu, Sakata, Inoue, & Misawa, 2009). The isothermal amplification techniques employ a uniform temperature to amplify targeted nucleic acid sequences (Zanoli & Spoto, 2013). The isothermal technique uses enzymes which are able to imitate DNA replication *in vitro* conditions and therefore does not require accurate temperature controls or thermo-cycling between 50 to 95°C which is demanded for the PCR assay (Gill & Ghaemi, 2013). Such isothermal amplification methods include helicase-dependent amplification (HDA), loop-

mediated isothermal amplification (LAMP), multiple displacement amplification (MDA), nucleic acid sequence-based amplification (NASBA), and recombinase polymerase amplification (RPA) (Notomi, Okayama, Masubuchi, Yonekawa, & Watanabe, 2000; Vincent, Xu, & Kong, 2004; Gill & Ghaemi, 2013; Yan, Zhou, Zheng, Gamson, Roembke, Nakayama, & Sintim, 2014).

RPA is a novel isothermal amplification method that uses a recombinase to form a nucleoprotein complex with oligonucleotide primers and facilitate the insertion of the primers into their complement double stranded DNA (Piepenburg, Williams, Stemple, & Armes, 2006). The primers allow the amplification of a targeted region of DNA in a manner that is similar to the extension step in PCR. The exo-probe enables the RPA assay to monitor real-time detection (TwistDx, 2013). In previous studies using RPA assays, amplified products are typically detected at a relatively low temperature, 37-42°C, within only 6-10 min runtime (Euler et al., 2013; Kersting, Rausch, Bier, & von Nickisch-Rosenegk, 2014; Santiago-Felipe, Tortajada-Genaro, Puchades, & Maquieira, 2014).

The RPA assay has been applied to detect various viruses and a few pathogens (Boyle, et al., 2013; Aebischer, Wernike, Hoffmann, & Beer, 2014; Zaghloul & El-Shahat, 2014; Glais & Jacquot, 2015; Teoh et al., 2015; Xia et al., 2015), but there were few attempts to detect the pathogenic bacteria from foods using an RPA assay (Santiago-Felipe, Tortajada-Genaro, Morais, Puchades, & Maquieira, 2015; Kim & Lee, 2016). In addition, to the best of our knowledge, there are no published reports in which the *Campylobacter* spp. have been detected in poultry products using the multiplex RPA assay. In a previous study, we successfully established the real-time RPA method for detecting and quantifying *Salmonella* Enteritidis (Kim & Lee, 2016). In this study, we have developed a rapid and specific multiplex real-time RPA assay to simultaneously detect *C. coli* and *C. jejuni*. Also, the

93 multiplex RPA assay was applied to several food models including eggs, chicken meat, and
94 chicken broth.

96 **2. MATERIALS AND METHODS**

97 **2.1 Bacterial culture and DNA template preparation**

98 *Campylobacter coli* and *C. jejuni* strains were obtained from the United States Department
99 of Agriculture, Eastern Regional Research Center (USDA-ERRC, Wyndmoor, PA). The
100 *Campylobacter* spp. stored at -80°C were recovered and grown on Brucella agar (Becton,
101 Dickinson and Company, Sparks, MD, USA) with the Campylobacter Growth Supplement
102 (Oxoid, Basingstoke, UK) under microaerophilic conditions (GasPak EZ Campy Container
103 System, BD) at 42°C for 48 h (Murray et al., 2003). A single colony was inoculated into
104 Brucella broth (BD) and microaerobically incubated at 42°C for 22-24 h until reaching a pre-
105 determined optical density. The numbers of *Campylobacter* spp. were evaluated by colony
106 counts on the supplemented Brucella agar. The genomic DNA from the *Campylobacter* spp.
107 cultures was obtained for the amplification reactions using the previous method (Kim & Lee,
108 2014). Approximately, 1×10^7 CFU/mL of diluted bacterial culture (1 mL) was centrifuged
109 for 5 min at $10,000 \times g$ and the supernatant was discarded. The pellets were suspended in 400
110 μ L of TZ buffer (20 g/L Triton X-100 and 2.5 mg/mL sodium azide in 0.1 mol/L Tris-HCl,
111 pH 8.0) and boiled for 10 min. Cell debris were discarded by centrifugation for 5 min at
112 $10,000 \times g$ and supernatants containing crude DNA extract were transferred to a fresh tube.
113 Twenty microliters of Quick-Precip Plus Solution (EdgeBio, Gaithersburg, MD, USA) and
114 800 μ L of absolute ethanol (Fisher Scientific, Fair Lawn, NJ, USA) were added to the
115 supernatants to precipitate genomic DNA. After centrifugation (5 min, $13,000 \times g$), the
116 pellets were washed with 70% ethanol and dried at room temperature. The pelleted DNA was

dissolved in nuclease-free water.

2.2 Primer design for multiplex real-time RPA assay

For detection of *C. coli* and *C. jejuni*, we used species-specific genes *ceuE* and *hipO*. The *ceuE* gene, which encodes siderophore enterochelin, a 34.5- to 36.2-kDa lipoprotein, has been used previously as a PCR target to identify *C. coli* (Gonzalez, Grant, Richardson, Park, & Collins, 1997; Mateo, Cárcamo, Urquijo M, Perales, & Fernández-Astorga, 2005; Hong et al., 2007). The unique nucleotide regions within the *ceuE* open reading frame (GenBank accession number X88849) were used for designing *C. coli*-specific PCR primers and probes in this study. The *hipO* gene (GenBank accession number Z23940), which is responsible for the hippurate hydrolase activity exclusively found within the *C. jejuni* genome, has been used as an effective identification marker for *C. jejuni* detection by PCR (Lawson, Shafi, Pathak, & Stanley, 1998; LaGier, Joseph, Passaretti, Musser, & Cirino, 2004; Persson & Olsen, 2005; Hong et al., 2007). The Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) was used to design the RPA primers recommended as TwistAmp (TwistDX, Cambridge, UK). From the amplicon sequence region among the primer candidates, the exo-probe regions which satisfied specific exo-probe conditions were manually selected according to the guidelines of probe design (TwistDx, 2013). Table 1 shows the primers and probe designed in this study.

2.3 Real-time RPA assay conditions for multiplex detection of *C. coli* and *C. jejuni*

Primers and probe concentrations, as well as reaction temperatures, were optimized for the multiplex real-time detection of *C. coli* and *C. jejuni* using the StepOne Plus real-time PCR platform (Applied Biosystems, Foster City, CA, USA). Optimal reaction mixtures consisted

of 200 nmol/L Camc-F, R primers, 60 nmol/L Camc probe, 400 nmol/L Camj-F, R primers, 240 nmol/L Camj probe, nuclease free water, template DNA, TwistAmp Exo Kit (TwistDX) containing dehydrated mastermix, 14 nmol/L Magnesium acetate, and TwistAmp rehydration buffer. All reagents except for the template DNA and the magnesium acetate were prepared in mastermix tubes. Subsequently, 3 μ L of template DNA and the magnesium acetate were added to each reaction tube. Twenty two microliters of the mixture was immediately transferred to the reaction tubes (Applied Biosystems) using a multi-channel pipette and the tubes were placed into the StepOne Plus system. Following temperature gradient multiplex reaction optimization from 35 to 51°C, the system was constantly set at 45°C and fluorescence intensity was read at every 30-second cycle.

2.4 Generation of standard curves

For the determination of detection and quantitative capabilities of *C. coli* and *C. jejuni* from food samples, standard curves for each target, *ceuE* (*C. coli*) and *hipO* (*C. jejuni*), were generated by the multiplex real-time RPA assay. Each DNA template was prepared from each dilution series of *C. coli* and *C. jejuni* cultures. The assays using the DNA templates were run in duplicate for five independent experiments. The standard curves were determined by plotting the Ct values against the Log CFU/reaction and the slope was generated by linear regression of the plotted points.

2.5 Determination of specificity and sensitivity for the multiplex Rti-RPA assay

The multiplex Rti-RPA assay was tested for *C. coli* and *C. jejuni* species specificity and sensitivity against a total 33 strains including 12 *Campylobacter* spp. and 21 species of non-*Campylobacter* organisms (Table 2). In order to assess the detection sensitivity of the assay in

food samples, *C. coli* and *C. jejuni* in 0.85% saline buffer were spiked into egg, chicken, and chicken broth samples to achieve a final concentration of 5 Log to 0 Log CFU/g or CFU/mL.

2.6 Food models

2.6.1 Eggs

For the practical application of the multiplex Rti-RPA assay to detect *C. coli* and *C. jejuni* from foods, eggs, chicken meats, and chicken broth were artificially inoculated with the different gradients of bacterial concentration of *C. coli* and *C. jejuni*. Fresh, unfertilized eggs were obtained from a local retail source in Dover, DE, USA. To detect *C. coli* and *C. jejuni* in egg yolk and white, each egg was washed with deionized water and the washed eggs were subsequently sanitized by submersion in 70% ethanol (vol/vol) for 30 min. Sanitized shell eggs were aseptically dried at room temperature for approximately 30 min before inoculation. Eggshells were then broken, the contents containing egg yolks and whites were transferred into sterile stomacher bags (Interscience, St. Nom, France) and the weight of the contents was measured. Each diluted *C. coli* and *C. jejuni* were spiked at five levels of inoculum from 5 Log to 1 Log CFU/g in three egg samples per inoculum level. The control egg sample was not exposed to the *Campylobacter* spp. artificially. In the stomacher bags, 0.85% saline buffer was added to the egg sample in a 9:1 ratio and the egg samples were homogenized using a Bag Mixer (Interscience, St. Nom la Breteche, France) for 5 min. To detect low levels under sensitive limits, *C. coli* and *C. jejuni* from 3 Log to 0 Log CFU/g were spiked in sensitized eggs and then samples were enriched in 1:10 volume of Brucella broth (BD) with *Campylobacter* Growth Supplement at 42 °C for 24 h. Ten milliliters of each egg sample was centrifuged at $500 \times g$ for 5 min to remove solid particles. Each supernatant was re-centrifuged at $12,000 \times g$ for 10 min and the supernatants were discarded. The pellets were

resuspended in 1 mL of saline buffer and centrifuged at $13,000 \times g$ for 5 min. The pellets were used for quantitative detection of *C. coli* and *C. jejuni*. The artificial inoculation trials at each dilution range were conducted as quadruple independent experiments to verify data reproducibility.

2.6.2 Chicken meat

Fresh packaged chicken thighs were obtained from local retailers in Dover, DE, USA. Chicken meat samples of skin and muscle, amounting to 25 g, were taken from multiple points on the thighs and placed into stomacher bags (Interscience). *C. coli* and *C. jejuni* were added to three meat samples per inoculum level in the stomacher bags at five levels of inoculation from 5 Log to 1 Log CFU/g. The inoculated and non-inoculated control chicken samples were homogenized in 225 mL of 0.85% saline buffer using a Bag Mixer (Interscience) for 2 min. Like the egg sample preparations, *C. coli* and *C. jejuni* were spiked from 3 Log to 0 Log CFU/g in chicken meats and the spiked and control chicken samples were enriched for 24 h in Brucella broth (BD) added Campylobacter Growth Supplement at 42°C. To remove chicken tissues, the homogenized samples were filtered twice with filter papers. Ten milliliters of the filtrates were centrifuged at $12,000 \times g$ for 10 min and the supernatants were discarded. The pellets were resuspended in 1 mL of saline buffer and centrifuged at $13,000 \times g$ for 5 min. The pellets were used for quantitative detection using the multiplex real-time RPA assay. The artificial inoculation trials were conducted as quadruple independent experiments.

2.6.3 Chicken broth

Chicken broth products were purchased from local retailers in Dover, DE, USA. Five levels of *C. coli* and *C. jejuni* from 4 Log through 0 Log CFU/mL were inoculated in each 50 mL of chicken broth. Chicken broth without *Campylobacter spp.* inoculation was used as a control.

The inoculated and control samples were vacuum filtered through polyethersulfone membrane filters (0.22 µm pore size, 25 mm diameter; Whatman, Clifton, NJ, USA) and the filters were put into microcentrifuge tubes to extract bacterial DNA. A DNA extraction method was employed and modified from our previous study (Kim & Lee, 2014). The artificial inoculation trials at each dilution range were conducted as triple independent experiments.

2.7 DNA extractions from *C. coli* and *C. jejuni* in chicken products for the multiplex real-time RPA assay

Two hundred microliters of 2 × TZ buffer was added to microcentrifuge tubes containing the sample pellets or filter membrane and 200 µL of 6% Chelex solution (Bio-Rad) was added and vortexed for 2 min. The tubes were boiled for 10 min and centrifuged at 10,000 × g for 5 min. And then the supernatants were retained. The following precipitation processes of genomic DNA were the same as the DNA template preparation from the pure culture and the multiplex real-time RPA assay was conducted as described above except 2 µL of sample template used. Quantification of *C. coli* and *C. jejuni* was evaluated using the standard curves as described above.

2.8 Culture-dependent enumeration of natural and artificial inoculated *Campylobacter* spp. in food samples

In parallel with the multiplex Rti-RPA assessments for the quantification of *C. coli* and *C. jejuni*, cultivable *Campylobacter* spp. in eggs, chicken meat, and chicken broth were also determined. The resuspended 1 mL of bacterial suspension retrieved from the *Campylobacter* spiked egg and chicken meat samples and non-spiked controls, as well as the *Campylobacter*

inoculated chicken broth and control chicken broth samples were directly plated on *Campylobacter* selective charcoal cefoperazone deoxycholate agar (CCDA; Neogen, Lansing, MI, USA) using the Eddy Jet 2 spiral plater (Neutec Group Inc., Farmingdale, NY, USA) for microaerobic incubation at 42°C. Colonies were enumerated on CCDA at 2-day cultivation.

2.9 Statistical analysis

Student's t-test was used to evaluate a significant difference of bacterial counts between the multiplex Rti-RPA and culture dependent assays. All bacterial numbers were Log₁₀ transformed and the IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA) was used to explore the statistical significance. A confidence interval at the 95% level ($P < 0.05$) was considered in all cases.

3. RESULTS

3.1 Optimal conditions for the multiplex real-time RPA assay

Primer and probe concentrations were optimized for the detection of low numbers of bacteria as well as maximization of primer and probe efficiencies. In the multiplex assay, the relative fluorescence curve of *C. jejuni* was suppressed by that of *C. coli* under the same condition of primer (400 nmol /L) and probe (120 nmol/L) concentrations for the multiplex detection (Table 3). Therefore, the primer and probe concentrations were optimized by reducing the primer and probe concentrations of *C. coli* and increasing those of *C. jejuni* gradually. The optimum conditions were 200 nmol/L of Camc primer, 400 nmol/L of Camj primer, 60 nmol/L of Camc probe, and 240 nmol/L of Camj probe obtaining the most efficient Cycle threshold (Ct) values (Table 3).

To optimize the reaction temperature, we performed a temperature gradient assay from 35 to

51°C. The lowest temperature (35°C) produced the highest Ct values from the two targets and the Ct values were reduced as the reaction temperature was increased up to 45°C (Table 4). At 49°C, the amplifications were retarded and the amplification curves were not detected at 51°C (Table 4). Therefore, the optimum reaction temperature was fixed at 45°C for rapid detection.

3.2 Specificity and sensitivity of the multiplex real-time RPA

The multiplex real-time RPA assay was tested for specificity against 33 bacteria including 12 *Campylobacter* spp. strains (Table 2). *C. coli* was positive for *CeuE* but negative for *hipO* and *C. jejuni* generated Ct values for only *hipO*. Other *Campylobacter* spp. and non-*Campylobacter* strains showed negative results in both *CeuE* and *hipO*.

In order to verify detection sensitivity of the method and the consistency of quantitative measurement, DNA templates were prepared from each 10-fold diluted pure culture of *C. coli* and *C. jejuni* for the multiplex real-time RPA. The two standard curves of the specific targets obtained from each *Campylobacter* strain in the range of 0 Log to 5 Log CFU per RPA assay are shown in figure 1. The standard curves generated from the target sequences showed excellent correlations of linear regression; r^2 coefficients of *CeuE* and *hipO* were 0.989 and 0.975, respectively from five independent experiments. In addition, this multiplex RPA assay detected 1 CFU/mL of pure cultured *C. coli* and *C. jejuni*.

3.3 Detection of *C. coli* and *C. jejuni* from eggs and chicken meats using the multiplex real-time RPA

The multiplex real-time RPA was applied to *C. coli* and *C. jejuni* spiked egg and chicken meat samples for quantitative detection and the results were compared with bacterial counts

by a *Campylobacter* spp. specific CCDA plate count method. Overall, for the egg and chicken meat samples spiked with *C. coli* and *C. jejuni* from 5 Log to 3 Log CFU/g, the *C. coli* and *C. jejuni* were proximately quantified as the spiked levels by the RPA (Table 5). Also, the colony counts on CCDA plates, which cultivated both *C. coli* and *C. jejuni*, were similar with the number of RPA results or a little higher than the each bacterial count of multiplex RPA results (Table 5). However, from the egg and chicken meat samples inoculated with *Campylobacter* spp. at 2 Log and 1 Log CFU/g, both *C. coli* and *C. jejuni* were not detected in the RPA assay. Therefore, the detection limit of *C. coli* and *C. jejuni* in non-enriched raw egg and chicken meat was 3 Log CFU/g.

3.4 Detection of *C. coli* and *C. jejuni* from enriched eggs and chicken meats using the multiplex real-time RPA

To detect low levels of *C. coli* and *C. jejuni* below the detection limit from raw samples, the egg and chicken meat samples were enriched in Brucella broth. After 24 hr enrichment, *C. coli* and *C. jejuni* were detected from all egg and chicken samples challenged with 0 Log to 3 Log CFU/g (Table 6). In the non-challenged control samples, *C. coli* was detected from 9 out of 12 egg samples and 6 out of 18 chicken meat samples. *C. jejuni* was detected from 3 out of 12 egg samples and 12 out of 18 chicken meat samples (Table 6) after enrichment. The non-inoculated food samples showing positive results can be considered naturally contaminated.

3.5 Detection of *C. coli* and *C. jejuni* from chicken broth using the multiplex real-time RPA

The RPA assay was also screened for detection efficiency in chicken broth samples spiked

with *C. coli* and *C. jejuni* from 5 Log to 0 Log CFU/mL and the RPA results were compared with the *Campylobacter* spp. counts on CCDA plates. *C. coli* and *C. jejuni* were detected as low as 0 Log CFU/mL by the RPA assay and the quantified levels were similar with spiked *Campylobacter* levels (Table 7). In addition, the colony counts on CCDA plates were slightly higher than each quantified *C. coli* and *C. jejuni* number by the RPA assay; the plate counts might represent total *Campylobacter* spp.

4. DISCUSSION

Previous case control studies reported that the most important vehicles for human infections with *Campylobacter* are contaminated poultry products (Rosenquist, Nielsen, Sommer, Norrung, & Christensen, 2003; Wingstrand et al., 2006). To control *Campylobacter* contaminations in poultry products, rapid detection is the most logical strategy. Therefore, it would be beneficial to have a rapid, sensitive, and specific method to detect *Campylobacter* in chicken and related products. Santiago-Felipe *et al.* showed that the RPA assay could be used to detect pathogenic bacteria such as *Salmonella* spp. and *Cronobacter* spp. in a food matrix, especially in milk. The aim of our study is to develop a multiplex real-time RPA method as a rapid and simple detection assay to simultaneously diagnose *C. coli* and *C. jejuni* from chicken and related foods.

In the ISO method (ISO10272-2: 2006), a culture-dependent method using a *Campylobacter* selective agar media was employed for the quantification of *Campylobacter* spp. in foods. The number of *Campylobacter* spp. is determined by enumerating typical colonies on the agar and subsequently confirmed by morphological, biochemical, and growth property tests. However, this method is extremely time and labor intensive due to the 3-day cultivation and biochemical property confirmations. Also, it is challengeable to differentiate species level

through their morphological and biochemical examinations.

Nowadays, the real-time PCR technique is generally used for a quantitative detection of pathogenic bacteria including *Campylobacter* spp. Both PCR and RPA assays are culture-independent and DNA amplification based technologies. However, the amplification principles of target DNA sequences are basically different between the two methods. In the PCR assay, thermal cycling steps are required for DNA amplification. RPA operates on an enzyme-derived primer binding process; the recombinase and crowding agents form a nucleoprotein complex with primers and recognize their complementary target sequence. The primers are then extended by *Sau* polymerase and the amplification is accomplished by the cyclic repetition of the process at the isothermal condition (37 – 42 °C) (Piepenburg, Williams, Stemple, & Armes, 2006; Euler et al., 2013; Kim & Lee, 2016). While the real-time PCR method required a 4-h runtime for detecting *Campylobacter* spp. from previous studies (Toplak et al., 2012; de Boer et al., 2015), our real-time RPA technique needed only about two and half hours including DNA sampling and RPA reaction.

In our study, the RPA was applicable to the real-time PCR instrument for monitoring the real-time detection using exo probes (TwistDX, Cambridge, United Kingdom). The exo probe contains closely located internal fluorophore and quencher linked to thymine and a tetrahydrofuran (THF) residue. The THF is cleaved when the exo probe binds to the target sequence and thereby the quencher separates from the fluorophore (Piepenburg et al. 2006; TwistDX, 2013; Zanolli & Spoto, 2013).

The classical identification and differentiation procedure that can distinguish *C. jejuni* from *C. coli* relies solely on hippurate hydrolysis; therefore, we chose the *hipO* gene as a target for *C. jejuni*. In the *ceuE* genes of *C. coli* and *C. jejuni*, there is approximately 13% of a nucleotide sequence divergence and many studies demonstrated that *ceuE* can be used as a

sensitive and specific target gene for the differential identification of *C. coli* (Houng, Sethabutr, Nirdnoy, Katz, & Pang, 2001; Mateo, Cárcamo, Urquijo, Perales & Fernández-Astorga, 2005; Hong et al., 2007). In this study, no cross-reaction was observed between the two targets and this assay did not produce any false-positive signals from other related bacteria such as *Campylobacter concisus*, *Campylobacter fetus* subsp. *fetus*, *Campylobacter lari*, and *Salmonella* spp.

For the multiplex real-time detection, optimization of primer and probe concentrations is indispensable due to cross-talk between reporter dyes. In our previous study, development of a multiplex real-time PCR assay to detect *Vibrio* spp. and total bacteria, a target signal having lower relative fluorescence intensity was strongly suppressed by the other target signal producing higher relative fluorescence intensity (Kim & Lee, 2014). Likewise, the present study showed that the *ceuE* target having FAM fluorescence dye produced much higher fluorescence intensity than a *hipO* target represented by a TAMRA fluorescence dye and the amplification signal of a *hipO* target was strongly interfered by a *ceuE* signal. In order to reduce this signal interference, we modified the primer and probe concentrations of the two targets. Consequently, the signal interference was minimized at the concentration ratios of 1:2 of primers and 1:4 of probes (*ceuE*: *hipO*).

The optimal conditions for multiplex detection of *C. coli* and *C. jejuni* by the RPA assay were determined to be 45°C for 20 min in this study. It is known that the RPA assay typically operates at a wide range of isothermal points from 25 to 43°C (Lillis et al., 2014). The assay worked well at 35°C, which is the lowest temperature tested in this study, but the reaction rate was accelerated depending on the increase of the reaction temperature up to 45°C. Also, the Ct values of the RPA assay were significantly delayed because it did not operate at temperatures over 49°C. This might be due to enzyme inactivation. To achieve rapid

detection, the optimum reaction temperature was determined as 45°C. The 45°C reaction temperature is slightly higher than the typical RPA reaction temperature between 37-43°C used in other RPA studies (Euler et al., 2013; Kersting, Rausch, Bier, & von Nickisch-Rosenegk, 2014; Santiago-Felipe, Tortajada-Genaro, Morais, Puchades, & Maquieira, 2015) but notably lower compared with other isothermal amplification methods which generally maintain reaction temperatures between 60 and 65°C (Compton, 1991; Notomi, Okayama, Masubuchi, Yonekawa, & Watanabe, 2000; Vincent, Xu, & Kong, 2004; Fang, Liu, Kong, & Jiang, 2010; Zanolli & Spoto, 2013). At 45°C, this RPA assay was able to detect *Campylobacter* spp. targets at a low concentration (1 CFU/ reaction) as little as 18 cycles in 10 min. However, the optimum reaction time was set at 35 cycles, 20 min to ensure any positive detection with very low bacterial targets in food samples. From this study, the runtime of the multiplex RPA assay was shown to exhibit an extremely fast reaction in comparison to the multiplex real-time PCR assay which requires a 4 h runtime (Toplak et al. 2012). However, it is also significantly more rapid than other isothermal assays which have incubation times in the range of 40-60 min (Notomi, Okayama, Masubuchi, Yonekawa, Watanabe, 2000; Vincent, Xu, & Kong, 2004; Fang, Liu, Kong, & Jiang, 2010; Toplak, Kovač, Piskernik, Možina, & Jeršek, 2012; Zanolli & Spoto, 2013).

The detection limit of the multiplex real-time RPA assay was 1 CFU/mL in pure culture and in chicken broth samples without enrichment. The detection limit of the RPA assay in non-enriched egg and chicken meat samples was 3 Log CFU/g, but after enrichment, 1 CFU/g of *C. coli* and *C. jejuni* were detected from all the samples by the RPA assay. When DNA amplification methods are used to detect pathogens in food, bacterial enrichment of food samples is widely used. However, it is known that the enrichment process precludes the exact quantification of the pathogens (Postollec, Falentin, Pavan, Combrisson, & Sohier, 2011).

Using the real-time PCR technique, there have been many attempts to quantify *C. coli* or *C. jejuni* in foods without pre-enrichment. Botteldoorn et al. (2008) reported that the detection limit of *Campylobacter* spp. was 3.3 Log CFU in chicken sample by the real-time PCR, and this is similar with our results. Rantsiou et al. (2010) developed a real-time PCR assay which quantified *C. jejuni* in chicken and the detection limit of this method was 10 CFU/g in the foods. The drawback of the assay is its limitation to one single target species. The detection limits of the multiplex real-time RPA assay herein are comparable with other multiplex assays. Wolffs et al. (2007) obtained a detection limit of 3 Log CFU/mL using the multiplex real-time PCR for quantification of *Salmonella* and *Campylobacter* spp. in chicken rinse samples. Toplak et al. (2012) established a multiplex real-time PCR assay that was able to detect 2 Log – 3 Log CFU/mL of *C. jejuni* in chicken juice. However, Toplak et al. did not conduct quantitative detections of both *C. coli* and *C. jejuni* in a food sample, thus they reported only the detection limit of *C. jejuni*. Consequently, our multiplex real-time RPA assay, which can simultaneously detect 0 Log or 3 Log CFU/mL or g of *C. coli* and *C. jejuni* in eggs, chicken, and chicken broth within 20 min of reaction time, is an exceptional approach when compared with other real-time PCR studies.

Until recently, the LAMP assay has been considered a representative isothermal assay for rapid and simple detection (Asidello & Baeumner 2011). *C. coli* or *C. jejuni* were detected from foods, feces, or environmental samples using real-time LAMP assays at 65°C within 30 – 60 min of reaction time (Yamazaki et al., 2008, 2009; Dong, Cho, Hahn, & Cho, 2014). Yamazaki et al. (2008 & 2009) reported that detection limits for LAMP assays targeting *C. jejuni* and *C. coli* were 5.6×10^3 and 4.8×10^3 CFU/g⁻¹ in human stool. Dong et al. (2014) established a LAMP assay to detect *C. jejuni*, which showed 2.5×10^2 CFU/mL of detection sensitivity in cattle farm samples using an overnight enrichment step. Therefore, the LAMP

studies for *Campylobacter* spp. required a longer reaction time, a higher reaction temperature, and showed lower detection sensitivity compared with the RPA assay of this study. Also, while the present RPA assay can detect multiple targets of *C. coli* and *C. jejuni*, the LAMP assays are single target detecting techniques. The LAMP assay typically needs six primers leading to a longer amplicon and undoubtedly requires more technical considerations to design primers; whereas the RPA assay uses three oligonucleotides including a probe for real-time detection and offers the same flexibility as real-time PCR. Hence, a higher multiplexing ability can be implemented for detecting different targets using the RPA assay.

5. CONCLUSION

C. jejuni and *C. coli* contamination in chicken products is one of the most important public health hazards to the industry (Waage, Vardund, Lund, & Kapperud, 1999; Sails, Fox, Bolton, Wareing, & Greenway, 2003). This study established a rapid and highly sensitive multiplex real-time RPA assay for the simultaneous detection of *C. jejuni* and *C. coli* from eggs, chicken meat, and chicken broth. Through the results, we can conclude that this RPA assay is considerably faster than real-time PCR and LAMP assays and there are no losses in sensitivity or specificity in comparison with other diagnostic systems. The reliable and sensitive results suggest that the multiplex real-time RPA assay can be useful to survey egg or chicken products for *C. coli* and *C. jejuni* contamination and consequently contribute to reducing the incidence of *Campylobacter* infection before contaminated foods are consumed.

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Fig 1.

(A) (B)

(C) (D)

Figure 1. Standard curves of two targets: (A) amplification plot of *Campylobacter coli*, (B) amplification plot of *C. jejuni*, (C) a slope generated by linear regression for *C. coli*, and (D) a slope generated by linear regression for *C. jejuni*. The slopes were plotted by Ct values and Log CFU. Pure cultured *C. coli* and *C. jejuni* were used ranges from 0 Log CFU to 5 Log CFU per reactions

Table 1. Primers and probes targeting the *Campylobacter coli* (*ceuE*) and *Campylobacter jejuni* (*hipO*) designed in this study

Target	Primer/ probe name	Sequence (5' – 3')	Amplicon size (bp)
<i>ceuE</i>	Camc-F	TTTTGCATTCTTTTAAAGTCTAATTTTAACAGCTT	241
	Camc-R	GTTAAGTCTTAGGGCATGGAAAGTATCTAAAATTC	
	Camc-probe	CAATAAAACTAGCACTACGGTAAAGGTTA[T(FAM)]A[THF]C[T(BHQ-1)]ATTAGTATGAGTGAT-SpacerC3	
<i>hipO</i>	Camj-F	GTTTGAAAAATTTGATAGTGATTATGTTTTTGGAT	128
	Camj-R	CAATAACTTCAATGCTATAACTATCCGAAGAAG	
	Camj-probe	AATATGCCTTTTGGTAGCGATAAGAAATTT[T(TAMRA)]A[THF]C[T(BHQ-2)]TAAAAAAGGTGCGAT-SpacerC3	

646 Table 2. Bacterial strains used for the specificity and sensitivity test in this study

Taxon	Strain	Source	Real-time RPA results (+/-)	
			<i>ceuE</i>	<i>hipO</i>
<i>Campylobacter coli</i>	RM1403	USDA-ERRC	+	-
	RM1529	USDA-ERRC	+	-
	RM1840	USDA-ERRC	+	-
	RM2221	USDA-ERRC	+	-
<i>Campylobacter jejuni</i>	14076	USDA-ERRC	-	+
	9368	USDA-ERRC	-	+
	RM1188	USDA-ERRC	-	+
	RM1246	USDA-ERRC	-	+
	RM1449	USDA-ERRC	-	+
<i>Campylobacter concisus</i>	ATCC51562	ATCC	-	-
<i>Campylobacter fetus</i> subsp. <i>fetus</i>	ATCC27372	ATCC	-	-
<i>Campylobacter lari</i>	ATCC35221	ATCC	-	-
Non-Campylobacter spp.				
<i>Salmonella</i> Enteritidis	RE-155350A	Carolina	-	-
<i>Salmonella</i> Heidelberg	SA 42	USDA-DSU	-	-
<i>Salmonella</i> Montevideo	SA 40	USDA-DSU	-	-
<i>Salmonella</i> Newport	SA41	USDA-DSU	-	-
<i>Salmonella</i> Typhimurium	SA34	USDA-DSU	-	-
<i>Bacillus cereus</i>	5056	USDA-DSU	-	-
<i>Bacillus subtilis</i>	RE-154921	Carolina	-	-
<i>Enterococcus faecalis</i>	RE-155600A	Carolina	-	-
<i>Escherichia coli</i> 0111	JB 1-95	USDA-DSU	-	-
<i>Escherichia coli</i> O157:H7		USDA-DSU	-	-
<i>Lactococcus lactis</i>	RE-155610	Carolina	-	-
<i>Lactobacillus casei</i>	Cab18	Kim et al., 2015	-	-
<i>Lactobacillus plantarum</i>	Cab39	Kim et al., 2015	-	-
<i>Leuconostoc mesenteroides</i>	W51	Kim et al., 2015	-	-
<i>Pseudomonas aeruginosa</i>	ATCC 10145	ATCC	-	-
<i>Pseudomonas fluorescens</i>	ATCC 13525	ATCC	-	-
<i>Serratia marcescens</i>	SM51	USDA-DSU	-	-
<i>Staphylococcus aureus</i>	ATCC 12598	ATCC	-	-
<i>Vibrio parahaemolyticus</i> O1:Kuk		USDA-DSU	-	-
<i>Vibrio vulnificus</i>	MLT 364	USDA-DSU	-	-
<i>Vibrio anguillarum</i>	RE-155721	Carolina	-	-

ATCC: American Type Culture Collection; Carolina: Carolina Biological Supply Co.; USDA-DSU: US Department of Agriculture, Agricultural Research Service at Delaware State University; USDA-ERRC: US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center.

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650 Table 3. Ct values for *C. coli* and *C. jejuni* obtained from different primers and probe
 651 concentrations by the multiplex Rti-RPA assay

Primers concentrations (nmol L ⁻¹)		Probe concentrations (nmol L ⁻¹)		Ct values	
Camc	Camj	Camc	Camj	<i>C. coli</i>	<i>C. jejuni</i>
400	400	120	120	7.86±0.04	nd
200	400	60	120	9.27±0.21	8.71±0.15
200	400	60	180	9.85±0.06	8.20±0.23
200	400	60	240	10.37±0.03	7.59±0.20
200	600	60	180	12.94±0.19	7.72±0.02
200	600	60	360	14.96±0.06	nd
100	600	40	240	19.74±0.24	18.27±0.18

652 Nd, not detected

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Table 4. Ct values for *C. coli* and *C. jejuni* obtained from different reaction temperatures by the multiplex Rti-RPA assay

Reaction temperatures (°C)	Ct values	
	<i>C. coli</i>	<i>C. jejuni</i>
35	11.79±0.30	8.83±0.16
37	10.43±0.67	7.61±0.20
39	8.90±0.12	7.27±0.39
41	7.78±0.11	6.47±0.21
43	6.87±0.02	6.28±0.19
45	6.33±0.01	6.31±0.07
47	6.66±0.05	6.62±0.08
49	18.84±0.37	10.66±0.33
51	nd	nd

Nd, not detected

Table 5. Quantification of *C. coli* and *C. jejuni* in spiked eggs and chicken meat analyzed by the multiplex Rti-RPA and cultural methods

Spiked levels of mixed <i>C. coli</i> and <i>C. jejuni</i> (Log CFU g ⁻¹)	Eggs (Log CFU g ⁻¹)			Chicken meat (Log CFU g ⁻¹)		
	RPA		CCDA	RPA		CCDA
	<i>C. coli</i>	<i>C. jejuni</i>	<i>Campylobacter</i> spp.	<i>C. coli</i>	<i>C. jejuni</i>	<i>Campylobacter</i> spp.
5	4.95±0.09	5.14±0.19	5.26±0.07	5.05±0.19	5.12±0.32	5.10±0.09
4	3.88±0.10	4.21±0.06	4.48±0.02	4.13±0.16	3.94±0.09	4.02±0.07
3	3.06±0.13	3.33±0.07	3.55±0.01	3.17±0.39	3.23±0.27	3.46±0.07
2	nd	nd	2.52±0.02	nd	nd	2.50±0.04
1	nd	nd	1.46±0.26	nd	nd	1.38±0.16
Control	nd	nd	nd	nd	nd	nd

Nd: not detected

Table 6. Detection of *C. coli* and *C. jejuni* in spiked and enriched eggs and chicken meat analyzed by the multiplex Rti-RPA

Spiked levels of <i>C. coli</i> and <i>C. jejuni</i> (Log CFU g ⁻¹)	Enriched eggs		Enriched chicken meat	
	No. of positives (%)		No. of positives (%)	
	n = 12		n = 18	
	<i>C. coli</i>	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. jejuni</i>
3	12 (100)	12 (100)	18 (100)	18 (100)
2	12 (100)	12 (100)	18 (100)	18 (100)
1	12 (100)	12 (100)	18 (100)	18 (100)
0	12 (100)	12 (100)	18 (100)	18 (100)
Control	9 (75)	3 (25)	6 (33)	12 (66)

Table 7. Quantification of *C. coli* and *C. jejuni* in spiked chicken broth analyzed by the multiplex Rti-RPA and cultural methods

Spiked levels of <i>C. coli</i> and <i>C. jejuni</i> (Log CFU mL ⁻¹)	RPA (Log CFU mL ⁻¹)		CCDA (Log CFU mL ⁻¹)
	<i>C. coli</i>	<i>C. jejuni</i>	<i>Campylobacter</i> spp.
Log 5	4.87±0.00	5.12±0.12	5.06±0.05
Log 4	4.13±0.11	4.03±0.37	4.36±0.11
Log 3	3.40±0.01	2.87±0.02	3.46±0.15
Log 2	1.97±0.19	1.86±0.15	2.45±0.05
Log 1	1.06±0.56	0.71±0.15	1.33±0.08
Log 0	0.01±0.32	0.01±0.17	nd
Control	nd	nd	nd

Nd: not detected