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Real-time recombinase polymerase amplification assay for the rapid and sensitive detection of *Campylobacter jejuni* in food samples

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ABSTRACT

Campylobacter jejuni (*C. jejuni*), a foodborne pathogen, is a major contributor to human bacterial gastroenteritis worldwide and detrimental to public health. It is crucial for initiating appropriate outbreak control strategies to rapidly detect *C. jejuni*. As a novel isothermal gene amplification technique, recombinase polymerase amplification (RPA) has been developed for the molecular detection of diverse pathogens. In this study, we developed a real-time RPA assay so as to achieve the rapid and efficient detection of *C. jejuni* by targeting the *hipO* gene. The specificity and senstivity of real-time RPA was validated and the practical applicability of the method for the detection of *C. jejuni* in artificially contaminated milk and chicken breast samples was proved by comparing their reaction time, sensitivity, and efficacy with those of real-time PCR and culture-based methods. Based on the real-time RPA assay, analysis time was reduced to approximately 13 mins from 60 mins and the results were as reliable as those of the real-time PCR assay. Taken together, in terms of the detection of *C. jejuni*, the real-time RPA method was simple, rapid, sensitive, and reliable.

1. Introduction

Being a member of the *Campylobacter* genus, *Campylobacter jejuni*, is the most frequently reported bacterial foodborne pathogen in human gastrointestinal infections worldwide (Allos, 2001; Kaakoush et al., 2015; Rokosz et al., 2014). It is *C. jejuni* infection that gives rise to more cases of diarrhea than foodborne *Salmonella*, *Listeria* or *Shigella*. With wide distribution in the intestines of wild and domestic animals, particularly birds, *C. jejuni* spreads mainly through the fecal-oral route (Humphrey et al., 2007; Jacobs-Reitsma et al., 1995; Sahin et al., 2003; Vadalasetty et al., 2018; van Gerwe et al., 2009). The most important route of human infection is attributed to the handling and consumption of raw or undercooked poultry products, although increasing evidence has associated raw milk and untreated water with campylobacteriosis (Denis et al., 2011; Heuvelink et al., 2009). Headache, fever, abdominal pain and watery to bloody diarrhea are all typical symptoms of campylobacteriosis. Although campylobacteriosis is generally self-limiting, some severe cases associated with *C. jejuni* infection may be linked to other complications such as reactive arthritis, Guillain–Barré syndrome (GBS), and irritable bowel syndrome (IBS) (de Boer et al., 2015; Moore et al., 2005; Nachamkin et al., 1998; Pike et al., 2013). In recent years, outbreaks of *C. jejuni* infections are becoming of increasing concerns for public health in both developed and developing countries (Kaakoush et al., 2015; Pike et al., 2013). In addition, the economic loss caused by the pathogen is serious (Gillespie et al., 2002; Romero et al., 2016). Therefore, a method for the rapid, sensitive and accurate diagnosis of *C.*

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jejuni infection would help the government implement food safety policies or intervention strategies as a way of eliminating or mitigating the risk to the consumer and preventing outbreaks of the foodborne illness.

C. jejuni grows under microaerobic conditions. Conventional culture detection methods, with pre-enrichment, isolation, and identification at the species level included, are labor-intensive and time-consuming, requiring 8 days to reach a conclusive result (Mayr et al., 2010; Saiyudthong et al., 2015). As biotechnology advances, a number of gene amplification-based molecular detection technologies have been developed for *C. jejuni* diagnosis, such as polymerase chain reaction (PCR) (Kulkarni et al., 2002), real-time PCR (Oliveira et al., 2005), multiplex PCR (Kamei et al., 2016), loop-mediated isothermal amplification (LAMP) (Yamazaki et al., 2008), PCR-ELISA (Sails et al., 2001), and DNA hybridization (Chuma et al., 1993). Although these methods are clearly sensitive and specific, high skill levels and expensive equipment is required. Furthermore, implementation of these approaches is limited in the resource-limited or the point-of-need (POD) settings. For the molecular detection of diverse pathogens such as viruses, bacteria, parasites and so on. Recombinase polymerase amplification (RPA), a novel isothermal gene amplification technique, has been explored (Abd El Wahed et al., 2015a, 2015b, Boyle et al., 2013, Daher et al., 2016, Gao et al., 2018, Lai et al., 2017, Piepenburg et al., 2006). It mainly depends on three enzymes including a recombinase (UvsX), a single-stranded DNA binding protein (SSB), and a DNA polymerase. The amplification of DNA templates can be achieved by simulating the enzyme reaction of DNA duplication. Firstly, the recombinase can be closely combined with the primers of DNA to form duplexes. Then, when the primers find the completely complementary sequence in the DNA template, the template unwinds because of SSB and new DNA complementary strand comes into being with the help of the polymerase. Using two opposing primers, exponential amplification of the target sequence with RPA can be obtained at a constant temperature in 10 to 20 mins without denaturation which is much faster than PCR (Piepenburg et al., 2006). Furthermore, the RPA product can be measured in real-time through using different probes with fluorescence detection devices, which is viewed as the most applicable method for resource-limited and POD diagnoses of infectious diseases. In this study, we developed a real-time RPA method so as to simply and rapidly detect *C. jejuni*. RPA amplification products were detected in real time using exo probes. In addition, using a detection device, fluorescence signals were monitored in real time.

2. Materials and methods

2.1. Bacterial reference strains and growth conditions

Serving as the reference strains and field isolates, a total of 8 *C. jejuni* strains, 7, non-*C.jejuni* Campylobacters and 24 additional non-*Campylobacter* stains were tested (Table 1). *C. jejuni* was grown in Brucella medium (Becton Dickinson Co., Sparks, MD) at 42 °C microaerobically in a tri-gas incubator with 85% N_2, 10% CO_2, and 5% O_2. Other bacterial strains were incubated either aerobically or anaerobically as appropriate at 37 °C.

2.2. Preparation of template DNA

Based on the manufacturer's instructions, bacterial genomic DNA was extracted through a bacterial DNA extraction kit (TIANGEN, Beijing, China). The concentration of *C. jejuni* genomic DNA was quantified with the ND-2000c spectrophotometer (NanoDrop, Wilmington, USA), and the DNA copy number was calculated using the following formula: amount (copies/μL) = [DNA concentration (g/μL)/(bacterial genome length in base pairs × 660)] × 6.02 × 10^{23} (Yun et al., 2006).

Table 1
Bacterial strains used in the specificity test.

Strain	Origin	Real-time RPA	Real-time PCR
Campylobacter jejuni	ATCC 33291	+	+
Campylobacter jejuni	ATCC 33560	+	+
Campylobacter jejuni	ATCC 29428	+	+
Campylobacter jejuni	CICC 22936	+	+
Campylobacter jejuni	CICC 22936	+	+
Campylobacter jejuni	Isolated by lab	+	+
Campylobacter jejuni	Isolated by lab	+	+
Campylobacter jejuni	Isolated by lab	+	+
Campylobacter coli	CICC 23925	−	−
Campylobacter coli	ATCC 33559	−	−
Campylobacter coli	Isolated by lab	−	−
Campylobacter lari	ATCC 35223	−	−
Campylobacter lari	Isolated by lab	−	−
C. fetus subsp. *fetus*	ATCC 27374	−	−
C. upsaliensis	ATCC 4395	−	−
Vibrio parahaemolyticus	CICC 21617	−	−
Vibrio parahaemolyticus	ATCC 17802	−	−
Bacillus cereus	ATCC 11778	−	−
Bacillus cereus	CICC 10648	−	−
Citrobacter freundii	ATCC 10787	−	−
Enterobacter sakazakii	ATCC 29544	−	−
Enterobacter sakazakii	ATCC 21548	−	−
Escherichia coli O157:H7	CICC 21530	−	−
Escherichia coli	CMCC 44102	−	−
Enterococcus faecalis	ATCC 29212	−	−
Klebsiella pneumoniae	ATCC 4352	−	−
Listeria monocytogenes	ATCC 19114	−	−
Listeria monocytogenes	ATCC 7644	−	−
Proteus mirabilis	ATCC 29906	−	−
Providencia	ATCC 29944	−	−
Pseudomonas aeruginosa	Isolated by lab	−	−
Salmonella Typhimurium	CICC 22956	−	−
Serratia marcescens	ATCC 14756	−	−
Shigella sonnei	CICC 21679	−	−
Shigella sonnei	ATCC 25931	−	−
Shigella flexneri	CICC 21678	−	−
Staphylococcus aureus	ATCC 6538	−	−
Staphylococcus aureus	ATCC 25923	−	−
Yersinia enterocolitica	CICC 21609	−	−

+, positive result; −, negative result.

2.3. RPA primers and probe

In accordance with the reference sequences of different *C. jejuni* genotypes (accession numbers CP002029, AL111168, CP000814, AY944168, AY944149, GQ249183), three pairs of primers were designed, targeting the conserved region of the *hipO* gene. By testing for the combination that yielded the highest sensitivity, the real-time RPA primers and probes were selected (Table 2). Primers and exo probes were synthesized by a commercial company (Sangon, Shanghai, China). To select suitable RPA templates and to design primers and probes, users can refer to the criteria suggested in the TwistAmp™ reaction kit manual.

2.4. RPA assay

The real-time RPA assay was performed on the Genie III scanner device (OptiGene Limited, West Sussex, UK) described previously (Liu et al., 2017). The TwistAmp™ exo kit (TwistDX, Cambridge, UK) was used in the real-time RPA, and the reaction began at 38 °C. The fluorescence signal was recorded in real-time and increased markedly upon successful amplification.

2.5. Real-time PCR for C. jejuni

Real-time PCR for *C. jejuni* was performed on the ABI 7500 instrument. Premix Ex Taq™ (Takara Co., Ltd., Dalian, China) was used in the real-time PCR. The total reaction volume was 25 μL, comprising

Table 2
Primer and probe sequences for the *C. jejuni* real-time PCR, RPA and real-time RPA assays.

Method	Name	Sequence 5′–3′ Amplication	Size (bp)
Real-time RPA	RPA-FP	AGAAGTAGTATGTCCATCATGACCGCAAGC	254
	RPA-RP	CTGAGCTTGGTTTTGATGAATTATGTACTGC	
	exo Probe	ATTTGTGCATTCTTGTAAAGGCAAAGCATCCA	
		[FAM-dT]-THF-[BHQ1-dT]CTGCACGAAGTCCTAT	
Real-time PCR	PCR-FP	CAAAAAAATCCAAAATCCTCACTTG	132
	PCR-RP	TGCACCAGTGACTATGAATAACGA	
	PCR-Probe	JOE- TTGCAACCTCACTAGCAAAATCCACAGCT-Eclipse	

Fig. 1. Analytical sensitivity of the real-time PCR assay for detecting *C. jejuni*. Different concentrations of the *C. jejuni* DNA template (1.0×10^6 to 1.0×10^0 copies/reaction) were amplified by real-time RPA. As shown in this figure, the detection limit for the real-time RPA was 1.0×10^2 copies/reaction. The concentrations used as templates for reactions 1–7 were 1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3, 1.0×10^2, 1.0×10^1 and 1.0×10^0 copies/reaction, respectively. One representative plot out of five independent reactions for real-time RPA is shown.

2 × Premix Ex Taq, 10 μM of each primer, 5 μM of each probe, a passive reference (50 × ROX), 1 μL of genomic DNA template and ddH2O. The cycling conditions were 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s and 60 °C for 34 s. All probes were labeled with the reporter 6-FAM (6-CarboxyFluorescein) and the fluorescence quencher BHQ1 (Black Hole Quencher 1).

2.6. Specificity and analytical sensitivity analysis

Using a panel of pathogens which are considered important in food, the analytical specificity was evaluated by cross-reactivity analysis (Table 1). In order to conduct the sensitivity analysis, ten-fold serial dilutions of *C. jejuni*, ranging from 1.0×10^6 to 1.0×10^0 copies/μL, were analyzed. One microliter of each DNA dilution was amplified by the real-time RPA assay as a way of determining the detection limit of the method in eight replicates. The threshold time was plotted against the molecules detected.

2.7. Traditional bacterial culture assay

It is typical to use the GB 4789.9-2014 (2014) method for the microbiological investigation of food hygiene (National Food Safety Standards of China). With the method employed for the isolation of *C. jejuni*, the results were regarded as the standards for the sensitivity, specificity and accuracy analyses. The original rinse solution from the collected samples was aseptically spread on *Campylobacter* blood-wet selective agar base (modified CCDA-Preston; Oxoid, UK) plates containing antibiotics in a parallel trial. The *Campylobacter* plates were incubated under microaerophilic conditions (10% CO_2, 5% O_2 and 85% N_2 gas mixture) at 42 °C for 48 h, after which several presumptive colonies with similar moist and gray colony morphologies were selected for inoculation on blood-enriched Mueller Hinton (Difco, Becton Dickinson) agar plates using a loop and subcultured for 48 h at 42 °C. Finally, a multiplex PCR assay was performed to identify *Campylobacter* at the species level (Nielsen et al., 1997).

2.8. Validation with artificially contaminated samples

Commercially available milk and/or chicken breasts were used to assess the potential use and suitability of the real-time RPA assay. These samples were purchased from a local supermarket and confirmed to be *C. jejuni* – negative on the basis of the traditional culture assay and PCR. Twenty-five milliliters/g of milk/chicken breasts and 10, 100, and 1000 CFU of *C. jejuni* (ATCC 33291) were added into a sterile stomaching bag containing 225 mL Bolton broth, mixed well to get the homogeneous samples, and then incubated for 38 h or 48 h at 42 ± 1 °C microaerobically described as above,to increase the bacterial concentrations. Then the bacterial genomic DNA extraction and the real-time RPA reactions were performed as described previously (Liu et al., 2017). Each experiment was repeated at least three times, and similar results were obtained.

3. Results

The specificity of the real-time RPA assay was appraised with a group of bacterial foodborne pathogens. They are considered vital in terms of food safety, i.e., *Campylobacter coli, Campylobacter lari*, *Campylobacter fetus* subsp. *fetus*, *Campylobacter upsaliensis*, *Escherichia coli* O157, *Staphylococcus aureus*, *Enterobacter sakazakii*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Shigella*, and *Bacillus cereus*. With the tools including an exo probe and a portable, user-friendly point-of-need (POD) tube scanner, the real-time RPA reaction was initiated. As exhibited in Table 1, the 8 *C. jejuni* bacterial strains were all detected, whereas the other bacterial strains were not. Similar results were

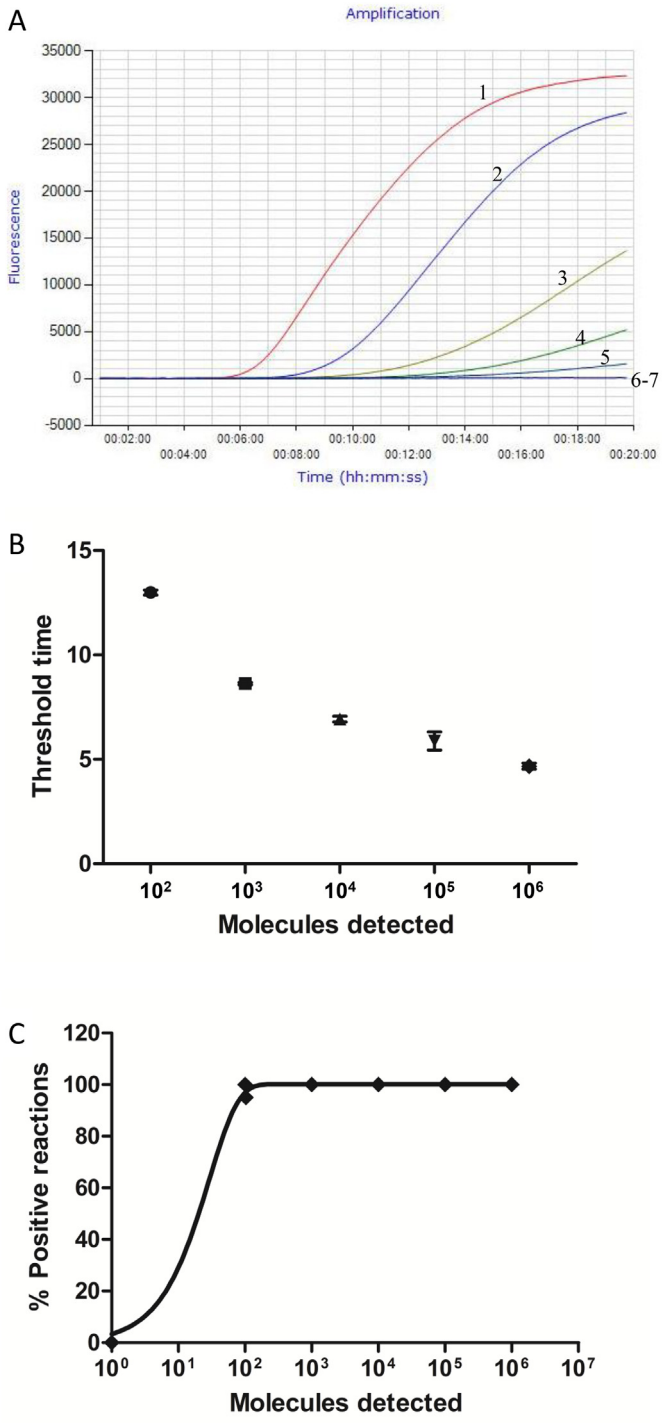

Fig. 2. Performance of the *C. jejuni* real-time RPA assay. Real-time RPA was carried out at 38 °C for 20 mins. (A) Similar to Fig. 1, fluorescence development was monitored over time using a dilution range of 1.0×10^6 to 1.0×10^0 copies/reaction of *C. jejuni* genomic DNA. The numbers for the amplification curves were designated the same as those above. (B) Semi-logarithmic regression analysis of data collected from eight *C. jejuni* real-time RPA test runs on standard DNA using Prism Software 7.0. The run times of the real-time RPA assay ranged from 4 mins-12 mins to detect 10^6 to 10^2 copies. (C) Probit regression analysis using SPSS software on data from eight runs. The detection limit at 95% probability (10^2 copies) is depicted by a rhomboid.

gained after five independent RPA runs were performed, demonstrating well the sufficient specificity of the real-time PRA assay for *C. jejuni* detection.

Utilizing a dilution range of 10^6 to 10^0 copies/μL of genomic DNA as templates, real-time RPA and real-time PCR were performed simultaneously to evaluate the sensitivity of this assay. It can be seen from Fig. 2A that the detection limit of real-time RPA was 10^2 copies/μL, which was the same as that determined for real-time PCR (Fig. 1). The real-time RPA assay was performed for eight runs on quantitated DNA, and 1.0×10^6 to 1.0×10^2 DNA molecules were detected in 8/8 runs. In contrast, 1.0×10^0 and 1.0×10^1 DNA molecules were detected in 0/8 runs (Fig. 2B). Due to the inconsistency in the results, probit regression analysis was accomplished with the help of SPSS software (IBM, Armonk, USA), and the sensitivity in 95% of the cases was determined to be 10^2 DNA molecules (Fig. 2C). Semi-log regression analysis of the real-time RPA method was carried out by collecting data from eight runs according to the quantitative DNA standards. The run times of the real-time RPA assay were approximately 4 min–12 min for 10^6–10^2 copies, respectively, while those of the real-time PCR assay were about 32 min–54 min, with Ct values ranging from 21 to 36 to get the final, similar results.

For determining the practicability of the real-time RPA approach in detection of *C. jejuni* in food, the RPA methodology was examined by artificially adding different amounts of *C. jejuni* into milk and/or chicken breasts for different enrichment times. To detect *C. jejuni*, comparison was made between the diagnostic ability of the real-time RPA assay and that of various other detection approaches, i.e., real-time PCR and the National food safety standard of China (2014) (GB4789.9-2014). The latter method for *C. jejuni* examination was used to make sure that the food samples were contaminated successfully. It turned out to be that the real-time RPA assay results were highly agreeable with those of real-time PCR. However, when it comes to time savings, real-time RPA had an overwhelming advantage. After 38-h enrichment, real-time RPA obtained positive results within 12.28 mins and 8.42 mins when milk samples were contaminated with 100 and 1000 CFU/25 mL of *C. jejuni*, respectively, however a requirement of approximately 51 mins and 38 mins respectively for real-time PCR with Cq values of 33.26 and 25.09 was needed (Table 3). As for chicken breast samples, they yielded similar results. Additionally, the detection of *C. jejuni* in chicken breasts at a concentration as low as 100 CFU/25 g could be detected within 12.57 mins after enriching using the real-time RPA but not by the other methods, showcasing the higher sensitivity of the real-time RPA assay (Table 3). After 38 or 48 h of enrichment, *C. jejuni* could not be detected in spiked samples with 10 CFU/25 mL (g) of *C. jejuni* contamination by any of the four methods tested. As such, these results strongly suggested the distinct advantages the real-time RPA technique has for detecting the rapidity and sensitivity of *C. jejuni*.

4. Discussion

In recent years there has been increasing concern about and focus on diseases caused by foodborne pathogens. *C. jejuni*, a major foodborne disease pathogen that contaminates food products, has become a major concern for food safety (Allos, 2001). Accordingly, efficient and sensitive methods for the rapid detection of *C. jejuni* in food are required at present.

Herein, a real-time RPA assay was developed to rapidly detect *C. jejuni* by targeting the *hipO* gene which has been used as a molecular diagnostic probe in many other detection and differentiation methods of *C. jejuni* such as PCR (Van et al., 2018), real-time PCR (Abu-Halaweh et al., 2005; Caner et al., 2008), and LAMP (Pham et al., 2015). The specificity of real-time RPA was examined with DNA from bacterial strains. Among them, only *C. jejuni* strains were correctly amplified, with no positive signals observed from non-target pathogens. We also analyzed the target sequence we amplified in this study with the *hipO* gene of *Campylobacter jejuni subsp.jejuni* (GenBank number: CP002029, AL111168, CP000814) and *Campylobacter jejuni subsp.doylei* (GenBank number: LS483295.1, CP027403.1, CP000768.1), which was 100% identical in reference genomes from the two subspecies, so this method

Table 3
Comparison of the reaction times of different methods to detect contaminated milk/chicken breasts.

Spiked cells (CFU/25 mL(g) of milk/chicken breasts)	Enrichment time (h)	*exo*-RPA (TT) min	Real-time PCR (Ct)	GB4789.9-2014	Viable cell count (CFU/g)
10	38	ND/ND	ND/ND	No/No	< 10/ < 10
	48	ND/ND	ND/ND	No/No	< 10/ < 10
100	38	12.28/12.57	33.26/ND	Yes/No	1.7×10^3/ < 10
	48	9.85/11.22	28.94/27.89	Yes/Yes	2.1×10^4/1.1×10^3
1000	38	8.42/9.62	25.09/26.27	Yes/Yes	2.2×10^3/1.4×10^3
	48	7.02/6.93	21.21/21.45	Yes/Yes	5.8×10^5/9.4×10^4

ND: not detected.

we developed should amplify both taxa. The template, *C. jejuni* genomic DNA, was detected between 6 mins and 16 mins for 10^6–10^2 copies of DNA. 10^2 copies/reaction was the detection limit of the real-time RPA assay, similar to that of conventional real-time PCR with the same diluted *C. jejuni* DNA. Using the artificially contaminated milk and chicken breast samples, this real-time RPA method had a diagnostic agreement of 100% with real-time PCR, and the former (6.93 min–12.57 min) was faster than the latter (32 min–51 min). As an isothermal DNA amplification method, RPA has been widely explored for the molecular detection of diverse pathogens (Gao et al., 2018; Abd El Wahed et al., 2015a; Lai et al., 2017), as real field testing has also been achieved (Abd El Wahed et al., 2015a; Abd El Wahed et al., 2015b). Compared to previous reports on *C. jejuni* detection, the real-time RPA method described herein has several merits. For instance, for detection of *C. jejuni*, the real-time RPA assay was a lot faster than that of real-time PCR and LAMP assays. The real-time RPA reaction could be completed within 13 mins, while approximately 54 mins was needed in the real-time PCR reaction and 60 mins for LAMP described in our study and in the literature, all exclusive of the enrichment time (de Boer et al., 2015; Dong et al., 2014; Jackel et al., 2017).

In addition, the sensitivity and specificity of the real-time RPA had either similarity or superiority to PCR methods. Moreover, compared with agarose gel electrophoresis-dependent PCR assays, such as LAMP (Dong et al., 2014; Velusamy et al., 2010), the results of the real-time RPA assay were obtained on the real-time fluorescence detection platform, which can reduce the potential impacts of matrix-associated inhibitors. Last but not the least, the portable POD tube scanner (Genie III) used in the study is rather lighter, smaller and cheaper than the real-time PCR machine, making on-site *C. jejuni* detection feasible, which is important for clinical diagnoses at the site of outbreaks, especially in resource-limited settings. However, it is entailed to utilize the real-time RPA for *C. jejuni* detection from practical samples with sufficient samples, if this method is to be applied in practice in the future.

5. Conclusions

The present study described a real-time RPA assay that allowed the sensitive and specific detection of *C. jejuni*. The real-time RPA assay was as reliable as real-time PCR and National food safety standard of China (2014) (GB4789.9-2014) and entailed a significantly reduced amount of time to detect contaminated milk and chicken breast food samples, as the reaction time was shortened to approximately 13 mins except for the enrichment time. The portable feature of the real-time RPA assay makes it promising for a rapid, reliable and sensitive detection of *C. jejuni* in the diagnosis of food testing and infectious diseases.

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Availability of data and materials

The data supporting our findings are contained within the manuscript.

Authors' contributions

YYG, BHZ and JCW designed and conducted the experiments. JCW, BHZ, GHL, QED, LWZ, LBL and XXS performed the experiments and analyzed the data. YYG drafted the manuscript. All authors read, revised, and approved the final manuscript.

Competing interests

The authors declare that they have no conflicts of interest with the research described herein and/or the publication of this article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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