Direct detection of *Campylobacter jejuni* in human stool samples by real-time PCR

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Abstract: Our purpose was to establish a quick and accurate real-time PCR (rtPCR) method to detect *Campylobacter jejuni* directly from human diarrheal stool as an alternative to traditional culture methods. To determine the consistency of rtPCR and culture method, 256 clinical diarrheal stool samples and 50 normal stool samples from healthy individuals were examined, and the whole process was double-blinded. Our data showed that the sensitivity of rtPCR in pure cultures and stool was 10^2 CFU·mL^{-1} and 10^3 CFU·g^{-1}, respectively. Of the 256 diarrheal samples, 10 specimens were successfully detected by both methods, whereas two specimens were PCR positive but culture negative. No positive results were found by these two methods in 50 normal specimens. Our data suggested that rtPCR was convenient in operation and time-saving (turnaround time 3.5–4 h), so it could be used for clinical diagnostic and epidemiological purposes.

Key words: Campylobacter jejuni, culture, diarrhea, human, real-time PCR.

Résumé : Notre but était de mettre au point une méthode rapide et fiable de PCR en temps réel (rtPCR) permettant de détecter *Campylobacter jejuni* directement à partir d'échantillons de selles chez l'humain comparativement aux méthodes de culture traditionnelles. Afin de déterminer le niveau d'uniformité de la rtPCR et de la méthode de culture, 256 échantillons cliniques de selles diarrhéiques et 50 échantillons de selles normales d'individus en bonne santé ont été examinés dans un processus à double-insu. Nos résultats ont démontré que la sensibilité de la rtPCR des cultures pures et des échantillons de selles était de 10^2 CFU·mL^{-1}et 10^3 CFU·g^{-1} respectivement. Des 256 échantillons diarrhéiques, 10 spécimens ont été détectés avec succès par les deux méthodes alors que deux spécimens étaient positifs en PCR mais négatifs en culture. Aucun résultat positif n'a été obtenu par ces deux méthodes à partir des 50 échantillons normaux. Nos résultats ont suggéré que la rtPCR était pratique et rapide (temps d'exécution de 3,5–4 h) de telle sorte qu'elle pourrait être utilisée à des fins de diagnostic clinique ou des fins épidémiologiques.

Mots-clés : Campylobacter jejuni, culture, diarrhée, humain, PCR en temps réel.

[Traduit par la Rédaction]

Introduction

Campylobacter infections have been recognized as major food-borne diseases in developed (Friedman et al. 2000) and developing countries (Oberhelman and Taylor 2000). The genus *Campylobacter* includes many species, of which *Campylobacter jejuni* is the most common pathogen and accounts for the majority of diagnosed human *Campylobacter* infections (Skirrow 1994; Allos and Blaser 1995). Overall, the high incidence of clinical disease associated with *C. jejuni* and its potentially serious sequelae underlines its importance as a significant public health hazard (European Commission 2000; Tauxe 2002).

Routine detection of *C. jejuni* in clinical laboratories is based on culture method using selective media and subsequent phenotypic identification. Presumptive results may be available after 2 days. However, definitive species-level identification based on phenotypic methods may require 5–6 days. And phenotypic identification can be challenging because of the fastidious growth requirements, the asaccharolytic nature, and possession of few distinguishing biochemical characteristics by *C. jejuni* (Goossens and Butzler 1992), which make the routine detection of *C. jejuni* a tedious and time-consuming job.

Molecular methods based on PCR amplification may provide an alternative to culture methods for the detection of *C. jejuni* in clinical specimens because of their relative ease of use, the fact that they are less time-consuming, and their potential application in large-scale screening programs by means of automated technologies. The application of PCR-based assays to the detection of *Campylobacter* species in clinical and food samples has been previously reported (Giesendorf and Quint 1995; Waegel and Nachamkin 1996; Linton et al. 1997; Lawson et al. 1999; O'Sullivan et al. 2000), but only a limited number of real-time PCR (rtPCR) methods have been reported for the specific detection of *C. jejuni* (Sails et al. 2003; Rudi et al. 2004; Oliveira et al. 2005; Debretsion et al. 2007; Rönner and Lindmark 2007; Schuurman et al. 2007). In this study, an rtPCR method was applied to detect *C. jejuni* in human faecal samples.

Received 3 March 2008. Revision received 21 May 2008. Accepted 26 May 2008. Published on the NRC Research Press Web site at cjm.nrc.ca on 26 August 2008.

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 doi:10.1139/W08-064

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this study included *C. jejuni* ATCC 33291 and *C. jejuni* ATCC 33560. The strains were used for sensitivity test of the primers–probe set and as positive controls in the culture method and rtPCR. They were inoculated on Columbia agar supplemented with 5% (*v*/*v*) defibrinated sheep blood (Oxoid Ltd., Basingstoke, Hampshire, England) and then incubated at 37 °C microaerophilically in an anaerobic incubator (model DY-2) (Yiwu refrigerating machine factory, China) with 80% N_2, 10% CO_2, 5% H_2, and 5% O_2.

Genomic DNA isolation of bacterial strains

Double-stranded DNA of the bacteria was extracted using the TIANamp Bacteria Genomic DNA isolation kit (TIANGEN Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. For each bacterial strain, a 10^6 CFU·mL^{-1} suspension (LaGier et al. 2004) determined by electronic nephelometer (B-D Crystal Stec, B-D Co. Ltd., USA) was prepared in deionized water from colonies grown on Columbia blood agar. Purity of isolated genomic DNA was assessed by examination of 260/280 nm optical density ratios (SpectraMax Plus^{384}, Molecular Devices). All DNA preparations having a 260/280 nm optical density ratio between 1.8 and 2.0 were classified as pure and were then stored at –20 °C prior to use.

Clinical stool samples

A panel of 256 stool specimens were collected from in- and outpatients with the syndrome of diarrhea who defecated more than three times in 1 day accompanied by changes in stool character, such as being watery. In addition, 50 stool specimens were collected from 50 healthy persons. The 50 healthy persons were all normal students of our medical university who had no fever, abdominal pain, diarrhea, or abdominal distension for 30 days at least. All of the sample suppliers had not been treated with antibiotics before the collection of stool. All samples were kept in Cary–Blair medium (Oxoid Ltd.) and were sent to the laboratory as soon as possible.

Culture methods

An aliquot of 0.5 g of diarrheal stool was weighed and then added into 4.5 mL of diluting solution (every 1000 mL contains 3.7 g of beef extract, 0.4 g of agar powder, and 2.0 g of 0.1% resazurin). After thorough homogenization, 100 μL was pipetted onto a 0.65 μm Millipore membrane filter placed on the surface of the culture medium. Three kinds of culture media were simultaneously used to ensure the reliability of the result: Columbia blood agar, *C. jejuni* selective agar supplemented with 5% (*v*/*v*) defibrinated sheep blood (Shanghai Reagent Providing and Research Center for Diarrheal Disease Control, Shanghai, China), and charcoal cefoperazone desoxycholate agar (Beijing Land Bridge Technology Co. Ltd., Beijing, China). After being well inoculated, the culture media with membrane filters were incubated in a candle jar at 42 °C for about 60 min, and then the membrane filters were removed and the agar plates were uniformly streaked. Finally, the three plates were incubated in a microaerobic atmosphere at 37 °C for at least 72 h (Fig. 1) (Steele and McDermott 1984).

Identification of *C. jejuni* was based on five aspects: colony morphology, Gram stain (–), motility (+), growth under microaerophilic atmosphere, and biochemical tests. Biochemical tests performed by us included oxidase (+), catalase (+), and urease (–) activities, hippurate hydrolysis test (+), indoxyl acetate hydrolysis test (+), nitrate reduction test (+), growth at 25 °C (–) and 42 °C (+) microaerophilically, growth at 37 °C (–) aerobically, and growth in media containing 1% glycine (+). All tests were performed in triplicate according to the manufacturer's instructions (Beijing Land Bridge Technology Co. Ltd.). The judgment of all results was accomplished by at least two experienced researchers together. All results of culture were secret to the researchers who performed the rtPCR assay before the accomplishment of the study.

Genomic DNA isolation from stool

DNA extraction from stool was performed using a QIAamp DNA stool minikit (QIAGEN Inc., USA) according to the manufacturer's instructions. The amount of specimen processed was 200 μL and the elution volume was also 200 μL. Extracted stool specimens were examined using a 16S rRNA based conventional PCR assay to confirm the presence of PCR-compatible bacterial double-stranded DNA. All extracted samples were stored at –20 °C prior to identification.

rtPCR assay

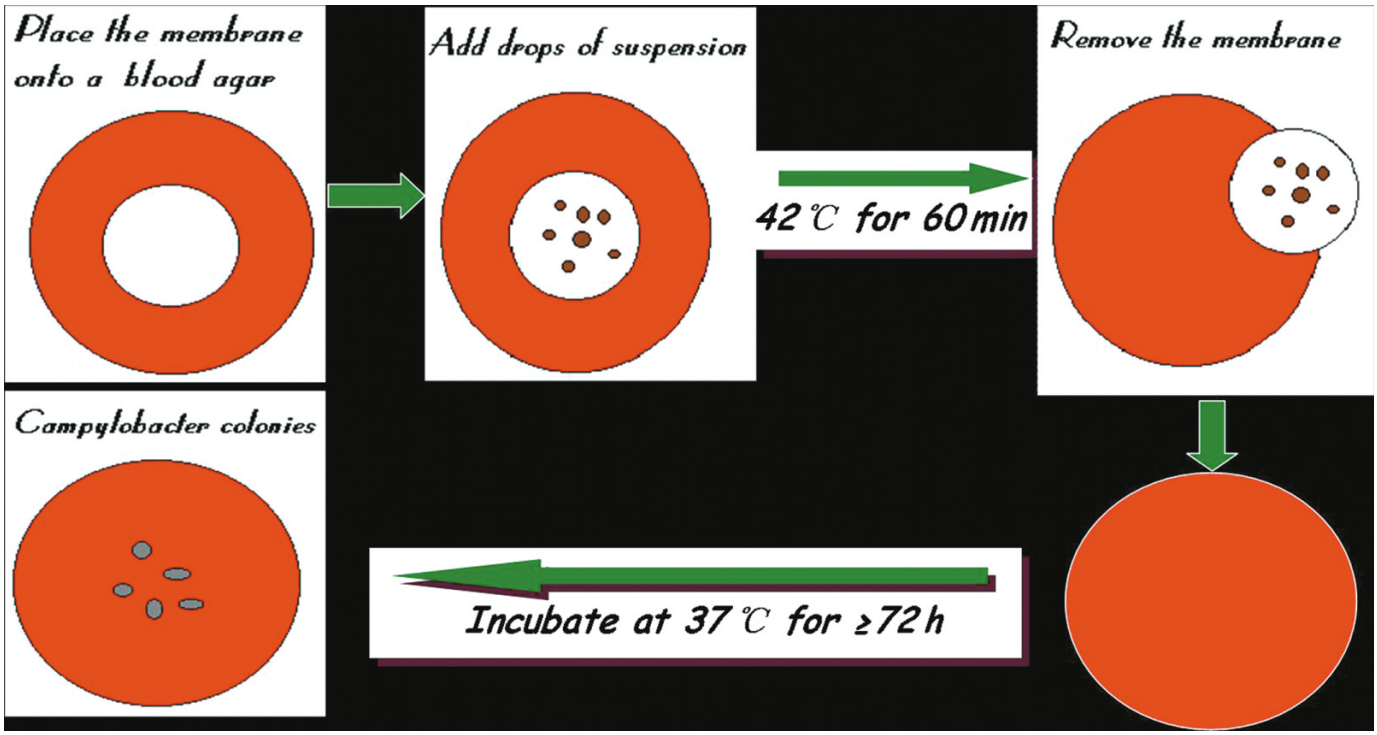
Primers and probe

A specific primer and TaqMan probe set targeting the *C. jejuni* specific region of the ORF-C sequence was used, which was previously described by Sails et al. (2003). The primers and probe were synthesized by Invitrogen Biotechnology Co. Ltd. (Shanghai, China). All oligonucleotides and probe were stored in the dark at –20 °C prior to use.

rtPCR conditions

The rtPCR assay was performed in a 50 μL volume on an Opticon-2 DNA engine (model:CFD-3220) (M-J Co. Ltd., USA) with a 96-well plate reader using the equation $\Delta RQ = RQ^+ - RQ^-$ (Bassler et al. 1995). Each rtPCR reaction contained primers (final concentration 300 nmol·L^{-1}), TaqMan probe (final concentration 300 nmol·L^{-1}), 25 μL of 2× rtPCR Premix (QIAGEN Inc.), 5 μL of DNA sample to be evaluated, and bovine serum albumin (final concentration 0.1 μg·μL^{-1}). The reaction was carried out in a 200 μL microtube (PCR tube strips) (BIORAD, USA) special for rtPCR, which was put in disposable 96-well optical reaction plates (Opticon-2 DNA engine). Thermal cycling conditions were as follows: one cycle at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each set of reactions included triplicate wells that were positive controls (containing 10^6 genome copies of *C. jejuni* DNA per PCR), no-template controls, and negative controls, respectively. A positive result for *C. jejuni* was based on a threshold of four times the average ΔRQ value of no-template controls (Sanchez-Vizcaino and Cambro-Alvarez 1987) or negative

Fig. 1. Filtration method. Culture methods are concisely presented below. After 0.65 μm membrane filter filtration, three kinds of media were uniformly streaked and then incubated at 37 °C microaerobically. All suspected colonies on the plates were observed by two or more experienced researchers.



controls (diarrheal stool without *C. jejuni*). All results of rtPCR were unknown to the researchers who performed the conventional culture before the accomplishment of the study.

Confirmatory real-time PCR assay

For confirmation of the results for the rtPCR-positive but culture-negative specimens, DNA was extracted from the stored fecal suspension (–20 °C) and subjected to another rtPCR targeting a different sequence of the *C. jejuni* genome. The primers–probe set used was previously described by Nogva et al. (2000). The primers and probe were synthesized by Invitrogen Biotechnology Co. Ltd.. The rtPCR assay was performed in a 50 μL volume on the Opticon-2 DNA engine as previously. The reaction mixture consisted of primers (final concentration 300 nmol·L^{-1}), TaqMan probe (final concentration 200 nmol·L^{-1}), 25 μL of 2× real-time PCR Premix (QIAGEN Inc.), 1 μL of DNA sample, and bovine serum albumin (final concentration 0.1 μg·μL^{-1}). Thermal cycling conditions were as follows: one cycle at 95 °C for 10 min followed by 40 cycles of 95 °C for 20 s and 60 °C for 1 min. Each reaction included triplicate wells that were positive controls, no-template controls, and negative controls as previously.

Results

Detection for *C. jejuni* in clinical samples by culture methods

Of the 256 diarrheal stool samples, the number of positive ones for Columbia blood agar, *C. jejuni* selective blood agar, and charcoal cefoperazone desoxycholate agar was 10/256, 10/256, and 9/256, respectively, and the 10 or 9 positive samples were identical. The concentration of *C. jejuni* in the positive samples was found to be 10^4–10^6 CFU·g^{-1} by plate counts (Table 1). Of the 50 normal feces, no *C. jejuni* was detected by any of the three media.

Sensitivity of the primers and probe

The sensitivity of the rtPCR assay for pure cultures was determined using 10-fold dilutions (10^6–10^1 CFU·mL^{-1} per PCR) of DNA isolated from the representative *C. jejuni* strain ATCC 33291. As a result, the detection limit was found to be 10^2 CFU·mL^{-1} with ΔRQ values greater than 5.2 (about 5.7 on average). This experiment was replicated three times (Fig. 2). Ground stool samples obtained from three healthy people were confirmed to be culture negative for *C. jejuni*. Then, 1 mL of each of the 10-fold diluted bacterial suspensions (10^6–10^1 CFU·mL^{-1}) was added into six tubes of specimens (1 g each), respectively, and then the compound was homogenized followed by DNA extraction and rtPCR. This experiment was performed in triplicate. Finally, the detection limit for *C. jejuni* in spiked stool was found to be 10^3 CFU·g^{-1} (ΔRQ = 7.7 on average) with a threshold ΔRQ value of 7.1, which was four times the average of negative controls (diarrheal stool without *C. jejuni* confirmed by culture methods) (Fig. 3).

Detection for *C. jejuni* in clinical samples by rtPCR

All specimens were directly detected by rtPCR after DNA extraction without a preenrichment step. Of the 256 diarrheal stool samples, 12 were found to be positive by rtPCR assay with ΔRQ values all above 7.1, 10 of which were consistent with the positive samples detected by culture. The

Table 1. Detection of *Campylobacter jejuni* in feces by rtPCR and agar plates.

			rtPCR		
Sample No.	Concordant[a]	Culture result	ΔRQ	Result	Viable *C. jejuni* counts in feces (CFU·g^{-1})[b]
27	Yes	Positive	18.5	Positive	1.7×10^5
38	Yes	Positive	19.6	Positive	3.2×10^5
46	Yes	Positive	23.6	Positive	2.4×10^6
63	Yes	Positive	17.7	Positive	6.2×10^4
81	Yes	Positive	19.2	Positive	4.1×10^5
104	No	Negative	16.8	Positive	Not determined
128	Yes	Positive	18.9	Positive	1.4×10^6
130	Yes	Positive	16.5	Positive	8.7×10^4
165	Yes	Positive	16.6	Positive	5.1×10^4
197	No	Negative	15.9	Positive	Not determined
230	Yes	Positive	20.5	Positive	6.3×10^5
253	Yes	Positive	17.4	Positive	3.6×10^5

[a]McNemar test by SPSS13.0, $P > 0.05$.
[b]Concentration of *C. jejuni* determined by plate counts.

Fig. 2. Sensitivity of the rtPCR assay for detecting *Campylobacter jejuni* in pure cultures. Tenfold dilutions of *C. jejuni* were made in deionized water in triplicate. The average ΔRQ values for each dilution were plotted against the average number of CFU per millilitre as determined by electronic nephelometer. The adjusted ΔRQ threshold value was calculated to be 5.2 (horizontal line), which was four times the average of no-template controls. Error bars indicate the standard deviations of the means.

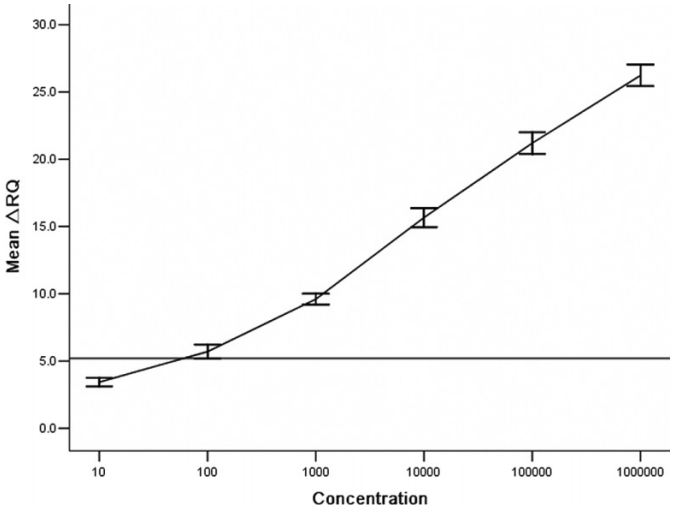
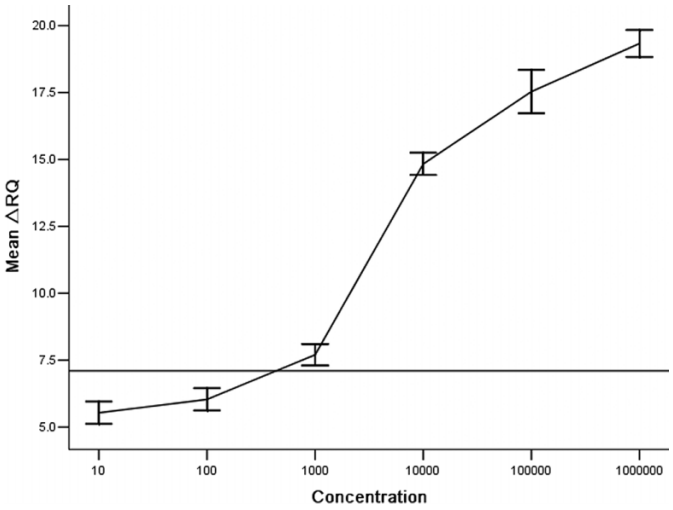


Fig. 3. Sensitivity of the rtPCR assay for detecting *Campylobacter jejuni* in spiked feces. Tenfold dilutions of *C. jejuni* determined by electronic nephelometer were made in deionized water in triplicate. The average ΔRQ values for each dilution were plotted against the average number of CFU per gram. The adjusted ΔRQ threshold value was calculated to be 7.1 (horizontal line), which was four times the average of negative controls (stools without *C. jejuni* confirmed by culture methods). Error bars indicate the standard deviations of the means.



two rtPCR-positive but culture-negative samples were confirmed to contain *C. jejuni* by the confirmatory rtPCR assay. Of the 50 healthy specimens, no *C. jejuni* was found by rtPCR, which was consistent with the result of the culture method.

Discussion

Campylobacter species are recognized as the most common bacterial agents causing acute gastroenteritis (Friedman et al. 2000; Allos 2001), and *C. jejuni* is the most common one among *Campylobacter* species associated with human diarrheal disease. There are so many sporadic cases caused by *C. jejuni* that from a public health perspective, the rapid identification of *C. jejuni* is required to prescribe appropriate therapeutic interventions and to provide epidemiologic data for disease control. As known, the conventional methods to identify *C. jejuni* are tedious and time-consuming and are prone to misdiagnoses. Studies on the rapid detection of *C. jejuni* have focused mainly on contaminated foods (O'Sullivan et al. 2000; Sails et al. 2003; Josefsen et al. 2004; Rudi et al. 2004; Oliveira et al. 2005; Debretsion et al. 2007; Rönner and Lindmark 2007), so to provide necessary information for clinicians, we hoped to develop a rapid and accurate rtPCR assay in this study for *C. jejuni* identification in human diarrheal feces.

The development of rtPCR has obviated the need to manipulate postamplification PCR products, thereby reducing

the risk of false-positive results generated by amplicon contamination. The turnaround time is also reduced, and our rtPCR assay takes 3.5–4 h compared with 5–6 days by culture methods. Therefore, this assay can provide necessary information in time for clinical prescription and disease control.

Sensitivity studies involving pure cultures and spiked feces demonstrated that the detection limits of the assay were 10^2 CFU·mL^{-1} and 10^3 CFU·g^{-1} for pure cultures and stool, respectively. During the process of culturing, the concentration of *C. jejuni* in the diarrheal feces was also determined by plate counts on *C. jejuni* selective blood agar. Finally, the concentration of *C. jejuni* in the positive samples was found to be 10^4–10^6 CFU·g^{-1}. Therefore, the assay was reliably sensitive to identify *C. jejuni* in the diarrheal feces and enrichment cultures.

Although having its disadvantages, the culture method is still the golden standard for the detection of *C. jejuni*. In this study, three media were employed to isolate *C. jejuni* from diarrheal feces to increase the reliability of the results. During the whole process of culturing, we employed at least two researchers to further confirm the reliability of the results. However, in this study, two samples were rtPCR positive but culture negative for *C. jejuni*. The two samples were confirmed to be *C. jejuni* positive by the confirmatory rtPCR assay. Thus, we speculated that *C. jejuni* in the two samples might be too few to be isolated or might be in the state of viable but nonculturable. Statistic analysis of the results showed that the difference between results of the two methods was not significant (Table 1).

The detection of *C. jejuni* in the 256 diarrheal samples and 50 healthy samples was completed by two different groups of researchers: one was in charge of the rtPCR assay and the other of the culture. The results of the two groups were not revealed until the accomplishment of the whole study so that it could obviate the bias of the results and make the study more reliable.

PCR inhibitors found in tissues and feces can have significant adverse effects on the efficiency and sensitivity of PCR-based assays (Chen et al. 1997). So the QIAamp DNA stool minikit was chosen for this study because a proprietary polysaccharide mixture is included in the protocol to bind and thus minimize PCR inhibitors in fecal matrices. To confirm the presence of PCR-compatible bacterial double-stranded DNA, extracted stool specimens were examined using a 16S rRNA based conventional PCR assay (data not shown). If the PCR replicated three times (including DNA extraction) did not produce any strip on the gel, the sample tested was considered to contain PCR inhibitors. In addition, the rtPCR system contained bovine serum albumin (0.1 μg·μL^{-1}), which can facilitate the amplification of target DNA and thus increase the robustness of the rtPCR assay (Abu and Rådström 2000; Kreader 1996).

In summary, the rtPCR assay reported here was demonstrated to be as sensitive as conventional culture methods but significantly reduced the time taken for *C. jejuni* detection (only 3.5–4 h). The rtPCR assay described in this article has the potential to complement or replace other phenotypic methods for identification of *C. jejuni* in clinical samples.

Acknowledgements

Dr. Wei Gong and Dr. Biao Nie are appreciated for technical insight and support throughout the course of this study.

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