INTRODUCTION

Campylobacter jejuni (C. jejuni) is recognized worldwide as leading cause of diarrhea and food borne gastroenteritis [1, 2]. This organism is carried in the intestinal tract of a wide variety of wild and domestic animals, especially birds. In most cases, the host is a carrier that does not exhibit symptoms, but it may have acquired immunity through an earlier C. jejuni infection [3]. C. jejuni cells may enter the environment, including drinking water, through the feces of animals, birds, or infected humans. Consumption of contaminated food and water with untreated animal or human waste accounts for 70 percent of C. jejuni-related illnesses each year. Unpasteurized milk, meat, and poultry have the potential to serve as the source of contamination [4-7]. C. jejuni was predominantly associated with gastrointesti-
nal infections. Some of the occasional complications of the infection could be manifested as meningitis, pneumonia, miscarriage, and a severe form of Guillain-Barré syndrome [3, 8]. This is especially prevalent in developing countries [3].

The infective dose of *C. jejuni* cells is very small, and it has been estimated that 500 cells could cause human illness [9, 10]. This means that even a very small number of *C. jejuni* cells in water or food may be a potential health hazard. Thus, sensitive methods are needed to detect *C. jejuni* contamination in food and drinking water sources.

Unfortunately, there are several problems concerning detection of *C. jejuni* cells in food and water using cultural methods. Among these difficulties, the two most important ones are the small numbers and the slow growth rate of the organism. The traditional methods currently used are time-consuming and laborious, requiring prolonged incubation (1 to 2 days) and selective enrichment to reduce the growth of the background flora, and biochemical identification. Moreover, *C. jejuni* cells may also enter a viable but nonculturable state due to starvation and physical stress [11].

Polymerase chain reaction (PCR) techniques have been developed for the detection of *C. jejuni* [12, 13]. These methods have reportedly improved sensitivity and comparable specificity in relation to culture methods. However, most of the PCR-based methods require a minimum of five hours before a result is available. The advent of real-time PCR technology offers the potential for PCR results to be available much more rapidly [14]. The LightCycler System (Roche Molecular Biochemical, Version 3.5, Indianapolis, Indiana) facilitates rapid cycling using hot air via a thermal chamber, and measures fluorescence in specially designed glass capillaries that contain the PCR reaction components [15]. When SYBR Green I is used as the fluorescent dye, a subsequent melting curve analysis of PCR products generates a specific profile. This real-time PCR assay works on the principle of measuring fluorescence signals at the end of the extension phase of each PCR cycle that are generated following the binding of SYBR Green I to PCR products. As the number of product increases, so too does the fluorescence signal resulting in a characteristic sigmoidal-shaped curve. The specificity of the fluorescent signal can be determined during subsequent melting curve analysis which results in a melting peak profile specific for the amplified target DNA [16]. This profile is dependent upon the length and the G–C content of the PCR product. In the present study, we have established a real-time assay targeting the 358 bp ampli-con of the VS1 gene for quantitative detection of *C. jejuni* using SYBR Green I and the LightCycler System.

**MATERIALS AND METHODS**

**Bacterial isolates and media**

Eleven isolates of *C. jejuni*, including the *C. jejuni* type strain, *C. jejuni* (ATCC 33560, American Type Culture Collection, Manassas, Virginia), were used to test the specificity of the primers and probe. The isolates were collected mostly from poultry in China from 1996 to 1998. The following bacterial strains were used as negative controls: *C. coli* (ATCC 33559), *C. lari* (ATCC 358221), *C. fetus* (ATCC 27374), *E. coli* (ATCC 25922), *Helicobacter canis* (NCTC 12739\(^7\)), *Helicobacter hepaticus* (ATCC 51448\(^7\)). Media: *Campylobacter* selective agar base (MERCK, CM02248, Darmstadt, Germany), *Campylobacter* selective supplement (MERCK, CM02249), defibrinated sheep blood, and *Campylobacter* enrichment broth (Bolton formula, CM0983, Oxoid, Basingstoke, Hampshire, United Kingdom). Oxoid Bolton *Campylobacter*
enrichment broth was made up using the defibrinated ship blood, Oxoid Bolton enrichment broth base and selective supplement according to the manufacturer’s instructions. *Arcobacter butzleri* and *E. coli* were grown aerobically on Oxoid Bolton enrichment broth without *Campylobacter* selective supplement at 37°C. *Helicobacter* spp. were grown microaerobically on Oxoid Bolton enrichment broth without *Campylobacter* selective supplement at 37°C.

**DNA extraction for the real-time PCR**

The analysis for the real-time PCR was performed without an enrichment step. A short procedure of treatment for the contaminated poultry, milk, and environmental water samples was as described above. Template DNAs were extracted from pre-enrichment cultures using a Wizard genomic DNA purification kit (Promega, Madison, Wisconsin) without prior enrichment. Control strain DNAs were extracted from pure cultures. DNA concentrations were determined with the spectrophotometer. The DNA was stored at 2°C to 8°C.

**Primers of PCR**

Oligonucleotide primers VS15 and VS16 [17] from the *C. jejuni* VS1 sequence (X71603) were used in the real-time PCR assay. The primers’ sequences were as follows: VS15, 5'-GAATGAAATTTAGAGGGG-3' and VS16, 5'-GATATGTATGATTTTATCCTGC-3'. The VS1 gene sequence was aligned with other sequences reported in GenBank using CLUSTAL W (Version 1.8). When reconstituted in 50 mM monovalent cations were calculated to be 62°C and 66.8°C, respectively, with the optimal PCR annealing temperature calculated to be 56°C. The primers were synthesized by TaKaRa Biotechnology (Dalian) Co., Ltd. (Otsu Shiga, Japan)

**Cloning and sequencing of PCR fragments.**

The PCR product from *C. jejuni* was gel-excised and purified using gel purification kit (Nanjing Biotechnology, China). It was subsequently cloned to pMD-T Vector plasmids (TaKaRa Biotechnology) according to the protocol described by the manufacturer. The sequences of the inserts were determined by dye termination using an Applied Biosystems (Foster City, California) model 373 DNA sequencer and GENESCAN 672 Software (Applied Biosystems).

**Preparation of quantification standard**

The Recombinant pMD-T Vector plasmids were transformed in *E. coli* Top F10 competent cells (Invitrogen, Carlsbad, California) and grown on LB-medium. After plasmids were extracted using plasmid extraction kit (Nanjing Biotechnology, China), plasmids were linearised with a unique cutting restriction enzyme digest (*Hind* III, or *Xba*I; provided by Gibco Life Technologies, Gaithersburg, Maryland). Linear double-stranded plasmids were quantified on a gel with lambda molecular weight standards and in a genequant spectrophotometer (Pharmacia [Pfizer, New York, New York]). The number of copies of target template was later calculated, and dilutions ranging from 10¹ to 10⁵ copies of this standard were prepared in TE buffer, aliquoted and frozen at -20°C.

**Real-time PCR assay**

Amplification reactions (20 µl) contained 2 µl DNA sample, 2 µl LightCycler Fast Start DNA Master SYBR Green I® (1U DNA Taq merase, 20 mM Mg²⁺, 20 µM dNTP, SYBR Green I, and Buffer [Roche Diagnostics, Mannheim, Germany]), 3 mM MgCl₂, 0.4 µM of each *C. jejuni*-specific primers and 0.5 U uracil-N-glycosylase. The reaction tubes were capillaries supplied by Roche. Capillaries
were closed, centrifuged, and placed into the rotor. Before amplification, the PCR mixture was heated to 95°C for 10 min to denature the template DNA. The amplification profile was as follows: 45 cycles of 95°C for zero sec, 56°C for 10 sec, and 72°C for 20 sec. The temperature transition rate was 20°C/sec. Acquisition mode was single, set at the end of elongation stage. To improve the specificity of the assay, the PCR was immediately followed by a melting curve analysis to determine the melting point of the double-stranded DNA product produced. This cycle consisted of heating at 20°C/sec to 95°C with a zero sec hold, cooling at 20°C/sec to 72°C with a 10 sec hold, heating at 0.1°C/sec to 95°C with a zero sec hold (continuous fluorescence measurements) and finally cooling at 20°C/sec to 40°C with a 30-min hold. Reactions and data analysis were performed in the LightCycler System (Roche Molecular Biochemical). PCR products were detected directly by monitoring the increase in fluorescence from the SYBR Green I, which was measured as a ratio of F1/1. The size of the PCR product was verified with ethidium bromide-stained 2 percent agarose gels in Tris-acetate-EDTA buffer. DL 2,000 Marker (TakaRa) was used as a size marker.

RESULTS

Sensitivity and detection limits of real-time PCR using SYBR Green I

Serial dilutions of purified DNA were made and a copy-standard curve was constructed. Dilutions ranging from 10⁰ to 10⁴ copies of target template per 2 µl were subjected to PCR. As shown in Figure 1A, there were 1.0 × 10⁰, 1.0 × 10¹, 1.0 × 10², 1.0 × 10³, 1.0 copies, and no template respectively in application curve for serial 10-time dilutions of C. jejuni DNA. As shown in Figure 1B, the standard curve based on the dilutions of DNA showed a linear relationship between log CFU and threshold cycles (Cₜ). The slope of the curve was -3.505, and the square regression coefficient after the linear regression was -1.00. When a new serial 10-time dilution from the same DNA purification was used in a separate PCR experiment, the slopes in the two runs were almost identical (3.493 vs. -3.512). When DNA from a separate isolation was used, the variation in the slopes of the standard curves was no larger than that between different serial dilutions from the same DNA isolation and the square regression coefficient (R²) remained constant. The DNA standard curves showed a higher degree of variability among the triplicates when the amount of template decreased. DNA standard curves showed that the detection limit of the PCR assay was approximately 1 copy per PCR from pure culture (Figure 1B). The number of C. jejuni copies can be described by the following equations: Cₜ = -3.505 × (Log copy) + 39.93 and copies = 10[(Cₜ - 39.93)/-3.505].

Specificity of PCR

Specific PCR primers were selected for C. jejuni. The 358-bp DNA fragment and the primers were subjected to homology searches, which reveal that the primers were specific to this organism. The specificity of the selected primers was subjected to an empirical screening. A total of 11 C. jejuni isolates, including the type strain, were tested and found to be positively responding to the chosen primers and probes. The specificity of the primers and probes was tested against three strains of other Campylobacter species as well as a set of four species belonging to other generation of genetically related or common food-born organisms/pathogens (see Materials and Methods), all of which were found to be negative. In addition, a qualitative PCR with the amplification primers was done for the selected strains (Figure 2A). The melting curve in Figure 2B for the select-
ed strains showed that a clear peak at 77.2°C was visible for four strains *C. jejuni*; and a half peak at 74.5°C was visible for other species and negative control. The sequence of PCR product has 100 percent homology with the VS-1 sequence published in GeneBank (X71603). These results confirmed that the amplification primers were specific to *C. jejuni*. PCR amplicons were analyzed on 2 percent agarose gels by standard horizontal gel electrophoresis (Figure 2C). The results showed a fragment of the expected length of 358 bp for *C. jejuni*. Moreover, no non-specific PCR products were detected.

**DISCUSSION**

*Specific detection and quantification of C. jejuni*

*C. jejuni* is recognized as a leading human food-borne pathogen. There is a requirement for rapid, quantitative, and accurate detection of the organism. In the present study, a real-time PCR system was constructed and applied to specifically detect and quantify *C. jejuni*. Colonization and infection appear to be dependent on motility and full-length flagella. In this regard, the flagellum gene *flaA* appears to be essential [18]. However, the variation between the different strains is too extensive; the *flaA* gene is unsuitable for design of the primers necessary for a real-time PCR assay [11]. A VS gene region (GeneBank accession no. X71603), an 1189-bp long DNA fragment was isolated from a *C. jejuni* CIP 70.2 (Collection de L’Institut Pasteur, France) Cosmid library and was found to be specific to this organism [17]. Oligonucleotide primers VS15 and VS16 from the *C. jejuni* VS1 sequence were used in the real-time PCR assay in the present study. When this assay was applied, it was positive for all isolates of *C. jejuni* tested (11 isolates, including type strain ATCC 33560) and negative for all other *Campylobacter* spp. (three isolates) and several other bacteria (four species
tested). This demonstrates the high specificity of the designed primer set. Furthermore, the amplification primers were specific as well for *C. jejuni*, avoiding potential artifacts in a mixed population.

In this assay a melting temperature, determined from the melting curve of the amplified product immediately following the termination of thermal cycling, confirmed that the product was that of *C. jejuni*. Agarose gel electrophoresis therefore was not necessary for identification of PCR products. The melting curve in Figure 2B for the selected strains showed that a clear peak at 77.2°C was visible for four strains of *C. jejuni*; and a half peak at 74.5°C was visible for other species and negative control. Primer dimmer may produce a half-peak at 74.5°C. The melting curve analysis improved the specificity of real-time PCR. Contaminated bacteria have no influence on the position of peak [14].

The square regression coefficients after the linear regression indicated a good correlation between the amount of template (Log copies) and the amount of product (represented by the Cts) in the standard curves ($R^2 = 1.00$). The linearity of the standard curves and the fact that the PCR operates with constant efficiency confirmed that the assay was well suited for quantitative measurements of *C. jejuni*. The detection limit of the present PCR assay was estimated to be approximately

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**Figure 2.** (A, top) Amplification curve from the *C. jejuni*-specific primers for a set of *Campylobacter* strains and other strains in real-time PCR. (B, middle) The melting curve of real-time PCR. A clear peak at 77.2°C was visible for 4 strains *C. jejuni*; and a half peak at 74.5°C was visible for other species and negative control. (C, bottom) The samples were subjected to electrophoresis in 2 percent agarose gel. Ten capillaries of the amplification product was loaded in each lane. Lane 1: Marker; Lane 2: *C. jejuni* ATCC 33560; Lane 3: *C. jejuni* 9832; Lane 4: *C. jejuni* 9816; Lane 5: *C. jejuni* (Z9614); Lane 6: *C. fetus*; Lane 7: *C. lari*; Lane 8: *C. coli*; Lane 9: *E. coli*; Lane 10: no template. Note: In Figure 2(A) and Figure 2(B): 1, *C. jejuni* ATCC 33560; 2, *C. jejuni* 9832; 3, *C. jejuni* 9816; 4, *C. jejuni* Z9614; 5, *C. fetus*; 6, *C. lari*; 7, *C. coli*; 8, *E. coli*; 9, no template; 10, blank, respectively.
one copy per PCR from bacterial culture. Our reported limit of detection was similar to those of others using fluorogenic 5'-nuclease PCR assay for endpoint detection. Jim and Colin obtained a detection level of approximately 25 CFU for Mycobacterium avium subsp. paratuberculosis/PCR [14], while Chen and colleagues showed a detection limit of as low as 2 CFU/PCR from a pure culture of Salmonella enterica serovar Typhimurium [19].

Advantages of real-time PCR detection of C. jejuni using SYBR Green I

The advent of the LightCycler and real-time PCR has provided the opportunity to develop a PCR assay, which meets the requirements of true rapid diagnostics [20]. In addition, the use of sealed capillary tubes in the LightCycler format combined with the absence of post-amplification manipulation of the PCR product significantly reduces the risk of contamination due to amplicon carry-over [15]. However real-time PCR using fluorescence resonance energy transfer probes was relatively expensive [21]. We have shown that with a little optimization, the much cheaper option of SYBR Green I can be used as an effective alternative for some laboratory applications. Jim and Colin found that the reagent cost per reportable result of performing the conventional PCR and the real-time PCR on the LightCycler using SYBR Green I were comparable [14]. A real-time PCR using SYBR Green I will become more prevalent in laboratories where rapid, sensitive, and high-throughput quantitative analysis is required.

In summary, the data presented here showed that the SYBR Green I was suitable for use in the LightCycler-based real-time PCR assay, which provided a specific, sensitive, and rapid method for quantitative detection of C. jejuni.

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