ORIGINAL CONTRIBUTION

A Real-time PCR Assay for the Detection and Quantitation of *Campylobacter jejuni* using SYBR Green I and the LightCycler

Chengbo Yang,[a,b] Yuan Jiang,[c] Kehe Huang,[a,*] Changqing Zhu,[c] Yulong Yin,[b] Jian-Hua Gong,[d] and Hai Yu[d]

[a]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, P.R. China;
[b]Key Laboratory of Subtropical Agro-ecology, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, P.R. China; [c]Jiangsu Entry/Exit Inspection and Quarantine Bureau, Nanjing, P.R. China; [d]Food Research Program, Agriculture and Agri-Food Canada, Guelph, Ontario, Canada

Campylobacter jejuni is recognized as a leading human food-borne pathogen. Traditional biochemical identification for *C. jejuni* is not reliable due to special growth requirements and the possibility that this bacterium can enter a viable but nonculturable (VNC) state. Nucleic acid-based tests have emerged as a useful alternative to traditional testing. In this article, we present fluorescent quantitative PCR assay for quantitative detection of *C. jejuni*, the assay was carried out using a LightCycler instrument and product formation was monitored continuously with the fluorescent double-stranded DNA binding dye SYBR Green I. When this assay was applied, the assay positive for all of the isolates of *C. jejuni* tested (11 isolates, including type strain ATCC33560) and negative for all other *Campylobacter* spp. (three isolates) and several other bacteria (five species tested). The total assay could be completed in 60 min with a detection limit of approximately 1 CFU, and a correlation coefficient was 1.000. Result indicated that fluorescent quantitative detection methods provided a special, sensitive, rapid, reproducible and accurate method for quantitative detection of *C. jejuni*.

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-

[*]To whom all correspondence should be addressed: Dr. Kehe Huang, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, P.R.China. Tel.: 86-25-4395507; Fax: 86-25-4442173; E-mail: khhuang@njau.edu.cn.
[†]Abbreviations: PCR, polymerase chain recation.

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 amplicon 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[T], National Collection of Type Culture), *Arcobacter butzleri* (NCTC 12481[T]), *Helicobacter hepaticus* (ATCC 51448[T]). Media: *Campylobacter* selective agar base (MERCK, CM02248, Darmstadt, Germany), Campylobacter selective supplement (MERCK, CM02249), defibrinated ship 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 manufacture'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'-GAAT-GAAATTTTAGAATGGGG-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 10^1 to 10^5 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 *T*aq merase, 20 mM Mg^{2+}, 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 10^0 to 10^5 copies of target template per 2 μl were subjected to PCR. As shown in Figure 1A, there were 1.0×10^5, 1.0×10^4, 1.0×10^3, 1.0×10^2, 1.0×10^1, 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 (C_t). 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 (R^2) 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: $C_t = -3.505 \times (\text{Log copy}) + 39.93$ and $\text{copies} = 10^{[(Ct - 39.93)/-3.505]}$.

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-
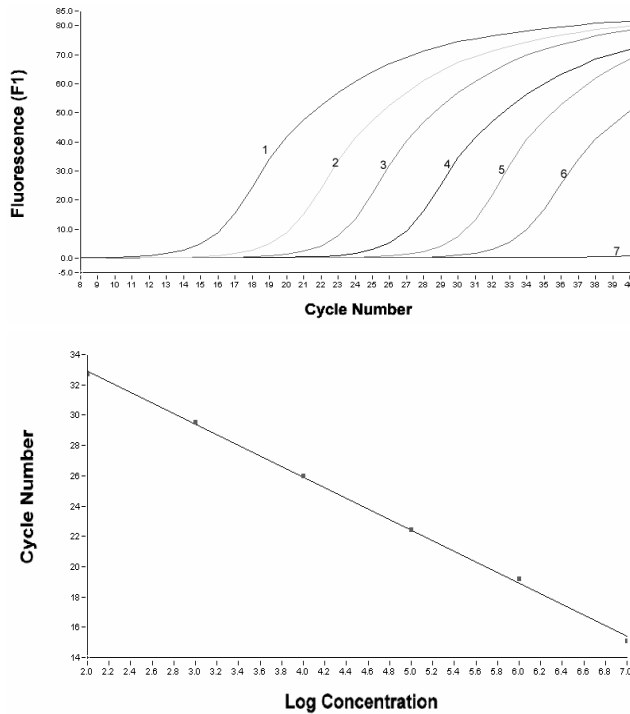
Figure 1. **(A, top)** Application curve for serial10-time dilutions of *C. jejuni* DNA in LightCycler. 1, 1.0 × 10^5 copies, 2, 1.0 × 10^4 copies, 3,1.0 × 10^3 copies, 4, 1.0 × 10^2 copies, 5, 1.0 × 10^1 copies, 6, 1.0 copies, 7, no template. **(B, bottom)** Standard curve for serial 10-time dilutions of *C. jejuni* DNA. Quantification was performed by determining the threshold cycle (C$_t$). C$_t$s were plotted against the calculated copies of bacterial DNA, i.e., a 10-time dilution of the bacterial DNA (1.0 × 10^5 copies/2 µl). The straight line, which was calculated by linear regression C$_t$ = -3.505 × (Log copy) + 39.93 and copies = 10$^{[(Ct -39.93)/-3.505]}$, shows a square regression coefficient (R^2) of 1.000.
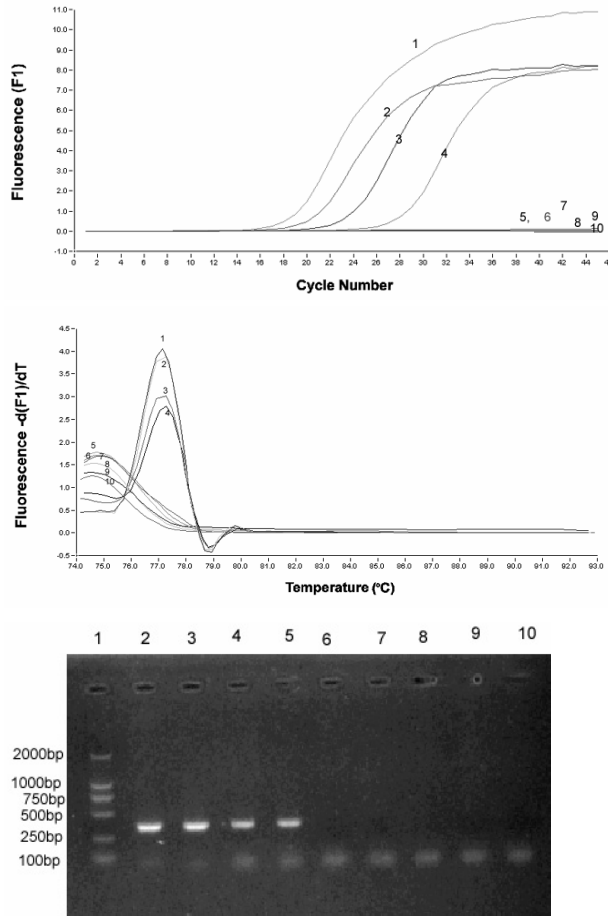
ed strains showed that a clear peak at 77.2°C was visible for four strains *C. jejuni*; and a half peak at 74.5°C was visible for other species and negative control. The sequence of PCR product has 100 percent homology with the VS-1 sequence published in GeneBank (X71603). These results confirmed that 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

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.

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 thermalcycling, 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 con-

trol. 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 C_ts) 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

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* sub**s**p. 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-throughout 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*.

REFERENCES

1. Dekeyser P, Gossuin-Detrain M, Butzler JP, and Sternon J. Acute enteritis due to related vibrio: first positive stool cultures. J Infect Dis 1972;125:390-2.
2. Allos BM and Blaser MJ. *Campylobacter jejuni* and the expanding spectrum of related infections. Clin Infect Dis 1995;20:1092-1101.
3. Nachamkin I, Blaser MJ, and Tompkins LS. *Campylobacter jejuni* current status and future trends. American Society for Microbiology, Washington D.C.; 1992.
4. Klein BS, Vergeront JM, Blazer MJ, Edmonds P, Brenner DJ, Janssen D, and Davis JP. *Campylobacter* infection associated with raw milk. JAMA 1986;225:361-4.
5. Mathewson JJ, Keswick BH, and DuPont HL. (1983) Evaluation of filters for recovery of *Campylobacter jejuni* from water. Appl Environ Microbiol 1983;46:985-7.
6. Castillo A and Escartin EF. Survival of *Campylobacter jejuni* on sliced watermelon and papaya. J Food Prot 1994;57:166-8.
7. Fricker CR and Park RWA. A two-year study of the distribution of thermophilic *Campylobacters* in human, environmental and food samples from the Reading area with particular reference to toxin production and heat-stable serotype. J Appl Bacteriol 1989;66:477-90.
8. Blaser MJ, Perez GP, Smith PF, Patton CM, Tenover FC, Lastovica AJ, and Wang WL. Extra-intestinal *Campylobacter jejuni* and *Campylobacter coli* infections: host factors and strain characteristics. J Infect Dis 1986;153:552-9.
9. Black RE, Levine MM, Clements ML, Hughes TP, and Blaser MJ. (1988) Experimental *Campylobacter jejuni* infection in humans. J Infect Dis 1988;157:472-9.
10. Robinson DA. Infective dose of *Campylobacter jejuni* in milk. Br Med J 1981;282:1584.
11. Hege KN, Anette B, Askild H, and Knut R. Application of the 5'-nuclease PCR assay in evaluation and development of methods for quantitative detection of

Campylobacter jejuni. Appl Environ Microbiol 2000;66:4029-36.

12. Purdy D, Ash CA, and Fricker CR. Polymerase chain reaction assay for the detection of viable Campylobacter species from potable and untreated environmental water samples, p. 147-153. In: Newell DG and Ketley JM, editors. *Campylobacter* VIII, *Campylobacters*, *Helicobacters*, and related organisms. London: Plenum Press; 1996.

13. Waage AS, Varund T, Lund V, and Kapperud G. Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. Appl Environ Microbiol 1999;65:1636–43.

14. Silvano P, Ian K, Rebecca, F, and James, F. Use of real-time PCR and the LightCycler system for the rapid detection of Pneumocystis carinii in respiratory specimens. Diagn Microbiol Infect Dis 2001;39:233–6.

15. Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, and Balis UJ. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 1997;22:176–81.

16. Jim OM and Colin H. A real time PCR assay for the detection and quantitation of Mycobacterium avium subsp. paratuberculosis using SYBR Green and the Light Cycler. J Microbiol Methods 2002;51:283-93.

17. Stonnet NJ and Guesdon JL. *Campylobacter jejuni*: specific oligonucleotides and DNA probes for use in polymerase chain reaction-based diagnosis. FEMS Immunol Med Microbiol 1993;7, 337-44.

18. Nachamkin I, Yang XH, and Stern NJ. Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. Appl Environ Microbiol 1993;59;1269-73.

19. Chen S, Yee A, Griffiths M, Larkin C, Yamashiro CT, Behari R, Paszko-Kolva C, Rahn K and DeGrandis SA. The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. Int J Food Microbiol 1997;35:239-50.

20. David F and Steven A. Quantitative immunocapture PCR assay for detection of *Campylobacter jejuni* in Foods. Appl Environ Microbiol 2000;66:4115-8.

21. Padungtod P, Hanson R, Wilson DL, Bell J, Linz JE, and Kaneene JB. Identification of *Campylobacter jejuni* isolates from cloacal and carcass swabs of chickens in Thailand by a 5' nuclease fluorogenic polymerase chain reaction assay. J. Food Prot. 2002;65, 1712-6.