DIAGNOSTIC MICROBIOLOGY

Development of a combined filtration-enrichment culture followed by a one-step duplex PCR technique for the rapid detection of *Campylobacter jejuni* and *C. coli* in human faecal samples

N. MISAWA, K. KAWASHIMA, H. KAWAMOTO and F. KONDO

Department of Veterinary Public Health, Faculty of Agriculture, Miyazaki University, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan

A new combined filtration-enrichment culture followed by a PCR technique for the rapid detection of *Campylobacter jejuni* and *C. coli* in human faeces has been developed. Only bacteria that passed through the membrane could multiply in the enrichment culture; target bacteria were detected by a one-step duplex PCR technique with combinations of primers that are specific for different *Campylobacter* spp., which should allow for the detection of a mixed infection in a single patient. A Falcon® cell-culture insert and 24-well tissue-culture plates were used. After 2 days, both *C. jejuni* and *C. coli* were reliably detected in diluted faeces that were seeded with as few as 10 cells which corresponds to a concentration of 10^3 cfu/g. Even allowing for the dilution of faecal samples, this represents an increase in sensitivity of two-to-three orders of magnitude over the conventional filtration method.

Introduction

Campylobacter jejuni and *C. coli* infections have been recognised as major food-borne diseases in developed [1] and developing [2] countries. As faecal or food samples, or both, are usually submitted to the laboratory for isolation of *C. jejuni* and *C. coli*, several approaches to isolating these organisms have been developed. [3, 4] Although selective media are used frequently for the primary isolation of campylobacters from faecal samples, selective media supplemented with antimicrobial agents may be inhibitory to some *Campylobacter* spp. and, therefore, may affect the isolation procedure. For this reason, a filtration method has been adopted to isolate these organisms from faecal samples [5]. However, the disadvantage of this method is its relatively low sensitivity [5]. To solve this problem, the organisms were enriched after filtration. This report describes a new assay in which a filtration-enrichment culture followed by a one-step duplex PCR technique was used for the rapid detection of *C. jejuni* and *C. coli* in human faecal samples.

Materials and methods

Filtration-enrichment culture

The filtration-enrichment culture system involved the use of a Falcon® cell-culture insert (Becton Dickinson and Company, Franklin Lakes, USA) and tissue-culture plates. The cell-culture insert consists of a plastic cylinder incorporating a polyethylene terephthalate track-etched membrane with a pore diameter of $0.4\ \mu\text{m}$. An sample of the enrichment broth was added to each well of a 24-well tissue-culture plate. The cell-culture insert with the diluted faecal sample was placed in a well, and the microtitration plate was incubated under micro-aerobic conditions. Only slender bacteria exhibiting motility and chemotaxis could pass through the membrane and multiply in the enrichment broth, which produced a high sensitivity for the detection of campylobacters. Finally, PCR with DNA extracted by alkaline and heat treatment was performed with two primer pairs. This protocol, which is a single (one-step) reaction method, allowed differentiation between *C. jejuni* and *C. coli*.

Bacterial strains

C. jejuni strains 81-176 and *C. coli* strain JCM2529 were used in this study. To determine the effect of

bacterial motility on detection and recovery, a non-motile *flaA/flaB* mutant (strain 98-248), constructed from strain 81-176 [6], was also used. Their motility was determined on a semi-solid agar plate composed of Brucella Broth (Becton Dickinson) containing agar 0.4%.

Recovery test

To assess the efficacy of the new filtration-enrichment method, the detection and recovery of *C. jejuni* and *C. coli* from artificially contaminated human faeces were measured. A fresh faecal specimen was obtained from a *Campylobacter*-negative person and diluted to 2% with 10 mM phosphate-buffered saline (PBS, pH 7.2). The bacterial cells were diluted serially and seeded into the diluted faeces at concentrations from 10^2 to 10^6 cfu/ml. The spiked faecal sample (100 μ l) was added to a cell-culture insert and placed in a well of a 24-well microtitration plate (Becton Dickinson) in which 600 μ l of Brucella broth supplemented with bovine fetal serum (Gibco-BRL, Gaithersburg, MD, USA) 5% had been added as an enrichment broth. A non-spiked faecal sample was used as a control. The air under the membrane was removed by tilting the insert. The microtitration plate was incubated in a jar at 37°C for a predetermined period under micro-aerobic conditions (N_2 80%, CO_2 10%, O_2 5% and H_2 5%).

One-step duplex PCR technique

The bacterial cells in the enrichment culture were harvested by centrifugation (8000 *g* for 10 min) and resuspended in 25 μ l of sterile distilled water. To extract bacterial DNA, 25 μ l of 50 mM NaOH were added to the bacterial suspension before it was incubated at 95°C for 5 min. After neutralisation with 4 μ l of 1 M Tris-HCl buffer (pH 7.5), the supernate was used as template DNA. One-step duplex PCR was used to enable the detection of both *Campylobacter* spp. in a single reaction. Primer combinations C1 (5'-CAAATAAAGTTAGAGGTAGAATGT-3')-C4 (5'-GGATAAGCACTAGCTAGCTGAT-3') and CC18F (5'-GGTATGATTTCTACAAAGCGAG-3')-CC519R (5'-ATAAAAGACTATCGTCGCGTG-3') were used to amplify 159-bp and 502-bp fragments for *C. jejuni* [7] and *C. coli* [8], respectively. PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, USA) in reaction mixtures of 25 μ l containing 5 μ l of extracted DNA, 2.5 μ l of 10 $\times$ reaction buffer (Qiagen, Tokyo, Japan), 160 nM of each primer, 0.625 U of Taq DNA polymerase (Qiagen) and 250 μ M of each deoxynucleotide (Amersham Pharmacia Biotech, Tokyo, Japan). The reaction was initiated by denaturation at 94°C for 1 min, and it was cycled 35 times at 94°C for 30 s annealed at 58°C for 30 s and 72°C for 1 min, with a final incubation at 72°C for 10 min to complete the extension.

Results and discussion

The species-specificity of the PCR process was verified (Fig. 1). The high sensitivity of PCR with known concentrations of bacterial cells was established by the detection of as few as 10^2 cells of both the *C. jejuni* and *C. coli* strains used in this study.

The PCR detection limits after the 24-h culture period for each wild-type strain of *C. jejuni* and *C. coli* were 10^2 and 10^1 cfu/100 μ l of diluted faecal samples seeded in the cell-culture insert, respectively (Fig. 2). After the 48-h culture period, as few as 10 cells spiked in the initial samples could be detected reliably for both species, which corresponds to 10^3 cfu/g of faeces (Fig. 2). In contrast to the wild type *C. jejuni* strains 81-176, the aflagellate mutant strain 98-248 was detected only with 10^4 cfu spiked in the initial sample, even after incubation for 72 h, suggesting that the PCR sensitivity depends on bacterial motility (Fig. 2). Although it remains unclear how the non-motile mutant could pass through the membrane when present in higher numbers, the vacuum step for replacing the gas of the jar may affect bacterial passage. No band was amplified from the enrichment cultures of non-spiked faecal samples. The recovery results obtained in three experiments were consistent with these outcomes.

A PCR-based assay for detecting *C. jejuni* and *C. coli* that is coupled with filtration enrichment of faecal samples exhibits high sensitivity. It was decided that filtration should be performed first to select bacteria that could pass through the filtration membrane, and that post-enrichment would allow the multiplication of target bacteria, thereby giving high sensitivity even with a low number of campylobacter cells in the faeces. As expected, a high sensitivity of 10^3 cfu/g was achieved in this study. Even allowing for the dilution of faecal samples, this represents an increase in sensitivity of two-to-three orders of magnitude over the conventional filtration method [3,5].

Overall, an efficient detection of campylobacter cells by PCR was obtained within a 48-h culture period at 37°C. Therefore, this assay system could reduce the time needed for diagnosis of confirmed *Campylobacter*

Fig. 1. *C. jejuni* and *C. coli* species-specific identification by a one-step duplex PCR amplification obtained with *C. jejuni*- and *C. coli*-specific primer combinations. Lane 1, *C. jejuni* 81-176; 2, *C. coli* JCM2529; 3, *C. jejuni* 81-176 and *C. coli* JCM2529; M, 100-bp DNA size marker.

Fig. 2. PCR detection of *C. jejuni* and *C. coli* spiked into diluted faecal samples. *C. jejuni* 81-176, *C. coli* JCM2529 and *C. jejuni* 98-248 (aflagellate mutant) constructed from strain 81-176 were used. The number of cells spiked into 100 µl of diluted faecal sample in a cell-culture insert is indicated below the lanes; lane **C**, a non-spiked faecal sample. Panels **a**, **b** and **c** represent 24-h, 48-h and 72-h cultures, respectively. **M**, 100-bp DNA size marker.

spp. in faecal samples. The present study used one-step duplex PCR for differentiating both species in a single tube, and the species specificity of the PCR was verified with spiked faecal samples. Patients infected with several different species of *Campylobacter* have been reported [9,10], so multiplex PCR protocols with combinations of primers that are specific for different *Campylobacter* spp. should allow for the detection of multiple infection in a single patient. Although the presence of PCR-interfering substances in faecal and food samples has been reported [11,12], the results of the present study indicate that a DNA extraction protocol involving alkaline-heat treatment of bacterial cells that pass through the membrane does not affect the PCR reaction. The simplification of the DNA recovery process for PCR amplification should also be emphasised.

It is important to isolate *Campylobacter* spp. in pure culture after filtration-enrichment culture, and the present technique may allow this. Improved isolation techniques have led to better isolation rates of these organisms [13] and filtration methods have been shown to be among the best available [3].

In a previous study, a short enrichment culture of pigeon faeces was performed before filtration to improve isolation rates [14]. Atypical *Campylobacter* spp. from stools are difficult to culture on the selective media that are used for *C. jejuni* and *C. coli* [3, 4] and

multiple techniques have been combined to improve the isolation rate of species other than *C. jejuni* and *C. coli* [13, 15, 16]. The combined use of selective and non-selective media with a filtration technique has been shown to improve isolation rates [9, 17]. Further application of this method, with some modifications to the combinations of the culture type and PCR protocol, may enable the rapid and sensitive detection of atypical *Campylobacter* spp. in various samples; this is currently being studied.

References

1. Friedman CR, Neimann J, Wegener HC, Tauxe RV. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In: Nachamkin I, Blaser MJ (eds) *Campylobacter*, 2nd edn. Washington, DC, ASM Press. 2000: 121–138.
2. Oberhelman RA, Taylor DN. *Campylobacter* infections in developing countries. In: Nachamkin I, Blaser MJ (eds) *Campylobacter*. Washington, DC, ASM Press. 2000: 139–153.
3. Goossens H, Butzler JP. Isolation and identification of *Campylobacter* spp. In: Nachamkin I, Blaser MJ, Tompkins LS (eds) *Campylobacter jejuni*: current status and future trends. Washington, DC, American Society for Microbiology. 1992: 93–109.
4. Nachamkin I, Engberg J, Aarestrup FM. Diagnosis and antimicrobial susceptibility of *Campylobacter* species. In: Nachamkin I, Blaser MJ (eds) *Campylobacter*. Washington, DC, ASM Press, 2000: 45–66.
5. Steele TW, McDermott SN. The use of membrane filters applied directly to the surface of agar plates for the isolation of *Campylobacter jejuni* from feces. *Pathology* 1984; **16**: 263–265.

6. Misawa N, Blaser MJ. Detection and characterization of autoagglutination activity by *Campylobacter jejuni*. *Infect Immun* 2000; **68**: 6168–6175.
7. Winters DK, Slavik MF. Evaluation of a PCR based assay for specific detection of *Campylobacter jejuni* in chicken washes. *Mol Cell Probes* 1995; **9**: 307–310.
8. Linton D, Lawson AJ, Owen RJ, Stanley J. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *J Clin Microbiol* 1997; **35**: 2568–2572.
9. Albert MJ, Tee W, Leach A, Asche V, Penner JL. Comparison of a blood-free medium and a filtration technique for the isolation of *Campylobacter* spp. from diarrhoeal stools of hospitalised patients in central Australia. *J Med Microbiol* 1992; **37**: 176–179.
10. Lawson AJ, Logan JMJ, O'Neill GL, Desai M, Stanley J. Large-scale survey of *Campylobacter* species in human gastroenteritis by PCR and PCR-enzyme-linked immunosorbent assay. *J Clin Microbiol* 1999; **37**: 3860–3864.
11. Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl Environ Microbiol* 1996; **62**: 1102–1106.
12. Russo F, Notarnicola M, Di Matteo G *et al.* Detection of *Helicobacter pylori cagA* gene by polymerase chain reaction in faecal samples. *Eur J Gastroenterol Hepatol* 1999; **11**: 251–256.
13. Lastovica AJ, le Roux E. Efficient isolation of campylobacteria from stools. *J Clin Microbiol* 2000; **38**: 2798–2799.
14. Mégraud F. Isolation of *Campylobacter* spp. from pigeon feces by a combined enrichment-filtration technique. *Appl Environ Microbiol* 1987; **53**: 1394–1395.
15. Bolton FJ, Hutchinson DN, Parker G. Reassessment of selective agars and filtration techniques for isolation of *Campylobacter* species from faeces. *Eur J Clin Microbiol Infect Dis* 1988; **7**: 155–160.
16. Engberg J, On SLW, Harrington CS, Gerner-Smidt P. Prevalence of *Campylobacter*, *Arcobacter*, *Helicobacter*, and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for campylobacters. *J Clin Microbiol* 2000; **38**: 286–291.
17. Piersimoni C, Bornigia S, Curzi L, De Sio G. Comparison of two selective media and a membrane filter technique for isolation of *Campylobacter* species from diarrhoeal stools. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 539–542.