Specific Detection and Confirmation of *Campylobacter jejuni* by DNA Hybridization and PCR

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Conventional detection and confirmation methods for *Campylobacter jejuni* are lengthy and tedious. A rapid hybridization protocol in which a 1,475-bp chromogen-labelled DNA probe (pDT1720) and *Campylobacter* strains filtered and grown on 0.22- μ m-pore-size hydrophobic grid membrane filters (HGMFs) are used was developed. Among the environmental and clinical isolates of *C. jejuni*, *Campylobacter coli*, *Campylobacter jejuni* subsp. *doylei*, *Campylobacter lari*, and *Arcobacter nitrofigilis* and a panel of 310 unrelated bacterial strains tested, only *C. jejuni* and *C. jejuni* subsp. *doylei* isolates hybridized with the probe under stringent conditions. The specificity of the probe was confirmed when the protocol was applied to spiked skim milk and chicken rinse samples. Based on the nucleotide sequence of pDT1720, a pair of oligonucleotide primers was designed for PCR amplification of DNA from *Campylobacter* spp. and other food pathogens grown overnight in selective Mueller-Hinton broth with cefoperazone and growth supplements. All *C. jejuni* strains tested, including DNase-producing strains and *C. jejuni* subsp. *doylei*, produced a specific 402-bp amplicon, as confirmed by restriction and Southern blot analysis. The detection range of the assay was as low as 3 CFU per PCR to as high as 10⁵ CFU per PCR for pure cultures. Overnight enrichment of chicken rinse samples spiked initially with as little as ~10 CFU/ml produced amplicons after the PCR. No amplicon was detected with any of the other bacterial strains tested or from the chicken background microflora. Since *C. jejuni* is responsible for 99% of *Campylobacter* contamination in poultry, PCR and HGMF hybridization were performed on naturally contaminated chicken rinse samples, and the results were compared with the results of conventional cultural isolation on Preston agar. All samples confirmed to be culture positive for *C. jejuni* were also identified by DNA hybridization and PCR amplification, thus confirming that these DNA-based technologies are suitable alternatives to time-consuming conventional detection methods. DNA hybridization, besides being sensitive, also has the potential to be used in direct enumeration of *C. jejuni* organisms in chicken samples.

Campylobacter jejuni accounts for $\geq 95\%$ of all human campylobacteriosis infections, which are mainly due to the consumption of raw milk and undercooked chicken (8). Due to the fastidious nature of campylobacters and their tendency to be easily suppressed by other enteropathogens, conventional detection of campylobacters in foods involves lengthy selective cultural enrichment (10). Subsequent identification and confirmation at the species level with traditional fermentation tests are limited since campylobacters are inherently biochemically inert. Discrimination between *C. jejuni* and *Campylobacter coli* is based solely on the hippurate hydrolysis test. The reliability of this test has been brought into question with the isolation of *C. jejuni* strains incapable of hydrolyzing hippurate (11). These limitations have resulted in efforts to develop more rapid, sensitive, and reliable alternatives to detect *C. jejuni* in foods.

Recently, molecular techniques, such as nucleic acid hybridization and nucleic acid amplification systems, have been ap-

plied to develop improved detection methods for *Campylobacter* spp. in stool and food samples. However, most of these methods do not distinguish among *C. jejuni*, *C. coli*, and *Campylobacter lari* (9, 14, 15). There are few previously reported DNA probes and/or primers that could distinguish among *C. jejuni*, *C. coli*, *C. lari*, and *Campylobacter upsaliensis* (3, 4).

We have previously used the digoxigenin-labelled (Boehringer Mannheim Canada [BMC], Laval, Québec, Canada) DNA probes pDT1719 and pDT1720 to detect *C. jejuni* and *C. coli* in stool specimens (13). In the present study, we used *C. jejuni*-specific probe pDT1720 and 0.22- μ m-pore-size hydrophobic grid membrane filters (HGMFs) to develop a rapid hybridization protocol to specifically detect *C. jejuni* in spiked food samples under stringent hybridization conditions. Furthermore, oligonucleotide primers selected from the DNA probe sequence were utilized in PCR studies to detect *C. jejuni*-specific amplicons in pure cultures, as well as in milk and chicken rinse samples.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains used in this study are shown in Table 1. *Campylobacter* spp. and related bacterial strains were subcultured regularly by using nonselective Mueller-Hinton broth (MHB) (Oxoid, Nepean, Ontario, Canada) and either Mueller-Hinton agar (MHA) or Preston (1) agar plates. All cultures were incubated at 37°C under microaerobic conditions with 85% N₂, 10% CO₂, and 5% O₂.

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TABLE 1. Specificity of DNA probe pDT1720 and PCR primers for the detection of *C. jejuni*

Organism(s)	No. of strains	Source(s) ^a	% Positive as determined by:	
			PCR	Hybridization
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i>				
Clinical isolates	22	1, 2	100	100
Chicken rinse isolates	13	3	100	100
Reference strains	11	3	100	100
<i>Campylobacter jejuni</i> subsp. <i>doylei</i>	3	3	100	100
<i>Campylobacter coli</i>	9	2, 3	0	0
<i>Campylobacter upsaliensis</i>	6	3	0	0
<i>Campylobacter lari</i>	7	3	0	0
<i>Campylobacter fetus</i> subsp. <i>fetus</i>	7	3	0	0
<i>Campylobacter fetus</i> subsp. <i>venerealis</i>	1	3	0	0
<i>Arcobacter nitrofigilis</i>	1	3	0	0
HPB panel of culture collection	310	4, 5	0	0
<i>Staphylococcus aureus</i> ATCC 13565	1	5	0	0
<i>Listeria monocytogenes</i> HPB81-681	1	4	0	0
<i>Shigella sonnei</i>	1	4	0	0
<i>Vibrio vulnificus</i> ATCC 27562	1	5	0	0
<i>Escherichia coli</i> VT1411	1	4	0	0

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Enumeration of *C. jejuni* organisms on HGMFs by using DNA probe. The *C. jejuni*-specific probe plasmid pDT1720 contained a 9.8-kb fragment cloned into pUC13. It was linearized by digestion with *Hind*III and labelled with digoxigenin by random primed labelling (BMC). Pure cultures of *Campylobacter* spp. and spiked skim milk and chicken rinse samples were deposited onto 0.22- μ m-pore-size HGMFs, and this was followed by incubation on Preston agar plates under microaerobic conditions for either 24 or 40 h. The HGMFs used were prepared in our laboratory by embossing melted country ski wax onto filter membranes with a homemade device (A. N. Sharpe, Food Directorate, Health Canada, Ottawa, Canada). The device has a zinc plate with an engraved square grid pattern (20 vertical and horizontal lines per in.) attached to an electrically heated block to keep the applied wax in liquid form. The device was mounted on a stand so that it could be lowered vertically to transfer the melted wax evenly onto the filter membrane when it was pressed gently onto the membrane surface. After cooling, the wax generated invisible hydrophobic grid lines. The HGMFs were then washed with pretreatment solution (50 mM sodium phosphate buffer [pH 6.0], 0.1 M NaHCO₃, 0.135% [vol/vol] Lugolvan G35). The bacterial cells on HGMFs were lysed by using 150 mM NaOH in 70% ethanol. These filters were heated in a 700-W microwave oven for 30 s at the high setting and then incubated with a solution containing 0.01% proteinase K, 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 0.1% sodium dodecyl sulfate at 37°C for 30 min. The HGMFs were washed at room temperature for 5 min each with 5 $\times$ SSC–0.1% sodium dodecyl sulfate and 2 $\times$ SSC. Air-dried HGMFs were exposed to UV light (UV Crosslinker; Bio-Rad, Mississauga, Ontario, Canada). The prehybridization and hybridization steps were carried out in solutions recommended for the digoxigenin-labelled probes (BMC) at 68°C for 30 min and 2 h, respectively. The hybrids were detected by a colorimetric immunoassay. The colored grids were enumerated as positive colonies with an automated HGMF counter (model MI-100 Interpreter; Richard Branker Research, Ltd., Ottawa, Canada).

DNA sequencing. DNA sequencing of a 1,418-bp fragment from pDT1720 was undertaken. Double-stranded dideoxy sequencing reactions were carried out by using α -³²S-dATP and Sequenase (United States Biochemicals, Cleveland, Ohio). The DNA sequencing strategy is shown in Fig. 1. DNA sequence analyses were performed with either PC/Gene (IntelliGenetics, Mountain View, Calif.) or programs and the database of the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Md.).

PCR mixture and conditions. All of the oligonucleotide primers used for PCR analysis, including CL1 (5'-ATTGTATTCTTGGCGTGGCCC-3'; coordinates 357 to 377), CL2 (5'-TGACGCTAGTGTGTAGGAG-3'; coordinates 644 to 663), CR1 (5'-ACTCCTACAACACTAGCGTC-3'; coordinates 664 to 645), CR2 (5'-GATTAGCGGTACGACTGTCT-3'; coordinates 795 to 776), and CR3 (5'-CCATCATCGCTAAGTGCAAC-3'; coordinates 1045 to 1026), were synthesized by using a model Oligo 1000 synthesizer (Beckman, Mississauga, Ontario, Canada). Optimization of PCRs with different combinations of forward (CL1 and CL2) and reverse (CR1 and CR3) primers included examinations of the effects of sample size (2- and 5- μ l portions of undiluted samples or cultures), dilution of cultures (10⁻¹ to 10⁻³), boiling of samples prior to addition to the PCR mixture, and primer concentration (0.18, 0.6, and 1.0 μ M). The cycling was performed with a thermocycler (model 9600; Perkin-Elmer, Applied Biosystems Division, Mississauga, Ontario, Canada), with denaturation at 95°C for 5 min, followed by 25 cycles consisting of melting at 95°C for 15 s, annealing at 48°C for 15 s, and extension at 72°C for 30 s and a final extension step at 72°C for 10 min. Amplicons were detected by loading of 10 μ l of sample onto 2% agarose (pulsed-field agarose; Bio-Rad) gels and subsequent electrophoresis in Tris-borate buffer performed for 1 h at 70 V. The amplicons were confirmed by restriction with *Dde*I and hybridization with pDT1720.

Effect of skim milk and chicken rinse on PCR. Chicken parts from supermarkets were either rinsed with phosphate-buffered peptone water (10 g of peptone per liter, 5 g of NaCl per liter, 3.5 g of dibasic sodium phosphate per liter, 1.5 g of monobasic potassium phosphate per liter; pH 7.2 $\pm$ 0.2) and stored at -20°C before analysis or rinsed in 0.1% peptone water (1 ml/g of chicken) and used immediately. The effect of skim milk and chicken rinse on the PCR was determined by amplifying *C. jejuni* suspended in skim milk or chicken rinse. The suspension was prepared by 10-fold serial dilution (10⁻¹ to 10⁻⁶) of *C. jejuni* cultures with either chicken rinse or Tween-saline buffer (1% Tween 80, 0.85% NaCl) as the diluent. A portion (5 μ l) of each dilution was transferred to a PCR tube. The amplification product from each reaction was visualized in agarose gels. The highest dilution that yielded an amplicon from each diluent was determined.

Development and evaluation of selective media. A preliminary study was conducted to check the inhibitory effects of brucella broth, MHB, Preston medium, Rosef medium, and charcoal used in media on the PCRs. In designing a selective MHB for enriching *C. jejuni* in chicken rinse, we tested cefoperazone (16 and 32 mg/liter), polymyxin B (1,250 and 2,500 U/liter), and vancomycin (5 and 10 mg/liter) as selective agents. Three concentrations of ferrous sulfate (0.0125, 0.025, and 0.05%) were also tested in combination with sodium pyruvate (0.025%) and sodium metabisulfite (0.025%) as growth supplements in MHB. The cultures were incubated at 37°C either in 5 to 7% CO₂ or under microaerobic conditions in a shaking incubator. The combination of selective agent (32 mg of cefoperazone per liter of medium), growth supplements (0.025% ferrous sulfate, 0.025% sodium pyruvate, 0.0125% sodium metabisulfite), and incubation under microaerobic conditions, which provided growth of campylobacters for optimum PCR results, was used in subsequent studies. MHA plates supplemented with 0.025% ferrous sulfate, 0.025% sodium pyruvate, and 0.025% sodium metabisulfite were used for enumeration of *C. jejuni* organisms in chicken rinses.

PCR detection of *C. jejuni* in spiked chicken rinses. Chicken rinses which had no detectable *C. jejuni* were obtained from the Animal Disease Research Institute, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada. The presence of *C. jejuni* in chicken rinses was determined in our laboratory by Rosef enrichment and isolation on selective medium (7). Each 5-ml chicken rinse portion was spiked with 100 μ l of 10-fold serial dilutions of an overnight culture

FIG. 1. Restriction map and sequencing strategy for *Sau*3AI fragment of pDT1720. The size of the fragment in pDT1720 (13a) is 1,418 bp. The open box labelled ORF shows the position of the partial open reading frame (positions 1 to 420). The box labelled Amplicon shows the amplified region (positions 644 to 1026) and the *Dde*I sites. The arrows above the *Sau*3AI fragment indicate the sizes and directions of the segments sequenced. The positions of the primers are indicated with short arrows. The *Dra*I and *Alu*I restriction sites are not included.

FIG. 2. HGMF containing *C. jejuni* growth after incubation on Preston medium, showing typical colored grids after hybridization with the *C. jejuni*-specific probe.

(10^{-9} to 10^{-7}) of *C. jejuni* HPB56 and added to an equal volume of $2\times$ MHB containing supplements, giving final concentrations of ≤ 1 to 10^6 CFU/ml. A positive control was prepared similarly, except that the chicken rinse was replaced with 0.1% peptone water or phosphate-buffered peptone. The inoculum size was determined by plating the overnight culture, as well as the spiked and unspiked chicken enrichment mixtures, onto MHA containing 2% agar to reduce the swarming of *C. jejuni* organisms. A 1-ml portion of each spiked and unspiked enrichment broth was boiled for 10 min to lyse the bacterial cells and to inactivate heat-sensitive nucleases. Then 5 μ l of each boiled sample was used for PCR. After 16 to 24 or 48 h of incubation with shaking at 37°C under microaerobic conditions, a PCR was performed for each sample, and *C. jejuni* organisms were enumerated by plating preparations onto selective MHA. The PCR products were detected by agarose gel electrophoresis, and all experiments were repeated three times. The method sensitivity for detecting *C. jejuni* in chicken rinses by PCR, with or without enrichment, was determined. To confirm the specificity of the PCR, the colonies were checked by several methods, including light microscopy, determination of susceptibilities to nalidixic acid and cephalothin, hippurate hydrolysis, and hybridization with DNA probe pDT1720.

Detection of *C. jejuni* in naturally contaminated chicken. Freezing may affect the viability of *C. jejuni* and/or the background flora. To further evaluate the protocol for enrichment and PCR, four fresh whole chickens or chicken parts were purchased from a supermarket. One milliliter of 0.1% peptone water per g of chicken parts or 30 ml per chicken was used to release *C. jejuni* from the chicken by shaking in a paint shaker for 1 min. Five milliliters of chicken rinse was mixed with either $2\times$ selective MHB or $2\times$ Rosef broth. PCRs were performed before and after incubation microaerobically at 37°C for 16 to 24 h. Five-microliter portions of an overnight culture of *C. jejuni* and uninoculated broth were used as positive and negative controls, respectively, in the PCR. To confirm the results, one milliliter of chicken rinse was filtered onto HGMF and hybridized with probe pDT1720 as described above. In addition, 20 μ l of enrichment broth was streaked onto Preston agar plates to obtain *C. jejuni* cultures for confirmation by microscopy and biochemical tests.

Nucleotide sequence accession number. The 1,418-bp sequence of pDT1720 has been deposited in the GenBank database under accession no. U27272.

RESULTS

Detection of *C. jejuni* on HGMFs. The hybridization results showed that the pDT1720 probe was specific for *C. jejuni* (Table 1) and did not cross-react with other bacterial strains. The probe did not distinguish subspecies within *C. jejuni*, and strains of both *Campylobacter jejuni* subsp. *jejuni* and *Campylobacter jejuni* subsp. *doylei* hybridized with the probe. The cloning vector did not affect the specificity and sensitivity of the probe; therefore, it is not necessary to remove the vector in the preparation of the DNA probe. It should be noted that non-specific background may appear on HGMFs and should not be considered a positive result. The HGMF required 40 h of incubation on Preston medium before any hybridization signal could be detected in the entire grid (Fig. 2).

DNA sequence. Analysis of the 1,418-bp sequence of pDT1720 deposited in the GenBank database showed the presence of an open reading frame corresponding to the carboxyl terminus at positions 1 to 420 [accession no. gi1881378 (U27272)]. Neither the DNA sequence nor the protein sequence showed any homology with any other sequence in the National Center for Biotechnology Information database. Prosite analysis (PC/Gene) showed that there were three N-glycosylation sites (amino acid positions 32, 33, and 99), four protein kinase C phosphorylation sites (amino acid positions 3, 114, 122, and 133), and one casein kinase II phosphorylation site (amino acid position 60). Nevertheless, we do not know if any functional products are encoded by these sequences.

Specificity of PCR amplification. Two unique left primers (CL1 and CL2) and three right primers (CR1, CR2, and CR3) were selected from the DNA sequence for PCR experiments. Amplification of 0.12 and 0.25 μ g of CsCl-purified chromosomal DNA of *C. jejuni* 37G by using either primers CL2 and CR2 or primers CL2 and CR3 yielded the expected 152- and 402-bp amplicons, respectively, but no amplicons were obtained from *C. coli* 8 DNA. No amplicons were detected from *C. jejuni* 37G with primers CL2 and CR1 or primer CL1 in combinations of CR1 and CR2. When amplification was performed with primers CL1 and CR3, one band and three bands were obtained from *C. jejuni* 37G and *C. coli* 8, respectively (data not shown). Primers CL2 and CR3 were evaluated further for specific amplification of *C. jejuni* by using a panel of test strains (Table 1). The primers produced a 402-bp amplicon from *C. jejuni* and *C. jejuni* subsp. *doylei* (Fig. 3, lanes B and C). The specific amplicons were confirmed by *Dde*I digestion, which generated 166-, 118-, 105-, and 13-bp fragments (Fig. 3, lanes D and E). One of the *C. jejuni* strains (HPB3) was cephalothin sensitive and nalidixic acid resistant. This strain hybridized with pDT1720 and also produced a 402-bp amplicon after PCR with primers CL2 and CR3 (data not shown). Sixteen clinical and six chicken isolates were also tested by using the same protocol. Amplicons were obtained only from isolates identified as *C. jejuni* by conventional methods. One of the clinical isolates that showed weak hippurate hydrolysis was positive by PCR. No amplicons were obtained from species other than *C. jejuni*.

Optimization of PCR conditions. Except for the DNase-producing *C. jejuni* strains tested, amplicons were obtained when primers at a concentration of 0.2 μ M were used to

FIG. 3. Agarose gel electrophoresis of PCR products from *C. jejuni* and *C. jejuni* subsp. *doylei* and their corresponding *Dde*I restriction profiles. Lane A, molecular size standard; lane B, *C. jejuni*; lane C, *C. jejuni* subsp. *doylei*; lane D, *C. jejuni* digested with *Dde*I; lane E, *C. jejuni* subsp. *doylei* digested with *Dde*I.

FIG. 4. Agarose gel electrophoresis of PCR products from chicken rinse samples spiked with different concentrations of *C. jejuni*. Lane A contained the molecular size standard. Lanes B through G contained the PCR products of unenriched chicken rinse samples in supplemented MHB spiked with the following concentrations of *C. jejuni*: 6×10^5 CFU/ml (undiluted) (lane B); 8×10^4 CFU/ml (10^{-1} dilution) (lane C); 9×10^3 CFU/ml (10^{-2} dilution) (lane D); 6×10^2 CFU/ml (10^{-3} dilution) (lane E); 4×10^1 CFU/ml (10^{-4} dilution) (lane F); and 10^{-5} dilution (lane G). Lane H contained a PCR-positive control spiked with 6×10^5 CFU of *C. jejuni* per ml in the absence of chicken rinse. Lane I contained a PCR-negative control with unspiked chicken rinse. Lane J contained a PCR-positive control with purified pDT1720 DNA in MHB. Lane K contained a PCR-negative control with MHB only. Lanes L through Q contained the PCR products of enriched chicken rinse samples in MHB spiked with 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} dilutions, respectively, of the *C. jejuni* culture suspension described above. Lane R contained a PCR-positive control spiked with 6×10^5 CFU of *C. jejuni* per ml in the presence of MHB and chicken rinse.

amplify DNA from 1×10^1 to 10^7 cells in the PCR. Amplicons were obtained from DNase-producing *C. jejuni* strains T4, T36, and JE710 when a primer concentration of $1.0 \mu\text{M}$ was used to detect DNA from 3 to 10^5 CFU in the PCR. Also, boiling samples for 5 to 10 min prior to the PCR produced more reproducible amplification of *C. jejuni* DNA from DNase-producing strains. The optimal PCR mixture ($50 \mu\text{l}$), which also allowed amplification of DNase-positive isolates, contained primers at a concentration of $1.0 \mu\text{M}$, 2.5 mM MgCl_2 , 56 mM KCl, 11 mM Tris-HCl (pH 9.0), each deoxynucleotide triphosphate at a concentration of $200 \mu\text{M}$, 1% Triton X-100, and 2.5 U of *Taq* polymerase.

Effect of skim milk or chicken rinse on PCR. The result of amplification of serial dilutions of *C. jejuni* 8G and 37G in either skim milk or peptone water showed that skim milk decreased the sensitivity of PCRs 10-fold. Similarly, amplification of serial dilutions showed that the sensitivity of detecting *C. jejuni* HPB56 in chicken rinses was one 10-fold dilution lower than the sensitivity in Tween-saline buffer (data not shown).

Development of media. Our preliminary studies showed that brucella broth containing trace amounts of charcoal inhibited the PCR. When $5\text{-}\mu\text{l}$ portions of overnight cultures in MHB or Rosef medium containing about the same number (10^7 CFU/ml) of *C. jejuni* HPB6, HPB56, and HPB34 cells were used in the PCR, amplicons were obtained from MHB but not from Rosef medium. However, 10^{-1} to 10^{-3} dilutions of the cultures grown in Rosef medium yielded PCR amplicons, and the sensitivity was about 10 times lower than the sensitivity observed with MHB. Neither 0.1% peptone water nor phosphate-buffered peptone water inhibited either the growth of *C. jejuni* or the PCR. However, phosphate-buffered peptone reacted with the growth supplement and formed a precipitate when it was mixed with the selective MHB. Using 0.1% peptone water is convenient because it does not form a precipitate which would interfere with filtration. Ferrous sulfate, sodium metabisulfite, and sodium pyruvate were not inhibitory to the PCR at any concentration tested. When 0.05% ferrous sulfate was used in

combination with sodium metabisulfite and sodium pyruvate, fewer amplicons were obtained after the PCR. Therefore, the optimum concentration of ferrous sulfate (0.025%) was subsequently used. The selective agents cefoperazone, polymyxin, and vancomycin had no inhibitory effect on the PCR at all concentrations tested. We found that in comparison to Preston medium and MHA incubated at 37 or 42°C in 5% CO_2 , cefoperazone alone resulted in reductions of 10^4 - and 10^5 -fold in the background flora when chicken rinse was plated onto the medium. The hazy growth of background flora on Preston medium was eliminated by microaerobic incubation. The background flora was further reduced when three antibiotics were used in MHA. However, when the growth of *C. jejuni* in spiked chicken rinse in selective MHB was examined, cefoperazone (32 mg/liter) alone gave better results than the three antibiotics combined. Without incubation, the sensitivity of the PCR performed with a culture in MHB supplemented with three antibiotics was 10 times lower than the sensitivity of the PCR performed with a culture in MHB supplemented with one antibiotic. After 24 h of incubation, the sensitivity of Mueller-Hinton medium supplemented with three antibiotics was 100 times lower than the sensitivity of Mueller-Hinton medium supplemented with one antibiotic. We were not able to enrich *C. jejuni* in chicken rinse in a 5 to 7% CO_2 incubator. Therefore, enrichment broth was incubated microaerobically (5% O_2 , 10% CO_2 , 85% N_2) with shaking.

PCR of spiked chicken rinse. Supplemented MHB supported the growth of *C. jejuni* at initial inocula as small as 10 CFU/ml to levels detectable by PCR (about 10^4 to 10^6 CFU/ml) after overnight incubation (Fig. 4). Without enrichment, the lowest level of detection of *C. jejuni* by PCR was 10^5 CFU/ml of chicken rinse or about 10^3 CFU per PCR tube (Fig. 4). After overnight enrichment of chicken rinse in MHB with supplements, all of the spiked chicken rinse samples were PCR positive for initial inoculum sizes ranging from 1.0×10^1 to 1.0×10^5 CFU/ml.

Detection of *C. jejuni* from naturally contaminated chicken. When four naturally contaminated chicken rinse samples were analyzed by PCR, amplicons were obtained only from two samples after overnight enrichment of the four samples in Rosef medium (Fig. 5, lanes L and M). None of the four

FIG. 5. Agarose gel electrophoresis of PCR products to detect *C. jejuni* in naturally contaminated chicken rinse samples. Lane A, molecular size standard; lane B, unenriched chicken rinse 1 in supplemented MHB; lane C, unenriched chicken rinse 2 in MHB; lane D, chicken rinse 1 after a 16-h enrichment in MHB; lane E, chicken rinse 2 after a 16-h enrichment in MHB; lane F, PCR-positive control with purified pDT1720 DNA in MHB; lane G, PCR-positive control with purified pDT1720 DNA in Rosef broth; lane H, PCR-negative control in MHB; lane I, PCR-negative control in Rosef broth; lane J, unenriched chicken rinse 1 in Rosef broth; lane K, unenriched chicken rinse 2 in Rosef broth; lane L, chicken rinse 1 after a 16-h enrichment in Rosef broth; lane M, chicken rinse 2 after a 16-h enrichment in Rosef broth.

naturally contaminated chicken rinse samples showed amplification after PCR prior to enrichment and after enrichment in selective MHB. When 1-ml portions of the preenriched and enriched chicken rinses from the naturally contaminated samples were filtered onto an HGMP and hybridized with pDT1720 after 48 h of incubation on Preston medium, two of the four samples showed positive grids on the HGMP. Unfortunately, the membrane contained too many positive grids to provide an estimate of the *C. jejuni* concentration. The results of the PCR and hybridization methods were in agreement with the results of the conventional culture method.

DISCUSSION

DNA-based methods have the advantage over conventional culture methods for the detection of *C. jejuni* because they are less time-consuming. Specific DNA probes should overcome the difficulty in identification of atypical campylobacters. Our hybridization protocol in which pDT1720 and HGMP were used led to specific hybridization of *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. The specificity of the probe was not affected by the ingredients in skim milk and chicken rinse and therefore can be used for simultaneous detection and identification of *C. jejuni*. The 0.22- μ m-pore-size HGMP minimized the loss of *Campylobacter* during filtration and reduced the spread of growth from grid to grid. The colorless grid lines improve the differentiation of positive grids from nonspecific background color. This method potentially can be used for direct enumeration of *C. jejuni* organisms in poultry, which has a high rate of carriage of this organism.

To further reduce the time required to detect and/or confirm *C. jejuni* cultures, we developed a PCR protocol by using specific primers CL2 and CR3 selected from the DNA sequence of pDT1720. When the protocol was used in combination with an enrichment medium containing cefoperazone and growth supplement containing ferrous sulfate, sodium metabisulfite, and sodium pyruvate in MHB, only *C. jejuni* isolates, including *C. jejuni* subsp. *doylei* isolates, were detected, and these organisms generated a single 402-bp band on agarose gels; no amplification products were produced by the background flora. In contrast, a nonspecific band(s) was observed when other primers were used to detect *C. jejuni* in chicken (16). The specificity of CL2 and CR3 allows for the replacement of agarose gel electrophoresis with other methods (e.g., the use of fluorescent labels and detection by fluorometers). In this aspect, CL2 and CR3 are superior to other previously described primers, which require gel electrophoresis to distinguish nonspecific amplification from other species (3).

The enrichment in selective MHB improved the sensitivity of detecting *C. jejuni* in chicken samples without showing an inhibitory effect in the PCR. Therefore, this method did not require sample pretreatment, medium removal, or isolation of DNA (which requires multiple steps and special reagents) prior to PCR, as is required by some previously described methods (2, 5, 16). This is advantageous because it reduces the cost, the processing time, and the risk of false positives.

For two of the previously described PCR protocols used with overnight enrichment of chicken samples the reported sensitivities of *Campylobacter* detection were 12.5 CFU per PCR (5) and 42 CFU/g of chicken (2). Our protocol achieved this level of sensitivity and also detected DNase-producing *C. jejuni*. Since most previous studies did not include DNase-producing strains, it is difficult to compare the performance of our protocol with the performance of other protocols. Therefore, we recommend that DNase-producing *C. jejuni* strains be included in future evaluation or method development studies because

these strains are more difficult to detect than other *C. jejuni* strains with nucleic acid-based methods.

Based on the results obtained with a small number of chicken samples, Rosef medium is more suitable than selective MHB. Although Rosef medium is more inhibitory to the PCR, it may allow *Campylobacter* strains to grow to higher numbers than selective MHB. The hybridization method has an advantage over the PCR protocol in that it can be used with a 200-fold larger sample size than the PCR protocol and detects lower levels of *C. jejuni* organisms. *C. jejuni* may be present in coccoid forms in chickens and may not multiply to high enough numbers in the presence of background flora during overnight enrichment. Also, the detection of the coccoid form of *C. jejuni* is less sensitive than the detection of spirals when the PCR method is used (6).

Both the DNA probe hybridization and PCR amplification protocols reported here represent rapid, specific, and sensitive methods for confirming *C. jejuni* isolates. The hybridization method has the potential to be used to enumerate campylobacters in highly contaminated samples of milk and chicken (6). Although the PCR protocol shows high sensitivity for detecting *C. jejuni* in spiked samples, it is less sensitive than hybridization for detecting low numbers (30 to 10³ CFU per ml of chicken rinse) of *C. jejuni* organisms.

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