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# Use of PCR for Direct Detection of *Campylobacter* Species in Bovine Feces†

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**This study reports on the use of PCR to directly detect and distinguish *Campylobacter* species in bovine feces without enrichment. Inhibitors present in feces are a major obstacle to using PCR to detect microorganisms. The QIAamp DNA stool minikit was found to be an efficacious extraction method, as determined by the positive amplification of internal control DNA added to bovine feces before extraction. With nested or seminested multiplex PCR, *Campylobacter coli*, *C. fetus*, *C. hyointestinalis*, and *C. jejuni* were detected in all fecal samples inoculated at ≈$10^4$ CFU $g^{-1}$, and 50 to 83% of the samples inoculated at ≈$10^3$ CFU $g^{-1}$ were positive. At ≈$10^2$ CFU $g^{-1}$, *C. fetus*, *C. hyointestinalis*, and *C. jejuni* (17 to 50% of the samples) but not *C. coli* were detected by PCR. From uninoculated bovine feces, a total of 198 arbitrarily selected isolates of *Campylobacter* were recovered on four commonly used isolation media incubated at three temperatures. The most frequently isolated taxa were *C. jejuni* (152 isolates) and *C. lanienae* (42 isolates), but isolates of *C. fetus* subsp. *fetus*, *Arcobacter butzleri*, and *A. skirrowii* also were recovered (≤2 isolates per taxon). Considerable variability was observed in the frequency of isolation of campylobacters among the four media and three incubation temperatures tested. With genus-specific primers, *Campylobacter* DNA was detected in 75% of the fecal samples, representing an 8% increase in sensitivity relative to that obtained with microbiological isolation across the four media and three incubation temperatures tested. With nested primers, *C. jejuni* and *C. lanienae* were detected in 25 and 67% of the samples, respectively. In no instance was DNA from either *C. coli*, *C. fetus*, or *C. hyointestinalis* detected in uninoculated bovine feces. PCR was more sensitive than isolation on microbiological media for detecting *C. lanienae* (17%) but not *C. jejuni*. Campylobacters are a diverse and fastidious group of bacteria, and the development of direct PCR not only will increase the understanding of *Campylobacter* species diversity and their frequency of occurrence in feces but also will enhance the knowledge of their role in the gastrointestinal tract of livestock and of the factors that influence shedding.**

*Campylobacter* species are recognized as one the most frequent causes of acute diarrheal disease in humans throughout the world; as much as 1% of the population is thought to be infected with *Campylobacter* species every year in North America (Centers for Disease Control and Prevention-U.S. Department of Agriculture-Food and Drug Administration Collaborating Sites Foodborne Disease Active Survey Network [Foodnet]). *Campylobacter* infections also can cause enteritis and abortions in cattle (37). Alberta, Canada, possesses a very large beef cattle population (>5 million head) in the southern region of the province, but relatively limited research has investigated the prevalence of *Campylobacter* species associated with beef cattle. The prevalence of *Campylobacter* infections in humans in this region is considerably higher than the national average (Health Canada Website, http://cythera.ic.gc.ca/dsol/ndis/ndex_e.html), but a definitive link to beef cattle as a source of *Campylobacter* infections has not been established. Numerous selective media are used for the isolation of campylobacters; almost all contain several antibiotics as inhibitory agents (8, 17). By use of enrichment and/or isolation of campylobacters on semiselective media, a number of studies have reported the association of *Campylobacter* species with cattle (1, 5, 14, 15, 20, 34, 38, 44, 45). Campylobacters are very fastidious, and antimicrobial agents incorporated into media used to selectively isolate *Campylobacter jejuni* and *C. coli* have been shown to inhibit the growth of other *Campylobacter* species, such as *C. upsaliensis*, *C. hyointestinalis*, and *C. fetus* (1, 4, 12, 25, 37). As a result, microbiological methods do not provide a true measure of the frequency and diversity of *Campylobacter* species associated with livestock and their feces.

The application of PCR may provide a more accurate description of the prevalence of *Campylobacter* species associated with livestock. However, the presence of inhibitors in fecal materials is a major obstacle limiting the usefulness of PCR for detecting microorganisms in feces. A number of inhibitors are present in human feces; these include bile salts, hemoglobin degradation products, and complex polysaccharides (33, 46). In addition, polyphenolic substances from plant tissues are very inhibitory to PCR (23). A variety of strategies for removing PCR inhibitors from human stool samples have been reported. For example, Lawson et al. (25) used polyvinylpyrrolidone to reduce the inhibitory effects of polyphenolic substances in the PCR detection of *C. upsaliensis* and *C. helveticus* in human feces. Largely because of high labor costs and time constraints in processing numerous clinical samples, a number of commercial kits have been specifically developed for extracting DNA from human feces; their utility for detecting bacteria (32), including *C. jejuni* and *C. coli* (6), has been demonstrated. The

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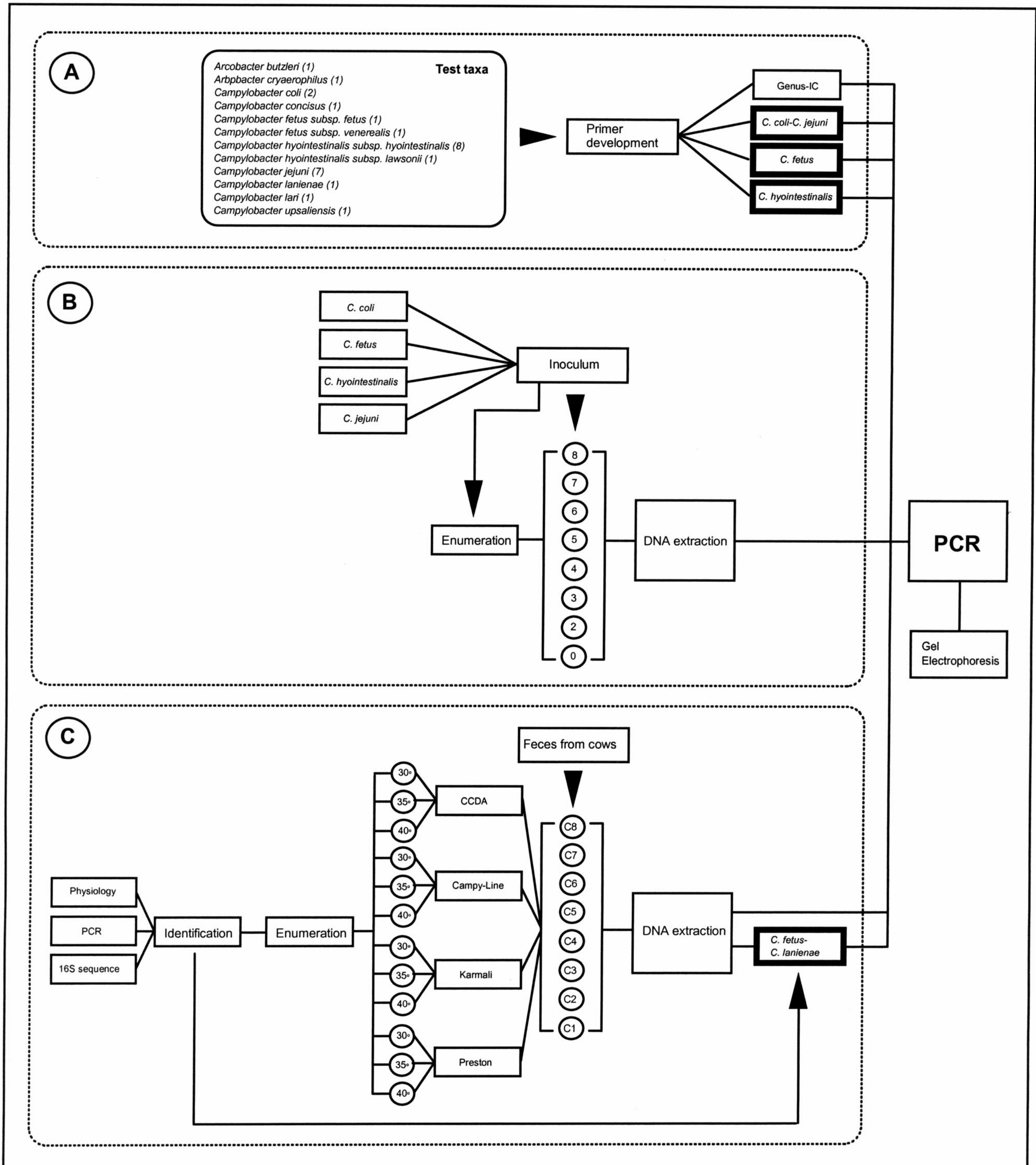


FIG. 1. Flow diagram of the experiments conducted in each objective. (A) In the first objective, an internal control (IC) and primers for the genus *Campylobacter*, *C. coli*, *C. jejuni*, *C. fetus*, and *C. hyointestinalis* were developed. The presentation of two taxon names in the same box (on the right) represents multiplex reactions. Boxes with thick walls represent nested PCR. Names in the box labeled "Test taxa" represent taxa that were used to determine the specificities of PCRs; numbers in parentheses following the taxon names represent the numbers of isolates tested. (B) In the second objective, bovine feces were inoculated with various concentrations of *C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. jejuni* (target densities in $\log_{10}$ CFU per gram are presented in circles); the control treatment ("0") was not inoculated with campylobacters. CFUs were enumerated in the inoculated and control feces by using dilution plating. Concurrently, DNA was extracted from all fecal treatments and subjected to PCR with the primers shown in objective A, and amplicons were electrophoresed. (C) In the third objective, uninoculated bovine feces were collected from eight dairy cows (C1 to C8). Campylobacters were isolated and enumerated on four media maintained at three temperatures. Representative colonies were selected, established in pure cultures, and identified by using physiological characters, PCR with primers for specific taxa and, if required, extraction of DNA and sequencing of a portion of the 16S rRNA gene. During the course of this experiment, *C. lanienae* was frequently isolated on Karmali medium at 40°C. As a result, a nested primer set was developed for this taxon, and the *C. lanienae* primers were used in a multiplex PCR with primers for *C. fetus*. DNA was extracted from fecal samples obtained from the eight cows and subjected to PCR, and amplicons were electrophoresed. The experiments in the second and third objectives were conducted three times on separate occasions.

TABLE 1. Primer sequences for the amplification of *Campylobacter* species DNA from bovine feces

| PCR target and gene[a] | Primer | $T_m$(°C) | Sequence (5′ to 3′) | Size (bp) | Reference or source |
|---|---|---|---|---|---|
| *Campylobacter* genus and internal control | | | | | |
| 16S rRNA | C412F | 58 | GGATGACACTTTTCGGAGC | 816 | Linton et al. (29) |
| | C1228R | | CATTGTAGCACGTGTGTC | | Linton et al. (29)[b] |
| *Campylobacter coli* and *C. jejuni* | | | | | |
| 16S rRNA | MD 16S1Upper | 58 | ATCTAATGGCTTAACCATTAAAC | 857 | Denis et al. (9); CCCJ609F modified from Linton et al. (30) |
| | MD16S2Lower | | GGACGGTAACTAGTTTAGTATT | | Denis et al. (9); CCCJ1442R modified from Linton et al. (30) |
| *Campylobacter coli* and *C. jejuni* primary multiplex | | | | | |
| *ceuE* (*C. coli*) | COL3Upper | 58 | ATTTGAAAATTGCTCCAACTATG | 462 | Gonzales et al. (16) |
| | MDCOL2Lower | | TGATTTTATTATTTGTAGCAGCG | | Denis et al. (10); COL2 modified from Gonzalez et al. (16) |
| *mapA* (*C. jejuni*) | MDmapA1 Upper | | CTATTTTATTTTTGAGTGCTTGTG | 589 | Denis et al. (10) |
| | MDmapA2Lower | | GCTTTATTTGCCATTTGTTTTATTA | | Denis et al. (10) |
| *Campylobacter coli* and *C. jejuni* nested multiplex | | | | | |
| *ceuE* (*C. coli*) | CCceuEN3F | 58 | AAGCGTTGCAAAACTTTATGG | 330 | New primer[c] |
| | CCceuEN3R | | CCTTGTGCGCGTTCTTTATT | | New primer[c] |
| *mapA* (*C. jejuni*) | CJmapAN3F | | TGGTGGTTTTGAAGCAAAGA | 413 | New primer[c] |
| | CJmapAN3R | | GCTTGGTGCGGATTGTAAA | | New primer[c] |
| *Campylobacter fetus* and *C. lanienae* primary multiplex | | | | | |
| 23S rRNA (*C. fetus*) | FET1 | 56 | CTCATAATTTAATTGCACTCATA | 784 | Bastyns et al. (3) |
| | HYOFET23SR | | GCTTCGCATAGCTAACAT | | New primer |
| 16S rRNA (*C. lanienae*) | CLAN76F | | GTAAGAGCTTGCTCTTATGAG | 920 | Logan et al. (31) |
| | CLANL521021R | | TC**G**TATCTCTA**CA**AGGTTCTTA[d] | | New primer; CLAN1021R modified from Logan et al. (31) |
| *Campylobacter fetus and C. lanienae* nested multiplex | | | | | |
| 23S rRNA (*C. fetus*) | FETNF | 56 | CGATAATTGATGTGAGAATCATC | 473 | New primer |
| | HYOFET23SR2 | | GGGAGTAAATCTTAATACAAAGTTAGG | | New primer |
| 16S rRNA (*C. lanienae*) | CLANNF | | TAGTTGGTGAGGTAATGGCTC | 360 | New primer |
| | CLANNR | | GCAGTTTAATGGTTGAGCCA | | New primer |
| *Campylobacter hyointestinalis* primary | | | | | |
| 23S rRNA | HYO1F | 54 | AT**AAT**CTAGGTGAGAATCCTAG[d] | 611 | New primer; HYO1 modified from Bastyns et al. (3) |
| | HYOFET23SR | | See HYOFET23SR above | | New primer |
| *Campylobacter hyointestinalis* seminested | | | | | |
| 23S rRNA | HYO1F | 54 | See HYO1F above | 468 | See above |
| | HYOFET23SR2 | | See HYOFET23SR2 | | New primer |

[a] Primary PCRs were also used to identify *Campylobacter* species isolated from feces.
[b] There was a typographical error in Linton et al. (29): primer 1288 should have read primer 1228.
[c] Developed with Primer3 software.
[d] Bold type indicates regions of the primer that were modified.

primary objective of this study was to develop a PCR-based method for detecting *Campylobacter* species directly in bovine feces without relying on an enrichment step. Specific objectives were to (i) develop primers and an internal control for amplifying *Campylobacter* DNA from feces, (ii) measure the sensitivity of PCR-based detection of four species of *Campylobacter* added to bovine feces, and (iii) compare PCR with conventional isolation for detecting campylobacters in bovine feces.

TABLE 2. Primer sequences used to identify *Campylobacter* species isolated from bovine feces

| PCR target and gene | Primer | $T_m$ (°C) | Sequence (5′ to 3′) | Size (bp) | Reference or source |
|---|---|---|---|---|---|
| *Arcobacter butzleri* 16S rDNA | BUTZ<br>ARCO | 61 | CCTGGACTTGACATAGTAAGAATGA<br>CGTATTCACCGTAGCATAGC | 401 | Houf et al. (19) |
| *Arcobacter cryaerophilus* 23S rDNA | CRY1<br>CRY2 | 61 | TGCTGGAGCGGATAGAAGTA<br>AACAACCTACGTCCTTCGAC | 257 | Houf et al. (19) |
| *Arcobacter skirrowii* 16S rDNA | SKIR<br>ARCO | 61 | GGCGATTTACTGGAACACA<br>See ARCO above | 641 | Houf et al. (19) |
| *Campylobacter helveticus* 16S rDNA | CHCU146F<br>CH1371R | 60 | GGGACAACACTTAGAAATGAG<br>CCGTGACATGGCTGATTCAC | 1225–1375 | Linton et al. (29) |
| *Campylobacter hyointestinalis* 16S rDNA | CFCH57F<br>CH1344R | 65 | GCAAGTCGAACGGAGTATTA<br>GCGATTCCGGCTTCATGCTC | 1,287 | Linton et al. (29) |
| *Campylobacter lari* 16S rDNA | CL594F<br>CL1155R | 64 | CAAGTCTCTTGTGAAATCCAAC<br>ATTTAGAGTGCTCACCCGAAG | 561 | Linton et al. (29) |
| *Campylobacter mucosalis* 23S rDNA | MUC1<br>MUC2 | 58 | ATGAGTAGCGATAATTGGG<br>ACAGTATCAAGGATTCGTC | 306 | Bastyns et al. (3) |
| *Campylobacter sputorum* 23S rDNA | SPUT1<br>SPUT2 | 58 | ATAAGTACCGAAGTCGTAGG<br>TCTAGGGCTTTAACACCC | 588 | Bastyns et al. (3) |
| *Campylobacter upsaliensis* 16S rDNA | CHCU146F<br>CU1024R | 60 | See CHCU146F above<br>CACTTCCGTATCTCTACAGA | 878 | Linton et al. (29) |
| Universal prokaryote 16S rRNA | UNI27F<br>UNI1492R | 50 | AGAGTTTGATCCTGGCTCAG<br>TACGG(C/T)TACCTTGTTACGACT | V[a] | Lane (24) |
| Universal prokaryote 16S rRNA | UNI338F<br>UNI1100R | 50 | ACTCCTACGGGAGGCAG<br>AGGGTTGCGCTCGTTG | V | Lane (24) |

[a] V, amplicon size was variable.

## MATERIALS AND METHODS

**Progression of experiments.** The experiments conducted for each objective are presented in Fig. 1. For the first objective (Fig. 1A), internal control DNA and primers for detecting *Campylobacter* species directly in feces were developed. For the second objective (Fig. 1B), the sensitivity of PCR for amplifying *Campylobacter* species directly from bovine feces inoculated with four taxa was compared to that of dilution plating. The efficacy of a commercial DNA extraction kit for removing PCR inhibitors also was ascertained by using internal control DNA designed for use with *Campylobacter* genus-specific primers. For the third objective (Fig. 1C), the detection of *Campylobacter* species in uninoculated bovine feces by PCR was compared to that by dilution plating. Due to the high frequency of isolation of *C. lanienae*, nested primers were developed for this taxon and subsequently used in a multiplex PCR with primers for *C. fetus*.

**Objective 1. (i) Test taxa and culture conditions.** Primers for detecting DNAs of the genus *Campylobacter*, *C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. jejuni* directly in bovine feces were developed (Table 1). We selected these taxa because of their association with bovine species. To increase sensitivity and specificity, primers for *C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. jejuni* were nested or seminested. Furthermore, *C. coli* and *C. jejuni* were tested by multiplex PCR.

The following reference strains were used to test the primers that we developed: *Arcobacter butzleri* (ATCC 49616 [American Type Culture Collection]), A. *cryaerophilus* (ATCC 49942), *C. coli* (HC 1111 [Health Canada] and ATCC 49941), *C. concisus* (ATCC 33237), *C. fetus* subsp. *fetus* (ATCC 25936), *C. fetus* subsp. *venerealis* (ATCC 19438), *C. hyointestinalis* subsp. *hyointestinalis* (ATCC 35217), *C. hyointestinalis* subsp. *lawsonii* (NCTC 12901 [National Collection of Type Cultures and Pathogenic Fungi]), *C. jejuni* (HC 1102, HC 1104, HC 1115, HC 1108, ATCC 29428, ATCC 49943, and ATCC 33291), *C. lanienae* (NCTC 13004), *C. lari* (ATCC 35221), and *C. upsaliensis* (LCDC 5424 [Laboratory Centre for Disease Control]). To obtain biomass, all isolates were grown on *Campylobacter* blood-free selective agar base (modified *Campylobacter* charcoal differential agar [CCDA]) (Oxoid, Nepean, Ontario, Canada) without antibiotic supplements or brucella agar (Difco, Detroit, Mich.) at 37°C in anaerobic gas jars (Oxoid). Microaerophilic conditions were generated with a CampyPak *Plus* microaerophilic system with a palladium catalyst (BBL, Becton Dickinson, Sparks, Md.).

**(ii) Genomic DNA extraction and PCR methods.** DNA was extracted from the reference strains by using a DNeasy kit (Qiagen Inc., Mississauga, Ontario, Canada) according to the manufacturer's protocol. The conditions for primary amplification were 1 cycle at 95°C for 15 min; 25 cycles of 30 s at 94°C, 90 s at the annealing temperature ($T_m$), and 60 s at 72°C; and extension for 10 min at 72°C. For multiplex reactions, mixtures consisted of a total volume of 20 μl containing reaction buffer, 0.2 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.5 μM each primer (Sigma-Genosys, Oakville, Ontario, Canada), 0.2 μg of bovine serum albumin (Promega, Madison, Wis.), and 1 U of HotStar *Taq* polymerase (Qiagen). Each PCR was performed with a total of 2 μl of a $10^{-5}$ dilution of genomic DNA (≈500 ng $\mu l^{-1}$). For nested and seminested amplifications, the reaction conditions were the same, with the exception that 35 cycles were used, 1 μl of the reaction mixture from the primary amplification step was used as a template, and bovine serum albumin was not included in the reaction mixture. All PCR products (10 μl) were electrophoresed in a Tris-borate-EDTA–2% agarose gel (Invitrogen Corp., Burlington, Ontario, Canada), visualized by staining with ethidium bromide, and viewed under UV light. A 100-bp ladder (Promega) was used to size products. The $T_m$s used and estimated product sizes are shown in Table 1.

**Objective 2. (i) Internal control construction.** An internal control designed to amplify under the same PCR conditions as those described for the *Campylobacter* genus-specific primer set was constructed by deleting a fragment of the *C. jejuni* (ATCC 49943) 16S rRNA gene by the strategy of Denis et al. (10). The deletion was achieved by PCR amplification of the 16S rRNA gene with mutagenic primer C1228RIC (5′-CATTGTAGCACGTGTGTCTCCCCAGGCGGTACACTTAATG-3′; the underlined sequence corresponds to C1228R). PCR amplification of the template with primers C412F and C1228RIC yielded a 475-bp product instead of an 816-bp product containing both the C412F and the C1228R primer sites. The 475-bp product was cloned into the pGEM-T EASY vector (Promega) and transformed into *Escherichia coli* JM109 cells. Transformed colonies were screened for the presence of an insert by PCR amplification with primers C412F

and C1228. Plasmid DNA was extracted with a QIAprep spin miniprep kit (Qiagen). The plasmid-IC was linearized by digestion with *Nco*I enzyme (Promega). The enzyme was removed by two extractions with Strataclean resin (Stratagene) according to the manufacturer's protocol. The concentration of linearized plasmid-IC was adjusted to 700 copies $\mu l^{-1}$ in 10 mM Tris-Cl (pH 8.5) buffer, and plasmid-IC was stored at −20°C until used.

**(ii) Extraction of DNA from bovine feces.** A QIAamp DNA stool minikit (Qiagen) was used to extract DNA from 200 ± 5 mg of bovine feces according to the manufacturer's protocol for the isolation of DNA from stools for pathogen detection. Briefly, the procedure involved lysis of the bacterial cells within the fecal material in ASL buffer, adsorption of impurities to InhibitEX reagent, and purification of the DNA on a spin column. Prior to extraction, the internal control was added to each sample at the rate of 10 μl per 200 mg of feces. Extracted DNA was stored at −20°C until processed.

**(iii) Inoculation.** Feces were collected from cattle in Lethbridge, Alberta, Canada. Fecal samples that were determined to be negative for *Campylobacter* DNA or that produced a weak amplicon with the *Campylobacter* genus-specific 16S rRNA gene primers were selected. Fecal samples that were free of *Campylobacter* DNA were infrequently obtained, and this situation necessitated the use of contaminated feces, albeit feces containing small amounts of campylobacters. Subsamples of fecal samples were stored at −20°C until required. Fecal samples were thawed once.

Isolates used to inoculate feces included *C. coli* ATCC 49941, *C. fetus* ATCC 25936, *C. hyointestinalis* ATCC 35217, and *C. jejuni* ATCC 49943 (i.e., taxon treatment). All bacteria were grown for 24 h at 37°C. In replicate 1, *C. coli*, *C. fetus*, and *C. jejuni* were grown on brucella agar, whereas *C. hyointestinalis* was grown on CCDA. In replicates 2 and 3, both *C. fetus* and *C. hyointestinalis* were grown on CCDA, and the other species were grown on brucella agar. In all instances, biomass production media were not amended with antibiotics. To obtain biomass, cells were scraped from the media and suspended in sterile brucella broth (Difco). The turbidity ($A_{600}$) of the suspension was adjusted to 0.5 (this represented a cell density of approximately $2 \times 10^9$ CFU $ml^{-1}$), and six 10-fold serial dilutions were prepared in brucella broth (i.e., inocula), representing a range of cell densities of approximately $10^3$ to $10^9$ CFU $ml^{-1}$ (i.e., density treatment). To enumerate cell densities in the suspensions, dilution spread plate counts were made on either brucella agar (*C. coli* and *C. jejuni*) or CCDA (*C. fetus* and *C. hyointestinalis*). Feces were inoculated with each taxon treatment at the rate of 2 ml per 18 g (wet weight) of feces for each density; sterile brucella broth was used for the control. Immediately after addition of the inocula, feces were thoroughly mixed with a metal spatula. The experiment was conducted on three separate occasions (i.e., replicates). For each replicate, two samples were prepared (i.e., subsamples).

**(iv) Detection of *Campylobacter* species by dilution plating.** To enumerate campylobacters in the inoculated feces by conventional microbiological plating, 2.5 g of feces was placed in 22.5 ml of phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer [pH 7.2]) in a 50-ml Falcon tube (DiaMed, Missassauga, Ontario, Canada), and the suspension was vortexed at the maximum setting for 1 min. The suspension was diluted in a 10-fold dilution series, and 100 μl was spread on CCDA containing selective supplement SR115E (Oxoid). Cultures were incubated microaerophilically at 37°C as described above. Colonies were enumerated at the dilution yielding 20 to 200 CFU after 48 h, and CFUs per gram of feces (fresh and dry weights) were calculated. To determine fecal dry weights, two aliquots of feces (approximately 20 g each) were dried at 50°C for 72 h. We observed no differences in water content (dry matter content ranged from 19.1 to 19.8%) among the fecal samples, and so the CFUs are presented per gram of fresh weight. A mean value (CFU per gram) for the two subsamples for each taxon treatment and density treatment was used to calculate the overall mean and SEM for the three replicates.

**(v) Direct detection of *Campylobacter* species by PCR.** DNA was extracted from the two subsamples for each taxon treatment and density treatment with the DNA stool minikit as described above. Amplification of *C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. jejuni* DNAs was achieved with the primary and nested or seminested PCR primers listed in Table 1. In all instances, the same reaction and amplification conditions as those described above were used, with the exception that 2 μl of fecal DNA was used as a template. The mean proportion of positive samples for the two subsamples for each taxon treatment and density treatment was used to calculate the overall mean and SEM ($n$ = 3).

**Objective 3. (i) Collection of feces.** Feces were aseptically collected from eight Holstein dairy cows on three separate occasions (i.e., replicates) at ca. 2-week intervals. All of the cattle used were early-lactation cows fed a total mixed ration consisting of barley, alfalfa, and corn silage. Fecal samples were obtained following the morning feeding.

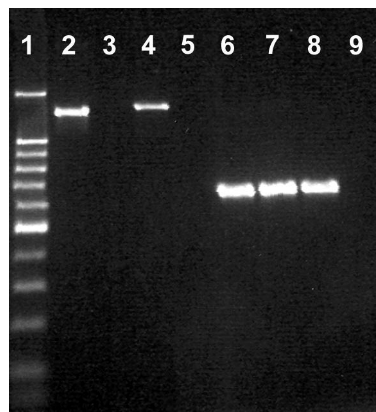


FIG. 2. Comparison of the 16S (29) and 23S rDNA primers developed in the current study for detecting *C. hyointestinalis*. Lane 1, 100-bp molecular weight marker (the dark band was at 500 bp); lane 2, 16S primers with *C. hyointestinalis* subsp. *hyointestinalis* HBF; lane 3, 16S primers with *C. hyointestinalis* subsp. *hyointestinalis* HBE; lane 4, 16S primers with *C. hyointestinalis* subsp. *hyointestinalis* ATCC 35217; lane 5, 16S primers with *C. hyointestinalis* subsp. *lawsonii* NCTC 12901; lane 6, 23S primers with *C. hyointestinalis* subsp. *hyointestinalis* HBF; lane 7, 23S primers with *C. hyointestinalis* subsp. *hyointestinalis* HBE; lane 8, 23S primers with *C. hyointestinalis* subsp. *hyointestinalis* ATCC 35217; lane 9, 23S primers with *C. hyointestinalis* subsp. *lawsonii* NCTC 12901. An amplicon was not observed in either of the negative control reactions, and so these treatments were removed.

**(ii) Detection of *Campylobacter* species by plating.** On each collection date, 2.5 g of feces from each cow was suspended in 22.5 ml of phosphate-buffered saline by vortexing as described above, and 100 μl of each suspension was spread on each of four test media. The media consisted of (i) Campy-Line agar (Dalynn Biologicals) (28); (ii) Karmali agar (Oxoid) (22) with selective supplement CM935 (Oxoid); (iii) CCDA with selective supplement SR115E; and (iv) Preston agar, comprised of CM689 campylobacter base (Oxoid), 50 ml of lysed horse blood (SR48; Oxoid) $liter^{-1}$, and selective supplement SR117E (Oxoid). Cultures were grown microaerophilically at 30, 35, and 40°C as described above. At 48- and 72-h intervals, CFUs of *Campylobacter* species were enumerated based on colony morphology and microscopic appearance. Arbitrarily selected colonies deemed to be campylobacters were transferred to the same medium from which they had been isolated and were streaked for purity. Colonies were selected based on colony appearance and frequency of occurrence. All strains were stored in brucella broth amended with 30% glycerol at −20 and −80°C until identified. In most instances, groupings based on colony appearance and morphology were determined to be monotaxic (based on the identification of representative strains), and the mean CFU per gram and SEM ($n$ = 3) were calculated for each taxon.

**(iii) Identification of *Campylobacter* species.** Presumptive identification of isolates recovered from bovine feces was accomplished with both physiological and molecular characters. All isolates were subjected to colony PCR with the *Campylobacter* genus-specific, *C. coli*-*C. jejuni*, *C. fetus*-*C. lanienae*, and *C. hyointestinalis* primary primer sets (Table 1). If required, *Campylobacter* isolates also were tested with primers for *C. helveticus*, *C. lari*, *C. mucosalis*, *C. sputorum*, and *C. upsaliensis* (Table 2). We also tested the *C. hyointestinalis* primer set of Linton et al. (29) (Table 2) but abandoned this primer set for a set described in Table 1. The same reaction and amplification conditions as those described above were used, with the exception that the DNA template consisted of 1 μl of a suspension containing 24- to 48-h-old cells of the isolate to be identified. For each isolate, cells from an individual colony were uniformly suspended in 100 μl of sterile brucella broth in 96-well microtiter plates with sterile toothpicks or pipette tips. Positive controls consisted of cells from reference strains, and the negative control consisted of brucella broth alone.

The abilities of strains to produce catalase and $H_2S$, to reduce nitrate and nitrite, and to hydrolyze hippurate and indoxyl acetate and their sensitivities to nalidixic acid (30 μg; BBL) and cephalothin (30 μg; BBL) were determined as described by Logan et al. (31). In addition, *C. fetus* strains were tested for their ability to grow on brucella agar containing 1% glycine. For *Arcobacter* strains, the ability to grow in atmospheric oxygen, on brucella agar containing 1% glycine,

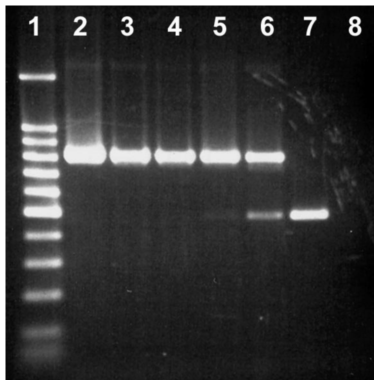


FIG. 3. Impact of *C. jejuni* L102 genomic DNA concentration on the expression of the internal control amplicon. Lane 1, 100-bp molecular weight marker (the dark band was at 500 bp); lane 2, $10^{-1}$ dilution; lane 3, $10^{-2}$ dilution; lane 4, $10^{-3}$ dilution; lane 5, $10^{-4}$ dilution; lane 6, $10^{-5}$ dilution; lane 7, no template; lane 8, no internal control.

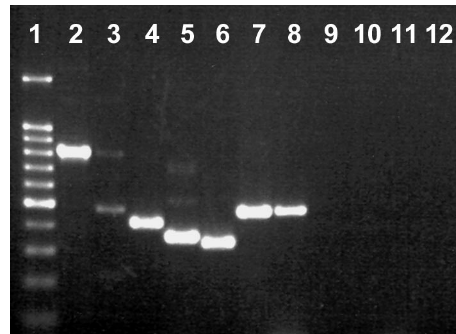


FIG. 4. Multiplex PCR for detection of *Campylobacter* species in bovine feces. Lane 1, 100-bp molecular weight marker (the dark band was at 500 bp); lane 2, DNA from uninoculated feces amplified with the *Campylobacter* genus-specific primer set; lane 3, DNA from uninoculated feces amplified with the *Campylobacter* genus-specific primer set (note the weak genus amplicon and the internal control amplicon at 465 bp); lane 4, DNA from uninoculated feces amplified with the *C. jejuni*-*C. coli* multiplex nested primer set (note the *C. jejuni* amplicon at 413 bp); lane 5, DNA from uninoculated feces amplified with the *C. fetus*-*C. lanienae* nested primer set (note the *C. lanienae* amplicon at 360 bp); lane 6, DNA from feces inoculated with *C. coli* ATCC 49941 at a density of $\approx 10^3$ CFU $g^{-1}$ and amplified with the *C. jejuni*-*C. coli* multiplex nested primer set (note the *C. coli* amplicon at 330 bp); lane 7, DNA from feces inoculated with *C. hyointestinalis* subsp. *hyointestinalis* ATCC 35217 at a density of $\approx 10^2$ CFU $g^{-1}$ and amplified with the *C. hyointestinalis* seminested primer set (note the *C. hyointestinalis* amplicon at 468 bp); lane 8, DNA from feces inoculated with *C. fetus* ATCC 25936 at a density of $\approx 10^2$ CFU $g^{-1}$ and amplified with the *C. fetus*-*C. lanienae* nested primer set (note the *C. fetus* amplicon at 473 bp); lane 9, negative control for the internal control and *Campylobacter* genus-specific primers (no template added); lane 10, negative control for *C. jejuni*-*C. coli* multiplex primers; lane 11, negative control for *C. fetus*-*C. lanienae* multiplex primers; lane 12, negative control for *C. hyointestinalis* subsp. *hyointestinalis* primers.

and on MacConkey agar was determined. For *C. jejuni* and *C. lanienae*, six and seven arbitrarily selected strains were subjected to physiological tests, respectively.

For isolates presumptively identified as *C. fetus*, *C. lanienae*, *A. butzleri*, or *A. skirrowii*, the complete or partial 16S rRNA gene was sequenced. The 16S rRNA gene was amplified by PCR with eubacterial primers UNI27F and UNI1492R (Table 2). For all amplifications, the same PCR mixtures and amplification conditions as those described above for colony PCR were used. To obtain a partial sequence for the 16S rRNA gene, primers UNI338F and UNI1100R were used (Table 2) with an ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, Calif.). Prior to sequencing, excess dye was removed with a Qiagen DyeEx spin kit. Sequences were obtained with an ABI PRISM 377 automated DNA sequencer. Contigs were constructed by using Staden (Medical Research Council, Laboratory of Molecular Biology, Cambridge, England), and all sequences were compared directly with the National Center for Biotechnology Information (NCBI) GenBank nonredundant nucleotide database by using BLASTN.

The nucleotide sequences for a presumptively identified isolate of *C. lanienae* (L52) were aligned with data retrieved from GenBank by using the multialignment program CLUSTAL W (40), and the alignments were refined visually by using GeneDoc (www.psc.edu/biomed/genedoc). Sequences (GenBank accession numbers) for *C. concisus* (L04322), *C. curvus* (L04313), *C. fetus* subsp. *fetus* (M65012), *C. fetus* subsp. *venerealis* (M65011), *C. gracilis* (L04320), *C. hyointestinalis* subsp. *hyointestinalis* (AF097681, AF097689 [type], and AF097691), *C. hyointestinalis* (AF219235 and M65010), *C. hyointestinalis* subsp. *lawsonii* (AF097683 and AF097685 [type]), *C. lanienae* (AB076675, AB076677, AF043423, and AF043425[type]), *C. mucosalis* (L06978), *C. rectus* (L04317), *C. sputorum* (L04319), and *C. jejuni* subsp. *jejuni* (L04315) were included in the analyses.

The sequence data were analyzed by using programs contained within PHYLIP (13). Phylogenetic estimates were based on methods for determining neighbor-joining distance, maximum parsimony, and maximum likelihood. Divergence (or distance) for each pair of sequences was calculated by using DNADIST with the Kimura two-parameter model. The NEIGHBOR program was used for estimating phylogenies from the distance matrices. DNAPARS was executed to perform maximum-parsimony analysis, and DNAML was used for maximum-likelihood analysis. Support for the internal branches within the resulting trees was obtained by bootstrap analysis. A total of 1,000 bootstrap replicates for the 16S ribosomal DNA (rDNA) data were generated by using SEQBOOT, majority-rule consensus trees were constructed by using the CONSENSE program, and the trees were visualized by using TreeView (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

**(iv) Direct detection of *Campylobacter* species by PCR.** For each cow on each occasion, an aliquot of feces was inoculated with the internal control, DNA was extracted with the DNA stool minikit, the PCR mixtures and amplification conditions were set up, and the PCR products were visualized as described above. Due to the frequent isolation of *C. lanienae* on Karmali agar, primers based on the 16S rRNA gene were developed for this taxon (Table 1) and used in a multiplex PCR with primers for *C. fetus*. The mean proportion of positive samples and SEM ($n$ = 3) were calculated for each taxon.

**Nucleotide sequence accession number.** The partial 16S rRNA gene sequence for *C. lanienae* L52 has been deposited in GenBank under accession number AY288304.

## RESULTS

**Objective 1. (i) Genus *Campylobacter*.** The genus-specific primer set based on the 16S rRNA gene (29) provided amplicon products for all of the taxa of campylobacters tested. In addition, we observed weak products for *A. butzleri* and *A. cryaerophilus*.

**(ii) *C. coli* and *C. jejuni*.** The primary primer sets for *C. coli* and *C. jejuni* were based on previously published results, and they were found to be highly specific. The nested primers that we designed for *C. jejuni* (i.e., *mapA*) were also highly specific, but the nested primers for *C. coli* (i.e., *ceuU*) did not confer specificity on their own. However, they also were found to be highly specific when used in conjunction with the primary primer set.

**(iii) *C. fetus*.** The *C. fetus* nested primer set based on the 23S rRNA gene was found to be highly specific for the species but did not distinguish between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. The nested primer set based on the 16S rRNA gene of *C. fetus* was also highly specific for *C. fetus* relative to other *Campylobacter* species in pure cultures. How-

TABLE 3. Microbiological isolation and direct PCR detection of *Campylobacter* populations in inoculated bovine feces

| Taxon and treatment[a] | Mean ± SEM log CFU $g^{-1}$ (fresh wt) ($n$ = 3)[b] | Mean ± SEM proportion of samples found positive by PCR for: | | | |
|---|---|---|---|---|---|
| | | Genus[c] | *C. coli* and *C. jejuni*[d] | *C. fetus* and *C. lanienae*[e] | *C. hyointestinalis*[f] |
| *Campylobacter coli* | | | | | |
| A | 8.19 ± 0.043 | 1.0 | 1.0 | —[g] | — |
| B | 7.21 ± 0.038 | 1.0 | 1.0 | — | — |
| C | 6.27 ± 0.061 | 1.0 | 1.0 | — | — |
| D | 5.28 ± 0.056 | 1.0 | 1.0 | — | — |
| E | 4.17 ± 0.084 | 1.0 | 1.0 | — | — |
| F | 3.28 ± 0.087 | 0.50 ± 0.29 | 0.83 ± 0.17 | — | — |
| G | 1.98 ± 0.490 | 0.67 ± 0.17 | 0.0 | — | — |
| N | 0.0 | 0.50 ± 0.29 | 0.0 | — | — |
| *Campylobacter fetus* | | | | | |
| A | 7.97 ± 0.030 | 1.0 | — | 1.0 | — |
| B | 7.09 ± 0.065 | 1.0 | — | 1.0 | — |
| C | 6.04 ± 0.037 | 1.0 | — | 1.0 | — |
| D | 5.08 ± 0.015 | 1.0 | — | 1.0 | — |
| E | 4.07 ± 0.004 | 1.0 | — | 1.0 | — |
| F | 3.13 ± 0.103 | 1.0 | — | 0.50 ± 0.29 | — |
| G | 2.48 ± 0.059 | 0.33 ± 0.33 | — | 0.17 ± 0.17 | — |
| N | 0.0 | 0.33 ± 0.17 | — | 0.0 | — |
| *Campylobacter hyointestinalis* | | | | | |
| A | 7.73 ± 0.080 | 1.0 | — | — | 1.0 |
| B | 6.52 ± 0.129 | 1.0 | — | — | 1.0 |
| C | 5.58 ± 0.157 | 1.0 | — | — | 1.0 |
| D | 4.79 ± 0.013 | 1.0 | — | — | 1.0 |
| E | 3.67 ± 0.037 | 1.0 | — | — | 1.0 |
| F | 2.95 ± 0.051 | 1.0 | — | — | 0.67 ± 0.33 |
| G | 2.13 ± 0.069 | 0.50 ± 0.29 | — | — | 0.50 ± 0.29 |
| N | 0.0 | 0.67 ± 0.33 | — | — | 0.0 |
| *Campylobacter jejuni* | | | | | |
| A | 8.14 ± 0.071 | 1.0 | 1.0 | — | — |
| B | 7.24 ± 0.029 | 1.0 | 1.0 | — | — |
| C | 6.21 ± 0.053 | 1.0 | 1.0 | — | — |
| D | 5.28 ± 0.030 | 1.0 | 1.0 | — | — |
| E | 4.28 ± 0.012 | 1.0 | 1.0 | — | — |
| F | 3.38 ± 0.091 | 0.83 ± 0.17 | 0.67 ± 0.17 | — | — |
| G | 2.49 ± 0.114 | 0.67 ± 0.17 | 0.16 ± 0.17 | — | — |
| N | 0.0 | 0.83 ± 0.17 | 0.0 | — | — |

[a] Treatments consisted of inoculation of feces with different concentrations of the target bacterium (10-fold dilutions); treatment N was uninoculated (control).
[b] Feces content ranged from 19.1% ± 0.80% to 19.8% ± 0.36% dry matter.
[c] Nonnested *Campylobacter* genus-specific primer set targeting the 16S rRNA gene.
[d] Nested multiplex primer sets for *C. coli* and *C. jejuni* targeting the *ceuE* and *mapA* genes, respectively.
[e] Nested multiplex primer sets for *C. fetus* and *C. lanienae* targeting the 23S and 16S rRNA genes, respectively.
[f] Seminested primer set for *C. hyointestinalis* targeting the 23S rRNA gene.
[g] —, not determined.

ever, this primer set was subsequently observed to provide an amplicon of the correct size (552 bp) for all bovine fecal samples. The sequence of this PCR product exhibited relatively poor similarity with the *C. fetus* 16S rRNA sequences deposited at NCBI (GenBank accession numbers AF219234, AF219233, and AJ306569), and this primer set was abandoned in favor of the primer set targeting the 23S rRNA gene.

**(iv) *C. hyointestinalis*.** One of 16S rRNA primers for *C. hyointestinalis* used by Linton et al. (29) binds in a polymorphic region (18), and we observed that these primers did not provide a PCR product for some of the isolates of *C. hyointestinalis* obtained from bovine feces at Lethbridge in a previous study (Fig. 2). To increase the comprehensiveness of detection, we modified a forward primer based on the 23S rRNA gene (HYO1F) from that of Bastyns et al. (3). We designed a new reverse primer (HYOFET23SR) to replace the reverse primer (69ar) used by Bastyns et al. (3); many of the sequences for the 23S rRNA gene of *C. hyointestinalis* did not include the binding region for 69ar. For the secondary reaction, a seminested primer set (HYO1F and HYOFET23SR2) was used. Both the primary and the seminested primer sets were found to be highly specific for all of the test strains of *C. hyointestinalis* subsp. *hyointestinalis*. Neither primer set provided an amplicon for *C. hyointestinalis* subsp. *lawsonii* (Fig. 2).

**Objective 2. (i) DNA extraction.** Genomic DNA was obtained from all fecal samples by using the DNA stool minikit. The concentration of *Campylobacter* DNA was observed to affect the intensity of the internal control amplicon (Fig. 3). However, in the minimum detection experiment (i.e., feces inoculated with various concentrations of *C. coli*, *C. jejuni*, *C.*

TABLE 4. Isolation of campylobacters from bovine feces[a]

| Taxon and medium | Temp (°C)[b] | Mean $\log_{10}$ CFU $g^{-1}$ from the following cow: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 593 | 790 | 791 | 842 | 850 | 870 | 985 | 986 |
| *Campylobacter jejuni* | | | | | | | | | |
| Campy-Line | 35 | 0.0 (0) | 1.23 (1) | 1.24 (1) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Karmali | 35 | 0.0 (0) | 2.82 (2) | 1.21 (1) | 1.49 (2) | 0.0 (0) | 2.14 (2) | 0.0 (0) | 0.0 (0) |
| CCDA | 35 | 0.0 (0) | 2.72 (2) | 1.26 (1) | 0.67 (1) | 0.0 (0) | 2.35 (2) | 0.0 (0) | 0.0 (0) |
| Preston | 35 | 0.0 (0) | 2.87 (2) | 1.39 (1) | 0.0 (0) | 0.0 (0) | 2.33 (2) | 0.0 (0) | 0.0 (0) |
| Campy-Line | 40 | 0.0 (0) | 2.84 (2) | 1.97 (2) | 0.67 (1) | 0.0 (0) | 1.77 (2) | 0.0 (0) | 0.0 (0) |
| Karmali | 40 | 0.0 (0) | 2.93 (2) | 2.12 (2) | 0.93 (1) | 0.0 (0) | 3.11 (3) | 0.0 (0) | 0.0 (0) |
| CCDA | 40 | 0.0 (0) | 2.82 (2) | 1.37 (1) | 0.83 (1) | 0.0 (0) | 3.56 (3) | 0.0 (0) | 0.0 (0) |
| Preston | 40 | 0.0 (0) | 2.98 (2) | 1.35 (1) | 0.77 (1) | 0.0 (0) | 3.70 (3) | 0.67 (1) | 0.0 (0) |
| *Campylobacter lanienae* | | | | | | | | | |
| Campy-Line | 35 | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Karmali | 35 | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| CCDA | 35 | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Preston | 35 | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Campy-Line | 40 | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Karmali | 40 | 4.83 (3) | 1.43 (1) | 2.98 (2) | 0.0 (0) | 2.61 (2) | 3.23 (2) | 3.16 (2) | 0.0 (0) |
| CCDA | 40 | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |
| Preston | 40 | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) |

[a] One isolate of *C. fetus* from cow 870 was recovered on CCDA at 40°C. Two isolates of *A. skirrowii* from cow 842 were recovered on Karmali agar at 40°C. One isolate of *A. butzleri* from cow 842 was recovered on Karmali agar at 35°C. Three replicates were conducted at ca. 2-week intervals; numbers in parentheses indicate the number of positive replicates.

[b] No isolates of *Campylobacter* or *Arcobacter* were recovered at 30°C.

*fetus*, and *C. hyointestinalis*), the 475-bp internal control amplicon was always observed in fecal samples for which no or a weak PCR product (816 bp) was detected by the *Campylobacter* genus-specific primer set (Fig. 4).

**(ii) Detection of campylobacters in bovine feces.** No amplification products were detected for the controls (uninoculated feces) for *C. coli*, *C. fetus*, *C. jejuni*, and *C. hyointestinalis* (Table 3). For all four taxa, all of the samples of feces inoculated at $\approx 10^4$ CFU $g^{-1}$ were positive (range of 3.67 ± 0.037 to 4.28 ± 0.012 log CFU $g^{-1}$ [mean and standard error of the mean]). At $\approx 10^3$ CFU $g^{-1}$, 83% ± 17%, 50% ± 29%, 67% ± 17%, and 67% ± 33% of the samples of feces were positive for *C. coli*, *C. fetus*, *C. jejuni*, and *C. hyointestinalis*, respectively (range of 2.95 ± 0.051 to 3.38 ± 0.091 log CFU $g^{-1}$). *C. coli* was not detected at a density of $\approx 10^2$ CFU $g^{-1}$. In contrast, 17% ± 17%, 17% ± 17%, and 50% ± 29% of the samples of feces inoculated with *C. fetus*, *C. jejuni*, and *C. hyointestinalis*, respectively, provided an amplification product at this population density (range of 2.13 ± 0.069 to 2.49 ± 0.114 log CFU $g^{-1}$).

Amplification of *Campylobacter* DNA (with the nonnested genus-specific primer set) was observed for all fecal samples inoculated with $\approx 10^4$ CFU $g^{-1}$ (Table 3). At a density of $\approx 10^2$ to $10^3$ CFU $g^{-1}$, *Campylobacter* DNA was detected in 33% ± 33% to 100% of the samples. Although DNAs of *C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. jejuni* were not detected in uninoculated feces, 33% ± 17% to 83% ± 17% of these samples were observed to be positive for *Campylobacter* DNA.

**Objective 3. (i) Detection of *Campylobacter* species by plating.** Considerable experience and diligence were frequently required to detect *Campylobacter* colonies on the test media. A total of 198 arbitrarily selected *Campylobacter* isolates were recovered from uninoculated bovine feces. Considerable variability was observed in the frequencies of isolation of *C. jejuni* and of presumptively identified *C. lanienae* isolates with the four media and incubation temperatures tested (Table 4). At 30°C and, to a lesser extent, at 35°C, considerable fungal contamination was encountered, making enumeration of and isolation of possible campylobacters at 72 h even more difficult; the most prevalent fungus observed was *Geotrichum candidum* (11). We did not observe any campylobacters at 30°C. In contrast, considerable numbers of *C. jejuni* isolates were recovered on all four media at 35 and 40°C. All isolates, including the *C. jejuni* reference strain, amplified with the *mapA* (*C. jejuni*) primer set and arbitrarily selected isolates (L36, L42, L69, L75, L92, and L97) all were able to produce catalase, did not produce $H_2S$, reduced nitrate but not nitrite, hydrolyzed hippurate and indoxyl acetate, were sensitive to nalidixic acid, and were resistant to cephalothin (Table 5). The Campy-Line medium was less effective at recovering *C. jejuni* than the other three media tested at 35°C. Of the seven fecal samples that were positive for *C. jejuni* at 35°C (isolated on at least one medium), two (29%), seven (100%), six (86%), and five (71%) samples were positive on Campy-Line, Karmali, CCDA, and Preston media, respectively. At 40°C, the media produced very similar results, and the efficiency of isolation ranged from 78 to 89%.

Considerable numbers of presumptively identified *C. lanienae* isolates also were recovered from test cattle, but only on Karmali medium at 40°C (Table 4). All of these isolates and the *C. lanienae* reference strain were amplified with the primer set for the *C. lanienae* 16S rRNA gene (Fig. 5) but not with primer sets for other *Campylobacter* species. In addition, the reference strain of *C. hyointestinalis* subsp. *lawsonii* (NCTC 12901) but not that of *C. hyointestinalis* subsp. *hyointestinalis* (ATCC 35217) was amplified by the primer set for the *C. lanienae* 16S rRNA gene (Fig. 5). Seven arbitrarily selected isolates of *C. lanienae* (L52, L54, L83, L85, L71, L90, and L94) from bovine feces produced catalase, did not produce $H_2S$,

TABLE 5. Results of physiological and molecular typing of arbitrarily selected isolates of *Arcobacter* and *Campylobacter* recovered from bovine feces

| Source and taxon (isolate) | Result[a] for: | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Catalase | $H_2S$ | Nitrate | Nitrite | Hippurate | Indoxyl acetate | Nalidixic acid | Cephalothin | Glycine (1%) | MacConkey agar | $O_2$ | PCR[b] | 16S[c] |
| Bovine feces | | | | | | | | | | | | | |
| *A. butzleri* (L111) | w | − | + | − | − | + | R | R | + | + | + | *A. butzleri* | + |
| *A. skirrowii* (L109) | + | − | + | − | − | + | S | S | + | − | + | *A. skirrowii* | + |
| *A. skirrowii* (L110) | + | − | + | − | − | + | S | S | + | − | + | *A. skirrowii* | + |
| *C. fetus* subsp. *fetus* (L49) | + | − | + | − | − | − | R | S | + | ND | ND | *C. fetus* | + |
| *C. jejuni* (L36) | + | − | + | − | + | + | S | R | ND | ND | ND | *C. jejuni* | |
| *C. jejuni* (L42) | + | − | + | − | + | + | S | R | ND | ND | ND | *C. jejuni* | |
| *C. jejuni* (L69) | + | − | + | − | + | + | S | R | ND | ND | ND | *C. jejuni* | |
| *C. jejuni* (L75) | + | − | + | − | + | + | S | R | ND | ND | ND | *C. jejuni* | |
| *C. jejuni* (L92) | + | − | + | − | + | + | S | R | ND | ND | ND | *C. jejuni* | |
| *C. jejuni* (L97) | + | − | + | − | + | + | S | R | ND | ND | ND | *C. jejuni* | |
| *C. lanienae* (L52) | + | − | + | −[d] | − | − | R | S | ND | ND | ND | *C. lanienae* | + |
| *C. lanienae* (L54) | + | − | + | −[d] | − | − | R | S | ND | ND | ND | *C. lanienae* | + |
| *C. lanienae* (L83) | + | − | + | −[d] | − | − | R | S | ND | ND | ND | *C. lanienae* | |
| *C. lanienae* (L85) | + | − | + | −[d] | − | − | R | S | ND | ND | ND | *C. lanienae* | |
| *C. lanienae* (L71) | + | − | + | −[d] | − | − | R | S | ND | ND | ND | *C. lanienae* | |
| *C. lanienae* (L90) | + | − | + | −[d] | − | − | R | S | ND | ND | ND | *C. lanienae* | |
| *C. lanienae* (L94) | + | − | + | −[d] | − | − | R | S | ND | ND | ND | *C. lanienae* | |
| Reference strains | | | | | | | | | | | | | |
| *A. butzleri* ATCC 49616 | w | − | + | − | − | + | S | S[e] | + | + | + | *C. butzleri* | |
| *C. fetus* subsp. *fetus* ATCC 25936 | + | − | + | + | − | − | R | S | + | ND | ND | *C. fetus* | |
| *C. fetus* subsp. *venerealis* ATCC 19438 | + | − | + | + | − | − | R | S | + | ND | ND | *C. fetus* | |
| *C. hyointestinalis* ATCC 35217 | + | + | + | +[f] | − | − | R | S | ND | ND | ND | *C. hyointestinalis* | |
| *C. jejuni* ATCC 49943 | + | − | + | − | + | + | S | R | ND | ND | ND | *C. jejuni* | |
| *C. lanienae* NCTC 13004 | + | − | + | −[d] | − | − | R | R | ND | ND | ND | *C. lanienae* | |

[a] w, weak; +, positive; −, negative; R, resistant; S, susceptible; ND, not determined.
[b] Identification based on species-specific primers.
[c] Identification confirmed by using the sequence of the 16S rRNA gene.
[d] Logan et al. (31) reported that *C. lanienae* was able to reduce nitrite.
[e] Vandamme et al. (42) reported that 17% of *A. butzleri* isolates were susceptible to cephalothin.
[f] Vandamme and De Ley (41) reported that *C. hyointestinalis* was not able to reduce nitrite.

reduced nitrate but not nitrite, did not hydrolyze hippurate or indoxyl acetate, were resistant to nalidixic acid, and were sensitive to cephalothin (Table 5). The reference strain of *C. lanienae* exhibited the same physiological profile as the isolates from bovine feces, with the exception that it was resistant to cephalothin. Contrary to our findings, Logan et al. (31) reported that *C. lanienae* was able to reduce nitrite.

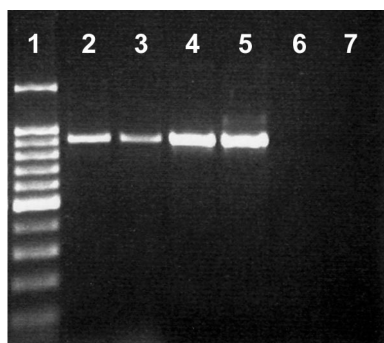


FIG. 5. Amplification of *C. lanienae* isolates with the 16S rRNA gene primers (31). Lane 1, 100-bp molecular weight marker (the dark band was at 500 bp); lane 2, *C. lanienae* L52; lane 3, *C. lanienae* L54; lane 4, *C. lanienae* NCTC 13004; lane 5, *C. hyointestinalis* subsp. *lawsonii* NCTC 12901; lane 6, *C. hyointestinalis* subsp. *hyointestinalis* ATCC 35217; lane 7, negative control.

A comparison of two isolates from bovine feces (L52 and L54) indicated that they were 100% similar for the partial 16S rRNA gene. A comparison with other *C. lanienae* strains deposited at NCBI (GenBank accession numbers AB076675, AB076677, AF043423, and AF043425) indicated 98% identity. However, we detected three polymorphic bases that occurred in the annealing region of the reverse primer of Logan et al. (31), accounting for the weaker amplification products that we observed for the *C. lanienae* isolates from bovine feces (Fig. 5).

Multiple-sequence analysis was used to produce neighbor-joining, maximum-parsimony, and maximum-likelihood trees based on the 16S rRNA gene sequence of *C. lanienae*. The trees produced by the neighbor-joining, maximum-parsimony, and maximum-likelihood methods grouped L52 with DNA sequences obtained for other *C. lanienae* strains, but there was only moderate bootstrap support (42 to 59%) for this clade (Fig. 6). For all of the phylogenetic models tested, the *C. lanienae* clade was disparate from the *C. hyointestinalis* strains. However, the closest relationship was with the *C. hyointestina-*

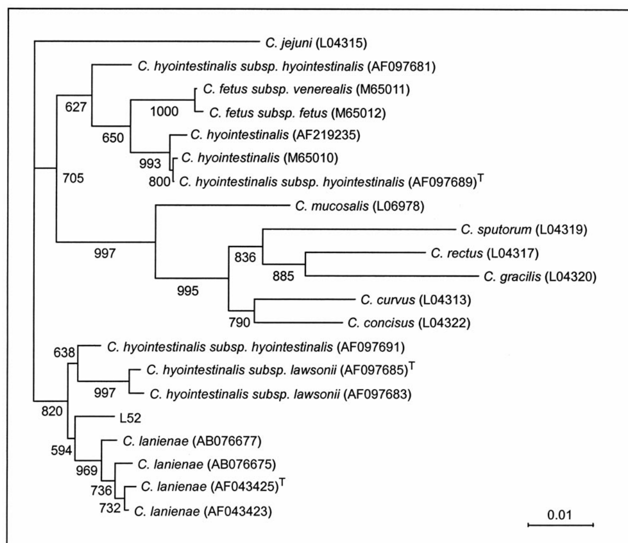


FIG. 6. Dendrogram based on a majority-rule consensus tree obtained from analyzing the partial 16S rRNA gene data set with the NEIGHBOR program (neighbor-joining option) and showing DNA sequence relatedness for campylobacters. The outgroup used in the analysis was *C. jejuni*, and isolates indicated by "T" represent type specimens. The bar represents 0.01 nucleotide substitution per base, and numbers at selected nodes indicate support obtained by bootstrap analysis (1,000 replicates) for the internal branches within the resulting trees.

*lis* subsp. *lawsonii* clade, which included one strain of *C. hyointestinalis* subsp. *hyointestinalis* (GenBank accession number AF097691). The other *C. hyointestinalis* strains included in the analysis formed a clade, but a clear relationship between the two subspecies of *C. hyointestinalis* was not resolved.

One isolate of *C. fetus* (L49) from cow 870 was recovered on CCDA at 40°C. This isolate, along with a *C. fetus* reference strain, was amplified with the *C. fetus* 23S rRNA primer set but not with primer sets for other species (Fig. 4). The two reference strains and the isolate of *C. fetus* from bovine feces produced catalase, did not produce $H_2S$, reduced nitrate but not nitrite, did not hydrolyze hippurate or indoxyl acetate, were resistant to nalidixic acid, and were sensitive to cephalothin (Table 5). Like the reference strain of *C. fetus* subsp. *fetus*, L49 exhibited positive growth on brucella agar containing 1% glycine. Analysis of the 16S rRNA gene sequence for L49 indicated 98 to 99% identity with *C. fetus* subsp. *venerealis* (GenBank accession number AF482990) and *C. fetus* subsp. *fetus* (GenBank accession numbers AF219234, AF219233, and AJ306569) sequences deposited at NCBI.

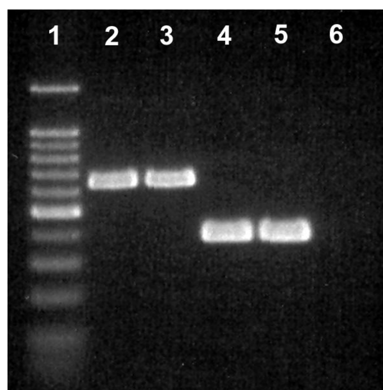


FIG. 7. Multiplex PCR of 16S and 23S rRNA genes for distinguishing *Arcobacter* species (19). Lane 1, 100-bp molecular weight marker ladder (the dark band was at 500 bp); lane 2, *A. skirrowii* L109 from bovine feces; lane 3, *A. skirrowii* L110 from bovine feces; lane 4, *A. butzleri* L111 from bovine feces; lane 5, *A. butzleri* ATCC 49616; lane 6, negative control.

Three isolates from cow 842 that were recovered on Karmali agar provided relatively weak amplicon bands with the *Campylobacter* genus-specific primer set and did not provide an amplification product with any of the *Campylobacter* species-specific primer sets. Multiplex PCR indicated that isolates L109 and L110 were *A. skirrowii* and that isolate L111 was *A. butzleri* (Fig. 7); the two presumptively identified *A. skirrowii* isolates were recovered on Karmali agar at 40°C, and the *A. butzleri* isolate was recovered on Karmali agar at 35°C. Analysis of the 16S rRNA genes for these three isolates indicated that L109 and L110 possessed 96% identity with *A. skirrowii* and that L111 possessed 99% identity with *A. butzleri*.

**(ii) Direct detection of *Campylobacter* species by PCR.** *Campylobacter* species DNA was detected in 75% ($n$ = 18) of the fecal samples tested, but none of the fecal samples obtained from cow 986 was positive (Table 6). In no instance was DNA from either *C. coli*, *C. fetus*, or *C. hyointestinalis* detected in bovine feces. In contrast, 25% ($n$ = 4) of the fecal samples were positive for *C. jejuni*, and 67% ($n$ = 16) of the samples were positive for *C. lanienae*. Relative to the efficacy of isolation on microbiological media (combined media and incubation temperatures), PCR was more sensitive for total campylobacters and *C. lanienae* in 8 and 17% of the fecal samples, respectively. Conversely, microbiological isolation was more sensitive than PCR for *C. jejuni* in 13% of the samples.

## DISCUSSION

We isolated *C. jejuni*, *C. fetus* subsp. *fetus*, *C. lanienae*, *A. butzleri*, and *A. skirrowii* from the feces of dairy cows. Interestingly, we detected multiple species (≥2) in feces from five of eight cows. To our knowledge, this is the first report of *C. lanienae* associated with a livestock source; this bacterium was first isolated from humans working in abattoirs in Switzerland that had been exposed to cattle carcasses, but its pathogenicity or virulence in humans has not been ascertained (31). Although we found some disparity, the isolates that we obtained from cattle feces clustered with known strains of *C. lanienae* based on the 16S rRNA gene. Interestingly, *C. hyointestinalis* subsp. *lawsonii* was observed to be more closely related to *C. lanienae* than it was to many strains of *C. hyointestinalis* subsp. *hyointestinalis* based on 16S rDNA sequences. The relatedness of these two taxa was further supported by our observation of positive amplification of *C. hyointestinalis* subsp. *lawsonii* but not *C. hyointestinalis* subsp. *hyointestinalis* with the *C. lanienae* primer set. Logan et al. (31) did not include *C. hyointestinalis* subsp. *lawsonii* in their description of *C. lanienae* as a new species, and Harrington and On (18) did not include *C. lanienae* in their phylogenetic analysis of *C. hyointestinalis* strains. The type specimen of *C. hyointestinalis* subsp. *lawsonii* (Gen-

TABLE 6. Microbiological isolation and PCR detection of campylobacters in bovine feces by replicate[a]

| Taxon and method | Positive results from the following cow: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 593 | 790 | 791 | 842 | 850 | 870 | 985 | 986 |
| Genus *Campylobacter* | | | | | | | | |
| Campy-Line | 0-0-0 | 1-1-0 | 0-1-1 | 0-1-0 | 0-0-0 | 1-1-0 | 0-0-0 | 0-0-0 |
| Karmali | 1-1-1 | 1-1-0 | 0-1-1 | **1**-1-**1** | 0-1-0 | 1-1-1 | 0-1-1 | 0-0-0 |
| CCDA | 0-0-0 | 1-1-0 | 0-1-0 | **1**-1-0 | 0-0-0 | 1-1-1 | 0-0-0 | 0-0-0 |
| Preston | 0-0-0 | 1-1-0 | 0-1-0 | 0-1-0 | 0-0-0 | 1-1-1 | 0-1-0 | 0-0-0 |
| PCR—*Campylobacter* | 1-1-1 | 1-1-**1** | 0-1-1 | 0-1-0 | **1**-1-**1** | 1-1-1 | **1**-1-1 | 0-0-0 |
| PCR—internal control | 0-0-0 | 0-0-0 | 1-0-0 | 1-0-1 | 0-0-0 | 0-0-0 | 0-0-0 | 1-1-1 |
| *Campylobacter jejuni* | | | | | | | | |
| Campy-Line | 0-0-0 | 1-1-0 | 0-1-**1** | 0-**1**-0 | 0-0-0 | 1-1-0 | 0-0-0 | 0-0-0 |
| Karmali | 0-0-0 | 1-1-0 | 0-1-**1** | **1**-**1**-0 | 0-0-0 | 1-1-1 | 0-0-0 | 0-0-0 |
| CCDA | 0-0-0 | 1-1-0 | 0-1-0 | **1**-**1**-0 | 0-0-0 | 1-1-1 | 0-0-0 | 0-0-0 |
| Preston | 0-0-0 | 1-1-0 | 0-1-0 | 0-**1**-0 | 0-0-0 | 1-1-1 | 0-**1**-0 | 0-0-0 |
| PCR—*C. jejuni* | 0-0-0 | 1-1-0 | 0-1-0 | 0-0-0 | 0-0-0 | 1-1-1 | 0-0-0 | 0-0-0 |
| *Campylobacter lanienae* | | | | | | | | |
| Campy-Line | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 |
| Karmali | 1-1-1 | 0-1-0 | 0-1-1 | 0-0-0 | 0-1-1 | 1-1-0 | 0-1-1 | 0-0-0 |
| CCDA | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 |
| Preston | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 | 0-0-0 |
| PCR—*C. lanienae* | 1-1-1 | 0-1-**1** | 0-1-1 | 0-0-0 | **1**-1-1 | 1-1-**1** | **1**-1-1 | 0-0-0 |

[a] No *C. coli*, *C. fetus,* or *C. hyointestinalis* was detected by PCR. For all media, results are compiled across the temperature treatment. Data represent a positive result for each of three replicates conducted over time (i.e., replicate 1 [R1]-R2-R3), where 1 is positive and 0 is negative. Bold type indicates a discrepancy between the results of the isolation and PCR detection methods. In all instances when there was a positive result for isolation and a negative result for PCR, very small numbers of CFU were recovered (<6).

Bank accession number AF097685) possesses 97% rDNA sequence identity with the type specimen of *C. lanienae* (GenBank accession number AF043425), and 95% identity with the type specimen of *C. hyointestinalis* subsp. *hyointestinalis* (GenBank accession number AF09769). Furthermore, considerable variation in 16S rDNA sequences was observed within the seven strains of *C. hyointestinalis* examined in the current study, and the phylogenetics of this species may require revision. For example, should *C. lanienae* be lowered to a subspecies of *C. hyointestinalis*, or should *C. hyointestinalis* subsp. *lawsonii* be elevated to species status?

Extreme care had to be taken to detect colonies of *Campylobacter* on the test media. Furthermore, considerable variability was observed in the frequency of isolation of species among the four media and incubation temperatures tested. The primary taxa that we recovered were *C. jejuni* and *C. lanienae*, but *C. fetus* subsp. *fetus*, *A. butzleri*, and *A. skirrowii* also were isolated infrequently. The media that we used were primarily developed for selectively isolating *C. jejuni* and *C. coli*, and the ineffectiveness of these media for the isolation of other taxa of campylobacters has been well documented by others (1, 4, 12, 37). *C. hyointestinalis* and *C. coli* are often isolated from bovine feces at low frequencies (1), and we have previously isolated these taxa on CCDA in Alberta, Canada. However, neither of these bacteria was recovered in the current study, a result which was likely a function of the small number of animals sampled and not of the isolation media that we used. We included 30 and 35°C incubation treatments in an attempt to facilitate the isolation of nonthermophilic taxa, such as *C. fetus*. We only recovered one isolate of *C. fetus* subsp. *fetus* on CCDA at 40°C. This finding contrasts with the much higher frequency of isolation of *C. fetus* subsp. *fetus* from bovine fecal samples by others. For example, Atabay and Corry (1) isolated this bacterium in feces from 11% of the dairy cattle they tested. Unfortunately, the effect of temperature on isolation frequency was confounded by the extensive growth of fungal contaminants. The most prevalent fungus that we observed on all four media was *G. candidum*, an arthroconidial fungus that is able to grow in low-oxygen environments (11). This fungus possesses a cosmopolitan distribution, it is commonly associated with feces, and it is a human pathogen causing geotrichoses affecting oral, bronchial, and bronchopulmonary epithelia and/or skin. In the current study, we relied on direct plating of feces and did not use enrichment methods which have been shown to be effective in selectively isolating a number of *Campylobacter* taxa from bovine feces, including *C. sputorum* (1). While enrichment methods may facilitate the isolation of some taxa, the enrichment medium used also is selective and will discriminate against some taxa.

As a result of the fastidiousness and diversity of campylobacters and arcobacters, it is clear that no one medium will provide an accurate measure of their occurrence. Furthermore, inexperienced personnel can easily overlook *Campylobacter* colonies, and species may be missed, particularly rarely occurring taxa that do not produce distinct colonies. For this reason, the use of PCR-based detection methods is very attractive. However, a variety of PCR inhibitors occur in feces, and the presence of these inhibitory compounds may inhibit the PCR and provide false-negative results (2, 7, 47). The QIAamp DNA stool minikit possesses a polysaccharide mixture that is used to remove PCR inhibitors of fecal origin, and it was found to provide the best performance in PCR for human feces compared to three other commercial kits (32). To determine whether PCR inhibitors have been removed, a number of researchers have designed internal primers and added them to feces prior to DNA extraction (33, 35, 39). We de-

signed our internal control based on the strategy of Denis et al. (10), but our internal control was designed to amplify with the *Campylobacter* genus-specific primer set. In all instances, we observed either amplification of the internal control or *Campylobacter* DNA from extracted bovine feces, indicating that the DNA stool minikit sufficiently removed PCR inhibitors present in the feces.

Considerable variation in the consistency (e.g., water content and presence of mucus) and composition (e.g., fiber versus grain) of the cattle feces used in the current study were observed, and these and other variables may have an impact on the efficacy of inhibitor removal. We used the amount of feces recommended by the manufacturer (200 mg [fresh weight]). However, very little is known about the spatial distribution of bacteria in feces, and if campylobacters are aggregated, the small amount of feces sampled may provide an erroneous measure of *Campylobacter* species prevalence. It may be possible to increase the amount of feces sampled, but increased sample size may overload the purification step. Complex polysaccharides are commonly encountered inhibitors of PCR found in feces (33). Furthermore, polyphenolic compounds of plant origin can be very inhibitory to PCR (23), and the high plant content of cattle feces would be expected to contribute large quantities of both complex polysaccharides and polyphenolic compounds, a factor which may limit the biomass of feces that can be efficaciously processed by the QIAamp DNA stool minikit. This possibility remains to be determined.

The quality of the extracted DNA is only one aspect of achieving highly specific and sensitive detection of a target bacterium. The amount of DNA present is also important, and nested PCR has been used with various degrees of success to increase the sensitivity of detection, particularly for genes with one or a small number of copies (21, 32, 36, 43). We observed that the nested and seminested primers were highly sensitive ($10^2$ to $10^3$ CFU $g^{-1}$) in detecting *C. coli*, *C. fetus*, *C. hyointestinalis* subsp. *hyointestinalis*, and *C. jejuni*. This sensitivity is substantially higher ($\approx$100 times) than that of nonnested PCRs used by others (25, 27). Given that only 200 mg of bovine feces was processed, we obtained a detection sensitivity of approximately 20 cells. In addition to the direct effects of nested PCR, the nested step may also increase sensitivity by diluting out inhibitors; some amplification would be expected to occur in the primary step even in the presence of PCR inhibitors, providing a sufficient template for the secondary reaction (32). Interestingly, we observed variability in detection sensitivities among the three sample times. Reasons for the variability among the replicates are uncertain, but the variability does not appear to be due to PCR inhibitors.

We observed that the nonnested 16S rDNA gene primers designed to detect campylobacters in feces were more sensitive in general than the nested primers for specific taxa. Even in uninoculated feces (i.e., minimum detection experiment), positive amplification of *Campylobacter* DNA was detected (33 to 83% of the samples tested). Analysis of the sequences of these amplicons demonstrated high similarity with *Campylobacter* (data not shown). A number of *Campylobacter* species not targeted in the current study (i.e., with nested or seminested primers) have been reported from bovine feces. For example, Giacoboni et al. (15) isolated *C. lari*, Atabay and Corry (1) recovered *C. sputorum*, and we recovered *C. lanienae* from bovine feces. Furthermore, we isolated *A. butlzeri* and *A. skirrowii* from bovine feces, and the genus-specific primers were observed to amplify DNA of these taxa, contrary to previous findings (29). Therefore, the apparently higher sensitivity of the genus-specific primers than of the species-specific primers may not be due to increased sensitivity but rather to the presence of additional *Campylobacter* species in the feces. In contrast to the minimum detection experiment, we included a primer set specific for *C. lanienae* in the direct PCR of campylobacters in the bovine feces experiment. The combined amplification frequency for *C. jejuni* and *C. lanienae* corresponded closely with that of the genus-specific primer set in this experiment. Nevertheless, the development and testing of nested primers for additional species of campylobacters and arcobacters in bovine feces are necessary, and we are currently pursuing this goal.

Relative to microbiological isolation, PCR was found to be more sensitive for detecting total campylobacters and *C. lanienae* but not *C. jejuni*. The media that we tested are designed to isolate *C. jejuni* and *C. coli* (1, 4, 12, 37), and this factor provides an explanation for the high isolation sensitivity that we observed for *C. jejuni*. Given the diversity of campylobacters associated with cows, the advantages of PCR over conventional isolation methods that we observed agree with the conclusions of Lawson et al. (26), who found that direct PCR of human feces provided unique data about mixed infections by non-*C. coli* and non-*C. jejuni* campylobacters.

We observed that the QIAamp DNA stool minikit effectively removed any inhibitors in bovine feces, as indicated by the amplification of an internal control designed to amplify under the same conditions as a 16S rDNA primer set for campylobacters and arcobacters. Using nested and seminested PCRs, we were able to detect *C. coli*, *C. fetus*, *C. hyointestinalis*, and *C. jejuni* at densities of $10^2$ to $10^3$ CFU $g^{-1}$ of feces (fresh weight). The PCR-based detection method was found to be substantially more effective in detecting *C. lanienae* than conventional culturing methods, and this is the first report of the detection of *C. lanienae* outside of humans, to our knowledge. Given the logistical advantages and high resolution for detecting campylobacters in bovine feces, PCR-based methods will facilitate the understanding of the interaction of this fastidious group of pathogens with cattle.

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