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Isolation of emerging *Campylobacter* species in working farm dogs and their frozen home-killed raw meat diets

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Abstract. We applied 7 culture methods to 50 working farm dog fecal samples and 6 methods to 50 frozen home-killed raw meat diet samples to optimize recovery of a wide range of *Campylobacter* spp. Culture methods combined filtration, enrichment broths, and agars at 37°C and 42°C in conventional and hydrogen-enriched microaerobic atmospheres. Overall, a prevalence of 62% (31 of 50) and 6% (3 of 50) was detected in dog and meat samples, respectively, based on *Campylobacter* genus PCR. A total of 356 *Campylobacter* spp. isolates were recovered from dogs, with successful isolation by individual methods ranging from 2 to 25 dogs. The species detected most commonly were *C. upsaliensis* and *C. jejuni*, and less commonly *C. coli* and *C. lari*. Species isolated that are rarely reported from dogs included *C. rectus*, *C. lari* subsp. *concheus*, *C. volucris*, and *Helicobacter winghamensis*. Six isolates from dogs positive by *Campylobacter* genus PCR were confirmed, using 16S rRNA sequencing, as *Arcobacter cryaerophilus* (1) and *Arcobacter butzleri* (5). *C. jejuni* multi-locus sequence typing results revealed a diversity of sequence types in working dogs, with several uncommonly reported from other *C. jejuni* sources in New Zealand. Overall, 20 isolates from 3 meat samples were positive by *Campylobacter* genus PCR; 1 meat sample was positive for *C. jejuni*, 1 for *C. rectus*, and 1 isolate was subsequently identified as *A. butzleri*. The method using *Campylobacter* enrichment broth in a hydrogen-enriched environment on nonselective agar resulted in significantly reduced recovery of *Campylobacter* spp. from both sample types.

Key words: *Campylobacteraceae*; dogs; farms; microbial culture; raw foods; zoonosis.

Introduction

Campylobacter-associated enteritis is one of the most common bacterial gastrointestinal diseases in humans, and the organisms are frequently detected in many animals, food, and the environment.[10] Campylobacteriosis is predominantly foodborne, especially from poultry meat, but is also significantly associated with travel, and contact with environmental water and animals, including pets.[20] *C. jejuni* and *C. coli* are the most common species associated with disease, but many other species have been implicated as pathogens (frequently referred to as "emerging" pathogens), and are generally considered underrepresented.[39,44] The underestimation of emerging *Campylobacter* spp. is mostly attributed to the bias of culture methods that are optimized for the recovery of *C. jejuni* and *C. coli.*[33] The narrow selection of available culture methods is related to the fastidious nature of these taxa and the vast diversity of growth requirements among them, such as incubation temperatures, atmospheric conditions, length of incubation, nutrient requirements, and differing susceptibilities to antimicrobial agents.[14] ELISA and PCR have enhanced sensitivity for the detection of *Campylobacter* spp. compared with culture methods[5,15,64] as well as the ability to detect a wider range of species, many of which are challenging to isolate.[9,38]

Dogs were first associated with campylobacteriosis in humans in 1960,[66] and the first species isolated from dogs was *C. jejuni* in 1977.[58] Since then, many studies worldwide have reported the frequent isolation of *C. jejuni* from sick and healthy dogs, with pathogenic involvement more likely to occur in young animals or precipitated by contributing factors such as stress, crowding, and concurrent diseases.[40] In New Zealand, an increased risk for campylobacteriosis in humans has been reported, with factors associated with farming and the rural environment.[8,59] Also in New Zealand, screening of client-owned dogs and retail raw meat for *Campylobacter* spp. has been reported.[6] Similar to humans,

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the distribution of species from dogs observed is significantly dependent on the methods of detection, but of all of the species isolated from the feces of dogs, *C. upsaliensis* is by far the most common, with *C. jejuni* and *C. helveticus* recovered relatively frequently; *C. coli, C. lari, C. hyointestinalis*, and *C. concisus* are rare.[1,25,31,55] The distribution of species detected by culture is in sharp contrast to those detected by PCR methods. A molecular study reported 14 different *Campylobacter* spp. in dogs.[12] For 7 of these *Campylobacter* species, there are no reports of isolation from dogs in the literature (*C. rectus, C. mucosalis, C. showae, C. gracilis, C. fetus, C. sputorum, C. curvus*), beyond identification of *C. rectus* and *C. showae* in saliva or dental plaques by molecular methods.[30,54]

A culture method described for the recovery of *Campylobacter* spp. from meat for human consumption enabled isolation of 17 *Campylobacter* spp.,[36] including all of the above species not previously recovered by culture in dogs. We applied this culture method (CB_H2_AB described below) and a combination of other culture methods to isolate a wide range of *Campylobacter* spp. from working farm dogs and their raw meat home-killed food; compared the performance of the methods; and determined the prevalences of *Campylobacter* spp. in the samples collected. We chose working farm dogs and their home-kill meat diet for study, because prevalences in these dog and meat types have not been evaluated previously, to our knowledge, and both could pose an infection risk to farmers. For comparison with previous studies in the region, *C. jejuni* isolates were subjected to multi-locus sequence typing (MLST) and were added to the ^{m}EpiLab *Campylobacter* Manawatu Sentinel Surveillance site study database that holds the results of recent studies.[23,46–48]

Materials and methods

Study design

Our study was a prospective cross-sectional study using convenience sampling. Participants were recruited from previous studies in which farmers agreed to be contacted for future investigations, and by telephone survey using data available on the New Zealand electoral roll in which the registrants' occupation was recorded as "farmer." The eligibility criteria for sampled premises were 1) location within the Manawatu region; 2) having a minimum of 3 working farm-herding dogs; and 3) feeding dogs home-killed raw meat at least once fortnightly. Sampling was performed July–August 2012 and March–May 2013. Fifty farms were visited in the morning and the dogs observed for defecation to allow sampling of freshly voided feces. If a dog did not defecate, feces were obtained by rectal digital recovery. Raw home-killed meat for feeding of working dogs was sampled either frozen from the freezer or from meat thawed that morning in order to feed the dogs. Dogs and meat were arbitrarily selected for sampling, and one sample from each was taken per farm. All samples were refrigerated and cultured within 4 h from sampling. The study was independently reviewed and approved by the Massey University Animal Ethics Committee under protocol MUAEC 12/23.

Campylobacter isolation

Culture methods consisted of anaerobe basal (AB) agar (Oxoid, Basingstoke, UK) prepared and supplemented in-house with 5% lysed horse blood (Venous supplies, Taukau, New Zealand); cefoperazone–amphotericin–teicoplanin (CAT) agar and modified charcoal–cefoperazone–deoxycholate agar (mCCDA; Fort Richard, Auckland, New Zealand); filtration using 0.6-µm pore size, mixed ester filter membranes (Whatman, Maidstone, UK) performed in biosafety cabinets; no or prior enrichment in Bolton broth (BB) or Campylobacter enrichment broth (CB; Lab M, Bury, UK), H_2-enriched microaerobic atmosphere (H_2-MA; 82% N_2, 10% CO_2, 5% H_2, 3% O_2) and a conventional MA (85% N_2, 10% CO_2, 5% O_2) in gas cabinets (MACS VA500 workstation, Don Whitley Scientific, Shipley, UK); and gas-jars using envelope-generated MA (CampyGen sachets, Oxoid) in a temperature-controlled room. Plates were checked daily for growth from day 2 (direct plating) or day 3 (if enriched) to day 6 of incubation. Control plates were used in all culture protocols. Colonies exhibiting morphologic features of *Campylobacter* spp. were checked for size and motility by dark-field microscopy and Gram-reaction using potassium hydroxide.[27] For presumptive *Campylobacter* spp., up to 2 individual colonies (as available), each of a different morphology, were subcultured using Columbia horse blood agar (Fort Richard). If, after an additional 2 or more days of culture, new colonies were detected that shared the same morphologic features with previously observed colonies from that agar plate, these colonies were also subcultured. Whole plates of pure colonies were harvested for storage in nutrient broth (Difco Laboratories, Bergen, NJ) containing 15% weight/volume (w/v) glycerol at −80°C. Plates were considered unreadable if over three-fourths of the streaked area was overgrown by non-target organisms.

Estimates of selectivity of culture methods for isolation of *Campylobacter* spp. were used to describe the workload with the different culture methods. The rationale was that culture methods are expected to grow only target organisms. Hence, suspect *Campylobacter* colonies that were negative by *Campylobacter* genus PCR were subcultured, stored, and retested for no benefit for the time and resources invested. Variation in colony morphology of *Campylobacter* spp. between agar plates has been reported.[49] However, this should not have influenced our estimates of selectivity, given that we defined selectivity to denote the ratio of PCR-confirmed *Campylobacter* colonies over presumptive *Campylobacter* colonies of all morphology types. A low selectivity result for a method suggests that the presumptive *Campylobacter* colonies should not be considered *Campylobacter*

spp. and should be confirmed by further identification tests because the true probability of an isolate being a *Campylobacter* is low. Conversely, high selectivity raises the confidence in the presumptive *Campylobacter* isolate being later confirmed by PCR. In this sense, selectivity was also used as a measure of confidence in laboratory practices that the colonies exhibiting morphologic features of *Campylobacter* spp. grown by selective media in an appropriate atmosphere may reliably be reported as *Campylobacter* species.[49]

Culture of fecal samples from dogs

Seven culture methods were performed. Cotton swabs were applied to fresh fecal samples and cultured as follows: 1) 4 swabs placed in CB for 48 h followed by passive filtration of 0.2 mL for 20 min to AB agar, with the inoculum distributed over the agar surface using sterile disposable hockey-stick spreaders, and grown in H_2-MA at 37°C (overall method termed CB_H2_AB); 2) same as method 1, but following enrichment, a swab was plated onto CAT agar (CB_H2_CAT); 3) a direct swab onto CAT agar at 37°C in envelope-generated MA (CAT_MA), 4) a swab placed in BB for 48 h followed by a swab onto mCCDA at 37°C in H_2-MA (BB_H2_mCCDA); 5) a swab placed in BB for 48 h followed by a swab onto mCCDA at 42°C in MA (BB_MA_mCCDA); 6) a direct swab onto CAT agar at 37°C in H_2-MA (CAT_H2); and 7) a swab suspended in 10 mL of phosphate-buffered saline (PBS, pH 7.3; Difco Laboratories) directly followed by passive filtration as in method 1 onto AB agar at 37°C in H_2-MA (AB_H2). Method CB_H2_CAT was performed on 38 samples and method AB_H2 on 21 samples.

Culture of meat samples

Six culture methods were performed. A 25-g sample of meat was initially stomached (Colworth stomacher 400, Seward, Worthing, UK) for 30 s with 225 mL of CB, and then divided into 5 aliquots of ~ 45 mL of meat suspensions in screw-top bottles. Three of the 5 meat suspensions had BB selective supplement added (SR0183 selective supplement, Oxoid). All meat suspensions were then given an initial enrichment of 48 h in the environmental conditions and processed as follows: 1) 0.2 mL of CB suspension passively filtered (procedure performed as for feces) onto AB agar at 37°C in H_2-MA (overall method termed mCB_H2_AB), 2) a swab of CB suspension onto CAT agar at 37°C in MA (mCB_H2_CAT), 3) a swab of CB suspension onto CAT agar at 37°C in MA (mCB_MA_CAT), 4) a swab of BB suspension onto mCCDA at 37°C in H_2-MA (mBB_H2_mCCDA), 5) a swab of BB suspension onto mCCDA at 42°C in MA (mBB_MA_mCCDA), and 6) a swab of BB suspension onto CAT agar at 37°C in MA (mBB_MA_CAT).

Given that the overgrowth of contaminants in our study could not be explained by the procedures in production, storage, and usage of the in-house prepared AB agar isolation media (all media were evaluated by quality control procedures), the mCB_H2_CAT methods were added during the study. Increasing the agar content of AB agar to 4% to limit the swarming growth of *Proteus* spp. was attempted in 12 samples, but was unsuccessful. To investigate if the overgrowth of contaminants was associated with the CB rather than the filtration technique in CB_H2_AB as compared with the swab to CAT agar in CB_H2_CAT, the AB_H2 method was added during the study.

As a check for fecal contamination, meat samples were also cultured for *Escherichia coli* by placing 25 g of meat in 225 mL of buffered peptone water (Difco Laboratories), with aerobic cultivation overnight at 37°C, from which 3 replicate 100-fold dilutions in 0.9% PBS (Difco Laboratories) were spiral-plated (aCOLyte spiral plater, Don Whitley Scientific) onto MacConkey agar (Fort Richard) with incubation at 37°C for an additional 24 h. Lactose-fermenting colonies, up to 4 as available, were subcultured onto blood agar for a spot indole test and, if positive, reported as *E. coli*.

Campylobacter identification and typing

Crude DNA extraction was performed by boiling a small loopful of fresh cultures for 10 min in a 2% w/v Chelex solution (Bio-Rad Laboratories, Hercules, CA) followed by centrifugation and decantation of the supernatant into a sterile tube with storage at −20°C until PCR was performed. Each fecal isolate was initially tested by the *C. upsaliensis* and *C. helveticus* components of a previously described multiplex PCR[35]; each food isolate was tested by a duplex PCR that consisted of the *C. jejuni* component of a previously described PCR[65] and the *C. coli* component of a previously described PCR.[18] If an isolate was negative in these initial assays, the *Campylobacter* genus–specific component of a previously described multiplex PCR[35] was performed. Subsequent to a positive genus PCR, fecal and food isolates were sequentially tested by the above species-specific PCRs as needed for the type of sample and thereafter, by species-specific PCRs for *C. lari, C. fetus*, and *C. hyointestinalis* (all based on a previously described multiplex PCR[35]) and *Arcobacter butzleri*.[28] Some of the assay parameters (annealing temperature and concentrations of dNTP, primers, and magnesium chloride) have been modified from the original publications as a result of in-house optimization procedures that involved validation with one target and one non-target strain (Supplementary Table 1). A subset of isolates negative by the species-specific PCRs was selected for 16S ribosomal RNA (rRNA) gene amplification and sequencing.[60] PCR assays were performed (Labcycler thermal cyclers, SensoQuest, Göttingen, Germany; Supplementary Table 1). The amplified products and a marker ladder (1 Kb Plus ladder, Invitrogen, Carlsbad, CA) were identified by electrophoresis in 1% w/v agarose gel in 0.5% w/v Tris–Borate–EDTA buffer (Duchefa Biochemie, Haarlem, The Netherlands), subsequently stained with 0.5 µg/mL of ethidium bromide, and exposed to

Table 1. Number of *Campylobacter*-positive working farm dogs ($n = 50$) from Manawatu, New Zealand using 7 culture methods and isolates identified by PCR.

Method	*Campylobacter* genus PCR	Confirmed *Campylobacter* spp.–specific PCRs			
		C. upsaliensis	*C. jejuni*	*C. coli*	Other
CB_H2_AB	2[a]	0[a]	0[a]	1	1
CB_H2_CAT	8[b]	7[b,c]	0[a]	0	2
CAT_MA	25[c]	21[d]	5[a,b]	0	2
CAT_H2	24[c]	18[d]	5[a,b]	1	2
BB_H2_mCCDA	21[c]	8[b]	8[b]	3	5
BB_MA_mCCDA	6[a,b]	3[a,b]	2[a]	1	0
AB_H2	11[c]	8[c,d]	3[a,b]	0	0
Overall	31	21	9	3	8

Shared superscript letters within each column denote no significance by McNemar test of paired data ($\alpha < 0.05$). Numbers of positive dogs in confirmed *Campylobacter* spp.–specific PCR columns (in rows for each method) do not necessarily add up to the number in *Campylobacter* genus PCR column because some of the dogs tested positive for multiple *Campylobacter* species. AB = nonselective anaerobe basal agar (Oxoid); BB = selective Bolton broth (Lab M); CAT = cefoperazone–amphotericin–teicoplanin agar (Fort Richard); CB = nonselective *Campylobacter* enrichment broth (Lab M); H2 = H_2-enriched microaerobic atmosphere (82% N_2, 10% CO_2, 5% H_2, 3% O_2); MA = microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2); mCCDA = modified charcoal–cefoperazone–deoxycholate agar (Fort Richard). All methods were performed at 37°C except BB_MA_mCCDA at 42°C. All methods were used on 50 dogs except CB_H2_CAT and AB_H2, which were used on 38 and 21 dogs, respectively.

ultraviolet light using a gel documentation system (Gel Doc +XR, Bio-Rad Laboratories).

The dog isolates confirmed as *C. jejuni* by PCR were genotyped using MLST of 7 housekeeping genes specific for *C. jejuni* and *C. coli*.[19] Amplifications were performed in a 25-µL volume reaction using a commercial master mix (AmpliTaq Gold master mix, Applied Biosystems, Foster City, CA) and 5 pmol of each primer; the products were sequenced (3130*xl* DNA sequencer, Applied Biosystems) following the manufacturer's instructions. Sequence data were collated; alleles and sequence types (ST) assigned using the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter/); and occurrence compared with other sources in the *m*EpiLab *Campylobacter* Manawatu Sentinel Surveillance site study data.[23,46–48] The Manawatu Sentinel Surveillance site study data contain over 3,500 samples (at the time of writing), a 10 plus year project for source attribution of campylobacteriosis using concurrent sampling of human cases, animals, food, and the environment.[23,46–48]

Statistical analysis

The results of the culture methods were compared using the McNemar test of symmetry for paired samples. Comparison of prevalence rates with those reported by others was performed using Fisher exact test of independence. Statistical and exploratory data analyses were performed using R (R software v.3.2.2, http://www.R-project.org/).

Results

An overall *Campylobacter* spp. prevalence of 62% (31 of 50) was confirmed in the dogs based on genus-specific PCR following bacterial isolation. Twenty-one dogs (42%) were positive for *C. upsaliensis* and 9 (18%) for *C. jejuni*. From 50 dog fecal samples, there were 408 presumptive *Campylobacter* isolates, with 356 positive by *Campylobacter* genus PCR, and identified by species-specific PCRs as *C. upsaliensis* (232 isolates), *C. jejuni* (81 isolates), *C. coli* (14 isolates), and *C. lari* (1 isolate; Table 1). Five isolates were PCR positive for *A. butzleri*. Fifteen isolates with negative species-specific PCRs from various dogs and from every culture method, as available, were identified as *Helicobacter winghamensis* (5 isolates), *C. upsaliensis* (4 isolates), *C. rectus* (2 isolates), *C. volucris* (2 isolates), *C. lari* subsp. *concheus* (1 isolate), and *A. cryaerophilus* (1 isolate) by 16S rRNA gene sequencing (identification accepted as the closest matching species). The remaining 8 isolates could not be identified. Culture methods CAT_MA, CAT_H2, and BB_H2_mCCDA confirmed the highest number of positive *Campylobacter* genus PCR dogs, with CAT_MA and CAT_H2 the highest number of confirmed *C. upsaliensis*, and BB_H2_mCCDA the highest number of dogs positive for *C. jejuni* (Table 1). Overall, 2, 5, and 24 dogs were positive for 3, 2, and 1 *Campylobacter* spp., respectively. Three dogs were positive for *Campylobacter* spp. by 1 method only, 6 by 2 methods, 9 by 3 methods, 10 by 4 methods, and 3 dogs by 5 methods. Combining all of the methods, 24 dogs were *Campylobacter* spp. positive on day 2, 4 were positive on day 3, 2 were positive on day 4, and 1 was positive on day 5 (*C. lari*) of incubation.

Species uncommonly isolated from dogs were as follows: *C. volucris* by the methods CB_H2_CAT and CAT_MA (on the fourth and second day of incubation, respectively), *C. lari* subsp. *concheus* by BB_H2_mCCDA (on the fourth day of incubation), *C. rectus* by CB_H2_AB (on the fourth day of incubation), and *H. winghamensis* by CB_H2_CAT, BB_H2_mCCDA, and CAT_H2 (on the third and fourth day of incubation). The proportion of readable

Table 2. Readability of culture plates and selectivity estimates of the culture methods for isolation of *Campylobacter* spp. used in 50 fecal samples of working farm dogs and 50 samples of their frozen home-killed raw meat diets in Manawatu, New Zealand.

	Feces			Home-killed meat
Method	Readability of plates*	Selectivity of method†	Method	Readability of plates*
CB_H2_AB	13 (26)	6/11 (55)	mCB_H2_AB	38 (76)
CB_H2_CAT	30 (79)	16/20 (80)	mCB_H2_CAT	34 (89)
CAT_MA	49 (98)	97/104 (93)	mCB_MA_CAT	47 (94)
CAT_H2	49 (98)	104/112 (93)	mBB_MA_CAT	49 (98)
BB_H2_mCCDA	48 (96)	72/77 (94)	mBB_H2_mCCDA	50 (100)
BB_MA_mCCDA	50 (100)	20/20 (100)	mBB_MA_mCCDA	50 (100)
AB_H2	21 (100)	41/65 (63)		

For explanation of abbreviations, see Table 1. All methods were performed at 37°C except mBB_MA_mCCDA at 42°C. All methods were used on 50 samples except mCB_H2_CAT and AB_H2, which were used on 38 and 21 samples, respectively. AB_H2 was only used on dog fecal samples. Numbers in parentheses are percentages.
* Plates were considered unreadable if more than three-fourths of the streaked area was overgrown by non-target organisms, and readability is given as the number of readable plates, with the % proportion of readable plates from the total number of plates given in brackets.
† Selectivity is given as the number of PCR-confirmed *Campylobacter* colonies/the number of presumptive *Campylobacter* colonies based on morphologic features on agar plates, size, and motility by dark-field microscopy and Gram-reaction using potassium hydroxide (% proportion of PCR-confirmed colonies from the presumptive *Campylobacter* colonies). Selectivity is presented only for culture methods used on fecal samples because very few isolates were obtained from meat.

Table 3. Occurrence of *Campylobacter jejuni* MLST types isolated in working farm dogs from Manawatu, New Zealand, across all sources from the ᵐEpiLab *Campylobacter* Manawatu Sentinel Surveillance site study data.

C. jejuni MLST	Human	Poultry	Ruminants	Water	Other
ST-42	54	7	53	10	2
ST-45	141	154	10	21	74
ST-50	102	68	74	8	1
ST-61	64	7	55	2	1
ST-137	6	1	0	1	5
ST-3232	2	0	6	0	0
ST-3610	3	0	3	1	0
ST-3676	11	1	1	1	0

Alleles and sequence types (STs) assigned using the *Campylobacter* PubMLST (multi-locus sequence typing) database (http://pubmlst.org/campylobacter/). Data contain over 3,500 samples in total (at the time of writing) from the Manawatu Surveillance Sentinel Site, a 10 plus year project for source attribution of campylobacteriosis using concurrent sampling of human cases, animals, food, and the environment,[23,46–48] of which 950 samples are of the 8 STs observed in our study.

plates, and selectivity estimate results for isolation of *Campylobacter* spp. were superior for culture methods CAT_MA, CAT_H2, BB_H2_mCCDA, BB_MA_mCCDA, and AB_H2 in fecal samples (Table 2).

Forty *C. jejuni* isolates from 8 dogs (2–9 isolates per dog) were subjected to MLST typing, and 33 (83%) returned full allelic profiles. Eight different STs, belonging to 5 different clonal complexes, were observed, and 2 dogs carried STs of different clonal complexes. Four of the 8 detected STs from the dogs were uncommonly detected compared to *C. jejuni* STs from human, poultry, ruminants, water, and other sources in the Manawatu Sentinel Surveillance site study database[23,46–48] (Table 3).

From 50 home-killed meat samples, 3 samples tested positive for *Campylobacter* spp. by genus-specific PCR (6%) following isolation, and 17 of 52 presumptive *Campylobacter* isolates from these 3 samples were positive by the *Campylobacter* genus PCR. Of the 17 isolates, 4 were positive by the *C. jejuni* PCR, and all were grown using method mBB_MA_mCCDA from 1 meat sample; 11 isolates grew from 1 other sample using mCB_H2_CAT, mCB_MA_CAT, mBB_H2_mCCDA, and mBB_MA_CAT, and all were positive for *A. butzleri* by PCR. Sequencing of the 16S rRNA gene for the 2 remaining isolates returned *C. rectus* as the most similar species. Both *C. rectus* isolates were obtained using mCB_MA_CAT in the third meat sample. Readability of the plates was over 90% for methods mCB_MA_CAT, mBB_MA_CAT, mBB_H2_mCCDA, and mBB_MA_mCCDA (Table 2). The selectivity of culture methods in meat samples was not calculated, given the low number of isolations. *E. coli* was detected in 24 meat samples.

Implementing the mCB_H2_CAT method resulted in increased readability of the plates: 79% for fecal samples and 89% for meat samples. Addition of the AB_H2 method during the study to 21 fecal samples resulted in increased readability of 100% (Table 2).

Discussion

Our main findings are the significant differences in isolation of *Campylobacter* spp. among the culture methods, and the isolation of 4 species rarely detected in dogs. All *Campylobacter* spp. identified in the dogs and their food are implicated as potential pathogens in people.[39] Of the emerging species, *C. volucris* was first described in black-headed gulls,[16] and since then has been reported in an immunocompromised human patient with bacteremia.[32] *C. lari* subsp. *concheus* was initially isolated from shellfish and subsequently in humans, seagulls, and river water.[17,61] *C. rectus* has been detected using molecular methods in dog feces[12] and oral swabs,[30] but the clinical significance in dogs is uncertain. In people, *C. rectus* is associated with periodontitis and gingivitis, various gastrointestinal diseases, and extra-intestinal infections; apart from dogs, no other potential sources have been identified.[39] *H. winghamensis* was described in people with clinical signs of gastroenteritis[41] and so far only one study, using molecular methods, has reported the organism from non-humans (rodents).[24]

All of the above species have been reported rarely, and it is not clear if the animals tested are the true reservoirs for the organism or if they are just transient carriers. With regard to fecal carriage of *C. jejuni* and *C. upsaliensis*, a longitudinal study in dogs reported short-term carriage of *C. jejuni* with genotypically diverse isolates (using pulsed-field gel electrophoresis), whereas the carriage of *C. upsaliensis* was of long duration and involved clonal strains.[25] A previous study in New Zealand reported a high diversity of *C. jejuni* STs in healthy dogs attending a veterinary clinic for elective procedures.[6] Four of 8 *C. jejuni* STs isolated in our study are very rarely observed in the Manawatu Sentinel Surveillance site study,[23,46–48] and the other 4 STs are common in several sources, which also supports the heterogeneous exposure of farm dogs. In addition, compared to 6 STs reported in the study of dogs attending the clinic,[6] only 2 STs (ST-45 and ST-61) were also isolated in working farm dogs in our study. Similarly, only ST-45 and ST-50 from 4 STs reported in another local study of environmentally deposited feces along dog walkways[43] were also isolated in working farm dogs in our study. These results suggest potential exposure of working farm dogs to additional sources compared to the urban dog population. Other studies using MLST also reported a high diversity of STs in dogs, including strains frequently seen in humans and food.[45,53]

In contrast to our study, a lower prevalence of *Campylobacter* spp. (13%) and *C. jejuni* (5%) was reported in 498 dog fecal samples in the Palmerston North area, mostly collected from the environment in dog walking areas.[42] Another study in the same region as our study also reported lower prevalences of *Campylobacter* spp. (36%), *C. jejuni* (13%), and *C. upsaliensis* (23%) in rectal swabs from 90 client-owned pet dogs attending a veterinary clinic.[6] Both studies employed a culture method similar to the BB_MA_mCCDA method used in our study, and the latter study[6] additionally used a method similar to CAT_MA. The comparison of prevalences between the 2 previous studies and our study using the Fisher exact test returns a significantly higher prevalence of *Campylobacter* spp. in dogs attending the clinic ($p < 0.001$)[6] and in working farm dogs in our study ($p < 0.001$), as well as of *C. jejuni* ($p < 0.01$), than the respective prevalences reported in environmental dog feces.[42] These differences between the studies are likely the result of the use of environmental dog feces[42] rather than differences in culture methods or the method of obtaining the fecal material (rectal vs. fecal swab). On the other hand, working farm dogs in our study had a significantly higher prevalence of *Campylobacter* spp. ($p < 0.01$) and of *C. upsaliensis* ($p = 0.03$) than dogs attending the clinic.[6] This difference does not apply for *C. jejuni* ($p = 0.47$). The variation in culture methods between the clinic-based study[6] and our study could explain the differences in prevalences of emerging *Campylobacter* spp. given that all emerging species except *C. volucris* in our study were isolated using methods not employed in the study of dogs attending the clinic.[6] On the other hand, the results of methods used by both our study and the study of dogs attending the clinic[6] indicate a similar prevalence of *C. jejuni* between working farm dogs and pet dogs attending the clinic, and a higher prevalence of *C. upsaliensis* in the former. In addition to exposure to farm environment, working farm dogs may have a higher prevalence of *Campylobacter* spp., including *C. upsaliensis*, given their communal lifestyle, given that intensive housing has been reported to be a risk factor.[4] Also, given that these factors would be expected to influence the prevalence of *C. jejuni*, it is not clear why there were similar prevalences of *C. jejuni* between our study and dogs attending the clinic. Possibly, it may be a spurious finding because of sampling variation from a relatively small sample size, or a random observation of similar prevalences because of variable fecal carriage dynamics in dogs between the 2 species. A longitudinal study[25] showed transient variations in short-term fecal carriage of *C. jejuni* compared to persistent fecal carriage of *C. upsaliensis*, hence there is a possibility for cross-sectional studies to give similar point prevalence estimates at a given time as a result of chance alone.

The benefit of applying multiple culture protocols is evident from the significant differences observed in pairwise comparison of methods, both in the overall isolation rate and for *C. upsaliensis* and *C. jejuni* in particular. For the less common species (*A. cryaerophilus, C. lari* subsp. *concheus, H. winghamensis*, and *C. rectus*) isolation in H_2-MA appeared to be the important factor. For *C. volucris* and *Arcobacter* spp., the use of CAT agar and BB_H2_mCCDA, respectively, appeared to be the most suitable. The requirement of hydrogen for isolation of many emerging *Campylobacter* spp. as well as the enhanced recovery of *C. jejuni* in a hydrogen-enriched atmosphere has been recognized previously.[33,52] Interestingly, *C. rectus* isolated from meat samples in our study grew in pure MA, although *C. rectus* is considered to

have a requirement for hydrogen.[63] In our study, too few of the emerging species were isolated for statistical comparisons. However, with regard to *C. jejuni* and *C. upsaliensis*, CAT_MA and CAT_H2 only differed in the presence of hydrogen and no significant differences in isolation were observed. A surprising finding was the rather large difference in isolation rate of *C. jejuni* between BB_H2_mCCDA and BB_MA_mCCDA. A difference in the isolation of *C. upsaliensis* for these 2 media also existed but it was smaller than that for *C. jejuni*. These 2 methods differed by both temperature and the presence of hydrogen; allocating the causative role of these 2 factors is not possible. Also, a synergistic or cumulative effect of both may have occurred as well. The improved recovery of *C. jejuni* with added hydrogen[33,52] and incubation at 37°C rather than at 42°C (with mCCDA) has been reported previously.[7] *C. upsaliensis* had the largest difference in isolation rate between methods in our study.

Comparisons of CAT agar and mCCDA for isolation of *C. upsaliensis* in veterinary studies have reported conflicting results. Although a higher isolation rate with CAT agar compared to mCCDA has been reported,[1,2] both an equivalent rate[31] and a lower rate[26] with CAT, for both *C. upsaliensis* and *C. jejuni*, have been reported. Similar to our study, mCCDA was outperformed by both CAT[3] and by the filtration method[7] for the isolation of *C. upsaliensis* from humans. Although filtration was reported as superior to CAT agar for isolation of *C. upsaliensis*,[34] we found that the methods were comparable. CAT agar was originally developed according to the antimicrobial-resistance profiles of several thermophilic *Campylobacter* spp.,[3] but was also shown to result in better growth and isolation of a greater diversity of *C. upsaliensis* strains than mCCDA[13] and enhanced detection of lower bacterial concentrations compared to mCCDA.[11] However, the latter study could not explain the difference in sensitivity between these agars, either by the absolute growth index for any length of incubation time, or the antimicrobial composition of the media.[11] Hence, the investigators speculated that the growth of *C. upsaliensis* is indirectly affected by the interaction of fecal microflora and culture agars.[11] In our study, mCCDA was always used in conjunction with BB, and a comparable rate of isolation of *C. upsaliensis* in CB_H2_CAT with both BB and mCCDA methods was observed, whereas direct plating on CAT agar outperformed all 3 of them. Improved recovery and motility of *C. upsaliensis* in CB compared to BB has been reported,[36] and our relatively small sample size may have had insufficient power to detect the difference between the 2 broths. The poor performance of CB_H2_AB is likely the result of overgrowth of contaminants, given that the modification to CB_H2_CAT both improved the readability of plates and the isolation of *Campylobacter*, although the isolation success was still significantly less than that of most other methods. This suggests that contaminating organisms may inhibit *Campylobacter* cells in CB given that the highest isolation rates were obtained using direct plating methods. We identified only one dog as positive after 4 d of incubation, but the relatively small number of samples is likely to preclude weighting of this observation, and incubation up to 6 d has been advised for higher isolation success.[33]

A major study objective was to isolate a diverse set of *Campylobacter* spp. using the culture method CB_H2_AB, which, with the exception of *C. rectus* isolated from one dog, failed in both fecal and meat samples. The failure was primarily ascribable to frequent overgrowth by contaminants (mostly *Proteus* spp. and less frequently *Pseudomonas* spp., both tentatively identified by morphologic features and the smell of the colonies). CB_H2_AB was developed using fresh beef samples only, with no report of overgrowth by contaminants.[36] Perhaps the home-killed meat (48% of which showed fecal contamination) and the dogs' feces used in our study contained too many non-target bacteria, making the antimicrobial-free method unsuitable. Overgrowth by contaminants on plates using the filtration method (without enrichment) and using selective plates has been reported at levels of usually <10% of plates,[2,11] similar to our results for methods using antimicrobial agents. CB_H2_AB with an enrichment duration reduced to 24 h was also successfully applied previously in many types of fresh meat products[37] and porcine samples including cecal contents.[56] Adding the (m)CB_H2_CAT methods to fecal and meat samples, and the AB_H2 method to fecal samples, resulted in increased readability of the plates. The marked increase in readability from 26% for CB_H2_AB, and 79% for CB_H2_CAT, to 100% in AB_H2, suggests that enrichment in CB resulted in a growth of contaminants to a level too high for the filtration method and partially too high for the CAT agar to allow detection of *Campylobacter* in the fecal samples.

A modification that could be useful in the isolation of *Campylobacter* spp. from food samples is the inclusion of a pre-enrichment step (usually up to 4 h) with delayed addition of antimicrobials to broth, a lower incubating temperature, or both. This step is suggested when a low number and/or injured *Campylobacter* cells are expected, such as in frozen products.[14,57] The absence of a pre-enrichment step in our study could explain the low prevalence of *Campylobacter* spp. observed in frozen home-killed meat by all methods used. The prevalence of *C. jejuni* was reported to be 8% in retail raw non-poultry meat pet food products in New Zealand[6] using a similar method to that of mBB_MA_mCCDA used in our study. In that previous study,[6] fecal contamination of meat was not reported, and it could be expected that home-killed meat has a greater contamination level than commercially available meat given a lack of hygienic measures in farming environments with regard to preparation, handling, and storage practices. However, the sample sizes of both studies were relatively small and limit the confidence that can be placed in comparisons of the 2 studies.

Selectivity estimates <90% in our study were observed with methods employing filtration and/or the nonselective CB enrichment (CB_H2_AB, CB_H2_CAT, and AB_H2),

whereas all methods employing antimicrobial agents had selectivity estimates >90%. It is possible that antimicrobials suppressed a wider range of species than the filtration method, thus providing less diversity of bacteria on the agar, of which even fewer were *Campylobacter*-like. In contrast, a greater diversity of bacteria passed through the filters, many of which grew on nonselective agar and more of which were *Campylobacter*-like. However, given that our aim was to isolate a variety of *Campylobacter* spp., a more flexible definition of "campylobacter-like" was used. Notably, *C. showae* resembles straight rods,[22] *C. mucosalis* has yellow colonies,[63] and *C. gracilis* is non-motile[62]; all are examples of isolates that would not be included if the focus was strictly on the phenotypic characteristics of the common *Campylobacter* species. Additional biochemical or phenotypic tests[50] could have been applied for presumptive isolates in our study, which could change the selectivity estimates by reducing the number of isolates passed to PCR testing. However, the addition of more screening tests for isolates increases both the workload and cost. The only method with 100% selectivity in our study was BB_MA_mCCDA, which could be in part because of the use of the 42°C incubating temperature compared to 37°C used with all other culture methods.

Cross-reaction of the PCR used in our study for *Campylobacter* spp. with *Arcobacter* spp. has been reported,[29] but cross-reaction with *H. winghamensis* is newly observed. *Arcobacter, Campylobacter*, and *Helicobacter* are closely related genera within *Campylobacteraceae* that can be isolated using similar culture methods.[21] The taxa have relatively high similarity both phenotypically and genotypically,[51] which makes their cross-reaction less surprising.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Acke E, et al. A comparison of different culture methods for the recovery of *Campylobacter* species from pets. Zoonoses Public Health 2009;56:490–495.
2. Aspinall ST, et al. A comparison of a new campylobacter selective medium (CAT) with membrane filtration for the isolation of thermophilic campylobacters including *Campylobacter upsaliensis*. J Appl Microbiol 1996;80:645–650.
3. Aspinall ST, et al. Selective medium for thermophilic campylobacters including *Campylobacter upsaliensis*. J Clin Pathol 1993;46:829–831.
4. Baker J, et al. *Campylobacter* species in cats and dogs in South Australia. Aust Vet J 1999;77:662–666.
5. Bessède E, et al. New methods for detection of campylobacters in stool samples in comparison to culture. J Clin Microbiol 2011;49:941–944.
6. Bojanić K, et al. Isolation of *Campylobacter* spp. from client-owned dogs and cats, and retail raw meat pet food in the Manawatu, New Zealand. Zoonoses Public Health 2017;64:438–449.
7. Bolton FJ, et al. Reassessment of selective agars and filtration techniques for isolation of *Campylobacter* species from faeces. Eur J Clin Microbiol Infect Dis 1988;7:155–160.
8. Bolwell CF, et al. Evaluation of the representativeness of a sentinel surveillance site for campylobacteriosis. Epidemiol Infect 2015;143:1990–2002.
9. Bullman S, et al. Emerging dynamics of human campylobacteriosis in southern Ireland. FEMS Immunol Med Microbiol 2011;63:248–253.
10. Butzler JP. *Campylobacter*, from obscurity to celebrity. Clin Microbiol Infect 2004;10:868–876.
11. Byrne C, et al. Basis of the superiority of cefoperazone amphotericin teicoplanin for isolating *Campylobacter upsaliensis* from stools. J Clin Microbiol 2001;39:2713–2716.
12. Chaban B, et al. Detection and quantification of 14 *Campylobacter* species in pet dogs reveals an increase in species richness in feces of diarrheic animals. BMC Microbiol 2010;10:73.
13. Corry JEL, Atabay HI. Comparison of the productivity of cefoperazone amphotericin teicoplanin (CAT) agar and modified charcoal cefoperazone deoxycholate (mCCD) agar for various strains of *Campylobacter, Arcobacter* and *Helicobacter pullorum*. Int J Food Microbiol 1997;38:201–209.
14. Corry JE, et al. Culture media for the isolation of campylobacters. Int J Food Microbiol 1995;26:43–76.
15. de Boer RF, et al. Improved detection of five major gastrointestinal pathogens by use of a molecular screening approach. J Clin Microbiol 2010;48:4140–4146.
16. Debruyne L, et al. *Campylobacter volucris* sp. nov., isolated from black-headed gulls (*Larus ridibundus*). Int J Syst Evol Microbiol 2010;60:1870–1875.
17. Debruyne L, et al. Novel *Campylobacter lari*-like bacteria from humans and molluscs: description of *Campylobacter peloridis* sp. nov., *Campylobacter lari* subsp. *concheus* subsp. nov. and *Campylobacter lari* subsp. *lari* subsp. nov. Int J Syst Evol Microbiol 2009;59:1126–1132.
18. Denis M, et al. Development of a m-PCR assay for simultaneous identification of *Campylobacter jejuni* and *C. coli*. Lett Appl Microbiol 1999;29:406–410.

19. Dingle KE, et al. Multilocus sequence typing system for *Campylobacter jejuni*. J Clin Microbiol 2001;39:14–23.
20. Domingues AR, et al. Source attribution of human campylobacteriosis using a meta-analysis of case-control studies of sporadic infections. Epidemiol Infect 2012;140:970–981.
21. Engberg J, et al. Prevalence of *Campylobacter, Arcobacter, Helicobacter*, and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for campylobacters. J Clin Microbiol 2000;38:286–291.
22. Etoh Y, et al. *Campylobacter showae* sp. nov., isolated from the human oral cavity. Int J Syst Evol Microbiol 1993;43:631–639.
23. French NP, et al. Molecular epidemiology of *Campylobacter jejuni* isolates from wild-bird fecal material in children's playgrounds. Appl Environ Microbiol 2009;75:779–783.
24. Goto K, et al. Epidemiology of *Helicobacter* infection in wild rodents in the Xinjiang-Uygur autonomous region of China. Curr Microbiol 2004;49:221–223.
25. Hald B, et al. Longitudinal study of the excretion patterns of thermophilic *Campylobacter* spp. in young pet dogs in Denmark. J Clin Microbiol 2004;42:2003–2012.
26. Hald B, Madsen M. Healthy puppies and kittens as carriers of *Campylobacter* spp., with special reference to *Campylobacter upsaliensis*. J Clin Microbiol 1997;35:3351–3352.
27. Halebian S, et al. Rapid method that aids in distinguishing gram-positive from gram-negative anaerobic bacteria. J Clin Microbiol 1981;13:444–448.
28. Houf K, et al. Development of a multiplex PCR assay for the simultaneous detection and identification of *Arcobacter butzleri, Arcobacter cryaerophilus* and *Arcobacter skirrowii*. FEMS Microbiol Lett 2000;193:89–94.
29. Inglis GD, Kalischuk LD. Use of PCR for direct detection of *Campylobacter* species in bovine feces. Appl Environ Microbiol 2003;69:3435–3447.
30. Kato Y, et al. Molecular detection of human periodontal pathogens in oral swab specimens from dogs in Japan. J Vet Dent 2011;28:84–89.
31. Koene MGJ, et al. Simultaneous presence of multiple *Campylobacter* species in dogs. J Clin Microbiol 2004;42:819–821.
32. Kweon OJ, et al. First case report of *Campylobacter volucris* bacteremia in an immunocompromised patient. J Clin Microbiol 2015;53:1976–1978.
33. Lastovica AJ. Emerging *Campylobacter* spp.: the tip of the iceberg. Clin Microbiol Newsl 2006;28:49–56.
34. Lastovica AJ, Le Roux E. Efficient isolation of *Campylobacter upsaliensis* from stools. J Clin Microbiol 2001;39:4222–4223.
35. Linton D, et al. Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. Res Microbiol 1996;147:707–718.
36. Lynch ÓA, et al. A method for the growth and recovery of 17 species of *Campylobacter* and its subsequent application to inoculated beef. J Microbiol Methods 2010;83:1–7.
37. Lynch ÓA, et al. Occurrence of fastidious *Campylobacter* spp. in fresh meat and poultry using an adapted cultural protocol. Int J Food Microbiol 2011;150:171–177.
38. Maher M, et al. Evaluation of culture methods and a DNA probe-based PCR assay for detection of *Campylobacter* species in clinical specimens of feces. J Clin Microbiol 2003;41:2980–2986.
39. Man SM. The clinical importance of emerging *Campylobacter* species. Nat Rev Gastroenterol Hepatol 2011;8:669–685.
40. Marks SL, et al. Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control. J Vet Intern Med 2011;25:1195–1208.
41. Melito PL, et al. *Helicobacter winghamensis* sp. nov., a novel *Helicobacter* sp. isolated from patients with gastroenteritis. J Clin Microbiol 2001;39:2412–2417.
42. Mohan V. Faeco-prevalence of *Campylobacter jejuni* in urban wild birds and pets in New Zealand. BMC Res Notes 2015;8:1.
43. Mohan V, et al. Characterisation by multilocus sequence and *porA* and *flaA* typing of *Campylobacter jejuni* isolated from samples of dog faeces collected in one city in New Zealand. N Z Vet J 2017;65:209–213.
44. Moore JE, et al. *Campylobacter*. Vet Res 2005;36:351–382.
45. Mughini Gras L, et al. Increased risk for *Campylobacter jejuni* and *C. coli* infection of pet origin in dog owners and evidence for genetic association between strains causing infection in humans and their pets. Epidemiol Infect 2013;141:2526–2535.
46. Müllner P, et al. Molecular epidemiology of *Campylobacter jejuni* in a geographically isolated country with a uniquely structured poultry industry. Appl Environ Microbiol 2010;76:2145–2154.
47. Mullner P, et al. Molecular and spatial epidemiology of human campylobacteriosis: source association and genotype-related risk factors. Epidemiol Infect 2010;138:1372–1383.
48. Mullner P, et al. Assigning the source of human campylobacteriosis in New Zealand: a comparative genetic and epidemiological approach. Infect Genet Evol 2009;9:1311–1319.
49. Nachamkin I, et al. Diagnosis and antimicrobial susceptibility of *Campylobacter* species. In: Nachamkin I, Blaser MJ, ed. Campylobacter. 2nd ed. Washington, DC: American Society for Microbiology, 2000:45–66.
50. On SL. Identification methods for campylobacters, helicobacters, and related organisms. Clin Microbiol Rev 1996;9:405–422.
51. On SL. Taxonomy of *Campylobacter, Arcobacter, Helicobacter* and related bacteria: current status, future prospects and immediate concerns. J Appl Microbiol 2001;90:1S–15S.
52. On SLW. Isolation, identification and subtyping of *Campylobacter*: where to from here? J Microbiol Methods 2013;95:3–7.
53. Parsons BN, et al. Typing of *Campylobacter jejuni* isolates from dogs by use of multilocus sequence typing and pulsed-field gel electrophoresis. J Clin Microbiol 2009;47:3466–3471.
54. Petersen RF, et al. A PCR-DGGE method for detection and identification of *Campylobacter, Helicobacter, Arcobacter* and related Epsilobacteria and its application to saliva samples from humans and domestic pets. J Appl Microbiol 2007;103:2601–2615.
55. Rossi M, et al. Occurrence and species level diagnostics of *Campylobacter* spp., enteric *Helicobacter* spp. and *Anaerobiospirillum* spp. in healthy and diarrheic dogs and cats. Vet Microbiol 2008;129:304–314.

56. Scanlon KA, et al. Occurrence and characteristics of fastidious *Campylobacteraceae* species in porcine samples. Int J Food Microbiol 2013;163:6–13.
57. Scotter SL, et al. Methods for the detection of thermotolerant campylobacters in foods: results of an inter-laboratory study. J Appl Bacteriol 1993;74:155–163.
58. Skirrow MB. *Campylobacter* enteritis: a 'new' disease. Br Med J 1977;2:9–11.
59. Spencer SEF, et al. The spatial and temporal determinants of campylobacteriosis notifications in New Zealand, 2001–2007. Epidemiol Infect 2012;140:1663–1677.
60. Suzuki MT, Giovannoni SJ. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 1996;62:625–630.
61. Van Dyke MI, et al. The occurrence of *Campylobacter* in river water and waterfowl within a watershed in southern Ontario, Canada. J Appl Microbiol 2010;109:1053–1066.
62. Vandamme P, et al. Chemotaxonomic analyses of *Bacteroides gracilis* and *Bacteroides ureolyticus* and reclassification of *B. gracilis* as *Campylobacter gracilis* comb. nov. Int J Syst Evol Microbiol 1995;45:145–152.
63. Vandamme P, et al. Revision of *Campylobacter, Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. Int J Syst Evol Microbiol 1991;41:88–103.
64. Vanniasinkam T, et al. PCR for the detection of *Campylobacter* spp. in clinical specimens. Lett Appl Microbiol 1999;28:52–56.
65. Wang G, et al. Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni, C. coli, C. lari, C. upsaliensis*, and *C. fetus* subsp. *fetus*. J Clin Microbiol 2002;40:4744–4747.
66. Wheeler WE, Borchers J. Vibrionic enteritis in infants. Am J Dis Child 1961;101:60–66.