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Large-Scale Survey of *Campylobacter* Species in Human Gastroenteritis by PCR and PCR–Enzyme-Linked Immunosorbent Assay

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A PCR-based study of the incidence of enteropathogenic campylobacter infection in humans was done on the basis of a detection and identification algorithm consisting of screening PCRs and species identification by PCR-enzyme-linked immunosorbent assay. This was applied to DNA extracted from 3,738 fecal samples from patients with sporadic cases of acute gastroenteritis, submitted by seven regional Public Health Laboratories in England and Wales over a 2-year period. The sending laboratories had cultured "*Campylobacter* spp." from 464 samples. The PCR methodologies detected 492 *Campylobacter*-positive samples, and the combination of culture and PCR yielded 543 *Campylobacter*-positive samples. There was identity (overlap) for 413 samples, but 79 PCR-positive samples were culture negative, and 51 culture-positive samples were PCR negative. While there was no statistically significant difference between PCR and culture in detection of *C. jejuni*-*C. coli* (PCR, 478 samples; culture, 461 samples), PCR provided unique data about mixed infections and non-*C. jejuni* and non-*C. coli* campylobacters. Mixed infections with *C. jejuni* and *C. coli* were found in 19 samples, and mixed infection with *C. jejuni* and *C. upsaliensis* was found in one sample; this was not apparent from culture. Eleven cases of gastroenteritis were attributed to *C. upsaliensis* by PCR, three cases were attributed to *C. hyointestinalis*, and one case was attributed to *C. lari*. This represents the highest incidence of *C. hyointestinalis* yet reported from human gastroenteritis, while the low incidence of *C. lari* suggests that it is less important in this context.

Gastroenteritis due to *Campylobacter jejuni* and *C. coli* is the principal cause of acute bacterial diarrhea (21). Several other species such as *C. upsaliensis*, *C. hyointestinalis*, and *C. fetus* have also been shown to be enteropathogenic for humans, but their significance remains unclear because the procedure for the isolation of *C. jejuni* and *C. coli* uses selective media that may inhibit their growth (1, 9, 15). Furthermore, clinical laboratories usually do not identify campylobacter isolates to the species level, since they have relatively fastidious growth requirements and lack easily distinguishable biochemical characteristics (9). Thus, non-*C. jejuni* and non-*C. coli* campylobacters may be underreported in human gastrointestinal illness (2, 15).

The target genes used for PCR identification of *Campylobacter* species from cultured isolates have included 16S rRNA (7, 17), 23S rRNA (4), *flaA* (flagellin) (29), GTP-binding protein (27), *ceuE* (iron transport protein) (8), and *hip* (hippuricase) (16). Flagellin gene and 16S rRNA gene (rDNA) PCR assays have been applied to foodstuffs (7, 30). The *flaA* (20), *hip* (16), and 16S rDNA (14) PCRs have been applied directly to small numbers of fecal samples without culture of an isolate. A recent study of 493 fecal samples (28) was based on enrichment culture and 16S rDNA PCR for detection of *C. jejuni*, *C. coli*, and *C. lari*.

To our knowledge, the present study represents the largest PCR-based survey of *Campylobacter* gastroenteritis yet undertaken and demonstrates the utility of this approach for investigation of the incidence and epidemiology of the full spectrum of enteropathogenic campylobacters.

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MATERIALS AND METHODS

Study design. Clinical samples were collected over a 2-year period by seven Public Health Laboratories (PHLs) in England and Wales, termed PHLs A, B, C, D, E, F, and G. The samples were from patients with acute gastroenteritis submitted from general practices and outpatient departments or collected by environmental health officers. Repeat, follow-up samples and samples from inpatients were not examined.

DNA was extracted from aliquots of fecal samples sent to the Central Public Health Laboratory not later than 10 days after initial receipt and culture at the collaborating laboratory. Samples were simply cultured on a selective medium, and no attempt was made to quantify the *Campylobacter* cells present; the lag between specimen culture and DNA extraction precluded direct quantitative comparison. Culture data collected by the collaborating laboratories were withheld until completion of blind PCR assays with DNA extracted from the corresponding fecal samples at the Central Public Health Laboratory. PCR data were then compared with the results of conventional selective culture performed by the contributing laboratories.

Bacterial reference strains. A large range of type and reference strains (19 *Campylobacter*, 12 *Helicobacter*, and 4 *Arcobacter* strains, and 11 strains of other enteropathogenic species) were used as controls as described previously (16).

Bacteriological investigation of clinical samples. Fecal samples were examined for *Campylobacter* spp., *Clostridium difficile*, *Escherichia coli* O:157, *Salmonella* spp., *Shigella* spp., and ova, cysts, and parasites by standard methods. With the exceptions of laboratories A and F, campylobacters were cultured on Campylobacter Blood Free Selective Agar Base (Oxoid CM739) with Charcoal Cefoperazone Desoxycholate Agar supplement (Oxoid SR155). Laboratory A used cefoperazone amphotericin B teicoplanin supplement (Oxoid SR174), while laboratory F used a cefoperazone and amphotericin B supplement (Prolab, Neston, United Kingdom). All plates were incubated for 48 h at 41 to 42°C (except Laboratory A [37°C]) under microaerobic conditions (5% O_2, 5% CO_2, 2% H_2, and 88% N_2, by volume). Isolates were identified to the genus level by morphology and Gram staining. Laboratory A further identified isolates by hippurate hydrolysis, indoxyl acetate hydrolysis, and urease production.

Extraction of nucleic acid from feces. Approximately 200 mg of each clinical fecal sample was homogenized in 2 ml of brucella broth (Life Technologies Ltd., Paisley, United Kingdom). Nucleic acid was extracted from a 100-μl aliquot of the fecal suspension as described previously by Lawson et al. (13). DNA extracts were stored at −20°C prior to screening by PCR.

PCR screening assays. Nucleic acid extracts were screened in batches of 96 samples (including positive and negative controls). Two 16S rDNA PCR assays were used to screen all fecal extracts. The first assay, termed pathgroup, was a newly designed assay specific for the *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and

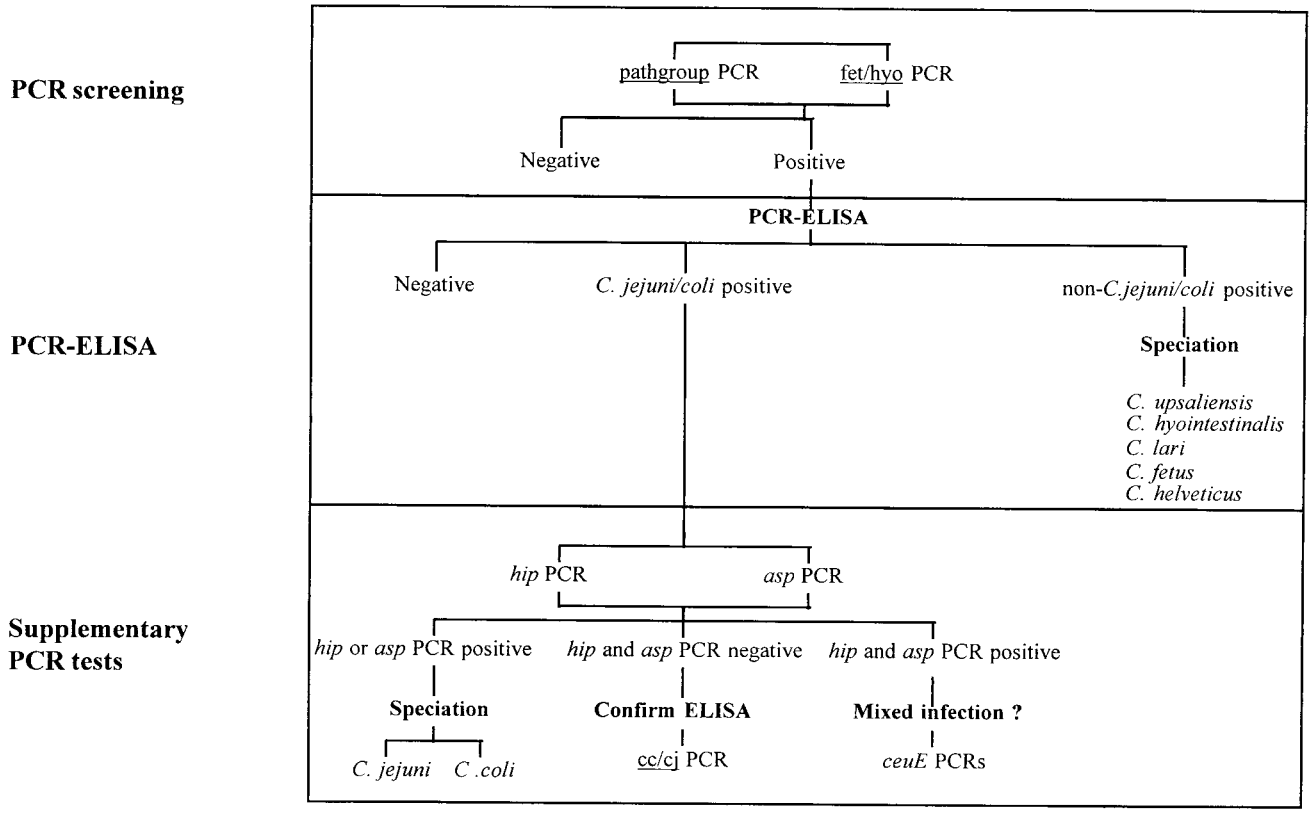

FIG. 1. Algorithm for *Campylobacter* detection and species identification (speciation) by PCR and PCR-ELISA.

C. helveticus group. The second assay, termed fet/hyo, was a duplex assay specific for *C. fetus* and *C. hyointestinalis* (17). The screening step is shown in the algorithm presented in Fig. 1.

Each 2.5-μl nucleic acid extract obtained from a fecal sample was amplified in a 25-μl reaction volume in a 96-well-format microtiter plate as described previously (14). Amplification conditions were denaturation at 94°C for 1 min, annealing at either 66°C for pathgroup or 65°C for fet/hyo for 1 min, and extension at 72°C for 1 min for 30 cycles in a RoboCycler thermocycler with a hot top assembly (Stratagene, La Jolla, Calif.). The products were analyzed by 96-well-format electrophoresis (on a 1% [wt/vol] agarose gel). The gels were stained with SYBR green I (Flowgen Instruments Ltd., Lichfield, United Kingdom).

Species identification. Samples positive by the screening PCR assays were identified by PCR-enzyme-linked immunosorbent assay (PCR-ELISA) (18) by using capture probes specific for *C. jejuni-C. coli*, *C. upsaliensis*, *C. hyointestinalis*, *C. lari*, *C. fetus*, and *C. helveticus*. Those samples identified as *C. jejuni-C. coli* by PCR-ELISA were further examined by supplementary PCR assays specific for the *hip* gene of *C. jejuni* and the aspartokinase (*asp*) gene of *C. coli* (16). In cases in which these two PCRs proved negative, a more sensitive PCR (cc/cj) for the multicopy 16S rRNA gene (16) was applied to confirm the PCR-ELISA data. In cases of potential mixed culture, two sets of *ceuE* gene primers (8) capable of distinguishing *C. jejuni* from *C. coli* were also used. The procedure used for species identification is summarized in the algorithm (Fig. 1).

Statistical analysis. The results of *Campylobacter* detection by the PCR screening assay and PCR-ELISA were compared with those obtained by culture on selective agar at contributing laboratories by McNemar's test (22).

RESULTS

Design and application of primers. Phylogenetic analysis was conducted with Megalign (Lasergene package; DNASTAR, Inc., Madison, Wis.). Sequences of 16S rDNA from *Helicobacter pylori*, *Bacteroides ureolyticus*, and 15 species of *Campylobacter* were recovered from the GenBank database and were aligned by the Clustal method (23). PCR primer pairs were designed from the alignment with the aid of the program Oligo (version 4.0; National Biosciences, Plymouth, Mass.). A set of pathgroup primers which inclusively detected *C. jejuni*, *C. coli*, *C. upsaliensis*, *C. lari*, and *C. helveticus* were designed in this manner. The forward pathgroup primer was 5′-ACA TGC AAG TCG AAC GAT GAA GC-3′ and the reverse pathgroup primer was 5′-TAT AGA TTT GCT CCA CCT CGC GG-3′. These yielded an amplicon of 1,195 bp from DNA prepared from reference strains of the five species mentioned above but not from the remaining type strains of *Campylobacter* and the other enteropathogenic species listed in Materials and Methods. The pathgroup primers were also tested with a set of 200 fecal samples which included 18 cc/cj-positive samples (14) known to contain *C. jejuni* or *C. coli*. With an annealing temperature of 66°C, pathgroup primers detected all 18 previous cc/cj-positive samples. Nonspecific mismatch products could be eliminated by raising the annealing temperature to 68°C, but at the expense of some loss of sensitivity. The lower annealing temperature (66°C) was retained for screening purposes in the full survey. For this and for the fet/hyo assay, any mismatch products were eliminated by the subsequent PCR-ELISA (cf. Fig. 1).

In the full survey of 3,738 samples, the pathgroup PCR was positive for 720 samples, while the fet/hyo duplex PCR was positive for 29 samples.

PCR-ELISA data. The 749 samples positive by the screening assays described above were subjected to PCR-ELISA according to the algorithm presented in Fig. 1. This identified campylobacters in 492 samples, as follows: *C. jejuni-C. coli* in 477 samples, *C. jejuni-C. coli* and *C. upsaliensis* in 1 sample, *C. upsaliensis* in 10 samples, *C. hyointestinalis* in 3 samples, and *C. lari* in 1 sample. The remaining 257 screening PCR-positive samples were PCR-ELISA negative. In this survey neither *C. fetus* nor *C. helveticus* was detected. A breakdown, by send-

TABLE 1. Detection of *Campylobacter* species

Source	No. of samples examined	No. of samples positive by specific technique/total no. of samples positive by both techniques[a]							
		C. jejuni-C. coli		*C. upsaliensis*		*C. hyointestinalis*		*C. lari*	
		PCR-ELISA	Culture	PCR-ELISA	Culture	PCR-ELISA	Culture	PCR-ELISA	Culture
Lab A	1,107	106/121	108/121	3[b]/3	1[c]/3	1/1	0/1		
Lab B	725	63/73	65/73	1/1	0/1	1/1	1[c]/1		
Lab C	1,178	186/203	189/203	5/5	0/5	1/1	0/1		
Lab D	300	55/57	35/57	1/1	0/1			1/1	0/1
Lab E	166	20/21	12/21						
Lab F	162	31/36	35/36	1/1	1[c]/1				
Lab G	100	17/18	17/18						
Total	3,738	478/529	461/529	11/11	2/11	3/3	1/3	1/1	0/1

[a] For example, in the case of *C. jejuni-C. coli*, a total of 461 of 3,738 isolates were detected by culture, while 478 of 529 were identified by the PCR algorithm described in Fig. 1 (PCR-ELISA). A total of 529 were found by a combination of methods; i.e., 68 were not detected by culture and 51 were not identified by PCR-ELISA.
[b] Includes one sample with a mixed infection with *C. upsaliensis* and *C. jejuni* detected by PCR-ELISA.
[c] For these samples, culture identified the strain only as a "*Campylobacter* sp." Subsequent species identifications were by PCR-ELISA.

ing laboratory, for detection of *C. jejuni-C. coli* and non-*C. jejuni*–non-*C. coli* campylobacters is given in Table 1.

Identification of *C. jejuni* and *C. coli*. PCR-ELISA detected 478 *C. jejuni-C. coli*-positive samples. These were subjected to supplementary PCRs, as outlined in Fig. 1 and Materials and Methods. With the *hip* primers, the campylobacters in 408 samples were positively identified as *C. jejuni*. With the *C. coli*-specific *asp* primers, the campylobacters in 16 samples were positively identified as *C. coli*. A further 19 samples were positive for both *hip* and *asp*, indicative of a mixed infection. These 19 samples were therefore investigated with different sets of *ceuE* primers, one specific for the *C. jejuni* sequence and one specific for the *C. coli* sequence. All 19 were positive by assays with both sets of primers, confirming mixed infections. From the remaining 35 samples positive for *C. jejuni-C. coli* by PCR-ELISA, no *hip* or *asp* amplicon was obtained. We were therefore unable to distinguish the two species in these samples but confirmed the PCR-ELISA result by the equivalent simple cc/cj PCR assay (Fig. 1).

Comparison of PCR-based and culture-based detection. The combination of results from the PCR screening, PCR-ELISA, and supplementary PCR assays allowed each sample to be assigned an overall molecular identification. This was then compared with the culture data: "*Campylobacter* spp." were cultured by the seven contributing laboratories from 464 of the 3,738 samples. Of these, the campylobacters in 413 samples (410 with *C. jejuni-C. coli*, 2 with *C. upsaliensis*, and 1 with *C. hyointestinalis*) had been identified by PCR-ELISA with the corresponding fecal sample. The remaining 51 were culture positive but PCR-ELISA negative (Table 1).

Seventy-nine culture-negative samples were positive by the screening PCR and PCR-ELISA (67 with *C. jejuni-C. coli*, 1 with a mixture of *C. jejuni-C. coli* and *C. upsaliensis*, 8 with *C. upsaliensis*, 2 with *C. hyointestinalis*, and 1 with *C. lari*; cf. Table 1). Thus, the combination of screening PCR and PCR-ELISA detected *Campylobacter* spp. in 543 of the 3,738 samples (528 with *C. jejuni-C. coli*, 1 with a mixture of *C. jejuni-C. coli* and *C. upsaliensis*, and 14 with other non-*C. jejuni*-non *C. coli* campylobacters).

For three of the samples reported by sending laboratories to be "*Campylobacter* sp. positive," PCR-ELISA detected *C. upsaliensis* (two samples) and *C. hyointestinalis* (one sample). Nine other *C. upsaliensis*-positive samples and two other *C. hyointestinalis*-positive samples were detected by PCR-ELISA but not by culture. One of those *C. upsaliensis* strains was detected by PCR-ELISA in a sample with a mixed infection with *C. jejuni*. The latter was detected by culture as well as by PCR-ELISA. A single *C. lari* infection was identified by PCR-ELISA but not by culture (cf. Table 1).

There was no statistical difference between the number of *C. jejuni-C. coli*-positive samples detected by PCR-ELISA or by culture in the study as a whole ($0.5 > P > 0.1$). In terms of individual sending laboratories, there was no statistical difference between the culture and PCR-ELISA for laboratories A, B, C, ($P > 0.5$), F ($0.5 > P > 0.1$), and G ($P = 0.5$). However, the detection rate was significantly higher by PCR-ELISA than by culture for laboratories D ($P < 0.001$) and E ($0.02 > P > 0.01$).

DISCUSSION

To our knowledge this study is the largest molecular survey of *Campylobacter* gastroenteritis yet undertaken and represents the first application of a PCR-based protocol to investigate the incidence of the enteropathogenic *Campylobacter* spp. in an epidemiologically valid context.

A key development in this study was the design of PCR assays specific for groups of enteropathogenic species rather than the use of a series of individual species-specific PCRs as we have described previously (14, 16). This was intended to reduce the number of PCR assays performed in the course of a large-scale survey. The basis for the screening PCRs described in Fig. 1 was phylogenetic trees drawn from alignments of *Campylobacter* 16S rDNA sequences (12, 24, 26). These trees all contained three distinct clades (species groups). The first contained *C. gracilis*, *C. sputorum*, *C. curvus*, *C. concisus*, *C. rectus*, and *C. showae*, organisms principally associated with niches in the periodontal cavities of humans and animals and which have as yet no association with human gastroenteritis. The second clade consisted of *C. fetus*, *C. hyointestinalis*, and *C. mucosalis*, which are historically associated with disease in farm animals, although the first two have also been occasionally implicated in human disease. The third clade consisted of *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*, species (other than *C. helveticus*) known to cause gastroenteritis in humans. In accordance with the requirements of this study, we developed primers for the third clade and used existing primers specific for *C. fetus* and *C. hyointestinalis* (17), the only other causative agents of human gastroenteritis that have been described.

In the course of the study several key practical issues of importance to any large-scale PCR-based survey were identified. These included the necessity of a dedicated PCR suite to reduce the risk of contamination, the robustness of the thermocyclers, and the regular monitoring of the performance and detection threshold for PCR primers (their titers and qualities could deteriorate over time or could vary between batches and manufacturers).

C. jejuni and *C. coli*, as expected, represented the largest proportion of PCR-positive samples. There was congruence between PCR and culture for 77.5% of the 529 positive samples, while 12.9% were found to be positive only by PCR and 9.6% were found to be positive only by culture. In any comparison of two detection methods with a sizeable sample number, one would not expect a complete correlation. Nonetheless, we note that in this investigation there are further factors to consider. Culture positive-only samples may have been PCR negative due to degradation of *Campylobacter* cells and DNA in the period (up to 10 days) between culture and receipt of the fecal sample for DNA extraction. In some cases inhibitory substances present in feces may have reduced the sensitivities of the PCR assays. There is also a possibility that certain wild-type *C. jejuni* or *C. coli* strains might have 16S rDNA sequences which are sufficiently divergent that they are not detected by PCR-ELISA, despite its detection of all Penner serotype reference strains (18). Isolates from a proportion of the specimens (laboratory C) which were negative by PCR were all successfully identified as *C. jejuni* with the PCR algorithm (Fig. 1). This suggests that culture-positive, PCR-negative samples occurred due to sampling factors rather than variations in target DNA sequences.

Culture has been found to be more sensitive than PCR in seeding experiments with logarithmic-phase cultures of laboratory strains of *C. jejuni* (14). That finding may be due to the amount of fecal material which is sampled when inoculating a selective agar plate, as opposed to the small volume of diluted material (2.5 μl) sampled by PCR.

Nonetheless, our study found more positive samples by PCR-ELISA than by culture alone. This probably reflects the detection of *Campylobacter* cells in metabolic states that are less amenable to culture on selective media (sublethally damaged cells, viable but nonculturable cells, or even dead cells). A key feature of the PCR algorithm was that it provided both detection and identification.

The 11 *C. upsaliensis* isolates detected by PCR-ELISA represent an incidence of 0.29%. Only two of these samples had been positive by culture, and for each sample the isolate was reported as a "*Campylobacter* sp." The sex/age (in years) distributions for the patients who provided *C. upsaliensis*-positive samples (m/2, m/5, m/10, f/23, m/25, f/27, f/33, m7/8, m/u, m/u, and u/u, where m is male, f is female, and u is unspecified) showed no evidence of an association with pediatric gastroenteritis, as has been previously reported in the literature (2, 10).

The three cases of *C. hyointestinalis* infection represent an incidence of 0.08%. Only one of these samples was reported as "*Campylobacter* sp." positive by culture. The sex/age distributions were m/39, f/66, and f/u. This represents the highest incidence of *C. hyointestinalis* yet reported as a cause of human gastroenteritis. The only other incidence figure available, based on culture, was 0.01% (2 of 15,185 cases) (11). Earlier pilot PCR studies by our group had detected *C. hyointestinalis* in 1 of 25 (16) and 1 of 200 (14) patients with gastroenteritis. Altogether, this would give an incidence of 0.13% (5 of 3,963). Although *C. hyointestinalis* is associated with proliferative enteritis in pigs (6), it has previously been considered to be only a very rare cause of gastroenteritis in humans (3, 5, 19). Our results suggest that further investigation of *C. hyointestinalis* as a human enteropathogen would be appropriate.

We detected *C. lari* (by PCR-ELISA alone) in only one sample (an incidence of 0.03%), although this species is often cited (15) as the third most commonly isolated enteropathogenic campylobacter from humans. The low incidence in our survey suggests that *C. lari* is less important than *C. upsaliensis* and *C. hyointestinalis* in human gastroenteritis.

In 19 samples there was evidence from supplementary PCR assays of a mixed infection with *C. jejuni* and *C. coli* not apparent from culture. Seven of these occurred in a laboratory (laboratory A) which had the capacity to identify some isolates to the species level and which reported that five were *C. jejuni* and two were *C. coli*. It is likely, therefore, that only the predominant colony type had been selected for identification. It is interesting that there were slightly more mixed *C. jejuni* and *C. coli* infections than *C. coli* infections alone. Mixtures of *Campylobacter* species (and serotypes) in human infections may be more common than was heretofore assumed. Another mixed *Campylobacter* infection noted in the survey was of *C. upsaliensis* and *C. jejuni*, which were codetected by PCR-ELISA; here, only the *C. jejuni* component was apparent from culture. There were also instances of coinfections of *C. jejuni-C. coli* with other enteropathogens detected by culture and/or microscopy at the contributing laboratories. There were mixtures of *C. jejuni* with both *Shigella sonnei* and *Blastocystis hominis* cysts (one sample), with a *Salmonella* sp. (two samples), and with *Cryptosporidium parvum* oocysts (three samples). The mixtures of *Cryptosporidium* and *C. jejuni-C. coli* are of interest since the presence of a coccidian, which is most frequently associated with waterborne infections (25), suggests that the route of transmission of the *C. jejuni* may have been water for these samples.

Although PCR is more expensive and labor-intensive than culture, it offers a nonselective way to monitor the incidence of enteropathogenic bacteria. We have shown that it is of value for epidemiological purposes, and it will ultimately be amenable to automation. The use of a broad-specificity screening PCR greatly reduces the number of assays required for a comprehensive survey. We have also demonstrated that our previously published PCR-ELISA (18) can be successfully applied in this context. In summary, the PCR algorithm presented here offers a different perspective on *Campylobacter* gastroenteritis than that provided by culture, giving information on the identity and occurrence of species that are not detected by culture. PCR-based analysis has a role in large-scale epidemiological surveys of *Campylobacter*.

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