PCR Detection, Identification to Species Level, and Fingerprinting of Campylobacter jejuni and Campylobacter coli Direct from Diarrheic Samples

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Campylobacter enteritis is the most common cause of acute bacterial diarrhea worldwide. Estimates of the true campylobacter infection rate in the United States and the United Kingdom are as high as 1% of the population per year (12, 22). Fifteen species of Campylobacter have been described. Two of these, Campylobacter jejuni and Campylobacter coli, account for the majority of human infections. Campylobacteriosis is currently diagnosed by isolation of the organism, which requires microaerobic incubation at 37 or 42°C for 48 h. A further 24 h is required for full phenotypic identification. Campylobacters are asaccharolytic, fastidious bacteria, and this limits the available phenotypic tests by which isolates may be differentiated (10). Identification to species level is hindered by variations in methodology and the subjective interpretation of biochemical test results. There are also isolates with atypical phenotypes. For example, the differentiation of C. jejuni from C. coli relies on the ability of C. jejuni to hydrolyze hippurate (18), but certain atypical C. jejuni strains fail to do so (16, 18), rendering identification based on this single test unreliable. These limitations might in principle be overcome by the use of PCR-based genotypic methods. Furthermore, it would be advantageous to identify campylobacters directly in a fecal sample, thereby avoiding the need for culture. The application of PCR to feces has been problematical, since many inhibitors are present (27). For this report we have designed new PCR assays, specific for C. jejuni-C. coli and for C. jejuni and C. coli alone, to be used with a protocol for the extraction of total DNA from feces suitable for PCR (5). We have evaluated these PCRs for detection, identification to species level, and typing of campylobacters directly from human fecal specimens.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The following 110 microaerobic bacterial control strains were tested with the PCR assays following culture on 5% Columbia blood agar plates at 37°C in a variable-atmosphere incubator (Don Whitley Scientific, Ltd.) which maintained a microaerobic atmosphere of 5% O2, 5% CO2, 2% H2, and 88% N2; C. jejuni subsp. jejuni NCTC 113517 and Penner serotype reference strains NCTC 12500, NCTC 12501, NCTC 12502, NCTC 12561, NCTC 12560, NCTC 12505, NCTC 12506, NCTC 12507, NCTC 12508, NCTC 12509, NCTC 12510, NCTC 12511, NCTC 12512, NCTC 12513, NCTC 12514, NCTC 12515, NCTC 12516, NCTC 12517, NCTC 12518, NCTC 12519, NCTC 12520, NCTC 12521, NCTC 12522, NCTC 12523, NCTC 12524, NCTC 12525, NCTC 12526, NCTC 12527, NCTC 12528, NCTC 12547, NCTC 12562, NCTC 12539, NCTC 12540, NCTC 12541, NCTC 12542, NCTC 12543, NCTC 12548, NCTC 12549, NCTC 12550, NCTC 12560, NCTC 12564, NCTC 12552, NCTC 12553, NCTC 12554, NCTC 12555, NCTC 12556, NCTC 12557, and NCTC 12558; C. jejuni subsp. jejuni hippurate-negative strains D 603, D 712, D 835, D 941, D 957, D 996, D 597, D 1713, D 1916, and D 2832; C. jejuni subsp. doylei NCTC 119517, D 2295, D 2722, D 2781, D 2990, D 3816, D 3818, D 3820, D 3836, and D 3886; C. coli NCTC 113667 and Penner serotype reference strains NCTC 12525, NCTC 12526, NCTC 12527, NCTC 12528, NCTC 12529, NCTC 12530, NCTC 12531, NCTC 12532, NCTC 12533, NCTC 12535, NCTC 12536, NCTC 12550, and NCTC 12551; C. concisus NCTC 114857, C. fetus subsp. fetus NCTC 108427, CCUG 7473, and CCUG 112866; C. fetus subsp. venenalis NCTC 103547, CCUG 7477, and CCUG 24260; C. helveticus NCTC 124707 and NCTC 12845; C. hylotoma RMT 32A; C. hyointestinalis NCTC 116877; CCUG 14915, and CCUG 112686; C. lan NCTC 113527, NCTC 11357, and NCTC 12144; C. mucosalis NCTC 110977; C. putorum subsp. putorum NCTC 115287; C. putorum subsp. facialis NCTC 114157; C. putorum subsp. ubalalus NCTC 113677; C. putalensis NCTC 115417; NCTC 11840, and NCTC 11926; Arcobacter cryaurophilus NCTC 118877; and Helicobacter pylori NCTC 116377.

The following five anaerobic species were tested following incubation on 5% Columbia blood agar plates at 37°C in an anaerobic work station (Don Whitley Scientific, Ltd.) which maintained an atmosphere of 5% CO2, 5% H2, and 90% N2; Bacteroides ureolyticus NCTC 109417; C. curvus NCTC 116497; C. gracilis NCTC 127387; C. rectus NCTC 114907; and C. showar NCTC 128437. The following 14 species were tested following aerobic culture on 5% Columbia blood agar plates at 37°C: Aeromonas hydrophila NCTC 80497; Escherichia coli NCTC 90017; NCTC 10448, and NCTC 10664; Vero-toxigenic E. coli serotype O157 strain NCTC 12079; Salmonella enteritidis NCTC 126947; Salmonella typhimurium NCTC 108887; Salmonella choleraeuis NCTC 102497; Salmonella dublin NCTC 118347; Salmonella typhosa NCTC 119767; Salmonella typhi NCTC 119767; Salmonella enterica NCTC 119767; Salmonella paratyphi A NCTC 119767; Salmonella paratyphi B NCTC 119767; Salmonella paratyphi C NCTC 119767; Salmonella paratyphi D NCTC 119767; Salmonella enterica serovar Typhimurium NCTC 119767; Salmonella enterica serovar Enteritidis NCTC 119767; Salmonella enterica serovar Agona NCTC 119767; Salmonella enterica serovar Bioul NCTC 119767; Salmonella enterica serovar Hadar NCTC 119767; Salmonella enterica serovar Infantis NCTC 119767; Salmonella enterica serovar Montevideo NCTC 119767; Salmonella enterica serovar Derby NCTC 119767; Salmonella enterica serovar Dublin NCTC 119767; Salmonella enterica serovar Typhi NCTC 119767; Salmonella enterica serovar Virchow NCTC 119767; Salmonella enterica serovar Kentucky NCTC 119767; Salmonella enterica serovar Mini NCTC 119767; Salmonella enterica serovar New Delhi NCTC 119767; and Salmonella enterica serovar Oranienburg NCTC 119767. These results were concordant with culture and phenotypic identification of the corresponding campylobacter isolate. Twenty-five Campylobacter-negative stool samples gave no reaction with the PCR assays. These PCR assays can rapidly and accurately identify campylobacters directly from human fecal specimens.
PCR FOR CAMPYLOBACTERS IN DIARRHEIC STOOLS

PCR primer design and amplification. Regions of the 16S RNA (trs) gene sequence which were identical for C. jejuni, C. coli, and a recently described (porcine) species, C. hyoilei, but different for other Campylobacter species, were sought. For the first PCR, a primer pair was designed from such a region with mismatched bases located at the 3' end of the primer-template duplex. The second PCR primer pair was based on the sequence of the hip gene. The PCR amplification conditions were as follows: denaturation at 94°C for 1 min, annealing at a temperature specific to the primer pair (see below) for 1 min, and extension at 72°C for 1 min. For C. jejuni-C. coli PCR, an annealing temperature of 58°C was used; for hip purpuricase PCR, an annealing temperature of 56°C was used. PCR products were analyzed by agarose gel electrophoresis (Life Technologies, Ltd.; μL) and 1 ng of genomic DNA/μL. Reaction mixes were overlaid with mineral oil and subjected to 25 cycles of amplification in a thermal cycle (Roche). The cycling was as follows: denaturation at 94°C for 1 min, annealing at a temperature specific to the primer pair (see below) for 1 min, and extension at 72°C for 1 min. For C. jejuni-C. coli PCR, an annealing temperature of 58°C was used; for hip purpuricase PCR, an annealing temperature of 56°C was used. 

RESULTS

The 16S rDNA-based PCR assay specific for C. jejuni and C. coli. Regions were identified from an alignment of 16S RNA (trs) gene sequences in which the sequences for C. jejuni and C. coli differed from those of other Campylobacter species. From two such regions, nucleotides 609 to 629 and 1442 to 1654 (according to the E. coli numbering scheme), a primer pair was designed for coidentification of the two species. The sequence of the forward primer, termed CCCJ609F, was 5'-ATT CTA ATG GCT TTA ACA CCA TTA-3', and the sequence of the reverse primer, termed CCCJ1442R, was 5'-GTA ACT AGT TTA GTA TTC CGG-3'. This assay was tested against the DNAs of the type strains of all species in the genus Campylobacter. The primer pair was employed in a PCR with an annealing temperature of 58°C, generating an amplicon of 854 bp from all tested strains of both C. jejuni subspecies and C. coli but not from all tested strains of the other Campylobacter species with the exception of C. hyoilei, a recently described species associated with proliferative enteritis of pigs (1). This species is a close phylogenetic relative of C. jejuni and C. coli (see below). There was no reaction with any other non-campylobacter species listed in Materials and Methods.

The hippuricase gene-based PCR assay specific for C. jejuni. The gene encoding hippuricase (hip) was recently cloned from C. jejuni, and a DNA probe derived from it hybridized to genomic DNAs of all strains of C. jejuni, including 11 phenotypically negative strains (11). On the basis of the nucleotide sequence (11), we designed a primer pair for amplification of the hip gene, which is absent from campylobacters other than C. jejuni. A forward primer, termed HIP400F, whose sequence was 5'-GAA GAG GTG TGG GGT G-3', was designed to anneal to nucleotides 400 to 418 of the hip sequence. A reverse primer, termed HIP1134R, whose sequence was 5'-AGC TAG ATT CAT ATT ACG C-3', was designed to anneal to nucleotides 1112 to 1134. The predicted product size was 735 bp. By using a PCR cycle with an annealing temperature of 66°C, an amplicon of this size was generated from all tested strains of C. jejuni and C. hyoilei, a probe that hybridized only to the type strain of C. jejuni. This probe did not hybridize to other serotype reference strains of that species. A clone (termed CCCH) that hybridized to all C. jejuni, C. coli, and C. hyoilei. No product was obtained from any other enteric bacteria tested (see above).

The PCR assay identifying C. coli. Genomic DNA from the type strain of C. coli was digested with HindIII, and those fragments sized between 0.5 and 4.0 kb were cloned into plasmid pUC19. Twenty such cloned fragments, amplified by PCR from cultures of E. coli containing recombinant plasmids (see Materials and Methods), were hybridized against genomic DNA from the type strains of C. coli, C. jejuni, and C. hyoilei. A probe that hybridized only to the type strain of C. coli failed to hybridize to other serotype reference strains of that species. A clone (termed CCCH) that hybridized to all C. coli Penner serotype reference strains, but not to those of C. jejuni, cross-reacted with the type strain of C. hyoilei.

Both strands of CCCH were sequenced. The 583 bp of DNA cloned from C. coli NCTC 113666 encoded an open reading frame of 405 bp (135 amino acids) immediately downstream of 178 bp of sequence with homology to the 3' end of aspartokinase genes. Primers were designed based on the sequence of

Nucleotide sequence accession numbers. The sequences of both strands of CCCH and of the PCR ampiclon from C. hyoilei have been deposited in GenBank under accession no. AF017758 and AF017759, respectively.
isolation of Campylobacter species on charcoal cefoperazone deoxycholate agar under microaerobic conditions.

Phenotypically identified to species level. One of the 20 culture-positive samples also gave a strong positive signal when tested with primers specific for *C. hyointestinalis*. Further investigation by culture confirmed a mixed infection of *C. jejuni* and *C. hyointestinalis*. NPI, no bacterial pathogens isolated.

PCR assays were carried out on fecal extracts. *rs*, 16S rRNA gene-based PCR assay; *hip*, hippuricase gene-based PCR assay; CCCH, *C. coli*-specific PCR assay.

**TABLE 1. PCR assays of fecal samples**

<table>
<thead>
<tr>
<th>Sample category</th>
<th>Isolate</th>
<th>No. of samples</th>
<th>Results of PCR assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture-positive diarrheic stools</td>
<td><em>C. jejuni</em></td>
<td>18</td>
<td>+ + + -</td>
</tr>
<tr>
<td>Culture-positive diarrheic stools</td>
<td><em>C. coli</em></td>
<td>2</td>
<td>+ - + +</td>
</tr>
<tr>
<td>Culture-negative diarrheic stools</td>
<td>NPI</td>
<td>5</td>
<td>- - - -</td>
</tr>
<tr>
<td>Culture-negative healthy stools</td>
<td>NPI</td>
<td>20</td>
<td>- - - -</td>
</tr>
</tbody>
</table>

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Application to fecal samples. The clinical samples provided by the Tooting Public Health Laboratory had been stored at 4°C for at least 7 days. From 20 of them, Campylobacter spp. had been isolated (Table 1), while from five others, no bacterial pathogens had been isolated (Table 1). Of the 20 isolates, 18 were identified phenotypically by us as *C. jejuni*, and 2 were identified as *C. coli*.

Following extraction of fecal DNA, these 25 samples were screened by PCR with the primer pairs specific for *C. jejuni*, for *C. coli*, and for *C. jejuni* and *C. coli* together. The 16S rRNA gene-based PCR (for *C. jejuni* and *C. coli* together) was positive for the 20 fecal samples from which a campylobacter strain had been isolated (Fig. 1A). For 18 of these 20 samples, the *C. jejuni*-specific PCR assay was also positive (Fig. 1B) while the *C. coli*-specific PCR was negative (Fig. 1C). For two samples (Fig. 1, lanes 9 and 14), the *C. coli*-specific PCR was positive (Fig. 1C) while the *C. jejuni*-specific PCR was negative (Fig. 1B). The five samples from which no bacterial pathogens had been isolated (Fig. 1) gave a negative result for all three PCR assays. The results of the PCR assays were in complete agreement with phenotypic methods for identification of the bacterial isolates to the species level. The 20 *C. jejuni* and *C. coli* isolates were then genotyped by PCR-restriction fragment length polymorphism (RFLP) of the flaA gene. The flaA gene fragments were as shown in Fig. 2A. When the same experiment was performed with fecal DNA as the substrate, identical flaA fingerprints were obtained for matching fecal samples (Fig. 2B) and isolates (Fig. 2A). The flaA fingerprints of the two *C. coli* isolates (Fig. 2A, lanes 9 and 14) matched those of a previously reported *C. coli* flaA fingerprint, designated fVI (20).

The 25 samples were PCR screened with primers specific for *C. lari*, *C. upsaliensis*, *C. helveticus*, *C. fetus*, and *C. hyointestinalis* (14). All samples but one were negative in these assays. Sample 6, from which *C. jejuni* had been isolated and detected by PCR, also gave an amplicon with primers which identify *C. hyointestinalis*. The primary culture from the corresponding fecal sample was reincubated at 25°C (conditions generally selective for *C. hyointestinalis*), and a small number of colonies were isolated. The pure culture which grew from these colonies was *H_{2}S* positive in triple sugar iron agar stabs, hippurate negative, resistant to nalidixic acid, sensitive to cephalothin, and unable to hydrolyze indoxyl acetate, and it grew at both 25 and 42°C. These phenotypes differentiate *C. hyointestinalis* from *C. jejuni*. The isolate gave the appropriate PCR amplicon with primers specific for *C. hyointestinalis*. Twenty further fecal samples provided by healthy volunteers were screened with the whole range of PCR primers as described above, and all were found to be negative.

**DISCUSSION**

PCR assays described in this report were designed for rapid and definitive identification to the species level of *C. jejuni* and *C. coli*. A major application is the detection and identification to the species level of *Campylobacter* in diarrheic stools. In

**FIG. 1. PCR identification to species level of campylobacters in 25 acute-phase diarrheic stools. (A) 16S rRNA gene PCR identifying *C. jejuni* and *C. coli* in the first 20 samples. (B) *hip* gene PCR identifies 18 of the above samples as positive for *C. jejuni*. Exceptions are samples for which results are shown in lanes 9 and 14. (C) *C. coli* specific PCR detects presence of this species in samples 9 and 14. The last five samples (lanes 21 to 25) gave no reaction with any primer pair. m, molecular size markers.
current laboratory practice, identification of campylobacters to the species level relies on relatively few phenotypic tests. For example, C. jejuni and C. coli are distinguished only by hippurate hydrolysis, while C. coli and C. upsaliensis are distinguished by the weak catalase activity and sensitivity to cephalothin of the latter species. Due to these limitations, clinical laboratories often report these enteropathogens simply as Campylobacter species. Even when a rapid hippurate hydrolysis phenotypic test is performed to identify C. jejuni isolates, significant difficulty remains in the identification of any hippurate-negative isolates, which could belong to other Campylobacter species or could indeed be hippurate-negative strains of C. jejuni (16). Other methods, which are definitive, reliable, and easy to use, are required to facilitate rapid identification of campylobacters to the species level.

PCR is such an alternative. PCR assays which co-identify C. jejuni and C. coli, based on the 16S rRNA gene sequence (9, 23), the flagellin (fla) gene sequence (17, 25), and an intergenic region of the tandemly arranged flagellin genes (26), have been previously reported, and PCR assays specific for C. jejuni, based on the 23S rRNA gene sequence (7) or that of a gene (mapA) coding for an outer membrane protein (21), have also been reported. The fla gene-based assays (17, 25) have been successfully applied to fecal material, but it should be noted that, unlike the assays reported in the present study, they do not offer a primary distinction between C. jejuni and C. coli.

In this study we have designed and evaluated three new PCR assays which can be employed in several ways. The first of these (based on 16S rDNA) provides for co-identification (from a pure culture or from fecal material) of the major human enteropathogens C. jejuni and C. coli. By current isolation and culture methods, they are estimated to cause approximately 99% of campylobacter infections in England and Wales or the United States (3, 22), and this assay can therefore match current reporting procedures for most clinical laboratories. Clearer understanding of the relative disease significance of campylobacters requires reliable identification to the species level. This is an important first step in epidemiological studies of campylobacter infection of humans. To this end, we have designed and evaluated PCR assays specific for C. jejuni and for C. coli. The respective PCR targets are unique to strains of C. jejuni (hippuricase gene) and to strains of C. coli or C. hyointestinalis (partial clone of the aspartokinase gene of C. coli), as is shown here in tests on a range of strains broadly representative of the species as measured by serotype diversity (48 Penner serotype reference strains of C. jejuni, 11 hippurate-negative strains of C. jejuni, 10 strains of C. jejuni subsp. doylei, and 14 Penner serotype reference strains of C. coli). The fact that the rs and aspartokinase PCRs cross-react with C. hyointestinalis should be noted but is not likely to provide an obstacle to practical application, since this is a species which has not been isolated from humans.

Negative PCR results were obtained from all control bacterial strains tested; these included a wide range of non-jejuni, non-coli campylobacters, related genera, and unrelated enteric species. Twenty-five control stool samples (20 from healthy individuals and 5 from diarrheic patients) which were negative by culture for campylobacters were also PCR negative by the new assays. These samples would have contained a complete range of bacterial species native to the human gastrointestinal tract, as undefined fecal flora. This demonstrates the specific specificity of the assays. Cultivable bacteria represent only a proportion of the total flora native to the gastrointestinal tract (4), and campylobacter culture-negative feces thus represent a comprehensive specificity control for these PCR assays.

With respect to the application of the PCR assays to fecal samples, we note that they avoid the necessity for culture and for subsequent multiple phenotypic tests. The relative frequency of C. jejuni (18 per 20 clinical samples) and C. coli (2 per 20 fecal samples) in this study is in agreement with previous prevalence data (3, 22). We note that laboratory identification of C. coli is problematical since the species can be distinguished from C. jejuni only by the absence of hippurate hydrolysis. Since there is no positive phenotypic test specific for C. coli, we used flaA gene fingerprinting to substantiate the standard identification and to further validate our coli-specific PCR.

Even in this limited sample, C. hyointestinalis was identified by PCR and an isolate could be retrospectively cultured. C. hyointestinalis is considered only a rare cause of human enteritis (6, 8, 15). However, in this study C. hyointestinalis was present in a sample where C. jejuni was assumed to be the sole campylobacter present. A PCR assay from fecal DNA detected C. hyointestinalis although this organism went unrecognized by current culture protocols. This result suggests that fecal DNA PCR could, if applied to large numbers of samples, quantify the proportion of human diarrheic stools containing non-jejuni, non-coli campylobacters. This would clarify the role of campylobacters in human enteric disease.

We were also able to use PCR-RFLP to obtain fla gene fingerprints of the isolates, as well as of campylobacters in the fecal samples. In each case the fecal sample and the isolate had the same genotype (shared the same fingerprint), and two of those corresponded to previously described C. coli fingerprints (20). The approach of direct genotyping of fecal and environmental material would allow rapid investigation of outbreaks and assist other epidemiological studies. The feasibility of PCR-based typing of C. jejuni in stools was recently demonstrated by using a commercial extraction procedure which is now unavailable (25), and the authors of this report remarked on the need for a rapid fecal DNA extraction protocol to facilitate routine PCR detection of C. jejuni in stools. The
results provided in the present study show that PCR-based detection, identification to the species level, and typing of campylobacters directly from fecal samples are indeed possible for clinical laboratories.

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