Discrimination among Thermophilic *Campylobacter* Species by Polymerase Chain Reaction Amplification of 23S rRNA Gene Fragments

MARK EYERS,¹ SABINE CHAPELLE,¹ GUY VAN CAMP,^{1†} HERMAN GOOSSENS,²
AND RUPERT DE WACHTER^{1*}

Departement Biochemie, Onderzoeksgroep Moleculaire Biologie,¹ and Departement Geneeskunde, Laboratorium voor Microbiologie,² Universiteit Antwerpen (UIA), Antwerp, Belgium

Received 18 May 1993/Returned for modification 27 July 1993/Accepted 23 September 1993

By comparing nucleic acid sequences determined for one of the most variable areas of 23S rRNA genes of 23 *Campylobacter* strains, we were able to identify regions specific for thermophilic *Campylobacter* strains. Oligonucleotide primers corresponding to these unique regions were synthesized and used in the polymerase chain reaction. One primer pair selectively detected all thermophilic *Campylobacter* species, while four other primer pairs allowed discrimination among the thermophilic species *Campylobacter coli*, *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis*. All primer sets were tested successfully on a large number of clinical isolates.

Thermophilic *Campylobacter* species are recognized as a major cause of acute diarrheal disease in humans throughout the world (16, 18, 21). The three species most commonly regarded as being involved in enteritis are, in descending order of importance, *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari*. Although the association with diarrhea of a fourth species named *Campylobacter upsaliensis* has been reported (10, 20), more studies are needed to determine its epidemiological significance. Isolation of campylobacters from clinical samples requires long incubation times and special growth conditions (4). Also, the discrimination among closely related thermophilic *Campylobacter* species has, until today, been a time-consuming and difficult task. The differentiation between *C. jejuni* and *C. coli* is especially delicate because it is based solely on the hippurate hydrolysis test (16). This distinction, however, is accurate in only approximately 90% of all cases (22).

The polymerase chain reaction (PCR) (17) is by now widely used in clinical settings. This technique allows a rapid amplification of target sequences and can be a highly specific and sensitive detection method (14). Because of its sensitivity, PCR allowed direct identification of fastidious bacteria in clinical samples, without prior isolation and purification of the organisms (5, 11). Genes for 16S rRNA (16S rDNA) have often been used as target sequences in these PCR assays for the identification of fastidious bacteria (5, 11), including *Campylobacter* spp. (6, 24). rRNA genes have a typical mosaic structure of phylogenetically conserved and variable regions (3). The latter may vary considerably among different bacterial species and therefore are excellent targets for species- or even subspecies-specific primers. In this paper we report the use of a 23S rDNA fragment as a target for PCR amplification. This allows for the first time discrimination among four thermophilic *Campylobacter* species.

The bacterial reference strains used in this work are listed in Table 1. Clinical isolates used in this study were obtained

from diarrheic stool specimens at the Department of Microbiology in the St. Pieters University Hospital in Brussels, Belgium (10), and analyzed by phenotypic characterization as described by Goossens et al. (9). We used 30 strains identified as *C. jejuni* subsp. *jejuni*, 31 identified as *C. coli*, 13 identified as *C. lari*, and 35 identified as *C. upsaliensis*. *Campylobacter* species were grown on blood agar as described by Vandamme et al. (26).

The PCR was carried out in accordance with instructions supplied with the reagents (Perkin-Elmer Cetus, Norwalk, Conn.) in a DNA thermal cycler (Perkin-Elmer Cetus) with chromosomal DNA concentrations of 0.08 ng/μl. DNA extractions for the PCR assay were done as described elsewhere (24). Alternatively, PCRs were done directly on bacterial colonies (colony PCR). Oligonucleotides were synthesized by Eurogentec S.A., Seraing, Belgium. The reaction mixture was covered with a drop of mineral oil (Sigma) to prevent evaporation and subjected to 27 cycles of amplification. Each cycle consisted of 1 min at 94°C, 1 min at 54°C, and 1 min at 74°C. Amplified samples were analyzed by electrophoresis on 1.5% agarose gels with ethidium bromide staining. Solid-phase sequencing of biotinylated PCR products was done with magnetic beads coated with streptavidin (Dynal, Oslo, Norway). The immobilization of PCR products on magnetic beads was performed as described by Hultman et al. (13). Sequencing reactions were performed on DNA bound to the beads by using the Sequenase kit for DNA sequencing (U.S. Biochemical Corp., Cleveland, Ohio).

The nucleotide sequences of 23S rDNA fragments, located between helices 43 and 69 (helix numbering according to Höpfl et al. [12]), from 17 *Campylobacter* strains (Table 1) have been published recently (25). These fragments are either approximately 875 bp or approximately 975 bp in size and compose one of the most variable regions of the 23S rRNA. The corresponding sequence was determined, by the same methods, for six additional *Campylobacter* strains that are listed in Table 1. The complete set of partial 23S rDNA sequences was aligned by using the DCSE (Dedicated Comparative Sequence Editor) computer program with a Vax work station (2).

* Corresponding author. Electronic mail address: DWACHTER@REKS.UIA.AC.BE.

† Present address: Centrum Medische Genetica, Universiteit Antwerpen (UIA), Antwerp, Belgium.

TABLE 1. Bacterial strains used in this study

<i>Campylobacter</i> sp.	Strain ^a
<i>C. coli</i>	LMG 6440 (ATCC 33559)* LMG 7535 (CCUG 10369)*
<i>C. jejuni</i> subsp. <i>doylei</i>	LMG 7790 (ATCC 49350)*
<i>C. jejuni</i> subsp. <i>jejuni</i>	LMG 6444 (ATCC 33560)* LMG 6629 (CCUG 17696)* ATCC 33250*
<i>C. lari</i>	LMG 8846 (ATCC 35221)*
<i>C. upsaliensis</i>	LMG 7533 (ATCC 14913)* LMG 8854 (NCTC A614/87)* LMG 8853 LMG 8852 LMG 8851
<i>C. concisus</i>	LMG 7788 (ATCC 33237)*
<i>C. fetus</i> subsp. <i>fetus</i>	LMG 6442 (ATCC 27374)*
<i>C. fetus</i> subsp. <i>venerealis</i>	LMG 6570 (CCUG 7477)* LMG 6443 (ATCC 19438)
<i>C. hyointestinalis</i>	LMG 7817 (ATCC 35217)*
<i>C. mucosalis</i>	LMG 7794 (CCUG 10771)*
<i>C. sputorum</i> bv. <i>bubulus</i>	LMG 6447 (ATCC 33562)*
<i>C. sputorum</i> bv. <i>fecalis</i>	LMG 6617 (CCUG 12015)*
<i>C. sputorum</i> bv. <i>sputorum</i>	LMG 7795 (ATCC 35980)*
<i>C. curvus</i>	LMG 7609 (ATCC 35224)
<i>C. rectus</i>	LMG 7614

^a The strain designation is given for the culture collection from which the strain was obtained. Other strain designations are given in parentheses. LMG, Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, Central Public Health Laboratory Services, London, United Kingdom; CCUG, Culture Collection of the University of Göteborg, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden. For the strains indicated by an asterisk, the sequence of a 23S rDNA fragment has been published (25). The corresponding sequence for the remaining strains was determined in the present study (see text).

By comparing these partial 23S rDNA sequences, we were able to design primers (named THERM1 and THERM2) that specifically allow DNA amplification in thermophilic *Campylobacter* species. The positions of these primers within the 23S rDNA fragments are shown in Fig. 1, and their sequences are as follows: THERM1, 5'-TATTCCAA TACCAACATTAGT-3'; THERM2, 5'-GATTACAACGGG CATGGC-3'; THERM3, 5'-TAAAGTAAGTACCGAAGC TG-3'; UPS, 5'-AAAGTAAGTACCGAAGCTT-3'; LARI, 5'-CTCTTAACGACTACGGCA-3'; COLI, 5'-GCGAAG CATAATCCTAAAT-3'; JEJ1, 5'-TCGAAACATAATCCT AAATG-3'; and JEJ2, 5'-TCGAAGCATGATCCTAAAT-3'. Primers THERM1 and THERM2 were tested in the PCR with target DNA purified from 23 strains (Table 1). All thermophilic strains gave visible bands corresponding to 290 bp (results not shown). All nonthermophilic *Campylobacter* strains and the negative control yielded no amplification product. These primers also failed to generate a detectable PCR product with DNA from strains of *Arcobacter cryaerophilus*, *Arcobacter butzleri*, *Helicobacter cinaedi*, *Helicobacter pylori*, *Helicobacter mustelea*, *Salmonella cholerae-suis*, *Shigella flexneri*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, and *Citrobacter freundii*.

Further comparative studies of partial 23S rRNA sequences allowed us to design primer pairs that discriminate among four different thermophilic *Campylobacter* species. Primer pairs THERM2-UPS, THERM1-LARI, THERM3-JEJ1, and THERM1-COLI (Fig. 1) were tested by PCR with target DNA isolated from the 23 strains listed in Table 1. Each primer pair resulted in specific amplification of a 23S rDNA fragment of a single *Campylobacter* species. As an example, the primer pair THERM1-COLI gave a band corresponding to 390 bp with *C. coli*, whereas the remaining

FIG. 1. Schematic representation of the positions of PCR primers and the sequencing primer 69BR along the 23S rDNA fragment. Primers 43A and 69AR are universal primers (23) allowing amplification in all bacteria of an rDNA fragment bounded by nucleotides corresponding to positions 1060 and 1935 in the *Escherichia coli* 23S rRNA. The use of the specific PCR primers and of the sequencing primer 69BR is explained in the text. *C. JEJ. subsp. JEJ.*, *C. jejuni* subsp. *jejuni*. Striped boxes represent an internal transcribed spacer present in certain species.

Campylobacter species and other control species, as well as the negative control, yielded no amplification product. Similarly, the primer pair THERM1-LARI1 specifically amplified a 260-bp fragment of *C. lari* strains. In the case of primer pair THERM2-UPS, some *C. upsaliensis* strains yielded a band of 520 bp whereas others yielded a band of 620 bp. These size differences can be explained by the presence or absence of an internal transcribed spacer in helix 45 of the 23S rRNA (25). A similar phenomenon was observed with the primer pair THERM3-JEJ1, which yielded a band of 710 or 810 bp, depending on the *C. jejuni* strain detected. All other strains and the negative control yielded no amplification product.

By using colony PCR, the described primer pairs were further evaluated with a large number of clinical isolates. As shown in Fig. 2A and C through E, electrophoretic analysis of the obtained PCR products confirmed most of the results that we obtained with the reference strains. Only in PCR experiments using primer pair THERM3-JEJ1 were no PCR products generated with 10 of 30 *C. jejuni* subsp. *jejuni* isolates. Therefore we determined the nucleotide sequences of these 10 strains in the region of the 23S rDNA corresponding to the primer JEJ1 (Fig. 1). A sequencing primer (named 69BR) located near this region (Fig. 1) was designed and used in solid-phase sequencing experiments. 23S rDNA fragments of the 10 isolates, generated by colony PCR with primer 69AR and a biotinylated version of primer 43A, were used as templates. Interestingly, the sequence analysis showed that the 10 strains carry identical sequences that differ in two positions from those of *C. jejuni* strains that are positive in PCR experiments using primer pair THERM3-JEJ1. This enabled us to design a second *C. jejuni* subsp. *jejuni*-specific primer, named JEJ2 (Fig. 1). By combining both *C. jejuni*-specific primers with the THERM3 primer in PCR, we were able to detect all *C. jejuni* subsp. *jejuni* isolates (Fig. 2B). This set of three primers was completely species specific except for the occurrence of a very weak band of 750 bp, observed in 2 of 12 tested *C. upsaliensis* strains. Such a band is visible in lane 12 of Fig. 2B, but it is clearly distinguishable on the basis of its chain length from the much stronger *C. jejuni*-specific signal. Additionally, these colony PCRs revealed that the transcribed spacer is present in 90% of all *C. upsaliensis* isolates, while it is present in only 10% of the *C. jejuni* subsp. *jejuni* isolates (results not shown).

In order to determine the sensitivity of the PCR assays, serial 10-fold dilutions of *Campylobacter* DNA were subjected to PCR amplification with the different primer pairs, and the products were subjected to electrophoresis on 1.5% agarose gels (data not shown). The results indicate that the PCR products generated with on average as little as 0.062 pg of DNA can be visualized properly on the agarose gel. On the basis of a genome size for *Campylobacter* spp. of 1,700 kbp and the presence of three copies of the 23S rDNA per chromosome (19), this corresponds to 12 bacteria at most.

All primer sets were designed so that they function under similar thermocycling conditions. This offers the possibility of performing the different assays simultaneously in one PCR machine. This set of PCR assays offers a sensitive and rapid epidemiological tool for the identification of thermophilic *Campylobacter* spp. In industrialized nations, *C. jejuni* subsp. *jejuni* represents the majority of *Campylobacter* species reported in clinical samples (18), and many clinical laboratories report isolates simply as *Campylobacter* sp. (7). However, the ability to differentiate *C. jejuni* subsp. *jejuni* from *C. coli* is useful in some situations, e.g., for

FIG. 2. Agarose (1.5%) gel electrophoresis of 10- μ l samples of PCR-amplified DNAs from different *Campylobacter* clinical isolates. The control lane (lane 16) contains a sample without DNA. (A) Thermophilic species-specific 23S rDNA amplification with primers THERM1 and THERM2. (B) *C. jejuni* subsp. *jejuni*-specific 23S rDNA amplification with primers THERM3, JEJ1, and JEJ2. (C) *C. coli*-specific 23S rDNA amplification with primers THERM1 and COLI. (D) *C. lari*-specific 23S rDNA amplification with primers THERM1 and LARI. (E) *C. upsaliensis*-specific 23S rDNA amplification with primers THERM2 and UPS. Lanes: 1, *C. jejuni* subsp. *jejuni* K227; 2, *C. jejuni* subsp. *jejuni* B788; 3, *C. jejuni* subsp. *jejuni* D562; 4, *C. coli* R268; 5, *C. coli* A625; 6, *C. coli* B786; 7, *C. lari* L31; 8, *C. lari* L34; 9, *C. lari* L35; 10, *C. upsaliensis* A475; 11, *C. upsaliensis* E293; 12, *C. upsaliensis* G203; 13, *C. sputorum* LMG 8531; 14, *C. fetus* LMG 6442; 15, *C. mucosalis* LMG 7794; 16, control; 17, 1-kb ladder (Bethesda Research Laboratories).

identification of *C. coli*, because more than 50% of these organisms are resistant to erythromycin, which is a standard therapy for treatment of *Campylobacter* enteritis (8). These assays would be particularly useful in outbreak situations in which large numbers of specimens need to be handled quickly.

Recently, a number of laboratories presented evidence that PCR-based assays can be successfully applied to the direct detection of *Campylobacter* spp. and other pathogens in human fecal material (1, 15). Besides being used for the detection of campylobacters in clinical stool samples, this assay could also theoretically be adapted to the detection of campylobacters in their animal reservoirs, as described recently by Giesendorf et al. (6).

This work was supported by the incentive program "Health Hazards" of the Office for Science Policy Programming of the Belgian State.

We are grateful to Jean-Marc Neefs and Peter De Rijk for help with the DCSE computer program and to Peter Vandamme, Hans Fierens, and Linda Vlaes for providing us with *Campylobacter* strains.

REFERENCES

1. Branstrom, A. A., D. J. Kopecko, M. Venkatesan, R. Sims, L. Banish, and M. Bush. 1991. Detection of *Shigella* in stools from asymptomatic monkeys and animal handlers using primers specific for *ipaH*. Abstr. 31st Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1087.
2. De Rijk, P., and R. De Wachter. Comput. Appl. Biosci., in press.
3. De Rijk, P., J.-M. Neefs, Y. Van de Peer, and R. De Wachter. 1992. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. 20:2075-2089.
4. Endtz, H. P., G. J. H. M. Ruijs, A. H. Zwinderman, T. van der Reijden, M. Biever, and R. P. Mouton. 1991. Comparison of six media, including semisolid agar, for the isolation of various *Campylobacter* species from stool specimens. J. Clin. Microbiol. 29:1007-1010.
5. Gaydos, C. A., T. C. Quinn, and J. J. Eiden. 1992. Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. J. Clin. Microbiol. 30:796-800.
6. Giesendorf, B. A. J., W. G. V. Quint, M. H. C. Henkens, H. Stegeman, F. A. Huf, and H. G. M. Niesters. 1992. Rapid and sensitive detection of *Campylobacter* spp. in chicken products by using the polymerase chain reaction. Appl. Environ. Microbiol. 58:3804-3808.
7. Goossens, H., and J.-P. Butzler. 1991. Isolation and identification of *Campylobacter* sp., p. 93-109. In I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
8. Goossens, H., H. Coignau, L. Vlaes, C. Verbrugge, I. Galand, C. Van den Borre, and J.-P. Butzler. 1988. Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 397.
9. Goossens, H., B. Pot, L. Vlaes, C. Van den Borre, R. Van den Abbeele, C. Van Naelten, J. Levy, H. Cogniau, P. Marbehan, J. Verhoef, K. Kersters, J.-P. Butzler, and P. Vandamme. 1990. Characterization and description of "*Campylobacter upsaliensis*" isolated from human feces. J. Clin. Microbiol. 28:1039-1046.
10. Goossens, H., L. Vlaes, M. De Boeck, B. Pot, K. Kersters, J. Levy, P. De Mol, J.-P. Butzler, and P. Vandamme. 1990. Is "*Campylobacter upsaliensis*" an unrecognised cause of human diarrhoea? Lancet 335:584-586.
11. Gumerlock, P. H., Y. J. Tang, F. J. Meyers, and J. Silva, Jr. 1991. Use of the polymerase chain reaction for the specific and direct detection of *Clostridium difficile* in human feces. Rev. Infect. Dis. 13:1053-1060.
12. Höpfl, P., W. Ludwig, K. H. Schleifer, and N. Larsen. 1989. The 23S ribosomal RNA higher-order structure of *Pseudomonas cepacia* and other prokaryotes. Eur. J. Biochem. 185:355-364.
13. Hultman, T., S. Bergh, T. Moks, and M. Uhlén. 1991. Bidirectional solid-phase sequencing of *in vitro*-amplified plasmid DNA. BioTechniques 10:84-93.
14. Mullis, K. B., and F. A. Faloona. 1987. Specific synthesis of DNA *in vitro* via a polymerase chain reaction. Methods Enzymol. 155:335-350.
15. Oyofe, B. A., S. A. Thornton, D. H. Burr, T. J. Trust, O. R. Pavlovskis, and P. Guerry. 1992. Specific detection of *Campylobacter jejuni* and *Campylobacter coli* by using polymerase chain reaction. J. Clin. Microbiol. 30:2613-2619.
16. Penner, J. L. 1988. The genus *Campylobacter*. Clin. Microbiol. Rev. 1:157-172.
17. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.
18. Tauxe, R. V. 1991. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations, p. 9-19. In I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
19. Taylor, D. E., M. Eaton, W. Yan, and N. Chang. 1992. Genome maps of *Campylobacter jejuni* and *Campylobacter coli*. J. Bacteriol. 174:2332-2337.
20. Taylor, D. E., K. Hiratsuka, and L. Mueller. 1989. Isolation and characterization of catalase-negative and catalase-weak strains of *Campylobacter* species, including "*Campylobacter upsaliensis*," from humans with gastroenteritis. J. Clin. Microbiol. 27:2042-2045.
21. Taylor, D. N. 1991. *Campylobacter* infections in developing countries, p. 20-30. In I. Nachamkin, M. J. Blaser, and L. S. Tompkins (ed.), *Campylobacter jejuni*: current status and future trends. American Society for Microbiology, Washington, D.C.
22. Totten, P. A., C. M. Patton, F. C. Tenover, T. J. Barrett, W. E. Stamm, A. G. Steigerwalt, J. Y. Lin, K. K. Holmes, and D. J. Brenner. 1987. Prevalence and characterization of hippurate-negative *Campylobacter jejuni* in King County, Washington. J. Clin. Microbiol. 25:1747-1752.
23. Van Camp, G., S. Chapelle, and R. De Wachter. 1993. Amplification and sequencing of variable regions in bacterial 23S ribosomal RNA genes with conserved primer sequences. Curr. Microbiol. 27:147-151.
24. Van Camp, G., H. Fierens, P. Vandamme, H. Goossens, A. Huyghebaert, and R. De Wachter. 1993. Identification of enteropathogenic *Campylobacter* species by oligonucleotide probes and polymerase chain reaction based on 16S rRNA genes. Syst. Appl. Microbiol. 16:30-36.
25. Van Camp, G., Y. Van de Peer, S. Nicolai, J.-M. Neefs, P. Vandamme, and R. De Wachter. Structure of 16S and 23S ribosomal RNA genes in *Campylobacter* species: phylogenetic analysis of the genus *Campylobacter* and presence of internal transcribed spacers. Syst. Appl. Microbiol., in press.
26. Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley. 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. Int. J. Syst. Bacteriol. 41:88-103.