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Development of a Simple and Rapid Method Based on Polymerase Chain Reaction–Based Restriction Fragment Length Polymorphism Analysis to Differentiate *Helicobacter*, *Campylobacter*, and *Arcobacter* Species

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Abstract. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of amplified DNA fragment of the 16S and 23S rRNA genes was performed on 35 *Helicobacter*, 24 *Campylobacter*, and 15 *Arcobacter* strains. PCR amplification generated a 1004-bp fragment of 16S rDNA and a 2.6-Kbp fragment of 23S rDNA from each strain. The amplicons were digested with *Dde*I and *Hpa*II, respectively. For both assays, distinctive profiles were obtained for each genus. 23S rDNA PCR-RFLP analysis with *Hpa*II enzyme identified *Campylobacter* and *Helicobacter* strains at the species level. Analysis of 16S rRNA gene with *Dde*I enzyme was not useful for the specific identification of *Campylobacter* and *Arcobacter*, although it discriminated among *Helicobacter* species. The PCR-RFLP technique allowed for the discrimination among these three related genus with only one restriction enzyme; therefore it can be a simple, rapid, and useful method for routine identification.

The genera *Helicobacter*, *Campylobacter*, and *Arcobacter* form a phylogenetically distinct group included in the epsilon-subdivision of proteobacteria [36]. These spiral-shaped, Gram-negative microorganisms are microaerophilic and fastidious in culture media [38].

Identification of these bacteria has become increasingly important because many of them are now recognized as human and/or animal pathogens [27]. Thermophilic campylobacters, particularly *C. jejuni* and *C. coli*, are the most isolated bacteria causing diarrhoeal diseases in humans [12]. Foods of animal origin and drinking water are widely regarded as the main source of food-borne infection because of the presence of those organisms as part of the intestinal flora of many animals [30]. Currently, three *Arcobacter* species-including *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*-are considered to be pathogenic for domestic animals and humans [21]. The route of transmission from animals to humans is not clear, but the bacterium has been isolated from meat products and water, suggesting that *Arcobacter* is a possible food-borne pathogen [23].

Helicobacter genus includes a group of organisms that colonize the mucus layer covering the epithelial surface of the gastrointestinal tract of humans and a variety of animals. An important number of *Helicobacter* species have been related to human and animal diseases, with *H. pylori* being the most notable. It is the main causative agent of chronic superficial gastritis and peptic ulcer disease; it is also a risk factor for gastric cancer [5] and has been designated as a class I carcinogen by the World Health Organization [18]. *H. pylori* infection occurs worldwide at a high prevalence rate, and it can be eradicated in up to 90% of patients using current triple therapies, of which clarithromycin and metronidazole antibiotics are key components [6].

A growing number of enterohepatic *Helicobacter* species are being reported to be associated with diseases in humans [10, 29, 33]. *H. pullorum* has been shown to occur in poultry as well as in humans in association with gastroenteritis. This organism is especially difficult to differentiate from *Campylobacter* species [25]. *H. fennelliae* is closely related to *H. pullorum* (95.9%

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sequence similarity of 16S rRNA gene; Shen et al. [32]) and has been isolated from rectal swabs and blood from homosexual men [14]. *H. fennelliae* may cause gastroenteritis, cellulitis, septic arthritis, and bacteremia, most commonly in patients infected with human immunodeficiency virus (HIV) [37].

The differentiation of *Helicobacter* species from each other and from *Campylobacter* and *Arcobacter* species by phenotypic analysis is difficult because of the lack of standardized procedures, the well-known biochemical inertness of these organisms, the great number of cross-species phenotypic similarities, and the prevalence of atypical strains. These difficulties increase the interest in molecular approaches to identification [28].

During past decades, several molecular techniques have been applied to identify these bacteria [28]. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RLFP) analysis of amplified rDNA fragments is one of the most frequent methods used to differentiate among *Helicobacter*, *Campylobacter*, and *Arcobacter* species [3, 20, 25] because it is more discriminatory, faster, and more cost-effective than phenotypic tests [26]. rDNA molecules present conserved regions coexisting with variable sequences that make them suitable targets for molecular identification methods. However, most of the PCR-RFLP identification schemes developed in previous works are too complex for routine use because they are unable to distinguish *C. jejuni* from *C. coli* in one step [24] or because they need more than one restriction enzyme to discriminate among *Helicobacter*, *Campylobacter*, and *Arcobacter* species [15]. Moreover, most of them have not been simultaneously applied to the identification of the three genera [8, 16, 19, 34]. Therefore, the objective of this study was the development of a rapid and easy-to-perform identification system based on PCR-RFLP analysis with the ability to differentiate among *Helicobacter*, *Campylobacter*, and *Arcobacter* species.

Material and Methods

Bacterial strains and culture conditions. A total of 74 strains were used in this study, including 31 *Helicobacter* strains isolated from gastric biopsies, 20 *Campylobacter* strains, and 12 *Arcobacter* strains isolated from poultry and water samples; 11 reference strains were provided by the National Collection of Type Cultures (NCTC; London, UK): *H. pylori* NCTC 11637, *H. pylori* NCTC 11638, *H. pullorum* NCTC 13153, *H. fennelliae* NCTC 11612, *C. jejuni* NCTC 11828, C. *jejuni* NCTC 12521, *C. upsaliensis* NCTC 11540, *C. coli* NCTC 11366, *A. butzleri* NCTC 12481, *A. cryaerophilus* NCTC 11885, and *A. skirrowii* NCTC 12713.

Helicobacter strains were grown on Columbia agar base supplemented with 10% (vol/vol) defibrinated horse blood (Oxoid; Basingstoke, UK); *Arcobacter* and *Campylobacter* were cultured on blood agar base no. 2 containing 5% (vol/vol) defibrinated sheep blood (Oxoid). Agar plates were incubated at 37°C under microaerophilic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) for 48 to 72 hours, except for *Arcobacter*, which was incubated in an aerobic atmosphere for 48 hours at 30°C. Cultures were preserved at −80°C in nutrient broth (Merck; Darmstadt, Germany) containing 10% (vol/vol) glycerol.

Biochemical characterization. *Helicobacter* strains isolated from biopsy specimens were characterized by colony morphology, Gram staining reaction, urease (Microkit S.L.; Madrid, Spain), catalase, and oxidase (Bactident oxidase Kit; Merck) activities. *Campylobacter* and *Arcobacter* were characterized by morphology, Gram stain, and API-*Campy* (Biomérieux, France) identification profiles. *Arcobacter* were differentiated from campylobacters by their ability to grow under aerobic conditions.

DNA isolation and PCR identification. Bacterial strains biochemically characterized as *Helicobacter*, *Campylobacter*, and *Arcobacter* were identified by PCR. Chromosomal DNA was extracted by cetyltrimethyl-ammonium bromide [39].

Primers C97 (5′-GCTATGACGGGTATCC-3′) and C05 (5′-ACTTCACCCCAGTCGCTG-3′) were used to amplify a 1200-bp PCR fragment of 16S rDNA from all *Helicobacter* species [11]. PCR was performed in 50 µl of the mixtures containing 5 µl template DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, and 0.5 µM each primer along with 2.5 U of Taq polymerase. The temperature profile for PCR was as follows: an initial step of 5 minutes at 95°C, followed by denaturation for 1 minute at 94°C, annealing for 2 minutes at 60°C, and primer extension for 2 minutes at 72°C. After the 33rd cycle, the extension step was prolonged for 5 minutes to complete synthesis of all strands, and then the samples were kept at 4°C until analysis.

Primers HP1 (5′-CCTAACCAATTGAGCCAAGAAG-3′) and HP2 (5′-CTTTCTAACACTAACGCGCTCA-3′), which amplified a 398-bp PCR fragment of 16S rDNA specific to *H. pylori* [4], were also used to identify the *Helicobacter* strains isolated from biopsy specimens. The amplification were carried out in a volume of 50 µl with 5 µl template DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, and 200 µM each dNTP, 0.4 µM each primer, 2 U of Taq polymerase; the mixture was then submitted to an initial denaturation at 95°C for 5 minutes, followed by 33 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute, and a final extension step at 72°C for 5 minutes.

PCR identification of *Campylobacter* species was carried out according to Fermér and Engvall [9] with THERM1 (5′-ATTCCAATACCAACATTAGT-3′) and THERM4 (5′-CTTCGCTAATG CTAACCC-3′) primers, which amplified a 491-bp fragment of 23S rDNA for *Campylobacter*-termotolerant species.

Simultaneous identification of *C. jejuni* and *C. coli* was performed by multiplex PCR (mPCR) with three different pairs of primers. The primers used amplified a 857-bp fragment of 16S rRNA gene for *Campylobacter* genus [22], a 589-bp fragment of *mapA* gene for *C. jejuni* species [35], and a 462-bp fragment of *ceuE* gene for *C. coli* species [13]. mPCR was based on the assay developed by Denis et al. [7] A final reaction volume of 30 µl was made by addition of 5 µl each sample. The PCR reaction mixture contained 0.6 U Taq polymerase, 1 × PCR buffer, 200 µM each dNTP, 1.5 mM $MgCl_2$, 0.5 µM MD16S1 and MD16S2 primers, and 0.42 µM MDmapA1, MDmapA2, COL3, and MDCOL2. The amplification consisted of an initial denaturation step at 95°C for 10 minutes followed by 35 cycles (denaturation at 95°C for 30 seconds, specific primer annealing at 59°C for 90 seconds, and extension at 72°C for 1 minute), ending with a final extension at 72°C for 10 minutes.

Arcobacter identification was performed by PCR amplification as described by Bastyns et al. [2] using ARCO1 (5′-GTCGTGCCAA GAAAAGCCA-3′) and ARCO2 (5′-TTCGCTTGCGCTGACAT-3′) primers, which amplified a 331-bp fragment of 23S rDNA.

All PCR reactions were performed with an automatic thermal cycler (Techne-Progene; Cambridge, UK). DNA templates from reference strains were used as positive controls. In addition, negative controls in which DNA was replaced with sterile distilled water were also included. All of the reagents used in the PCR reactions (Taq polymerase, dNTP, and $MgCl_2$) were provided by Ecogen (Spain), and all of the primers employed were prepared by TIB MOLBIOL (Germany).

PCR products were detected by electrophoresis on 1.2% (wt/vol) agarose gel in 1 × Tris-acetate-EDTA (TAE) buffer at 100 V for approximately 60 minutes and visualized by staining with ethidium bromide (0.5 μg/mL) and ultraviolet (UV) transillumination. GeneRuler 100-bp DNA Ladder Plus (MBI Fermentas) was used as molecular marker.

PCR-RFLP analysis. A 1004-bp 16S rDNA fragment from all *Helicobacter*, *Campylobacter*, and *Arcobacter* strains was amplified using CAH16S 1a and CAH16S 1b primers with sequences 5′-AATA CATGCAAGTCGAACGA-3′ and 5′-TTAACCCAACATCTCACG AC-3′, respectively, according to the Marshall et al. assay [24]. For amplification of a 2.6-kbp fragment within the coding region of the 23S rRNA gene of *Campylobacter*, *Arcobacter*, and *Helicobacter*, primers LS1 (5′-GGATTTCCGAATGGGGCAACCC-3′) and LS2 (5′-GTTTCGTGCTTAGATGTTTC-3′) were used [15]. The PCR reaction was carried out as described by Hurtado and Owen [16], including an initial denaturation step at 95°C for 5 minutes and a final extension at 72°C for 5 minutes to ensure full extension of the product. In both cases, amplified products were visualised by 1% (wt/vol) agarose gel electrophoresis and stained with ethidium bromide (0.5 μg/mL).

PCR products (10 μl) were digested with 10 U restriction enzymes in a final volume of 20 μl at 37°C for 3.5 hours. Restriction endonucleases were selected by computer analysis using REbsites (available at: www.http://rebase.neb.com). *Dde*I (Roche) and *Hpa*II (MBI Fermentas) were used to digest 16S rDNA and 23S rDNA fragments, respectively. The reaction was stopped by adding 3 μl stop-mix solution (50 mM EDTA, 0.3% Ficoll, and 0.3% bromophenol blue).

Restriction fragments were separated on 2% (wt/vol) agarose gel electrophoresis in TAE 1 × buffer at 90V for 3 hours and visualized after staining with ethidium bromide and UV transillumination. GeneRuler 100-bp DNA Ladder Plus was used as a standard for molecular size determination. To assess reproducibility for both 16S and 23S PCR-RFLP analysis, all of the strains were analysed at least two times in different experiments.

Results and Discussion

Biochemical identification. All of the clinical *Helicobacter* strains included in this study were Gram negative and positive for urease, catalase, and oxidase test. In previous genotypic analysis, 16 of the 20 *Campylobacter* strains were identified as *C. jejuni* and 2 as *C. coli* by the API-*Campy* system. The remaining two strains presented unacceptable profiles. *Arcobacter* strains were tested for growth at 30°C in aerobic conditions to confirm their aerotolerance, and after 24 to 48 hours of incubation, growth was observed in all of the plates. The API-*Campy* system was also applied, although all the isolates, including reference strains, were identified as *A. cryaerophilus* (Table 1). These results confirm the ineffectiveness of this system to identify *Arcobacter* strains. This fact has been previously reported by other investigators; Atabay et al. used the API-*Campy* scheme to study the diversity and prevalence of *Arcobacter* spp. in broiler chickens and found that only 20% of the isolates were correctly identified at the species level [1].

PCR identification. *Helicobacter* clinical and reference strains showed positive results by PCR assay using *Helicobacter* genus-specific primer sets C97 and C05. All of the clinical strains were identified as *H. pylori* by species-specific PCR. No amplification products were obtained from *Arcobacter* and *Campylobacter* reference strains.

A 491-bp fragment was obtained after amplification with genus specific primers only for *Campylobacter* spp. strains. After mPCR, 14 of the 20 *Campylobacter* strains were identified as *C. jejuni* and the remaining 6 as *C. coli*. For aerotolerant bacteria and *Arcobacter* reference strains, a 331-bp specific fragment was amplified with the primer set ARCO1/ARCO2 (Table 1).

PCR-RFLP differentiation. Bacterial strains previously identified as *Helicobacter*, *Campylobacter*, and *Arcobacter* were typed by PCR-RFLP of 16S rDNA and 23S rDNA technique (Table 1). The results of RFLP analysis resolved by agarose gel electrophoresis were almost identical to the predicted fragments based on the nucleotide sequence data.

A 1004-bp PCR product of the 16S rDNA was amplified in all of the strains. Differentiation to the genus level was achieved using *Dde*I because digestion of the amplicon with this restriction enzyme generated six different specific patterns: three for *Helicobacter*, two for *Campylobacter*, and one for *Arcobacter* (Fig. 1).

Digestion with restriction enzyme *Dde*I yielded different specific patterns for *H. pylori* (495, 257, 102, and 75 bp); *H. pullorum* (750 and 230 bp); and *H. fennelliae* (750, 280, 170, and 140 bp). The 31 clinical isolates produced fingerprints that were identical to that of *H. pylori* reference strains.

Analysis of the 16S rRNA gene with *Dde*I enzyme was not useful for the specific identification of *Campylobacter* and *Arcobacter*. Identical fingerprints were obtained for all of the *Arcobacter* species tested (421, 353, and 183 bp). The method also failed to distinguish *C. coli* from *C. jejuni*. These two species shared the same *Dde*I profile (272, 247, 153, 120, and 95 bp). Only *C. upsaliensis* showed a species-specific pattern (425, 248, 145, and 108 bp). All of the *Campylobacter* isolates presented the same profile as *C. jejuni* and *C. coli* reference strains.

For 23S rDNA PCR-RFLP analysis, an internal region of the 23S rRNA gene of approximately 2.6-Kbp was amplified from all of the strains. The PCR products,

Table 1. Strains of *Campylobacter* and *Arcobacter* used in this study and test results

Strain	Origin	Biochemical identification	PCR	16S PCR-RFLP pattern	23S PCR-RFLP pattern
Cl	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C2	Food	*Cjj*I	*Cj*	*Cj/Cc*	*CJ*
C3	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C4	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C5	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C6	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C7	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C8	Water	*Cjj*II	*Cc*	*Cj/Cc*	*Cc*
C9	Water	*Cjj*II	*Cc*	*Cj/Cc*	*Cc*
C10	Water	*Cc* (unacceptable)	*Cc*	*Cj/Cc*	*Cc*
C11	Water	*Cc*	*Cc*	*Cj/Cc*	*Cc*
C12	Food	*Cc*	*Cc*	*Cj/Cc*	*Cc*
C13	Water	*Cjj*I	*Cc*	*Cj/Cc*	*Cc*
C14	Water	*Cc* (unacceptable)	*Cj*	*Cj/Cc*	*Cj*
C15	Water	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C16	Water	*Cjj*II	*Cj*	*Cj/Cc*	*Cj*
C17	Water	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C18	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C19	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
C20	Food	*Cjj*I	*Cj*	*Cj/Cc*	*Cj*
A1	Food	*Ac*	*A* sp.	*Ab/Ac/As*	*Ab*
A2	Food	*Ac*	*A* sp.	*Ab/Ac/As*	*Ab*
A3	Food	*Ac*	*A* sp.	*Ab/Ac/As*	*Ab*
A4	Food	*Ac*	*A* sp.	*Ab/Ac/As*	*Ab*
A5	Food	*Ac*	*A* sp.	*Ab/Ac/As*	*Ab*
A6	Water	*Ac*	*A* sp.	*Ab/Ac/As*	*Ac/As*
A7	Water	*Ac*	*A* sp.	*Ab/Ac/As*	*Ac/As*
A8	Water	*Ac*	*A* sp.	*Ab/Ac/As*	*Ac/As*
A9	Water	*Ac*	*A* sp.	*Ab/Ac/As*	*Ac/As*
A10	Water	*Ac*	*A* sp.	*Ab/Ac/As*	*Ac/As*
A11	Food	*Ac*	*A* sp.	*Ab/Ac/As*	*Ab*
A12	Water	*Ac*	*A* sp.	*Ab/Ac/As*	*Ac/As*

Cj, C. jejuni; Cc, C. coli sp., Arcobacter sp.; Ab, A. butzleri; Ac, A. cryaerophilus; As, A. skirrowii.

showing a single band of the expected size, were subjected to restriction analysis with *Hpa*II. The digestion produced different patterns that allowed for the discrimination among these three related genera (Fig. 2).

23S rDNA analysis of *Helicobacter* with *Hpa*II enzyme also allowed for the identification of strains at the species level. Three different profiles were obtained, one for each one of the species studied: *H. pylori* (820, 600, 550, 320, and 230 bp); *H. pullorum* (600, 550, 480, 350, 305, and 230 bp); and *H. fennelliae* (110, 620, 320, 250, and 230 bp). Most of the clinical strains showed the same pattern (*Hp*I) as *H. pylori* NCTC 11637 and NCTC 11638, but 3 of the 31 strains had a different profile (*Hp*II: 1150, 600, 550, and 230 bp), which has not been described previously.

When applying the scheme to *Campylobacter*, this method allowed for the differentiation at the species level between *C. jejuni* and *C. coli*. Three different species-specific patterns were obtained: *C. coli* (682, 627, 558, and 456 bp); *C. jejuni* (627, 586, 558, 357, and 305 bp); and *C. upsaliensis* (1958 and 558 bp) profiles could be distinguished. Fourteen of the 20 *Campylobacter* strains were identified as *C. jejuni* and 6 as *C. coli*. The 2 strains that could not be identified by the API-*Campy* system were recognized by PCR-RFLP as *C. jejuni* and *C. coli*. In contrast, 3 strains characterized as *C. jejuni* by API-*Campy* presented the typical *C. coli* pattern, confirming the results previously obtained by mPCR. The API-*Campy* system seemed to have major problems in identifying *C. coli* strains because only 2 of the 6 such strains, on the basis of mPCR and 23S rDNA PCR-RFLP products, gave concordant results in API. Therefore, this system may not be reliable for the routine identification of *Campylobacter* species in accordance with other studies [17, 31]. *A. butzleri* showed a unique *Hpa*II pattern (861, 615, 550, 320, and 240 bp), but *A. skirrowii* and *A. cryaerophilus* shared the same pattern (1107, 615, 550, and 240 bp). Seven of the 12 *Arcobacter* strains were identified as *A. butzleri* and the remaining 5 as *A. skirrowii* or *A. cryaerophilus*.

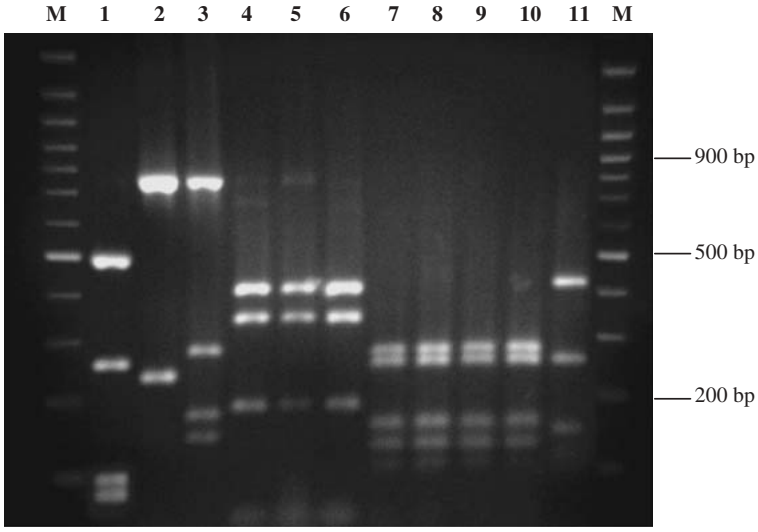

Fig. 1. 16S PCR-RFLP patterns of *Helicobacter*, *Campylobacter*, and *Arcobacter* reference strains generated by digestion with *Dde*I. Lane M = 100-bp DNA Ladder Plus with sizes indicated on right (bp). Lane 1 = *H. pylori* NCTC 11637. Lane 2 = *H. pullorum* NCTC 13153. Lane 3 = *H. fennelliae* NCTC 11612. Lane 4 = *A. butzleri* NCTC 12481. Lane 5 = *A. cryaerophilus* NCTC 11885. Lane 6 = *A. skirrowii* NCTC 12713. Lane 7 = *C. jejuni* NCTC 11828. Lane 8 = C. *jejuni* NCTC 12521. Lanes 9 and 10: *C. coli* NCTC 11366. Lane 11 = *C. upsaliensis* NCTC 11540.

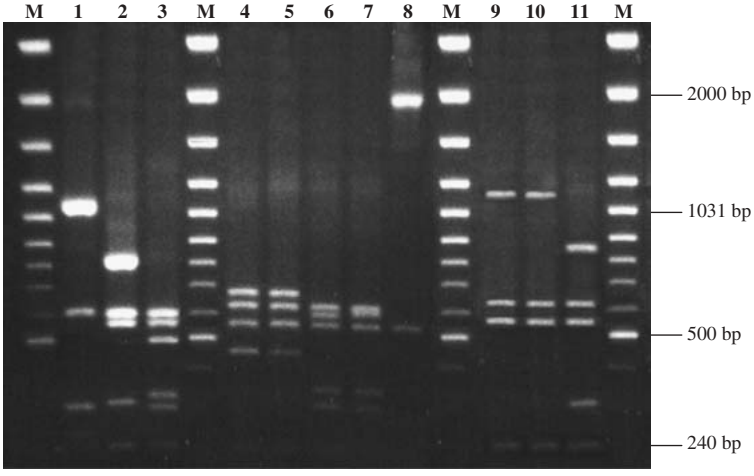

Fig. 2. 23S PCR-RFLP patterns of *Helicobacter*, *Campylobacter*, and *Arcobacter* reference strains generated by digestion with *Hpa*II. Lane M = 100-bp DNA Ladder Plus with sizes indicated on right (bp). Lane 1 = *H. fennelliae* NCTC 11612. Lane 2 = *H. pylori* NCTC 11638. Lane 3 = *H. pullorum* NCTC 13153. Lanes 4 and 5 = *C. coli* NCTC 11366. Lane 6 = *C. jejuni* NCTC 11828. Lane 7 = *C. jejuni* NCTC 12521. Lane 8 = *C. upsaliensis* NCTC 11540. Lane 9 = *A. cryaerophilus* NCTC 11885. Lane 10 = *A. skirrowii* NCTC 12713. Lane 11 = *A. butzleri* NCTC 12481.

In all of the cases, the RFLP profiles were reproducible when a different batch of DNA was used in both PCR-RFLP analysis, and no variation in the restriction profiles of strains belonging to the same species was observed.

Our results show that the PCR-RFLP method is more reliable than biochemical identification for *Helicobacter*, *Campylobacter*, and *Arcobacter*. We agree with other investigators [3, 16, 24] that it is also faster and simpler than other molecular techniques that require large quantities of cells and involve complex and time-consuming steps. In addition, it is more profitable because it does not require numerous specific PCRs or expensive equipment.

In conclusion, PCR-RFLP analysis of 16S and 23S rRNA gene sequences allows for genus identification and achieves a good level of species differentiation with only one set of primers and one restriction enzyme. This technique is a fast, simple, and suitable alternative method to conventional identification procedures for reliable characterization of *Helicobacter*, *Campylobacter*, and *Arcobacter* species.

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