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Molecular fingerprinting of *Campylobacter* and *Arcobacter* isolated from chicken and water

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Summary. The potential of a fingerprinting method based on the single-enzyme amplified fragment length polymorphism (s-AFLP) technique was evaluated for its efficacy in detecting foodborne *Campylobacter* and *Arcobacter* species. *Campylobacter* and *Arcobacter* isolates from chicken and water samples were subjected to s-AFLP and pulsed-field gel electrophoresis (PFGE) profiling. Molecular typing revealed a high degree of heterogeneity. AFLP was found to be appropriate for differentiating minimal genomic variations, which makes this technique a valuable tool for the identification of isolates. PFGE was effective in showing epidemiological relationships among closely related isolates. Either technique allowed the discrimination of *A. butzleri* from *A. cryaerophilus* and *A. skirrowii*. When used together, s-AFLP and PFGE can be applied to determine taxonomic and epidemiological relationships among campylobacteria. [**Int Microbiol** 2007; 10(2):85-90]

Key words: *Campylobacter* · *Arcobacter* · single-enzyme amplified fragment length polymorphism (s-AFLP) · pulsed-field gel electrophoresis (PFGE)

Introduction

Campylobacter jejuni and *Campylobacter coli* are common human enteric pathogens that cause acute bacterial diarrhea worldwide [9]. Most *Campylobacter* infections are foodborne, and the bacteria's presence in chickens has been demonstrated [30,37]. *Campylobacter* is also common in natural waters even though its sources are diverse and include discharges from wastewater treatment plants, runoff from pastures after rainfall, and direct fecal contamination by wild birds. Moreover, these bacteria can survive for several days in moist, cool environments, including wells and groundwater [21].

While *Campylobacter* is recognized as a major foodborne pathogen, only within the last decade has it become evident that *Arcobacter* may also be pathogenic to humans [16,34]. The disease caused by *Arcobacter* is clinically similar to that caused by *Campylobacter* [32]. *Arcobacter* has been isolated from poultry [35], surface water, ground water, sewage and activated sludge [8,19,29] and is thought to be a zoonotic foodborne as well as waterborne pathogen [1,19]. However, the epidemiology of human infection as well as the colonization of food animals is poorly understood, in part because of the lack of suitable typing methods [13].

The differentiation of *Campylobacter* and *Arcobacter* species by phenotypic analysis is difficult due to several reasons: (i) the lack of standardized procedures, (ii) partial biochemical inertness, (iii) their numerous phenotypic similarities, and (iv) the prevalence of atypical strains. These difficulties have increased interest in the development of molecular identification approaches [27]. Phenotypic typing schemes are still in use today, but they have been supplemented with a large variety of genotype-based methods of considerably higher typing ability and discriminatory power [4,33]. These methods include restriction fragment length polymorphism (RFLP) [26], randomly amplified polymorphic DNA-PCR [28], and PCR-RFLP [23,24]. Recently, a multilocus sequence typing (MLST) scheme has been developed and validated for *C. jejuni* [7]. However, most of the

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identification techniques are neither standardized nor are they broadly accepted. The European Research Group Campynet [http://campynet.vetinst.dk] has addressed this problem and is currently engaged in developing three standardized molecular typing methods for *Campylobacter*: flagellin gene RFPL analysis (*fla*-PCR), pulse-field gel electrophoresis (PFGE)-DNA profiling, and amplified fragment length polymorphism (AFLP) analysis with two enzymes. Nonetheless, despite their potential for epidemiological typing purposes [5,20], PFGE and classical AFLP are complex techniques and *fla*-PCR is not reliable for *Arcobacter* species. A few studies have described suitable typing methods for this genus, most of which have focused on *A. butzleri* [3,15,31]. As for *Campylobacter*, substantial intra-species genetic heterogeneity has been reported [2].

Although an optimal method for differentiation and epidemiological investigation of the main *Campylobacter* and *Arcobacter* species is not yet available, the single-enzyme-AFLP (s-AFLP) technique for genotyping of *Campylobacter* and *Helicobacter* is efficient, rapid, and easily performed [10,25]. Therefore, the main goal of this study was to evaluate the potential of s-AFLP-based profiling for identification and characterization of foodborne *Campylobacter* and *Arcobacter* species.

Material and methods

Bacterial strains and culture conditions. The 40 strains used in this study consisted of 20 *Campylobacter* and 12 *Arcobacter* strains isolated from poultry and water samples, and eight reference strains provided by the National Collection of Type Cultures (NCTC, London, UK): *Campylobacter jejuni* NCTC 11828 (RC1), *C. jejuni* NCTC 12521 (RC2), *C. lari* NCTC 11352 (RC3), *C. upsaliensis* NCTC 11540 (RC4), *C. coli* NCTC 11366 (RC5), and *Arcobacter butzleri* NCTC 12481 (RA1), *A. cryaerophilus* NCTC 11885 (RA2) and *A. skirrowii* NCTC 12713 (RA3).

The isolates were cultured on Columbia agar (Oxoid, Basingstoke, UK) with 5% (v/v) defibrinated sheep blood. *Campylobacter* strains were incubated in a microaerophilic atmosphere at 42°C for 24–48 h, while *Arcobacter* strains were grown under oxic conditions at 30ºC for 24–72 h. All the isolates were purified and characterized by morphology, Gram stain, oxidase, catalase positivity, and the API-*Campy* identification system (Biomérieux, France).

PCR identification. Chromosomal DNA was extracted by the cetyltrimethyl-ammonium bromide (CTAB) method [36]. Multiplex PCR (mPCR) identification of *C. jejuni* and *C. coli* was carried out according to Denis et al. [6]. An *Arcobacter* species-specific multiplex-PCR assay [17] was done using a set of primers that amplify a 401-bp fragment of the *A. butzleri* 16S rRNA gene, a 641-bp fragment of the *A. skirrowii* 16S rRNA gene, and a 257-bp fragment of the *A. cryaerophilus* 23S rRNA gene.

PCR products were detected by electrophoresis on a 1.2% (w/v) agarose gel in 1× Tris-acetate-EDTA (TAE) buffer at 100 V. Gels were stained with ethidium bromide (0.5 µg/ml) and inspected visually under UV light. As a molecular marker, the GeneRuler 100 bp DNA Ladder Plus (MBI Fermentas, Canada) was used. DNA from reference strains and negative controls, in which DNA was replaced with sterile distilled water, were included in all the assays.

AFLP analysis. AFLP fingerprinting protocol was adapted from Gibson et al. [10]. Briefly, 10 µg of genomic DNA was digested with 20 U of *Hin*dIII (BioLabs) and 5 mM spermidine trihydrochloride (Sigma) in a final volume of 20 µl at 37ºC. A 5-µl aliquot of digested DNA was incubated with 0.2 µg each of ADH1 (5´ ACGGTATGCGACAG 3´) and ADH2 (5´ AGCTCTGTCGCATACCGTGAG 3´) adapters and 1 U *T4*-DNA ligase (BioLabs) in a total volume of 20 µl of reaction buffer for 3–4 h at 37°C, after which the mixture was heated to 80°C for 10 min to inactivate the enzyme. Ligated DNA (5 µl) was used as template in a 50-µl PCR mix containing 2.5 mM $MgCl_2$, dNTPs solution (200 mM each), 200 ng of HIG primer (5´ GGTATGCGACAGAGCTTG 3´), and 1 U of *Taq* Polymerase (Ecogen, Spain) in PCR buffer. The cycling parameters were: 94°C for 1 min, 60°C for 1 min, and 72°C for 2.5 min repeated 33 times, preceded by a 2-min incubation at 95°C and followed by an additional 2 min incubation at 72°C. Amplified fragments were size-separated by electrophoresis at 85 V in 2.5% (w/v) agarose gels (Agarose MS-8, Pronadisa) in 1×TAE buffer with ethidium bromide (0.5 µg/ml). A 100-bp DNA ladder (MBI Fermentas) was used as a molecular mass marker.

PFGE analysis. The preparation and enzymatic digestion of bacterial DNA for PFGE analysis were as described previously [11]. Briefly, bacterial cells were harvested and treated with formaldehyde to inactivate endogenous nucleases. A mixture of 500 µl of cell suspension and 500 µl of 2% low-melting-point agarose (Agarose LM-2, Pronadisa) was poured into PFGE molds and allowed to solidify. Next, the plugs were incubated with 2 ml of lysis buffer (0.5 M Na_2EDTA and 1% sodium lauryl sarcosine) and 20 µl of proteinase K (50 mg/ml) overnight at 56°C, washed with TE buffer, cut in 3- by 5-mm pieces, and digested with 20 U of *Sma*I (Biolabs) as recommended by the manufacturer.

Restriction fragments were electrophoretically separated in 1% (w/v) PFGE-grade agarose gel (Agarose D-5, Pronadisa) in 0.5× Tris-borate-EDTA (TBE) buffer for 20 h at 14°C in a CHEF DR-III apparatus (Bio-Rad Laboratories, USA) at 6 V/cm with initial and final pulse times of 0.5 and 25 s, respectively. After electrophoresis, the gels were stained with ethidium bromide for 30 min, rinsed in distilled water, and photographed under UV light. A lambda ladder PFG marker (BioLabs) was included in each gel.

Fragment size estimation and computation of strain similarities. Molecular fragment sizes were estimated by comparison with molecular mass markers, using the computer program TDI-Lane Manager. The patterns were numerically analyzed using the NTSYS-PC program (Applied Biostatistics Inc., Setauket, NY, USA). Computed similarities among isolates were measured by calculating the Dice coefficient, and clustering was based on the unweighted pair-group; dendrograms were generated with the mathematical average (UPGMA) method.

Results and Discussion

Phenotypic and PCR identification. All *Campylobacter* isolates were gram-negative spiral-shaped cells, rapidly motile, oxidase and catalase-positive, and unable to grow in air after incubation at 30ºC for 48-72 h. Of the 20 *Campylobacter* isolates, 16 were identified as *C. jejuni* and two as *C. coli* by the API-*Campy* system. The remaining two isolates (C10 and C14) presented unacceptable profiles (Table 1). All *Arcobacter* isolates grew under aerobiosis and were gram-negative, motile, and oxidase- and catalase-positive. When the API-*Campy* system was used to identify *Arcobacter* isolates and reference strains, all were determined to be *A. cryaerophilus*.

Table 1. Identification of *Campylobacter* and *Arcobacter* isolates

Isolate	Origin / Sample	API-*Campy*	mPCR
C1, C2, C3, C4	Chicken/S1	*C. jejuni jejuni* I	*C. jejuni*
C5, C6	Chicken/S2	*C. jejuni jejuni* I	*C. jejuni*
C7	Chicken/S3	*C. jejuni jejuni* I	*C. jejuni*
C8	Water/W1	*C. jejuni jejuni* II	*C. coli*
C9	Water/W2	*C. jejuni jejuni* II	*C. coli*
C10	Water/W2	*C. coli* (unacceptable)	*C. coli*
C11	Water/W3	*C. coli*	*C. coli*
C12	Chicken/S4	*C. coli*	*C. coli*
C13	Water/W4	*C. jejuni jejuni* I	*C. coli*
C14	Water/W5	*C. coli* (unacceptable)	*C. jejuni*
C15, C16	Water/W6	*C. jejuni jejuni* I	*C. jejuni*
C17	Water/W7	*C. jejuni jejuni* I	*C. jejuni*
C18, C19	Chicken/S5	*C. jejuni jejuni* I	*C. jejuni*
C20	Chicken/S6	*C. jejuni jejuni* I	*C. jejuni*
A1, A3	Chicken/S7	*A. cryaerophilus*	*A. butzleri*
A2	Chicken/S8	*A. cryaerophilus*	*A. butzleri*
A4	Chicken/S9	*A. cryaerophilus*	*A. butzleri*
A5, A6	Water/W8	*A. cryaerophilus*	*A. butzleri*
A7, A8, A9, A10	Water/W9	*A. cryaerophilus*	*A. cryaerophilus*
A11	Chicken/S10	*A. cryaerophilus*	*A. butzleri*
A12	Water/W10	*A. cryaerophilus*	*A. cryaerophilus*

Molecular identification was considered the most accurate system for identification [6,12]. After mPCR, 14 of the 20 *Campylobacter* isolates were identified as *C. jejuni* and the remaining six as *C. coli* (Table 1). The two isolates that could not be identified by the API-*Campy* system, C10 and C14, were recognized by mPCR as *C. coli* and *C. jejuni*, respectively. Three isolates characterized as *C. jejuni* by API-*Campy* (C8, C9, and C13) yielded the 462-bp fragment of the *ceuE*

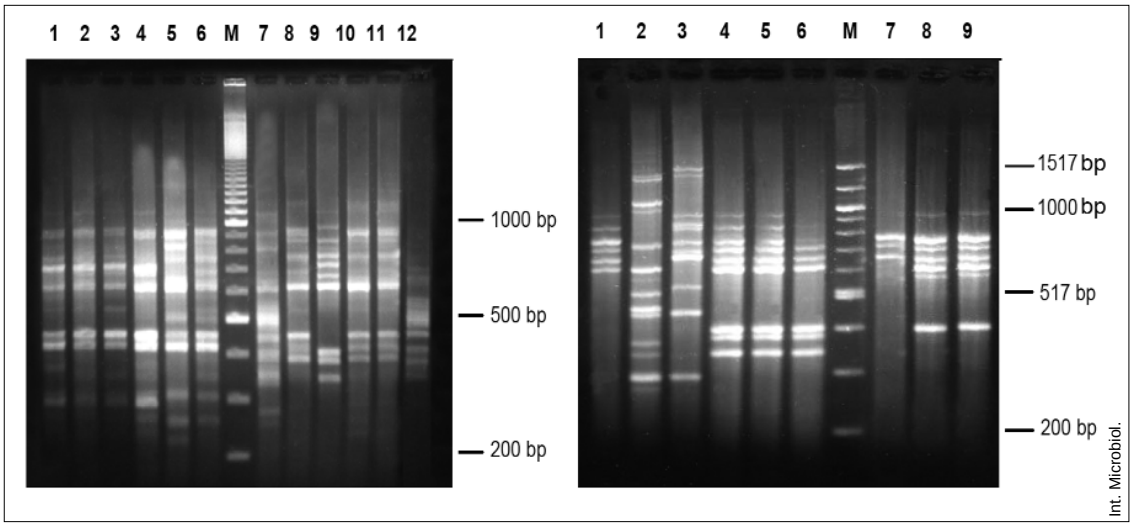

Fig. 1. AFLP patterns. (**A**) *Campylobacter*. Lane 1, C1; lane 2, C2; lane 3, C3; lane 4, C4; lane 5, C 5; lane 6, C6; lane 7, C9; lane 8, C13; lane 9, C14; lane 10, C15; lane 11, C16; lane 12, C10; lane M: DNA ladder with sizes indicated at right (bp). (**B**) *Arcobacter*. Lane 1, *A. butzleri* NCTC 12481; lane 2, *A. cryaerophilus* NCTC 11885; lane 3. *A. skirrowii* NCTC 12713; lane 4, A1; lane 5, A3; lane 6, A2; lane 7, A4; lane 8, A5; lane 9, A6; lane M, DNA ladder with sizes indicated at right (bp)

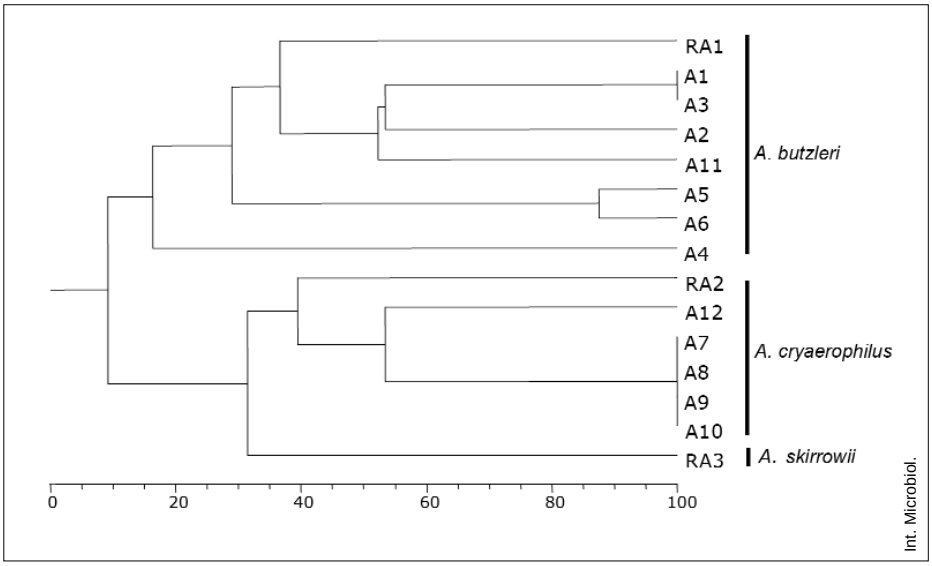

Fig. 2. Dendrogram of AFLP-profiles of *Arcobacter* strains. The degree of similarity (%) is shown on the scale bar.

gene, specific for *C. coli* species. Seven out the 12 *Arcobacter* isolates were identified as *A. butzleri* and the remaining five as *A. cryaerophilus*. All of the chicken isolates were identified as *A. butzleri*. Only one of the water isolates was identified as *A. butzleri* whereas the other five were *A. cryaerophilus* (Table 1). These results confirm the ineffectiveness of biochemical tests to identify *Arcobacter* and *Campylobacter* species [3,18].

AFLP analysis. *Campylobacter* AFLP patterns consisted of 4–11 fragments, ranging in size from 200 to 1300 bp (Fig. 1A). All strains exhibited unique AFLP profiles. Two main clusters could be defined, with as low as 5% similarity between them (dendrogram not shown). Other authors reported similar results in terms of this high degree of diversity by [14]. Cluster I comprised seven *C. jejuni* and four *C. coli* isolates. The *Campylobacter* reference strains also fell into this cluster. Cluster II comprised only a group of *C. jejuni* isolated from chicken.

Eleven distinct AFLP profiles were detected among *Arcobacter* strains, with 3–12 amplified DNA fragments ranging from 240 to 1300 bp (Fig. 1B). The DNA patterns

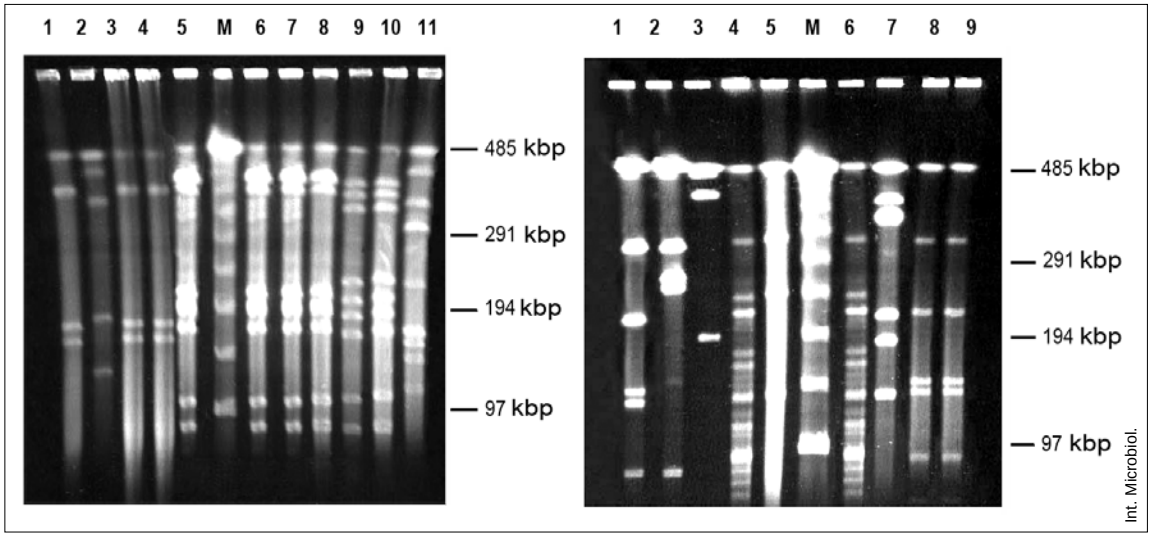

Fig. 3. PFGE patterns. (**A**) *Campylobacter*. Lane 1, C17; lane 2, C11; lane 3, C15; lane 4, C16; lane 5, C1; lane 6, C2; lane 7, C3; lane 8, C4; lane 9, C5; lane 10, C6; lane 11, C8; lane M, lambda ladder Plus with sizes indicated at right (kbp). (**B**) *Arcobacter*. Lane 1, A11; lane 2, *A. cryaerophilus* NCTC 11885; lane 3, *A. skirrowii* NCTC 12713; lane 4, A1; lane 5, A2; lane 6, A3; lane 7, A4; lane 8, A5; lane 9, A6; lane M: lambda ladder Plus with sizes indicated at right (kbp).

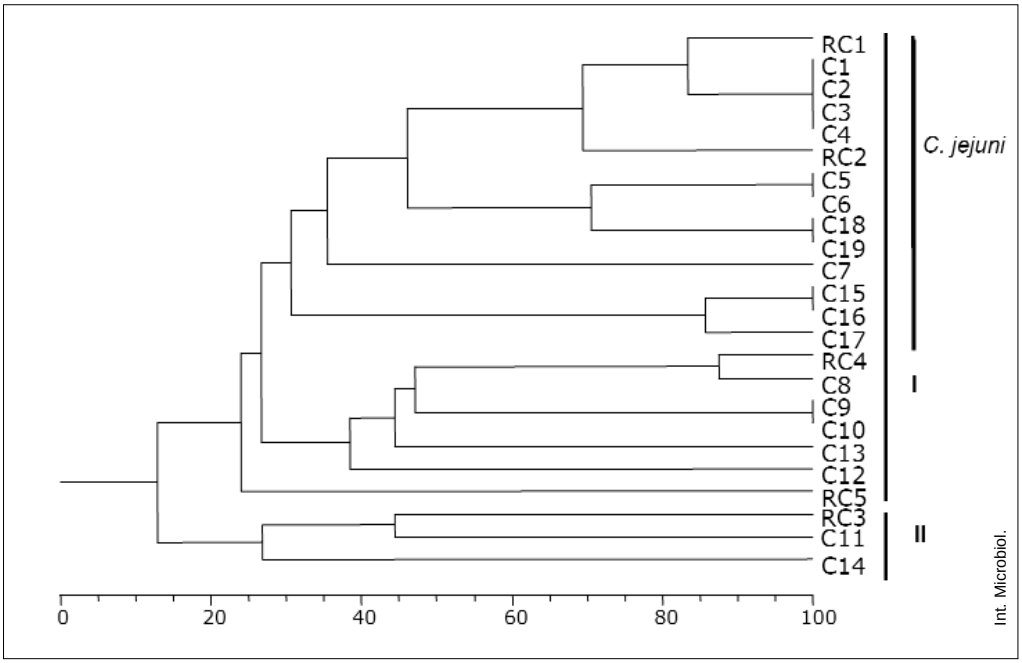

Fig. 4. Dendrogram of PFGE-profiles of *Campylobacter* strains. The degree of similarity (%) is shown on the scale bar.

varied greatly. Figure 2 shows the dendrogram obtained from *Arcobacter* AFLP data. Two different homology groups were determined, both grouped at 9% similarity: one included *A. butzleri* species, and the other *A. cryaerophilus* species and the *A. skirrowii* reference strain.

Identical patterns (100% homology) were obtained for A1 and A3, both isolated from the same chicken sample and identified by mPCR as *A. butzleri*. Four *A. cryaerophilus* strains isolated from the same water sample shared the same AFLP profile (A7–A10). These results confirmed the suitability of AFLP analysis for assessing epidemiological relatedness among isolates. The technique was also useful to differentiate *A. butzleri* from other *Arcobacter* species. However, in accordance with other authors [25], we found that AFLP did not allow for species differentiation of *Campylobacter*, as *C. coli* and *C. jejuni* did not group separately.

PFGE analysis. DNA fingerprints were obtained from all *Campylobacter* isolates after *Sma*I restriction except for C20, because its DNA was not cut with this enzyme. *Sma*I digestion produced 17 different PFGE profiles of *Campylobacter*, with between three and ten bands, sized approximately 70–450 kbp (Fig. 3A). PFGE did not allow for species differentiation of *Campylobacter*, although most *C. jejuni* strains grouped separately from *C. coli*. Figure 4 shows the dendrogram obtained from numerical analysis of all *Campylobacter* PFGE profiles. Seventeen types could be differentiated with 13% similarity between them. Identical patterns (100% homology) were observed for C1–C4, C5 and C6, C9 and C10, C15 and C16, and C18 and C19. In each case, the isolates had been obtained from the same sample.

Nine PFGE types were obtained for the 14 *Arcobacter* strains included in this work, with 3–12 bands ranging from 70 to 480 kbp (Fig. 3B). Isolate A12 yielded no DNA banding pattern at all due to extensive DNA degradation, despite three repetitions. High diversity was also observed in the profiles of *Arcobacter*, although two main groups could be defined with 12.5% similarity between them; *A. butzleri* clustered apart from the rest of *Arcobacter* species (dendrogram not shown). Isolates A1-A3 and A7-A8-A9-A10, which presented 100% homology on AFLP profiles, also had identical *Sma*I profiles. Moreover, two isolates (A5 and A6), which presented different AFLP patterns, were assigned to the same PFGE type. Both had been isolated from the same sample and were identified as the same specie. A second enzyme could be used to determine the relatedness between these two isolates [22].

In conclusion, molecular typing revealed a high degree of heterogeneity. PFGE was effective in showing epidemiological relationships between isolates. AFLP was more rapid and easier to perform. This technique is appropriate to differentiate minimal genomic variations, which makes it a valuable tool for the identification of isolates. The combined use of PFGE and single-enzyme AFLP methods can be useful to determine taxonomic and epidemiological relationships among campylobacteria.

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