Differentiation of *Campylobacter* species by AFLP fingerprinting

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The fluorescent amplified fragment length polymorphism (AFLP) fingerprinting method was tested for its ability to identify and subtype the most important *Campylobacter* species found in veterinary infections. Sixty-nine reference strains and 19 clinical isolates of *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter jejuni* subsp. *doylei*, *Campylobacter upsaliensis*, *Campylobacter coli*, *Campylobacter lari*, *Campylobacter fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *Campylobacter hyointestinalis* subsp. *hyointestinalis*, *C. hyointestinalis* subsp. *lawsonii*, *Campylobacter mucosalis*, *Campylobacter helveticus* and *Campylobacter sputorum* were subjected to analysis. The topology of the dendrogram obtained by numerical analysis of the AFLP profiles did not reflect the phylogenetic relationships as derived from 16S rDNA sequence comparison. However, except for *C. lari*, AFLP analysis grouped the strains that belonged to the same genomic species into distinct clusters. *C. lari* strains were separated into two distinct AFLP groups, which corresponded with nalidixic-acid-sensitive and -resistant variants of *C. lari*. These results correlated with data from whole-cell protein profiling. Within *C. jejuni*, *C. hyointestinalis* and *C. fetus*, strains could be identified at the subspecies level. AFLP analysis also allowed the subtyping of most species at the strain level. It is concluded that AFLP analysis is a valuable tool for concurrent identification of campylobacters at the species, subspecies and strain levels. In addition, the data confirm and extend previous reports showing that *C. lari* is a heterogeneous species that may comprise multiple taxa.

Keywords: taxonomy, typing, epidemiology, veterinary infections

INTRODUCTION

Campylobacter species have been recognized as important pathogens in humans and animals. Within the genus *Campylobacter*, the thermotolerant species *Campylobacter jejuni* and *Campylobacter coli* cause gastroenteritis in humans, and colonize poultry, pigs and cattle. *C. jejuni* is considered to be the most common cause of sporadic bacterial enteritis worldwide (Skirrow & Blaser, 1992). Other *Campylobacter* species can be considered as primary human pathogens, e.g. *Campylobacter upsaliensis*, *Campylobacter lari*, *Campylobacter fetus* subsp. *fetus* and *C. jejuni* subsp. *doylei*; because of inappropriate isolation and identi-

fication methods, they are thought to be significantly underdiagnosed causes of gastrointestinal diseases (Lastovica & Skirrow, 2000).

Several *Campylobacter* species are associated with infections in animals. *C. upsaliensis* strains have been isolated from healthy and diarrhoeic dogs and cats (Bourke *et al.*, 1998). The subspecies *C. fetus* subsp. *venerealis* causes abortion and infertility in cattle, whereas *C. fetus* subsp. *fetus* is an important cause of sporadic abortion in sheep and cattle (Garcia *et al.*, 1983; Lastovica & Skirrow, 2000). *Campylobacter hyointestinalis* and *Campylobacter mucosalis* have been isolated from pigs with proliferative enteritis and other enteric diseases. The species *C. hyointestinalis* is divided into two subspecies, namely *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* (On *et al.*, 1995). The latter has been isolated from pig

Abbreviations: AFLP, amplified fragment length polymorphism; UPGMA, unweighted pair-group method using arithmetic averages.

Table 1. *Campylobacter* strains used for AFLP analysis

Species and strain code*	Source	Country and isolation year
<i>C. jejuni</i> subsp. <i>jejuni</i>		
LMG 9081	Human faeces	South America, 1979
LMG 9221	Child faeces	Belgium
LMG 14244	Human faeces	The Netherlands
LMG 14317	Human faeces	The Netherlands
LMG 15906	Human faeces	Brazil
LMG 15907	Human faeces	Brazil
ATCC 29428	Child, diarrhoea	—
R-2631	Human faeces	Belgium
C356†	Chicken faeces	The Netherlands, 1990
C2555†	Chicken faeces	The Netherlands, 1992
C2412†	Chicken faeces	The Netherlands, 1992
C2651†	Chicken faeces	The Netherlands, 1993
C2345†	Chicken faeces	The Netherlands, 1992
C2143†	Chicken faeces	The Netherlands, 1992
C2172†	Human, diarrhoea	The Netherlands, 1993
<i>C. jejuni</i> subsp. <i>doylei</i>		
LMG 8556	Human stomach	UK, 1992
LMG 9243	Child, faeces	Belgium, 1986
LMG 8843	Child, diarrhoea	Australia, 1982–1983
LMG 8870	Child, blood	South Africa, 1986
LMG 9255	Child, faeces	Belgium, 1986
<i>C. coli</i>		
LMG 15909	Human faeces	Brazil
LMG 15908	Human faeces	Brazil
LMG 15882	Pig, enteritis	Australia, 1988
LMG 15884	Pig, enteritis	Australia, 1991
LMG 15904	Human faeces	Brazil
LMG 15905	Human faeces	Brazil
ATCC 33559	Pig faeces	Sweden
R-2153	Human faeces	Belgium
C2436†	Chicken faeces	The Netherlands, 1992
C2470†	Chicken faeces	The Netherlands, 1993
C2375†	Chicken faeces	The Netherlands, 1992
C2498†	Chicken faeces	The Netherlands, 1992
C2155†	Chicken faeces	The Netherlands, 1993
<i>C. upsaliensis</i>		
LMG 9124	Child, faeces	Belgium, 1987
LMG 9129	Child, diarrhoea	Belgium, 1986
LMG 8850	Canine faeces	Sweden, 1980
LMG 8854	Child, blood	South Africa, 1985–1988
LMG 9139	Child, diarrhoea	Belgium, 1986
LMG 13480	Child, faeces	Belgium
LMG 10783	Human faeces	Belgium
R-2343	Human faeces	Belgium
<i>C. lari</i>		
LMG 9889	Human	Canada
LMG 7929	Dog faeces	UK
LMG 9913	Human	—
LMG 8845	Child, faeces	—
LMG 9152	Horse intestine	Sweden, 1981
LMG 8846	Herring gull, cloacae	Canada

Table 1 (cont.)

Species and strain code*	Source	Country and isolation year
LMG 9914	Human	Canada
LMG 11760	Human	Canada, 1990
LMG 8844	Seawater	UK, 1982–1983
R-1189	Human faeces	Belgium
ATCC 35221	Herring gull, cloacae	–
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>		
LMG 8214	Pig intestine	France
LMG 9276	Child, diarrhoea	Belgium, 1986
LMG 7817	Pig intestine	USA, 1980
LMG 8464	Pig	–
LMG 8540	Beef calf, diarrhoea	USA, 1982
CCUG 14170	Pig intestine	USA, 1983
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>		
LMG 15912	Human	Brazil
LMG 14432	Pig stomach	UK
LMG 14433	Pig stomach	UK
LMG 14434	Pig stomach	UK
LMG 14435	Pig stomach	UK
<i>C. helveticus</i>		
CCUG 30563	Cat faeces	Switzerland, 1991
CCUG 30564	Cat faeces	Switzerland, 1991
CCUG 34016	Cat faeces	Sweden, 1995
CCUG 34042	Cat faeces	Sweden, 1995
LMG 12639	Cat, faeces	Switzerland, 1992
<i>C. fetus</i> subsp. <i>fetus</i>		
LMG 9150	Dog faeces	1980
LMG 14744	Human blood	Belgium
LMG 12689	Human blood	–
LMG 8620	Pig intestine	France
R-4117	–	Finland
ATCC 27374	Sheep fetus, brain	–
<i>C. fetus</i> subsp. <i>venerealis</i>		
ATCC 19438	Heifer, vaginal mucus	–
LMG 6443	Heifer, vaginal mucus	UK
5521‡	–	The Netherlands, 1993
5522‡	–	The Netherlands, 1993
<i>C. mucosalis</i>		
LMG 8266	Pig intestine	UK, 1972
LMG 7794	Pig intestine	UK, 1982
LMG 8433	Pig intestine	–
LMG 6265	Pig intestine	UK, 1973
LMG 6448	Pig	UK, 1972
<i>C. sputorum</i> bv. <i>paraurelyticus</i> LMG 11764	Human stool	Canada, 1991
<i>C. sputorum</i> LMG 14261	Human pus	Spain, 1993
<i>C. sputorum</i> bv. <i>fecalis</i> LMG 6617	Ovine faeces	Canada
<i>C. sputorum</i> bv. <i>sputorum</i> LMG 7795	–	–
<i>C. sputorum</i> bv. <i>sputorum</i> LMG 7975	Pig intestine	UK

* Strain abbreviations: LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium; ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden.

† Strains obtained from chickens during a study among randomly located farms in The Netherlands (Jacobs-Reitsma *et al.*, 1994).

‡ Strains obtained from the Animal Health Service, Boxtel, The Netherlands.

stomachs, but its role remains unknown (Gebhart *et al.*, 1985). The pathogenicity of the species *C. lari*, isolated from the intestines of gulls, shellfish, fish and other animals, and the species *Campylobacter helveticus*, isolated from the faeces of domestic cats and dogs, is unknown (Endtz *et al.*, 1997; Stanley *et al.*, 1992). The species *Campylobacter sputorum* is considered to be a commensal of the animals in which it can be found. It has been isolated from the genital tracts and the faeces of bulls, and has been associated with several diseases in humans (Borczyk *et al.*, 1988).

The classification of the genus *Campylobacter* has been significantly changed during the last decade. On the basis of phylogenetic results derived from DNA-rRNA hybridization and data from genotypic and phenotypic analyses of strains, the genus *Campylobacter* currently consists of 16 validly described *Campylobacter* species (Lawson *et al.*, 2001; Logan *et al.*, 2000; Vandamme *et al.*, 1991). Appropriate identification of *Campylobacter* species is often hampered by a lack of biochemical differences and also by unreliable test reactions (Goossens & Butzler, 1992; On, 1996). To study the prevalence of *Campylobacter* species in animal diseases, the natural distribution of species, and the incidence of these species in human infections, a method is needed that can concurrently identify *Campylobacter* strains to the species and strain levels. Amplified fragment length polymorphism analysis (AFLP), a random whole-genome fingerprinting method, has been used for high-resolution typing of several bacteria in both epidemiological and taxonomic studies (Savelkoul *et al.*, 1999). Recently, this method has been adapted for epidemiological typing of *C. jejuni* and *C. coli*. These studies demonstrated the subtyping of individual strains, and have already differentiated between two species (Duim *et al.*, 1999, 2000; Kokotovic & On, 1999; On & Harrington, 2000). In this study, the value of AFLP analysis was determined for the identification and differentiation, at strain level, of nine *Campylobacter* species and three subspecies belonging to well-defined taxa and representing *Campylobacter* species that are very important in a veterinary context.

METHODS

Bacterial strains and culture conditions. A total of 88 strains were analysed. Fifty-nine reference strains of *Campylobacter* species were obtained from the BCCM/LMG (the Bacteria Collection, Laboratorium voor Microbiologie, University of Gent, Gent, Belgium). These strains consisted of six *C. jejuni* subsp. *jejuni* strains, five *C. jejuni* subsp. *doylei* strains, six *C. coli* strains, seven *C. upsaliensis* strains, nine *C. lari* strains, five *C. hyointestinalis* subsp. *hyointestinalis* strains, five *C. hyointestinalis* subsp. *lawsonii* strains, four *C. fetus* subsp. *fetus* strains, one *C. fetus* subsp. *venerealis* strain, five *C. mucosalis* strains, one *C. helveticus* strain and five *C. sputorum* strains. Five reference strains (*C. jejuni* subsp. *jejuni*, *C. coli*, *C. lari*, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*) were obtained from the American Type Culture Collection (ATCC). Four *C. helveticus* strains and one *C. hyointestinalis* subsp. *hyointestinalis* strain were obtained from the CCUG (the Culture Collection of the University of

Göteborg, Göteborg, Sweden). Also included were seven recent isolates – one *C. jejuni* strain, one *C. coli* strain, one *C. upsaliensis* strain and one *C. lari* strain (University of Gent, Belgium), as well as one *C. fetus* subsp. *fetus* strain and two *C. fetus* subsp. *venerealis* strains (Animal Health Service, Boxtel, The Netherlands), all of which were identified by whole-cell protein profiling (P. Vandamme, unpublished results) – and 12 field isolates of *C. jejuni* ($n = 7$) and *C. coli* ($n = 5$) (Jacobs-Reitsma *et al.*, 1994). Strain numbers and available information are listed in Table 1. Bacteria were grown on blood-agar plates supplemented with 5% (v/v) sheep blood, at 37 °C for 2–3 d, under microaerophilic conditions. An anoxomat system (Mart) was used to produce the following final gas mixture: 6% O₂, 7% CO₂, 80% N₂, 7% H₂ (by vol.). Bacterial cultures were stored at –80 °C in 15% (w/v) glycerol in heart infusion broth.

AFLP analysis. For the isolation of chromosomal DNA, freshly grown cells were scraped from plates and washed with 1 ml TE (10 mM Tris/HCl, pH 8.0, 1 mM EDTA). DNA was isolated using the PUREGENE kit (Gentra Systems). DNA integrity was checked by agarose gel electrophoresis, and DNA preparations were stored at –20 °C. DNA concentrations were determined with a spectrophotometer and were standardized at 0.1 µg µl⁻¹.

AFLP analysis was performed according to the recently described AFLP method for *Campylobacter* genotyping, an adapted protocol of the AFLP Microbial Fingerprinting method of PE Applied Biosystems (Duim *et al.*, 1999). In short, chromosomal DNA was digested with *Hind*III and *Hha*I, and, in a simultaneous reaction, the fragments were ligated to restriction-site-specific adapters for 2 h at 37 °C. Then a preselective PCR using the adapter-specific primers *Hind*III (5'-GACTGCGTACCAGCTT-3') and *Hha*I (5'-GATGAGTCCTGATCGC-3') was performed. Next, an aliquot was subjected to a selective PCR in which both primers contained an additional A nucleotide at the 3'-end: *Hind*III primer 5'-GACTGCGTACCAGCTTA-3' and *Hha*I primer 5'-GATGAGTCCTGATCGCA-3'. The 5' end of the *Hind*III primer contained a fluorescent label. The final products were run on a 7.3% denaturing acrylamide sequencing gel for 5 h, using an ABI 373A automated DNA sequencer.

Data processing. After electrophoresis, the data for the fluorescently labelled banding patterns were collected by using ABI GENESCAN software (PE Applied Biosystems). Each gel track, ranging from the size-marker 100 bp band to the 500 bp band, was imported in the software package GelCompar 4.1 (Applied Maths) with the program ABICON (Applied Maths). Patterns were normalized by reference to the molecular mass of the internal ROX-labelled size standard that was included in each sample. After normalization, the levels of genetic similarity between the AFLP patterns were calculated with the Pearson product-moment correlation coefficient (r). For cluster analysis of AFLP banding patterns, the unweighted pair group method using arithmetic averages (UPGMA) was used (Vauterin & Vauterin, 1992).

RESULTS

The AFLP conditions that were initially developed for analysis of *C. jejuni* and *C. coli* were used to analyse 88 strains that belonged to different *Campylobacter* species (Duim *et al.*, 1999). The banding patterns of strains varied in terms of the distribution of bands ranging from 100 to 500 bp in size. Without adaptation of the method, banding patterns were obtained from all strains (Fig. 1).

Fig. 1. Dendrogram of AFLP fingerprints of *Campylobacter* species and subspecies. The distinct AFLP clusters are indicated by letters and bars, which correspond to the indicated species names. The percentage genetic similarity between banding patterns is indicated.

Reproducibility of the AFLP profiles

The reproducibility of the AFLP profiles was defined as a >90% similarity level between duplicated samples.

This was the mean similarity obtained between four independently obtained profiles for all 88 strains (i.e. four new DNA samples run on different gels) except for one strain of *C. upsaliensis* (R-2342) and two strains of

C. helveticus (CCUG 34242 and CCUG 34016). The repeated samples of these strains showed only 60–80% similarity, because of variation in band positions and a large number of faint bands, and were considered unreproducible. This can be explained by incomplete digestion by one of the restriction enzymes. The presence of restriction–modification systems acting on the restriction enzymes used for typing has been described for *Campylobacter* species (Newell *et al.*, 2000). Because of the aberrant patterns, these *C. helveticus* and *C. upsaliensis* strains were not included in the numerical analysis (Fig. 1).

Differentiation of *Campylobacter* species

To differentiate between species, the degrees of similarity of banding patterns were calculated using the Pearson correlation coefficient and were expressed as percentage similarities. After cluster analysis by UPGMA, a dendrogram was constructed (Fig. 1). Distinct AFLP clusters were identified that were defined by banding patterns that grouped together and showed a linkage level with other clusters of less than 20%. Altogether, 10 distinct AFLP clusters were observed, of which eight corresponded with the assigned taxonomic groups of these species (Fig. 1, clusters A to I). However, strains of the species *C. lari* were divided in two AFLP groups (Fig. 1, clusters B1 and B2). The AFLP classification of the clinical and field isolates correlated with data obtained from whole-cell protein profile analysis (data not shown). The strains of each *Campylobacter* species showed characteristic AFLP patterns, identified by the number and distribution of the AFLP bands (Fig. 1).

The *C. jejuni* subsp. *jejuni* cluster consisted of highly diverse AFLP fingerprints. The subspecies *C. jejuni* subsp. *doylei* showed highly homogeneous AFLP banding patterns, which were clearly differentiated from those of subsp. *C. jejuni* subsp. *jejuni*. The subspecies grouped together with a linkage level of $39.6 \pm 4.6\%$ (Fig. 1, clusters F1 and F2). The AFLP banding patterns of *C. coli* strains contained many closely distributed bands, which were more homologous than the patterns for *C. jejuni* subsp. *jejuni*, similar to that shown previously (Fig. 1, cluster I) (Duim *et al.*, 1999). The AFLP banding patterns of the *C. lari* strains were divided into two distinct clusters (Fig. 1, clusters B1 and B2). The strains in cluster B1 represented the nalidixic-acid-resistant thermophilic campylobacters (NARTC), the classical biotype of *C. lari*. The strains of cluster B2 were nalidixic acid sensitive (NASC). The banding patterns in the two *C. lari* clusters showed less than 20% similarity, which indicates that there is a difference at the DNA level for these *C. lari* variants.

The *C. upsaliensis* cluster showed banding patterns comprising a large number of bands that were highly heterogeneous (Fig. 1, cluster C). The fingerprints of *C. upsaliensis* contained a large number of bands and had

to be interpreted carefully because of the unreproducibility of the pattern of one strain. The three *C. helveticus* strains also showed patterns containing many bands. These patterns were specific and grouped the strains of this species in a separate cluster after numerical analysis (Fig. 1, cluster G).

Despite the highly similar fingerprints, a differentiation between the two subspecies *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* was obtained. At a high linkage level ($80.7 \pm 1.4\%$), the subspecies could be differentiated because the banding patterns consistently showed a few band differences (Fig. 1, clusters E1 and E2). For the field isolates R-4117, 5522 and 5521, the subspecies were also determined biochemically and confirmed using a subspecies-specific PCR (Hum *et al.*, 1997; Wagenaar *et al.*, 2001). These data correlated with the species identification obtained by AFLP analysis.

The *C. hyointestinalis* cluster consisted of banding patterns specific for the subsp. *hyointestinalis*, which were clearly separated (with a similarity level of $26.2 \pm 5.7\%$) from banding patterns that were specific for the subsp. *lawsonii* (Fig. 1, groups D1 and D2).

AFLP analysis of genome fragments of *C. sputorum* and *C. mucosalis* resulted in patterns with only 11–25 bands. The bands of both species showed considerable heterogeneity, and were separately clustered (Fig. 1, groups A and H). A differentiation between biovars of *C. sputorum* was not observed.

DISCUSSION

Several species of the genus *Campylobacter* are known to cause human and animal infections, and it is important for clinical and epidemiological studies that *Campylobacter* species are accurately differentiated. In this study, we present data showing that AFLP is a suitable genomic fingerprinting technique for the differentiation of the *Campylobacter* species tested. The reference strains (Table 1) showed highly distinct AFLP fingerprints between species, and when recent human and animal clinical isolates were included, the differentiation of species remained identical. Except for *C. lari*, strains that belonged to the same genomic species always grouped together in one particular AFLP cluster (Fig. 1).

AFLP data compared with taxonomic and phylogenetic data

The determination of total genomic DNA–DNA homology values has persisted as a dominant component of taxonomic analysis. However, recent studies have shown that AFLP genomic fingerprinting analysis is an accurate approach for determining bacterial taxonomy and for phylogenetic comparisons between bacteria (Huys *et al.*, 1996; Janssen *et al.*, 1997; Rademaker *et al.*, 2000). In contrast with AFLP studies of the genera *Xanthomonas*, *Acinetobacter* and *Aeromonas*, the present AFLP dendrogram did not correspond with the

phylogenetic relationships between *Campylobacter* species as deduced from comparative 16S rRNA gene-sequence analyses (Vandamme *et al.*, 1991; Vandamme, 2000). On & Harrington (2000) recently reported the same observation. However, all species except *C. lari* were separated into single clusters, and the two subspecies within *C. fetus*, *C. hyointestinalis* and *C. jejuni* were clearly distinguishable; this corresponded nicely with the present classification of these organisms. The results of the AFLP analysis of two strains (LMG 15882 and LMG 15884) initially described as a novel species (*Campylobacter hyoilei*; Alderton *et al.*, 1995) corroborated the reported synonymy between *C. hyoilei* and *C. coli* (Vandamme *et al.*, 1997). These data confirm the value of AFLP in studying relationships between bacteria at and below the species level.

The *C. lari* strains examined clustered into two distinct groups with a similarity level of 18.9%. The observed differences between these groups were much larger than that obtained between the subspecies of *C. jejuni*, *C. hyointestinalis* and *C. fetus*. The genetic diversity observed with AFLP fingerprinting confirms the reported heterogeneity among *C. lari* identified by using randomly amplified polymorphic DNA (RAPD) and protein analysis (Endtz *et al.*, 1997). In a recent study, On & Harrington (2000) used AFLP fingerprinting to demonstrate that the nalidixic-acid-resistant thermophilic and urease-positive thermophilic biovars of *C. lari* have substantive genetic differences. Our data identified genetic differences between nalidixic-acid-resistant thermophilic campylobacter strains and nalidixic-acid-sensitive campylobacter strains, and also suggest that this nomenespecies may comprise multiple distinct genetic groups. Also, protein-profile analysis confirmed this separation of the *C. lari* groups. These data suggest that the different AFLP clusters within *C. lari* represent distinct *Campylobacter* taxa.

The differences observed between the AFLP patterns of *C. upsaliensis* indicated a considerable degree of genetic diversity between strains of this species. These data confirm the reported genotypic heterogeneity among *C. upsaliensis* strains that was observed with PFGE typing (Bourke *et al.*, 1996). The species *C. helveticus* is phylogenetically the nearest neighbour of *C. upsaliensis*, as both species showed relative DNA homology (Stanley *et al.*, 1992). However, the AFLP profiles of *C. helveticus* and *C. upsaliensis* were distinct and showed no homology (Fig. 1, clusters C and H).

Identification of *Campylobacter* species

The AFLP clusters of all species tested were clearly distinct, and differentiation within the species *C. jejuni*, *C. hyointestinalis* and *C. fetus* at the subspecies level was also obtained. It can be concluded, therefore, that AFLP analysis can be used for the identification of *Campylobacter* strains at the species and subspecies levels. The similarity between AFLP patterns is calculated using the Pearson product-moment corre-

lation coefficient applied to the complete densitometric curves of the gel tracks; this method may be more reliable than band-based calculations for comparing large numbers of patterns (Rademaker *et al.*, 2000) and to provide a database for identification. However, for optimal species identification, it is necessary to include in the database a number of fingerprints for each species, as representations of strain relationships in dendrograms are subject to some degree of distortion and the clustering of a single pattern of a species may be incorrect.

The identification of the strains of the species *C. lari* may be complicated by the fact that this species belongs to distinct AFLP clusters that may represent distinct genetic groups. Of the closely related species *C. jejuni* and *C. coli*, which are the most important causes of human gastroenteritis, the identification can be problematic, because the overall phenotype and genotype of both taxa are remarkably similar. However, with AFLP analysis, the patterns show substantive differences which allow the identification of these species (Fig. 1). Also, for the species *C. fetus*, the currently available methods for identifying subspecies are not straightforward (Vandamme *et al.*, 1990). Both subspecies are highly related at the genetic level, but differ in terms of host-specificity and pathogenicity. In this study, AFLP analysis was able to separate the related subspecies from differences in only a few AFLP bands. In a larger study, it was demonstrated that AFLP analysis is able to separate field isolates of *C. fetus* subsp. *fetus* from *C. fetus* subsp. *venerealis* (Wagenaar *et al.*, 2001). In addition, with the AFLP method described by On & Harrington (2000), no differentiation between the subspecies was obtained.

The use of AFLP analysis as a species-identification method has the advantage that it enables species identification and shows the relationships between strains at both the taxonomic and epidemiological levels.

AFLP typing of *Campylobacter* species

For *C. jejuni*, the application of AFLP analysis to the typing of epidemiologically related and unrelated strains has been well established (Duim *et al.*, 1999, 2000). The AFLP patterns of the two subspecies of *C. hyointestinalis* were well distributed and highly diverse. Recently, Harrington & On (1999) demonstrated that extensive 16S rRNA gene sequence diversity exists between the two *C. hyointestinalis* subspecies. The AFLP analysis confirmed the diversity between subspecies but was also able to type individual strains, as the banding patterns of individual strains showed substantial diversity. The AFLP patterns of individual strains of the species *C. fetus* were highly homologous, which supports the reported genetic homogeneity of this species, but also limits the use of AFLP typing for studying the epidemiology of this species. In contrast, the patterns for *C. upsaliensis* and *C. helveticus* were very diverse but this could cause problems in the interpretation of the

patterns, as some bands were unreproducible. An additional analysis of epidemiologically related strains will be necessary in order to determine if AFLP analysis can differentiate these strains correctly and be of value for the typing of *C. upsaliensis* and *C. helveticus*. The patterns of *C. sputorum* and *C. mucosalis* showed, despite the small number of bands, sufficient diversity to permit strain typing. The differentiation of biovars of *C. sputorum* was not achieved with the small number of strains used in this study, but future analyses of larger numbers of strains may be more successful.

Conclusions

AFLP fingerprinting analysis of *Campylobacter* species is a reliable method that allows identification of strains at the species and subspecies levels, and it may be a suitable tool for the determination of strain relationships at the taxonomic and epidemiological levels.

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