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Amplified fragment length polymorphism based identification of genetic markers and novel PCR assay for differentiation of *Campylobacter fetus* subspecies

Marcel A. P. van Bergen,[1] Guus Simons,[2]† Linda van der Graaf-van Bloois,[1,3] Jos P. M. van Putten,[3] Jeroen Rombout,[2] Irene Wesley[4] and Jaap A. Wagenaar[1,3]

Correspondence
Jaap A. Wagenaar
jaap.wagenaar@wur.nl

[1]Animal Sciences Group, Division of Infectious Diseases, and OIE Reference Laboratory for Campylobacteriosis, PO Box 65, 8200 AB, Lelystad, The Netherlands

[2]Keygene N.V., Department of Microbial Genomics, Wageningen, The Netherlands

[3]Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, and OIE Reference Laboratory for Campylobacteriosis, Utrecht University, Utrecht, The Netherlands

[4]Pre-Harvest Food Safety and Enteric Diseases Research Unit, National Animal Disease Center, Agricultural Research Service, US Department of Agriculture, Ames, Iowa, USA

Differentiation of *Campylobacter fetus* into *C. fetus* subsp. *fetus* (Cff) and *C. fetus* subsp. *venerealis* (Cfv) is important for both clinical and economic reasons. In the past, several molecular typing methods have been used for differentiation, including amplified fragment length polymorphism (AFLP). In this study, AFLP was employed to identify *C. fetus* subspecies specific markers that can serve as a basis for design of novel PCR primer sets for Cfv. Four groups of *C. fetus* strains with different phenotypic or genotypic traits were examined by AFLP using 22 different *Dde*I/*Mbo*I primer combinations. Specific AFLP fragments were deduced and sequenced resulting in 41 sequences. Based on the obtained sequences, five potential subspecies-specific PCR assays were developed. Extensive evaluation of the five selected PCRs with a set of 65 diverse *C. fetus* strains identified primer set Cf C05 as subspecies Cfv-specific. This newly developed PCR is fully consistent with the AFLP subspecies differentiation results. The data indicate AFLP as a powerful tool for comparing closely related genomes and for exploiting this information to develop a specific PCR with extensive typing potential.

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INTRODUCTION

The species *Campylobacter fetus*, a micro-aerophilic, Gram-negative, curve-shaped bacterium, can be classified into two subspecies, *C. fetus* subsp. *venerealis* (Cfv) and *C. fetus* subsp. *fetus* (Cff) (Véron & Chatelain, 1973). Cfv causes bovine genital campylobacteriosis (Florent, 1959; Véron & Chatelain, 1973) and is considered a host-restricted pathogen. Its presence in the genital tract of cattle is associated with abortion and infertility, and it is therefore of economic importance. Cff is neither host- nor habitat-restricted; several animal species can carry Cff in the intestinal tract (Florent, 1959; Véron & Chatelain, 1973). Cff is associated with abortion in sheep, but generally causes less severe clinical symptoms in cattle compared to Cfv.

As some countries have eradicated Cfv from their cattle population, discrimination of both subspecies of *C. fetus* is crucial for veterinary control programmes. Subspecies differentiation is also of economic importance since Cfv-negative status of the animals is required by law for both the artificial insemination and embryo transfer industries (Anonymous, 2000; Wagenaar & Van Bergen, 2004).

The phenotypic test prescribed by the World Organisation for Animal Health (OIE) to differentiate the two *C. fetus* subspecies is the glycine-tolerance test (Wagenaar & Van Bergen, 2004). This test is based on the growth of Cff but not

†Present address: PathoFinder B.V., Canisius Wilhelmina Ziekenhuis, Nijmegen, The Netherlands.

Abbreviations: AFLP, amplified fragment length polymorphism; Cff, *Campylobacter fetus* subsp. *fetus*; Cfv, *Campylobacter fetus* subsp. *venerealis*.

The GenBank accession number for the target gene sequence of PCR Cf C05 is DQ146479.

Cfv in 1 % glycine. However, the reproducibility of the assay is poor and the test can give ambiguous results (Harvey & Greenwood, 1983; On, 1996; On & Harrington, 2001). Other phenotypic tests, such as selenite reduction and antibiotic susceptibility, are only indicative and do not completely discriminate between the subspecies (On, 1996; Vandamme, 2000). Several molecular typing methods have been applied to discriminate the two subspecies, including PFGE (On & Harrington, 2001), amplified fragment length polymorphism (AFLP) (Wagenaar *et al.*, 2001) and PCR (Hum *et al.*, 1997; Wang *et al.*, 2002). While numerical analysis of PFGE-DNA profiles is a valuable tool for differentiating *C. fetus* subspecies, it is time-consuming, difficult to interpret and therefore impractical for routine use. Similarly, the AFLP method is useful for differentiation of *C. fetus* subspecies (Wagenaar *et al.*, 2001) but it is laborious, time-consuming and not available in every diagnostic laboratory. PCR is fast and easily applicable, but the currently available assays have been reported to give erroneous results (Hum *et al.*, 1997; Wagenaar *et al.*, 2001) or have only been tested for a very limited set of *C. fetus* strains (Wang *et al.*, 2002). In the search for a robust, reliable and easy-to-use diagnostic PCR, we used the novel approach of DNA sequencing of *C. fetus* subspecies specific markers identified by AFLP to develop a PCR-based discriminative assay.

METHODS

Strains, DNA and typing methods. Sixty-five *C. fetus* strains (Table 1), comprising 62 field isolates and three reference strains, were grown under microaerobic conditions (6 % O_2, 7 % CO_2, 7 % H_2, 80 % N_2; Anoxomat, Mart Microbiology) on heart-infusion agar supplemented with 5 % sheep blood (Biotrading) for 3 days. All strains were typed by the subspecies-specific PCRs developed by Hum *et al.* (1997) and Wang *et al.* (2002) and by the *Hin*dIII/*Hha*I AFLP as previously described by Wagenaar *et al.* (2001). Strains used for the selection pools 1–4 (Table 1) were also tested for the ability to tolerate 1 % glycine (Table 2) (Wagenaar *et al.*, 2001).

Chromosomal DNA of all strains was isolated with the Puregene kit (Gentra systems; BIOzym) and diluted to a final concentration of 40–50 ng μl^{-1} for each pool. For PCR on individual strains, the DNA of each strain was diluted to a concentration of 1–10 ng μl^{-1}. PCR was also performed on cruder samples, i.e. on DNA prepared by 20 % Chelex-100 extraction (Engberg *et al.*, 2000), and on cell suspensions (OD_{600} = 0·1 in SuperQ water).

Group selection and *Mbo*I/*Dde*I AFLP. Based on results of AFLP genotyping and biochemical properties of the strains, four groups were identified (Table 2): group 1, Cff; group 2, Cff that differed from the first group in one *Hin*dIII/*Hha*I AFLP fragment; group 3, Cfv; and group 4, Cff strains that were biochemically aberrant compared to groups 1 and 2 [no growth in the glycine test indicating Cfv, whereas PCR (Hum *et al.*, 1997) and AFLP (Wagenaar *et al.*, 2001) indicated Cff]. From each identified group, two to four strains were selected from which DNA was pooled (indicated with pool number in Table 1). Each of the DNA pools was used as template in the AFLP analysis with restriction enzyme combination *Mbo*I/*Dde*I.

Identification of *C. fetus* subspecies specific markers. Pooled DNA of the four *C. fetus* groups was examined by AFLP using the restriction enzyme combination *Mbo*I/*Dde*I. This enzyme combination was chosen based on the AFLPinSilico band pattern for the sequenced *Campylobacter jejuni* NCTC strain 11168 using the REcomb program (Reijans *et al.*, 2003). AFLPinSilico mimics AFLP experiments and produces virtual fingerprints that enable the identification of fragments based on their length and the choice of selective nucleotides (Rombauts *et al.*, 2003). Since the genome of *C. fetus* is not in the public domain, no information about suitable restriction enzymes for AFLP of *C. fetus* was available when this study was conducted. As *C. jejuni* is its nearest genome-sequenced relative, AFLPinSilico was done using this strain.

AFLP was performed essentially as described by Vos *et al.* (1995) and Van den Braak *et al.* (2004). Briefly, chromosomal DNA of each group was digested with restriction enzymes *Mbo*I and *Dde*I (Roche) and specific *Mbo*I and *Dde*I adaptors were ligated (*Mbo*I: forward 5′-CTCGTAGACTGCGTACC-3′, reverse 5′-GATCGGTACGCAGTC TAC-3′; and *Dde*I: forward 5′-GACGATGAGTCCTGAG-3′, reverse 5′-TNACTCAGGACTCAT-3′). Pre-amplification was performed as described by Vos *et al.* (1995), using primers without selective nucleotides. In the following selective amplification, samples were amplified using a ^{33}P-labelled *Mbo*I primer containing one selective nucleotide, and a *Dde*I primer containing two selective nucleotides. In total, 22 different primer combinations were tested. Amplicons were run on 5 % denaturing polyacrylamide gels and fingerprints were visualized by phosphor imaging and screened automatically using AFLP QuantarPro software (Keygene Products). Fragments only commonly present in groups 1, 2 and 4 and absent in group 3 were potentially specific for Cff. Fragments only present in group 3 and absent in the other groups were potentially specific for Cfv. These candidate fragments were excised and re-amplified using AFLP primers without selective nucleotides. Successfully amplified products were sequenced on a capillary sequencer and analysed using the Basic Local Alignment Search Tool (BLASTX and BLASTN search) (http://www.ncbi.nlm.nih.gov/blast).

Development of subspecies-specific PCRs. For all 41 properly sequenced fragments, primer sets were developed to enable specific amplification of the fragments. Each set of primers was examined for its subspecies specificity by pre-screening the set with DNA from a well-defined panel of seven Cff and seven Cfv strains, indicated in Table 1 by † (Cff) and ‖ (Cfv). Primer sets yielding a subspecies-specific fragment were used with DNA of all strains shown in Table 1. PCR was performed using the following conditions. Each 50 µl of reaction mixture contained 32·7 µl SuperQ water, 5 µl 10× PCR buffer II (Applied Biosystems), 3 µl 25 mM $MgCl_2$ (Applied Biosystems), 0·3 µl 5 U μl^{-1} *Taq* polymerase (Applied Biosystems), 5 µl 2 mM dNTP (Amersham Biosciences), 1 µl 25 pmol μl^{-1} forward primer (Invitrogen, Life-Technologies), 1 µl 25 pmol μl^{-1} reverse primer and 2 µl chromosomal DNA solution (1–10 ng μl^{-1}). The PCR using primer set Cf C05 (forward, 5′-ATGATAAGATATATTTGTATCAG-3′; reverse, 5′-GAT GAAGAATATTACAAGATAAT-3′) was performed as described above, except that 0·25 µl 5 U μl^{-1} SuperTaq polymerase (HT Biotechnology) and the supplied 10× buffer, including $MgCl_2$, were used. All PCR reactions were performed in a Perkin Elmer 9700 thermocycler using the following conditions: 5 min incubation at 95 °C, 30 cycles of 1 min at 94 °C, 1 min at the calculated annealing temperature, and 2 min at 72 °C. Reactions were completed by extension at 72 °C for 10 min. The five selected PCRs (Cf B01, Cf B03, Cf B06, Cf C02 and Cf C05) with diagnostic potential were tested at both higher (+1 °C to +5 °C) and lower (−1 °C to −5 °C) annealing temperatures to assess the effect of temperature on the specificity of the PCR, and to reduce background. Higher annealing temperatures showing less background and identifying the same strains to be positive as the calculated annealing temperatures were selected. PCR Cf C05 had an optimum annealing temperature at 54 °C. All PCRs using the selected primer sets were performed in duplicate. PCRs that proved useful on chromosomal DNA were tested for their specificity on DNA extracted using the Chelex method and on cell suspensions. All PCR products exceeding 100 bp

Table 1. Comparison of subspecies differentiation results of AFLP and PCRs

F, Identified as *Campylobacter fetus* subsp. *fetus*; V, identified as *Campylobacter fetus* subsp. *venerealis*; –, no specific PCR product. T, type strain.

Strain	AFLP	PCR			Source	Country	Supplier
		Hum *et al.* (1997)	Wang *et al.* (2002)	Cf C05			
378/5	F	F	F	–	Bovine	Belgium	J. Godfroid, Veterinary and Agrochemical Research Centre, Brussels
4114	F	V	F	–	Human	Belgium	J. P. Butzler, University of Brussels
F128	F	F	F	–	Human	Belgium	J. P. Butzler, University of Brussels
L249093	F	F	–	–	Human	Spain	P. Idigoras, Hospital Donostia, San Sebastián
ATCC 27374T*†	F	F	F	–	Ovine	France	
F8135	F	F	F	–	Human	Germany	W. Kalka-Moll, University of Cologne
m*†	F	F	F	–	Bovine	Netherlands	Animal Sciences Group, Lelystad
110800 1-2	F	V	–	–	Bovine	Netherlands	Animal Sciences Group, Lelystad
938230	F	F	F	–	Bovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
5,5,42	F	F	–	–	Ovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
501340	F	F	F	–	Bovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
601	F	F	–	–	Ovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
Ru-13826	F	F	F	–	Bovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
Ru-17722-2 small	F	F	–	–	Bovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
Ru-9516-18	F	F	F	–	Bovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
Sz-1074	F	F	F	–	Ovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
0111-23289	F	V	F	–	Human	Netherlands	W. Ang, Erasmus Medical Center Rotterdam
10†‡	F	F	F	–	?	South Africa	M. Hinton, Onderstepoort Veterinary Institute
135‡	F	F	F	–	?	South Africa	M. Hinton, Onderstepoort Veterinary Institute
Cfv-za‡	F	F	F	–	?	South Africa	M. Hinton, Onderstepoort Veterinary Institute
8	F	F	F	–	?	South Africa	M. Hinton, Onderstepoort Veterinary Institute
136	F	F	–	–	?	South Africa	M. Hinton, Onderstepoort Veterinary Institute
248,1	F	F	F	–	?	South Africa	M. Hinton, Onderstepoort Veterinary Institute
5396/7	F	F	F	–	?	South Africa	M. Hinton, Onderstepoort Veterinary Institute
Zaf1	F	F	F	–	Bovine	South Africa	M. Hinton, Onderstepoort Veterinary Institute
122*†	F	F	–	–	Ovine	Turkey	L. Guler, Veteriner Kontrol ve Araştirma Enstitüsü, Konyn
68	F	F	F	–	Ovine	Turkey	L. Guler, Veteriner Kontrol ve Araştirma Enstitüsü, Konyn
74	F	F	–	–	Ovine	Turkey	L. Guler, Veteriner Kontrol ve Araştirma Enstitüsü, Konyn
V51/99†§	F	F	–	–	Bovine	UK	D. Newell & M. Toszeghy, Veterinary Laboratories Agency, Weybridge
98/v444†§	F	F	–	–	Bovine	UK	D. Newell & M. Toszeghy, Veterinary Laboratories Agency, Weybridge
XV-98*†	F	F	–	–	Ovine	UK	D. Newell & M. Toszeghy, Veterinary Laboratories Agency, Weybridge
0194-98	F	F	–	–	Ovine	UK	D. Newell & M. Toszeghy, Veterinary Laboratories Agency, Weybridge
98/v156	F	F	–	–	Bovine	UK	D. Newell & M. Toszeghy, Veterinary Laboratories Agency, Weybridge
98/v445	F	V	F	–	Bovine	UK	D. Newell & M. Toszeghy, Veterinary Laboratories Agency, Weybridge
DC 20C	F	F	F	–	Bovine	UK	J. Corry, University of Bristol
C036271	F	F	F	–	Human	UK	J. Frost, Health Protection Agency, London
C036959	F	F	–	–	Human	UK	J. Frost, Health Protection Agency, London
3051	F	F	F	–	Bovine	USA	I. Wesley, National Animal Disease Center, Ames

cont.

Table 1. cont.

Strain	AFLP	PCR			Source	Country	Supplier
		Hum *et al.* (1997)	Wang *et al.* (2002)	Cf C05			
3290	F	F	F	–	Ovine	USA	I. Wesley, National Animal Disease Center, Ames
3293	F	F	–	–	Bovine	USA	I. Wesley, National Animal Disease Center, Ames
3754	F	F	F	–	Human	USA	I. Wesley, National Animal Disease Center, Ames
6877	F	V	F	–	Bovine	USA	I. Wesley, National Animal Disease Center, Ames
3286 (small)	F	F	F	–	Ovine	USA	I. Wesley, National Animal Disease Center, Ames
3292	F	F	F	–	Bovine	USA	I. Wesley, National Animal Disease Center, Ames
87-364	F	F	F	–	Equine	USA	L. Schroeder-Tucker, National Veterinary Services Laboratories, Ames
87-72	F	V	F	–	Bovine	USA	L. Schroeder-Tucker, National Veterinary Services Laboratories, Ames
87-742-1	F	V	F	–	Bovine	USA	L. Schroeder-Tucker, National Veterinary Services Laboratories, Ames
97-365-1	F	F	F	–	Bovine	USA	L. Schroeder-Tucker, National Veterinary Services Laboratories, Ames
82-40	F	F	F	–	Human	USA	M. Blaser & Z. Tu, University of New York
84-104	F	F	F	–	Monkey	USA	M. Blaser & Z. Tu, University of New York
84-91	F	F	F	–	Human	USA	M. Blaser & Z. Tu, University of New York
D223	F	F	F	–	Human	USA	P. Fields, Centers for Disease Control and Prevention, Atlanta
D5605	F	F	F	–	Human	USA	P. Fields, Centers for Disease Control and Prevention, Atlanta
LMG 6570	V	V	–	V	Bovine	Belgium	
511‖	V	V	F	V	Bovine	Hungary	J. Varga, Faculty of Veterinary Science, Budapest
18156‖	V	V	–	V	Bovine	Netherlands	Animal Sciences Group, Lelystad
5.5.21‖¶	V	V	–	V	Bovine	Netherlands	M. Koene & I. Visser, Animal Health Service, Deventer
ATCC 19438^{T} ‖¶	V	V	–	V	Bovine	UK	
97/v549‖¶	V	V	–	V	Bovine	UK	D. Newell & M. Toszeghy, Veterinary Laboratories Agency, Weybridge
3288	V	V	–	V	Bovine	USA	I. Wesley, National Animal Disease Center, Ames
3295	V	V	–	V	Human	USA	I. Wesley, National Animal Disease Center, Ames
89-083	V	V	–	V	Bovine	USA	L. Schroeder-Tucker, National Veterinary Services Laboratories, Ames
90-152	V	F	–	V	Bovine	USA	L. Schroeder-Tucker, National Veterinary Services Laboratories, Ames
84-112‖	V	V	–	V	Bovine	USA	M. Blaser & Z. Tu, University of New York
99-257‖	V	V	–	V	Human	USA	M. Blaser & Z. Tu, University of New York

*Cff DNA used for pool 2.

†Cff DNA used for pre-screening of the primer sets.

‡Cff DNA used for pool 4.

§Cff DNA used for pool 1.

‖Cfv DNA used for pre-screening of the primer sets.

¶Cfv DNA used for pool 3.

Table 2. The four identified groups of *C. fetus* strains used for *Dde*I/*Mbo*I AFLP analysis

Group	Properties	Biochemical (1 % glycine-tolerance test)	PCR (Hum *et al.*, 1997)	AFLP (Wagenaar *et al.*, 2001)
1	Cff	Cff	Cff	Cff
2	Cff, differing from pool 1 in one *Hin*dIII/*Hha*I fragment	Cff	Cff	Cff
3	Cfv	Cfv	Cfv	Cfv
4	Cff, differing from pool 1 by being negative in the glycine assay	Cfv	Cff	Cff

were run on a 1·5 % agarose gel and PCR products smaller than 100 bp were run on a 3 % agarose gel. PCR Cf C05 revealed a PCR product of 54 bp. PCR products were visualized by ethidium bromide staining.

RESULTS

Identification of *C. fetus* subspecies specific markers

Analysis of the *C. fetus* subspecies specific region in the *Hin*dIII/*Hha*I AFLP (Wagenaar *et al.*, 2001) revealed three distinct genotype groups. In a search for subspecies-specific markers, these groups and a fourth group that showed an aberrant reaction in the glycine test were subjected to AFLP with 22 different *Mbo*I/*Dde*I primer combinations. This yielded 1161 fragments that were common to all four groups, and 274 fragments (markers) that were specific for one of each of the four groups. Thirteen markers appeared to be shared by groups 1, 2 and 4, and were considered Cff-specific (Table 3). Fifty-nine Cfv-specific AFLP markers were typical for group 3 (Table 3). All 72 (13 Cff + 59 Cfv) identified markers were excised from the AFLP gel, re-amplified by PCR, and sequenced. This yielded 41 (5 Cff + 36 Cfv) out of 72 potential sequences.

Development of subspecies-specific PCRs

To assess the use of the identified sequences for subspecies identification, PCR primer sets were designed for all 41 (5 Cff + 36 Cfv) sequences obtained and tested on a subset of *C. fetus* strains (Table 1, strains indicated by † or ‖). From the five selected Cff primer combinations, none were specific for Cff strains (Table 3). Yet, five of 41 primer combinations (Cf B01, Cf B03, Cf B06, Cf C02 and Cf C05) yielded reproducible PCR products for the specific identification of Cfv (Table 3). The diagnostic potential of these five Cfv-specific PCRs was further assessed with a set of 65 *C. fetus* strains, including three reference strains and 62 very diverse field isolates (Table 1). Strains originating from the same outbreaks displayed the same outcome for all individual PCRs (data not shown).

Comparison of the newly developed diagnostic PCRs with existing methods

To position the novel diagnostic PCR with existing AFLP and PCR assays, 65 strains were analysed with the different typing methods. In those assays, 24 out of 65 strains yielded a different classification in the various tests (Table 1). Reference strains ATCC 27374^T (= NCTC 10842^T), ATCC 19438^T (= NCTC 10354^T) and LMG 6570 (= CCUG 7477) gave similar results in all assays. With the other strains the results of the Cff-specific PCR described by Wang *et al.* (2002) gave the most variable results compared to all other tests. For this reason, this PCR method was not used further in the comparative study.

Further comparison of the other assays showed that the results of the newly developed primer sets Cf B01, Cf B03, Cf C02 and Cf B06 approached either those of the PCR described by Hum *et al.* (1997) or those of the AFLP. Primer sets Cf B01, Cf B03, Cf C02 and Cf B06 showed, respectively, two, four, three and nine discrepancies with the results of the PCR assay of Hum *et al.* (1997), and, respectively, eight, six, seven and one differences compared to the *Hin*dIII/*Hha*I AFLP. However, the results with the primer set Cf C05 were fully consistent (65/65) with AFLP, while eight of these strains yielded different results with the PCR of Hum *et al.* (1997).

As the PCRs with the primer set Cf C05 appeared to better reflect AFLP results, the use of this PCR was assessed with *C. fetus* target DNA extracted by Chelex and by cell suspensions

Table 3. Subspecies designation results based on AFLP identified specific markers

Specific for:	Specific for pool:	No. of unique markers	No. of sequences obtained	No. of potential specific PCRs	No. of specific PCRs that correlate with AFLP
Cff	1, 2, 4	13	5	0	0
Cfv	3	59	36	5	1
Total		72	41	5	1

rather than purified chromosomal DNA. All three DNA isolation methods yielded the same outcome for all individual strains resulting in acceptable PCR products (data not shown).

Sequence analysis of the subspecies-specific AFLP fragments

To gain more information about the nature of subspecies-specific genetic markers, the respective fragments were further analysed. All fragments amplified by primer sets Cf B01, Cf B03, Cf B06, Cf C02 and Cf C05 appeared to carry an open reading frame. BLAST analysis using publicly available databases, however, yielded no similarities with sequences present in the database. Furthermore, alignment of the DNA sequences of all five PCR products revealed no sequence similarities among the various sequences or with the Cfv-specific fragment amplified in the PCR by Hum *et al.* (1997). As the PCR Cf C05 was shown to be useful for differentiation, the target gene sequence was submitted to GenBank and given the accession number DQ146479.

DISCUSSION

AFLP fingerprinting indicates that the genetic diversity of *C. fetus* is very limited. This is in contrast with other *Campylobacter* species, including *C. jejuni*, *Campylobacter coli*, *Campylobacter lari*, *Campylobacter upsaliensis* and its closest taxonomic relative *Campylobacter hyointestinalis* (Roop *et al.*, 1984), which show more diversity in AFLP patterns within the species (Duim *et al.*, 2001). The close relatedness of *C. fetus* is also apparent from the similar 16S rRNA gene sequences in the different subspecies (On & Harrington, 2001; Wesley *et al.*, 1991), and the limited phenotype diversity. Despite these limited differences, initially classification into the two subspecies was done based on clinical presentation and the 1 % glycine-tolerance test; Cfv is venereally transmitted and causes infertility exclusively in cattle, whereas Cff is orally transmitted, inducing abortion in sheep and cattle, and rarely septicaemia in humans (Garcia *et al.*, 1983; Wesley *et al.*, 1991).

At this time, only one assay is accepted by the OIE to discriminate between the two subspecies. Unfortunately, the reproducibility of this assay is poor. This is at least partially due to the sometimes slow and poor growth of *C. fetus* isolates. Whether this poor reproducibility reflects genetic differences is unclear and may await unravelling of the molecular basis underlying the glycine-tolerance test. An additional problem with the glycine-tolerance test is that this phenotype can be transferred between strains by phage-mediated transduction (Chang & Ogg, 1971). This makes the assays less attractive for subspecies differentiation purposes.

Reliable discrimination of the *C. fetus* subspecies is of veterinary importance as eradication programmes have been put in place in several countries. Due to the serious reproductive problems caused by Cfv, countries must report the presence of the causative agent of bovine genital campylobacteriosis (BGC) once diagnosed. Eradication programmes are fully dependent on the correct identification of the subspecies. More robust and easy-to-use discriminatory assays would facilitate the control of BGC. Several molecular tests have been evaluated as alternatives for the glycine test (Hum *et al.*, 1997; On & Harrington, 2001; Wagenaar *et al.*, 2001; Wang *et al.*, 2002). Subspecies-specific PCRs have been described (Hum *et al.*, 1997; Wang *et al.*, 2002) but have been found to give erroneous results (Hum *et al.*, 1997; Wagenaar *et al.*, 2001). Development of new, more reliable, easy-to-perform tests is indispensable, and a multiplex PCR identifying a specific fragment for each subspecies would be a great asset. Due to the lack of the entire genome sequence of both subspecies, a genomic subtraction technique (Schober *et al.*, 2001) which has been useful for identification of differences between strains (Winstanley, 2002) may be a valuable tool to detect genetic differences. In the present study, we used the power of the AFLP technique to identify differences among closely related genomes (Van den Braak *et al.*, 2004) to successfully design a rapid PCR method for differentiation of *C. fetus* subspecies.

The first step towards the identification of subspecies-unique DNA fragments was the use of the appropriate restriction enzyme combinations in the AFLP. *Mbo*I/*Dde*I AFLP yielded 72 specific markers, including 59 for Cfv and 13 for Cff. The lower number of identified Cff-specific markers was probably due to the fact that the Cff-specific fragments must be shared by three different Cff groups, compared to one group for Cfv. AFLP differences may arise from large or minor genetic differences as well as from single point-mutations, which thus may cause false results. Although diagnostic PCR assays based on single nucleotide differences have been described (Misawa *et al.*, 1998), larger sequence differences are likely to give more reliable results. For 41 of the 72 DNA fragments sequence information was obtained each of which had the potential to serve as a basis for development of subspecies-specific PCRs.

A rather low yield of subspecies-specific markers compared to the observed differences in *Mbo*I/*Dde*I AFLP was apparent. From the 41 PCR primer sets that were designed on the basis of the obtained specific sequences only five showed subspecies specificity towards the seven Cff and seven Cfv screening strains. This rather low yield may be explained by heterogeneity in the targeted sequence in different isolates. Furthermore, for each sequence only one primer set was developed. As a result of unsuitable primer sets, initially promising sequences might have been discarded, presumably reducing the number of final potential candidates. Another reason that these primer sets appeared nonspecific could be assigned to weak fragments, which were regarded negative in the *Mbo*I/*Dde*I AFLP, resulting in selection of false candidate markers. A further reduction from the five potential primer sets Cf B01, Cf B03, Cf B06, Cf C02 and Cf C05 to the one Cfv-specific primer set Cf C05 was obtained when evaluation of the primer sets used a much larger set of different strains than that screened in the initial selection of specific fragments.

A key part of our study was the evaluation of the usefulness of novel PCRs in the classification of a large number of field isolates. This analysis is complex, as the existing methods to distinguish Cfv and Cff, such as the glycine-tolerance test, are not optimal. Comparison of the results of AFLP, previously described PCR methods (Hum *et al.*, 1997; Wang *et al.*, 2002), and the newly developed PCRs yielded similar results when reference strains were used. With the diverse set of field strains, however, differences between the methods became apparent, indicating considerable strain diversity for the genetic markers used in the various assays. Yet, in our hands, the PCR with the primer combination Cf C05 yielded identical subspecies differentiation to the AFLP, which is considered a very discriminatory technique. Positive results were obtained with chromosomal DNA as well as with crude extracts, indicating the robustness of the assay. These data indicate that the Cf C05-based PCR is an important asset in *C. fetus* subspecies differentiation. For future diagnostics, a combination of an existing species-specific PCR and the Cf C05 PCR for subspecies discrimination may be optimal for *C. fetus* diagnostics.

The identification of subspecies-specific DNA fragments may provide important information about differences in virulence properties. We found no similarities for the obtained subspecies-specific sequences with proteins in the databases. Apparently, the sequences on which the developed PCRs were based have not previously been reported or identified in other bacteria. This could indicate that the new Cfv-specific PCR Cf C05 can be used for development of a diagnostic assay in which samples can be tested in a relatively high background of other bacteria.

New Cff-specific markers remained unidentified, and erroneous results were obtained with the PCR described by Wang *et al.* (2002). Therefore, attempts to characterize new Cff-specific markers by this or other molecular methods seem worthwhile. In this context it should be noted that only 22 out of the possible 64 (+1, +2) primer combinations have been used in the AFLP screening. In addition, other restriction enzyme combinations might yield a larger pool of putative specific markers to be tested.

Finally, it should be noted that our successful approach of using AFLP to identify specific markers adds a new dimension to this technique that may enhance the identification of future diagnostic targets as well as virulence genes.

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REFERENCES

Anonymous (2000). Terrestrial Animal Health Code. http://www.oie.int. Edited by the Office International des Epizooties. Paris: Office International des Epizooties.

Chang, W. & Ogg, J. E. (1971). Transduction and mutation to glycine tolerance in *Vibrio fetus*. *Am J Vet Res* **32**, 649–653.

Duim, B., Vandamme, P. A., Rigter, A., Laevens, S., Dijkstra, J. R. & Wagenaar, J. A. (2001). Differentiation of *Campylobacter* species by AFLP fingerprinting. *Microbiology* **147**, 2729–2737.

Engberg, J., On, S. L., Harrington, C. S. & Gerner-Smidt, P. (2000). Prevalence of *Campylobacter*, *Arcobacter*, *Helicobacter*, *and Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for Campylobacters. *J Clin Microbiol* **38**, 286–291.

Florent, A. (1959). Les deux vibriosis génitales: la vibriose due à *V. fetus venerealis* et la vibriose d'origine intestinale due à *V. fetus intestinalis*. *Meded Veeartsschool Gent* **3**, 1–60.

Garcia, M. M., Eaglesome, M. D. & Rigby, C. (1983). Campylobacters important in veterinary medicine. *Vet Bull* **53**, 793–818.

Harvey, S. & Greenwood, J. R. (1983). Relationships among catalase-positive Campylobacters determined by deoxyribonucleic acid-deoxyribonucleic acid hybridization. *Int J Syst Bacteriol* **33**, 275–284.

Hum, S., Quinn, K., Brunner, J. & On, S. L. W. (1997). Evaluation of a PCR assay for identification and differentiation of *Campylobacter fetus* subspecies. *Aust Vet J* **75**, 827–831.

Misawa, N., Allos, B. M. & Blaser, M. J. (1998). Differentiation of *Campylobacter jejuni* serotype O19 strains from non-O19 strains by PCR. *J Clin Microbiol* **36**, 3567–3573.

On, S. L. W. (1996). Identification methods for *Campylobacters*, *Helicobacters* and related organisms. *Clin Microbiol Rev* **9**, 405–422.

On, S. L. W. & Harrington, C. S. (2001). Evaluation of numerical analysis of PFGE-DNA profiles for differentiating *Campylobacter fetus* subspecies by comparison with phenotypic, PCR and 16S rDNA sequencing methods. *J Appl Microbiol* **90**, 285–293.

Reijans, M., Lascaris, R., Oude Groeneger, A. & 9 other authors (2003). Quantitative comparison of cDNA-AFLP, microarrays and GeneChip expression data in *Saccharomyces cerevisiae*. *Genomics* **82**, 606–618.

Rombauts, S., Van de Peer, Y. & Rouzé, P. (2003). AFLPinSilico, simulating AFLP fingerprints. *Bioinformatics* **19**, 776–777.

Roop, R. M., II, Smibert, R. M., Johnson, J. L. & Krieg, N. R. (1984). Differential characteristics of catalase-positive *Campylobacters* correlated with DNA homology groups. *Can J Microbiol* **30**, 938–951.

Schober, C., Gorkiewicz, G. & Zechner, R. (2001). Identification of differential DNA fragments associated with the pathogenicity of *Campylobacter fetus* species by subtractive hybridization. *Int J Med Microbiol* **291** (Suppl. 13), 122–123.

Vandamme, P. (2000). Taxonomy of the family *Campylobacteraceae*. In *Campylobacter*, pp. 3–26. Edited by I. Nachamkin & M. J. Blaser. Washington, DC. American Society for Microbiology.

Van den Braak, N., Simons, G., Gorkink, R. & 7 other authors (2004). A new high-throughput AFLP approach for identification of new genetic polymorphism in the genome of the clonal microorganism *Mycobacterium tuberculosis*. *J Microbiol Methods* **56**, 49–62.

Véron, M. & Chatelain, R. (1973). Taxonomic study of the genus *Campylobacter* Sebald and Véron and designation of the neotype strain for the type species, *Campylobacter fetus* (Smith and Taylor) Sebald and Véron. *Int J Syst Bacteriol* **23**, 122–134.

Vos, P., Hogers, R., Bleeker, M. & 8 other authors (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407–4414.

Wagenaar, J. A. & Van Bergen, M. A. P. (2004). Bovine genital campylobacteriosis. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, pp. 439–450. Edited by the Office International des Epizooties. Paris: Office International des Epizooties.

Wagenaar, J. A., Van Bergen, M. A. P., Newell, D. G., Grogono-Thomas, R. & Duim, B. (2001). Comparative study using amplified fragment

length polymorphism fingerprinting, PCR genotyping, and phenotyping to differentiate *Campylobacter fetus* strains isolated from animals. *J Clin Microbiol* **39**, 2283–2286.

Wang, G., Clark, C. G., Taylor, T. M., Pucknell, C., Barton, C., Price, L., Woodward, D. L. & Rodgers, F. G. (2002). Colony multiplex PCR assay for identification and differentiation of *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus* subsp. *fetus*. *J Clin Microbiol* **40**, 4744–4747.

Wesley, I. V., Wesley, R. D., Cardella, M., Dewhirst, F. E. & Paster, B. J. (1991). Oligodeoxynucleotide probes for *Campylobacter fetus* and *Campylobacter hyointestinalis* based on 16S rRNA sequences. *J Clin Microbiol* **29**, 1812–1817.

Winstanley, C. (2002). Spot the difference: applications of subtractive hybridisation to the study of bacterial pathogens. *J Med Microbiol* **51**, 459–467.