Differentiation of the Subspecies of *Campylobacter fetus* by Genomic Sizing

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Campylobacter fetus subsp. *fetus* and *C. fetus* subsp. *venerealis* are currently differentiated by tolerance to glycine and by their epidemiology. Analysis of *C. fetus* DNA by pulsed-field gel electrophoresis, after digestion with the restriction endonucleases *Sma*I and *Sal*I, was used to differentiate between the subspecies. All strains presently identified as *C. fetus* subsp. *fetus* had a genomic size of 1.1 Mb, whereas the majority of the *C. fetus* subsp. *venerealis* strains had a genomic size of 1.3 Mb. An additional group of strains, which were previously described as *C. fetus* subsp. *venerealis* biovar "intermedius" and were able to tolerate higher concentrations of glycine than the rest of the *C. fetus* subsp. *venerealis* strains, had an average genome size of 1.5 Mb. We suggest that pulsed-field gel electrophoresis may be useful as an additional aid in the differentiation of *C. fetus* strains at the subspecies level.

Strains of *Campylobacter fetus* subsp. *fetus* are associated with abortion in sheep and cattle (23-26). Human infections, which include bacteremia and other systemic infections, especially in immunocompromised patients, have been reported previously (4). The virulence of *C. fetus* subsp. *fetus* has been attributed to the presence of an S layer, composed of a protein with a subunit molecular mass of 97 to 149 kDa (5, 18, 30). In contrast, strains of *C. fetus* subsp. *venerealis* have adapted to the bovine genital tract (bovine vibriosis) (23) and are of major concern to the cattle industry due to their ability to cause infertility of cows inseminated with contaminated bull semen (21). Human infections due to *C. fetus* subsp. *venerealis* have not been reported (14). Attempts to differentiate between the two subspecies on the basis of fluorescent-antibody assay (17), fatty acid contents (6), and DNA-DNA homology studies (3) have not been successful. Moreover, the ability of the strains to produce H₂S or to grow on media containing sodium selenite has also been examined, but the results were difficult to interpret (29). The degree of tolerance to glycine and the epidemiology of the organisms are the two main methods currently available for differentiation.

The aim of this study was to assess the use of genomic sizing with the aid of pulsed-field gel electrophoresis (PFGE) to differentiate strains of *C. fetus* at the subspecies level.

MATERIALS AND METHODS

Culture method for *C. fetus*. The sources and characteristics of the *C. fetus* strains are shown in Table 1. Cultures were stored in 20% glycerol (BDH, Toronto, Canada) in peptone broth (Oxoid Ltd., Basingstoke, United Kingdom) at -70°C. When required, the culture broths were thawed and streaked on plates of brain heart infusion agar (Oxoid Ltd.) containing 5% whole defibrinated horse blood (Gibmar Laboratories, Edmonton, Canada). The plates were incubated under microaerobic conditions (10% CO₂, 10% H₂, 80% N₂) at 37°C for up to 72 h. Although all strains have been identified by their original suppliers, their ability to grow at 25°C, microscopic morphology, and oxidase, cata-

lase, and nitrate activities were retested immediately prior to this study. The ability of the organisms to tolerate glycine was tested according to Florent's method (11). Briefly, 0.1 ml of the bacterial cultures, adjusted to a McFarland no. 1 turbidity, were inoculated into fluid Albimi broth containing 0.6, 1, 1.3, and 1.9% glycine, and readings were made after 2 to 3 days of incubation. The final identifications of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* strains were confirmed by investigation of the type strains ATCC 27374 (UA60) and ATCC 19438 (UA811), respectively.

Preparation of DNA inserts. DNA inserts were prepared as described by Chang and Taylor (7).

Restriction endonuclease digestion. Each DNA insert was washed for 20 min in 10 mM Tris-EDTA (BDH) containing 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Mo.). This step was carried out at least three times and was followed by washing three times in 10 mM Tris-EDTA only. Before enzyme digestion, the Tris-EDTA was removed and replaced by 100 µl of the appropriate digestion buffer and the insert was incubated at room temperature for 30 min, after which the buffer was replaced with 100 µl of fresh enzyme buffer and 50 to 70 U of the appropriate enzyme (*Sma*I or *Sal*I [Boehringer GmbH, Mannheim, Germany]) (7) was added. The reaction mixture was incubated at 37°C for 18 to 24 h. After incubation, the DNA inserts were washed in Tris-EDTA buffer and loaded into 1% agarose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) gel wells, which were then sealed with 1% agarose in gel buffer.

PFGE. The contour-clamped homogeneous electric field (CHEF) system (LKB Instruments Inc., Bromma, Sweden) of PFGE was used to separate the DNA fragments. Gels were subjected to electrophoresis for 24 h at 8°C with a voltage of 175 V and pulse times of 15 to 25 s in 0.05 M Tris-borate-EDTA buffer. Following electrophoresis, the gels were stained with ethidium bromide and the DNA fragments were visualized with a UV transilluminator. The sizes of the DNA fragments were determined by measuring distances of band migration compared with the DNA standards of a λΔ39 DNA ladder (Promega, Madison, Wis.) as well as DNA fragments of *Campylobacter jejuni* (UA580) and *Campylobacter coli* (UA417) of known size (7).

DNA-DNA hybridization. DNA fragments of the *C. fetus* strains were separated by CHEF agarose gel electrophoresis

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TABLE 1. Characteristics of *C. fetus* strains

Strain type ^a	ADRI ^b no.	UA ^c no.	Isolation source	Glycine tolerance (%)	Source ^d
<i>C. fetus</i> subsp. <i>fetus</i>	1025	UA777	Human	1.3	E. Holst
	802	UA778	Ovine	1.3	M. Corbel
	1028	UA779	Ovine	1.5	CCUG 10371
	1029	UA780	Bovine	1.3	CCUG 10770
	1030	UA781	Bovine	1.9	CCUG 17693
	1341	UA782	Bovine	1.9	A. Cipolla
	553 ^T	UA60 ^T	Ovine	1.9	ATCC 27374 ^T
<i>C. fetus</i> subsp. <i>venerealis</i> ^e	817	UA790	Bovine	1.0	M. Corbel
	544	UA791	Bovine	1.0	B. Clark
	545	UA813	Bovine	1.0	B. Clark
	546	UA814	Bovine	1.0	B. Clark
	812	UA815	Bovine	1.0	M. Corbel
<i>C. fetus</i> subsp. <i>venerealis</i>	1023	UA809	Human	<1	E. Holst
	1345	UA810	Bovine	<1	A. Cipolla
	1267 ^T	UA811 ^T	Bovine	<1	ATCC 19438 ^T
	543	UA816	Bovine	<1	B. Clark
	554	UA817	Bovine	<1	H. Lior
	555	UA818	Bovine	<1	B. Firehammer
	838	UA820	Bovine	<1	B. Firehammer

^a Strain identified on the basis of tolerance to >1% glycine.

^b ADRI, Animal Disease Research Institute, Nepean, Ontario, Canada.

^c UA, University of Alberta.

^d Sources of strains are as follows: E. Holst, Department of Medical Microbiology, University of Lund, Lund, Sweden; M. Corbel, Central University Laboratory, Ministry of Agriculture, Fisheries and Food, New Haw Weybridge, Surrey, United Kingdom; A. Cipolla, Instituto Nacional de Tecnología Agroparia-EEA, Balcarce, Argentina; CCUG, Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden; ATCC, American Type Culture Collection, Rockville, Md.; B. Clark, Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organization, Parkville, Victoria, Australia; H. Lior, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada; and B. Firehammer, Veterans Research Laboratory, Montana State University, Bozeman.

^e Biovar "intermedius."

after digestion with the restriction endonucleases *Sma*I and *Sal*I and were transferred to nitrocellulose filters (Bio-Rad, Richmond, Calif.) by the Southern transfer technique described previously (27). Purified chromosomal DNA of *C. fetus* subsp. *fetus* UA60^T (ATCC 27374^T) was nick translated and hybridized to the DNA in the nitrocellulose filters, prepared above, by the method described by Maniatis et al. (16). After hybridization, the nitrocellulose filters were exposed to X-ray film (XAR-5; Kodak, Rochester, N.Y.) for up to 1 week at -80°C, and the hybridization patterns were analyzed.

RESULTS

Phenotypic characterization. All *C. fetus* strains tested grew at 25 and 37°C but not at 42°C and were able to produce oxidase, catalase, and nitrate but failed to generate H₂S in triple sugar iron media. Differentiation of *C. fetus* subspecies strains was done by utilizing their tolerance to glycine. Table 1 shows the arrangement of the strains according to their ability to tolerate glycine. Strains which tolerated less than 1% glycine were referred to as *C. fetus* subsp. *venerealis*, while strains that tolerated more than 1% glycine were referred to as *C. fetus* subsp. *fetus* (12, 23). Those strains which tolerated up to 1% glycine were referred to as *C. fetus* subsp. *venerealis* biovar "intermedius" (10, 29).

Restriction endonuclease analysis of *C. fetus* genome DNA. Genomic DNA of *C. fetus* strains generated six to eight fragments when digested with the restriction endonuclease *Sma*I (Table 2). Figure 1 shows the PFGE patterns of chromosomal DNA of nine *C. fetus* strains digested with the restriction endonuclease *Sma*I. Strains UA778 and UA60^T

(Fig. 1, lanes B and C) produced identical patterns. Strains UA809 and UA811^T (Fig. 1, lanes D and E) also appeared similar. The rest of the *C. fetus* strains produced distinctly different patterns.

The restriction endonuclease *Sal*I, on the other hand, produced 7 to 10 fragments with genome DNA from the *C. fetus* strains. Genome DNA of *C. fetus* strains, which generated similar patterns when digested with the restriction endonuclease *Sma*I, appeared to be similar when digested with the restriction endonuclease *Sal*I (Fig. 2).

Determination of genomic sizes of *C. fetus* strains. The size of the genome DNA for each of the strains was calculated from more than six different repetitions of PFGE experiments carried out at different pulse times. The size of each of the DNA fragments was determined by calibration with reference to the λ 39 DNA ladder (Fig. 1 and 2). When digested with the restriction endonuclease *Sma*I, genomic DNA of *C. fetus* strains generated fragments which ranged in size from 50 to 407 kb. The total genome size of the strains was calculated from the sum of the fragment sizes generated by the digested genomic DNA. All *C. fetus* subsp. *fetus* strains, a *C. fetus* subsp. *venerealis* biovar "intermedius" strain (UA790), and two *C. fetus* subsp. *venerealis* strains (UA810 and UA818) formed a distinct group of genomic sizes which measured 1,134 (1,125 to 1,158) kb, while five *C. fetus* subsp. *venerealis* strains (UA809, UA811^T, UA816, UA817, and UA820) formed a genomic size group that measured 1,362 (1,329 to 1,394) kb. Four strains of *C. fetus* subsp. *venerealis* biovar "intermedius" constituted the third group of genomic sizes which measured 1,537 (1,519 to 1,581) kb. Similar results were obtained with the restriction endonuclease *Sal*I.

TABLE 2. Genomic sizes of *C. fetus*

UA no.	PFGE pattern with:			
	<i>Sma</i> I		<i>Sal</i> I	
	No. of fragments	Genome size (kb) ^a	No. of fragments	Genome size (kb) ^a
777	6	1,125 ± 3	8	1,141 ± 5
778	6	1,125 ± 3	10	1,137 ± 2
779	6	1,125 ± 3	8	1,125 ± 5
780	6	1,125 ± 3	7	1,141 ± 5
781	8	1,158 ± 2	7	1,146 ± 3
782	8	1,158 ± 3	8	1,139 ± 5
790	7	1,138 ± 5	7	1,125 ± 5
810	7	1,152 ± 5	7	1,124 ± 5
818	6	1,165 ± 7	9	1,139 ± 2
60 ^T	6	1,125 ± 2	9	1,137 ± 2
791	8	1,529 ± 4	10	1,572 ± 4
813	7	1,527 ± 2	10	1,579 ± 2
814	8	1,581 ± 4	9	1,522 ± 5
815	8	1,519 ± 3	9	1,579 ± 6
809	7	1,329 ± 5	7	1,329 ± 5
811 ^T	8	1,329 ± 6	7	1,334 ± 2
816	8	1,394 ± 2	7	1,354 ± 3
817	8	1,394 ± 3	7	1,362 ± 3
820	7	1,374 ± 5	7	1,368 ± 4

^a Determined from at least six different gel runs at different pulse times.

DNA-DNA hybridization. Whole chromosomal DNA of *C. fetus* subsp. *fetus* UA60^T was hybridized under high-stringency conditions of hybridization and washing to Southern blots prepared from pulsed-field gels containing DNA of different *C. fetus* subspecies. There appeared to be significant homology between UA60^T and the various *C. fetus*

FIG. 1. CHEF gel electrophoresis of *C. fetus* DNA digested with *Sma*I. DNA was subjected to electrophoresis for 24 h at 175 V and 8°C with a pulse time of 25 s in a 1% agarose gel. Bacteriophage λ Δ39 concatemers were used to determine the fragment sizes. Lanes: A, UA777; B, UA778; C, UA60^T; D, UA809; E, UA811^T; F, UA812; G, UA813; H, UA814; I, UA815. Lanes A to C, *C. fetus* subsp. *fetus*; lanes D to F, *C. fetus* subsp. *venerealis*; lanes G to I, *C. fetus* subsp. *venerealis* biovar "intermedius."

FIG. 2. CHEF gel electrophoresis of *C. fetus* DNA digested with *Sal*I. DNA was subjected to electrophoresis for 24 h at 175 V and 8°C with a pulse time of 15 to 20 s in a 1% agarose gel. Bacteriophage λ Δ39 concatemers were used to determine the fragment sizes. Lanes: A, UA777; B, UA778; C, UA60^T; D, UA809; E, UA811^T; F, UA812; G, UA813; H, UA814; I, UA815. Lanes A to C, *C. fetus* subsp. *fetus*; lanes D to F, *C. fetus* subsp. *venerealis*; lanes G to I, *C. fetus* subsp. *venerealis* biovar "intermedius."

subspecies. In none of the strains was any major region of nonhomology visible by this technique.

DISCUSSION

The current classification of the subspecies of *C. fetus* was proposed by Véron and Chatelaine (29) and Smibert (24) and is recognized by the recent editions of *Bergey's Manual of Systematic Bacteriology* (24) and the *Approved Lists of Bacterial Names* (22). This classification was based on two main criteria: the ability of these campylobacters to grow on media containing variable concentrations of glycine and the normal habitat of these organisms. Chang and Ogg (8, 9) have shown that the glycine characteristic may not be reliable for separating the etiologic agents of vibriotic abortion and bovine vibriosis into the subspecies *fetus* and *venerealis*, respectively. These workers have demonstrated that transduction of the glycine tolerance character was accomplished with a bacteriophage from a glycine-resistant donor to a glycine-sensitive recipient strain of *C. fetus*. Moreover, they showed that glycine resistance could occur as a result of spontaneous mutation (9). These facts emphasized the need for a more reliable method for identifying the two subspecies.

PFGE is capable of separating large fragments of chromosomal DNA, which have been digested with restriction endonucleases, to produce a pattern that can help in the differentiation of strains. It was applied successfully to differentiate organisms at the species level (15). Furthermore, this technique is used to differentiate strains within the species and has proved to be useful in epidemiological studies of a variety of microorganisms (1, 2, 12, 19, 28, 31, 32). Moreover, it facilitates the calculation of the DNA genome size (7). As with other campylobacters (7, 31, 32), we have been able to measure the sizes of genomic DNA from different *C. fetus* strains. The strains clustered in three distinct groups of genomic sizes, i.e., 1.1, 1.3, and 1.5 Mb. Genome sizes of strains of *C. fetus* subsp. *fetus*, including the type strain ATCC 27374 (UA60), measured 1.1 Mb;

however, three more strains (UA790, UA810, and UA818), which showed lower tolerance to glycine (up to 1.0%), also belonged to this group. The consistency of the genomic sizes and the findings of Chang and Ogg (8, 9) suggest that these strains may actually belong to the *C. fetus* subsp. *fetus* group. These findings are concordant with those of Chang and Taylor (7), who found that *C. fetus* subsp. *fetus* UA60^T has a smaller genomic size (1.2 Mb) than do the closely related *C. jejuni* (1.7 Mb) and *C. coli* (1.7 Mb). The findings of Chang and Taylor were described as preliminary and were made with one strain (7). During our study, we calculated the genome size of UA60^T from at least six repeated runs with different pulse times and concluded that 1.12 to 1.14 Mb, which is within the range of sizes for the rest of the *C. fetus* subsp. *fetus* strains (Table 2), represents the actual genomic size of this strain. Strains of *C. fetus* subsp. *venerealis*, which tolerated 0.6% glycine in the media, including the type strain ATCC 19438 (UA811), were clustered together by their genomic sizes and measured 1.3 Mb. This study confirms the current classification of *C. fetus* (24, 29). Moreover, strains of *C. fetus* subsp. *venerealis* biovar "intermedius" were differentiated from *C. fetus* subsp. *venerealis* by their genome size (1.5 Mb).

The results of the DNA-DNA hybridization experiments confirm what was previously observed (12), namely, that strains of *C. fetus* are very highly related at the DNA level. However, the degree of DNA-DNA and DNA-rRNA homology and sequence analysis of the 5S and 16S rRNAs are still needed to further confirm the classification of these microorganisms (20).

Since the genomic size of a species or a subspecies represents a relatively stable character (1, 2, 28, 31, 32), PFGE could provide, especially for the veterinary laboratory, a novel technique that will discriminate between the subspecies of *C. fetus* with more consistency than the phenotypic characterization of these relatively biochemically inert organisms. Furthermore, different strains produce different PFGE patterns, a phenomenon that can be of great value in epidemiological studies of *C. fetus*.

This study also provides genotypic confirmation of a human isolate (UA809) which was identified by its phenotypic characterizations as belonging to the subspecies *venerealis*. In fact, two more human isolates received recently were also identified as belonging to the subspecies *venerealis* (data not shown). This is the first report of a human isolate of *C. fetus* subsp. *venerealis*.

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