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Genetic Divergence of *Campylobacter fetus* Strains of Mammal and Reptile Origins

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***Campylobacter fetus* is a gram-negative bacterial pathogen of both humans and animals. Two subspecies have been identified, *Campylobacter fetus* subsp. *fetus* and *Campylobacter fetus* subsp. *venerealis*, and there are two serotypes, A and B. To further investigate the genetic diversity among *C. fetus* strains of different origins, subspecies, and serotypes, we performed multiple genetic analyses by utilizing random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), and DNA-DNA hybridization. All 10 primers used for the RAPD analyses can distinguish *C. fetus* strains of reptile and mammal origin, five can differentiate between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* strains, and four showed differences between type A and type B isolates from mammals. PFGE with SmaI and SalI digestion showed varied genome patterns among different *C. fetus* strains, but for mammalian *C. fetus* isolates, genome size was well conserved (mean, 1.52 ± 0.06 Mb for SmaI and 1.52 ± 0.05 Mb for SalI). DNA-DNA hybridization demonstrated substantial genomic-homology differences between strains of mammal and reptile origin. In total, these data suggest that *C. fetus* subsp *fetus* strains of reptile and mammal origin have genetic divergence more extensive than that between the two subspecies and that between the type A and type B strains. Combining these studies with sequence data, we conclude that there has been substantial genetic divergence between *Campylobacter fetus* of reptile and mammal origin. Diagnostic tools have been developed to differentiate among *C. fetus* isolates for taxonomic and epidemiologic uses.**

Campylobacter species are gram-negative, slender, spiral, curved rods. Sixteen valid *Campylobacter* species have been described (9, 18, 20, 28, 34), of which *Campylobacter fetus* is the type species (26). *C. fetus* includes two subspecies, *fetus* and *venerealis* (2, 28). *Campylobacter fetus* subsp. *fetus* causes abortion in sheep and sporadic abortion in cattle, and *Campylobacter fetus* subsp. *venerealis* causes abortion and infertility in cattle (11, 13, 24, 27). Either of the subspecies can be a human pathogen, causing enteritis, abortion, bacteremia, septicemia, endocarditis, or meningitis (3, 4, 27, 30, 37).

Based on the lipopolysaccharide structure and surface layer protein (SLP) composition, *C. fetus* strains may be designated either type A or type B (7, 19, 23); *C. fetus* subsp. *venerealis* strains are always type A, whereas *C. fetus* subsp. *fetus* strains can be either type A or type B, or rarely type AB (19, 23, 29). Sequence analyses of the housekeeping gene *recA* and the *C. fetus*-specific gene *sapD* suggested that *C. fetus* type A and type B strains diverged before *C. fetus* subspecies *fetus* and *C. fetus* subsp. *venerealis* diverged from one another (29). In contrast, *C. fetus* strains of reptile origin showed very high divergence from *C. fetus* strains of mammalian origin. The 16S rRNA sequence analysis also suggested that reptile *C. fetus* isolates form a distinct phylogenotype between *C. fetus* and *Campylobacter hyointestinalis* (29).

Analyses using specific gene sequences can provide only limited insights into differences between *C. fetus* strains at the genomic level, and the entire *C. fetus* genomic sequence has not been determined. In contrast, random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis (PFGE), and whole-genome DNA-DNA hybridization can be employed to aid in understanding genetic divergence among *C. fetus* strains at a more global level.

In the present study, we used such methods to further address questions regarding genetic divergence and evolutionary relationships for *C. fetus* strains of different subspecies, serotypes, and host origins.

MATERIALS AND METHODS

Bacterial strains and growth media. A total of 19 *C. fetus* strains were analyzed in this study, including 13 *C. fetus* subsp. *fetus*, 2 *C. fetus* subsp. *venerealis*, and 4 *C. fetus* strains of reptile origin. Of these 19 strains, 12 are type A, 6 are type B, and 1 is type A/B (Table 1). Details of the characterization of these strains have been reported elsewhere (29, 31, 32, 33). The strains were routinely grown on brucella broth (BBL Microbiology Systems, Cockeysville, Md.) or on Trypticase soy agar plates at 37°C in a 5% CO_2 incubator.

RAPD-PCR. RAPD-PCRs were performed using 10 different primers (Table 2). The PCR conditions for primers 1254, 1281, 1283, 1290, 1247, D8635, and D14307 (1), primers ERIC-1 and ERIC-2 (8), and primer OPA-11 (16) were all as originally described.

PFGE profiling. The preparation of bacterial DNA-agarose samples and subsequent enzyme digestion for PFGE was as described previously (5). DNA was digested with SmaI or SalI (New England Biolabs [NEB], Beverly, MA), and fragments were electrophoretically separated in 1% agarose gels at 14°C with the following variations according to the particular range of markers used: for low-range PFG Marker (NEB), 6 V/cm and switch times from 1 to 12 s for 15 h; for MidRange I PFG Marker (NEB), switch times ramped from 1 to 25 s for 24 h; and for Lambda Ladder PFG Marker (NEB), 4.5 V/cm and switch times ramped from 5 to 120 s for 48 h.

DNA-DNA hybridization. Dot blot DNA-DNA hybridizations were performed with 5 ng of whole bacterial genomic DNA. The probes consisted of fluorescein-

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TABLE 1. Characteristics and genomic sizes of the *C. fetus* strain studied

Strain no.	Strain designation	Origin	Type	Subspecies	SmaI		SalI	
					Genomic size (Mb)	Type (no. of bands)	Genomic size (Mb)	Type (no. of bands)
1	80-109	Mammal	A	*fetus*	1.55	A (15)	1.55	A (15)
2	82-40	Mammal	A	*fetus*	1.42	B (14)	1.47	B (14)
3	83-94	Mammal	A	*fetus*	1.42	C (14)	1.47	B (14)
4	84-32 (23D)	Mammal	A	*fetus*	1.60	D (15)	1.47	B (14)
5	84-86	Mammal	A	*fetus*	1.54	E (15)	1.47	B (14)
6	84-92	Mammal	A	*fetus*	1.54	E (15)	1.47	B (14)
7	99-256	Mammal	A	*fetus*[a]	1.56	A (15)	1.54	C (15)
8	84-87	Mammal	B	*fetus*	1.55	F (16)	1.49	D (16)
9	84-90	Mammal	B	*fetus*	1.42	B (14)	1.55	A (15)
10	84-91	Mammal	B	*fetus*	1.55	A (15)	1.55	A (15)
11	84-94	Mammal	B	*fetus*	1.55	A (15)	1.60	E (16)
12	84-104	Mammal	B	*fetus*	1.54	G (15)	1.59	F (15)
13	84-107	Mammal	B	*fetus*	1.55	A (15)	1.55	A (15)
14	84-112	Mammal	A	*venerealis*	1.58	H (17)	1.85	G (17)
15	99-257	Mammal	A	*venerealis*	1.64	I (18)	1.50	H (14)
16	85-388	Reptile	A	*fetus*	1.80	J (10)	NA[b]	NA
17	85-389	Reptile	A	*fetus*	1.44	K (8)	NA	NA
18	03-427	Reptile	A	*fetus*	1.72	L (9)	NA	NA
19	85-388	Reptile	A/B	*fetus*	1.83	M (10)	NA	NA

[a] Strain 99-256 is from the American Type Culture Collection (ATCC 33561) and has been considered a *C. fetus* subsp. *venerealis* strain. However, the present study provides evidence that it is *C. fetus* subsp. *fetus* rather than *C. fetus* subsp. *venerealis*, based on RAPD and PFGE.
[b] NA, not applicable, due to poor digestion.

ated genomic DNA prepared from *C. fetus* subsp. *fetus* mammalian strain 23D or *C. fetus* subsp. *fetus* strain 85-388 of reptile origin. Each sample of chromosomal DNA was heat denatured at 100°C for 5 min and applied to a positively charged nylon membrane, and the DNA was immobilized by UV irradiation. Labeling of the probes and the hybridization reactions were performed based on the protocol supplied with the Random Primer Fluorescein Labeling Kit with antifluorescein-AP (Perkin-Elmer Life Sciences, Inc., Boston, MA).

16S rRNA sequence analyses. Two *C. fetus* subsp. *venerealis* strains, 99-257 and 84-112, were analyzed using the universal 16S rRNA gene primers 8F and 1510R (22) and were compared with previously published sequences of other *C. fetus* strains involved in this study (29, 33). Sequence analyses were performed with the Genetics Computer Group programs (Madison, WI).

Nucleotide sequence accession number. The nucleotide sequences of 16S rRNA for strains 84-112 and 99-257 have been deposited in GenBank under accession number AY864915.

RESULTS

RAPD. To better detect DNA diversity at the level of the whole genome among different *C. fetus* strains, we screened the 19 isolates with 10 different primers in RAPD analyses (Table 2 and Fig. 1 and data not shown). Use of each of the 10 primers resulted in distinguishing the mammalian and reptilian *C. fetus* strains (Fig. 1 and data not shown), use of five of the primers permitted distinguishing between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, and four of the primers differentiated type A and type B strains. The use of primer ERIC-2, 1247, or D14307 each permitted distinguishing *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, type A and type B, and mammalian *C. fetus* and reptilian *C. fetus* strains (Fig. 1 and Table 2).

PFGE. The SmaI and SalI PFGE patterns of the 19 *C. fetus* strains are shown in Table 1 and Fig. 2. SmaI digestion produced 8 to 18 fragments with sizes varying from 3 kb to 436 kb (Fig. 2A and data not shown). Among the 13 mammalian *C. fetus* subsp. *fetus* strains, there exist seven SmaI digestion patterns designated A to G. The two *C. fetus* subsp. *venerealis* (H and I) and four reptile origin *C. fetus* (J to M) strains each

TABLE 2. RAPD identification of *C. fetus* strains

Primer	Sequence (5′ → 3′)	Reference	Distinguishing[d]		
			Cf vs-Cv[a]	*M vs R*[b]	*A vs B*[c]
1281	AACGCGCAAC	1	N	D	N
1283	GCGATCCCCA	1	N	D	N
1290	GTGGATGCGA	1	N	D	N
D8635	GAGCGGCCAAAGGGAGCAGAC	1	D	D	N
1247	AAGAGCCCGT	1	D	D	D
D14307	GGTTGGGTGAGAATTGCACG	1	D	D	D
1254	CCGCAGCCAA	1	N	D	N
OPA-11	CAATCGCCGT	16	D	D	N
ERIC-1	ATGTAAGCTCCTGGGGATTCA	8	N	D	D
ERIC-2	AAGTAAGTGACTGGGGTGAGCG	8	D	D	D

[a] Cf, *C. fetus* subsp. *fetus* (n = 13 strains); Cv, *C. fetus* subsp. *venerealis* (n = 2 strains).
[b] M, mammalian *C. fetus* strain (n = 15); R, reptile *C. fetus* strain (n = 4).
[c] A, type A *C. fetus* strain (n = 12); B, type B *C. fetus* strain (n = 6).
[d] N, not distinguishable; D, distinguishable.

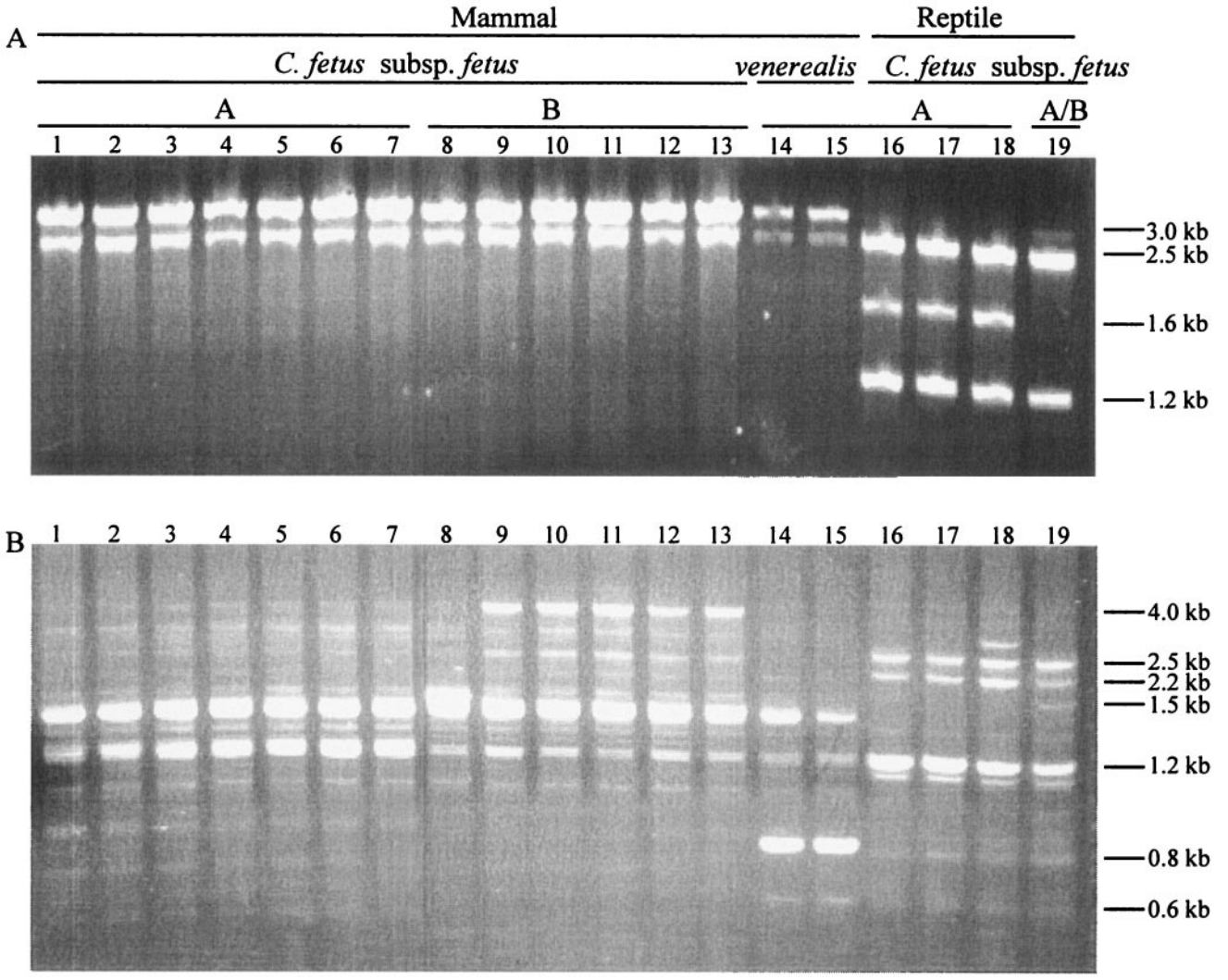

FIG. 1. RAPD profiles of 19 *C. fetus* strains generated using primers 1254 (A) and ERIC-1 (B). The lane numbers representing the strains correspond to the strain numbers in Table 1.

showed a different SmaI pattern. Based on the restriction patterns, we calculated the genome size for each strain. Using SmaI digestion, the 13 mammalian *C. fetus* subsp. *fetus* genomes varied from 1.42 to 1.60 Mb (mean, 1.52 ± 0.06), the 2 *C. fetus* subsp. *venerealis* genomes were 1.58 and 1.64 Mb (mean, 1.61 ± 0.05), and the 4 reptile strains were 1.80, 1.44, 1.72, and 1.83 Mb (mean, 1.70 ± 0.18). For the 13 mammalian *C. fetus* subsp. *fetus* strains, results of the SalI digestion confirmed the genomic size (1.52 ± 0.05). SalI digestion of the whole genome demonstrated six patterns for the 13 mammalian *C. fetus* subsp. *fetus* strains and two patterns for the 2 *C. fetus* subsp. *venerealis* strains. However, each of the four reptile origin *C. fetus* subsp. *fetus* strains showed a SalI digestion profile totally different from that for the mammalian *C. fetus* subsp. *fetus* strains; these appear to be due to incomplete digestion. The genome sizes calculated from both SmaI and SalI digestion showed intrastrain variation, in part because some of the digested fragments showed very similar migration characteristics and were not readily distinguishable.

DNA-DNA hybridization. The *C. fetus* subsp. *fetus* 23D genome probe showed a high degree of hybridization with all 13 mammal *C. fetus* subsp. *fetus* strains and with the 2 *C. fetus* subsp. *venerealis* strains but much less with the 4 reptile *C. fetus* strains. In contrast, the probe based on reptile strain 85-388 showed strong hybridization to all four reptile strains but none to any of the mammalian *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* strains. As expected, neither of the probes showed any significant hybridization signal with the control *Campylobacter jejuni* strain 11168 (Fig. 3). The hybridization experiments were each performed twice, yielding similar results.

16S rRNA analyses. Our previous study compared 16S rRNA sequences among strains, including *C. fetus* subsp. *fetus* type A (84-32) and type B (84-107), *C. fetus* subsp. *venerealis* (99-256), and four reptile strains (85-387, 85-388, 85-389, and 03-427) (29, 33). However, strain 99-256 (ATCC 33561), used as a representative *C. fetus* subsp. *venerealis* strain in the previous study (29), was found to be identical in 16S rRNA se-

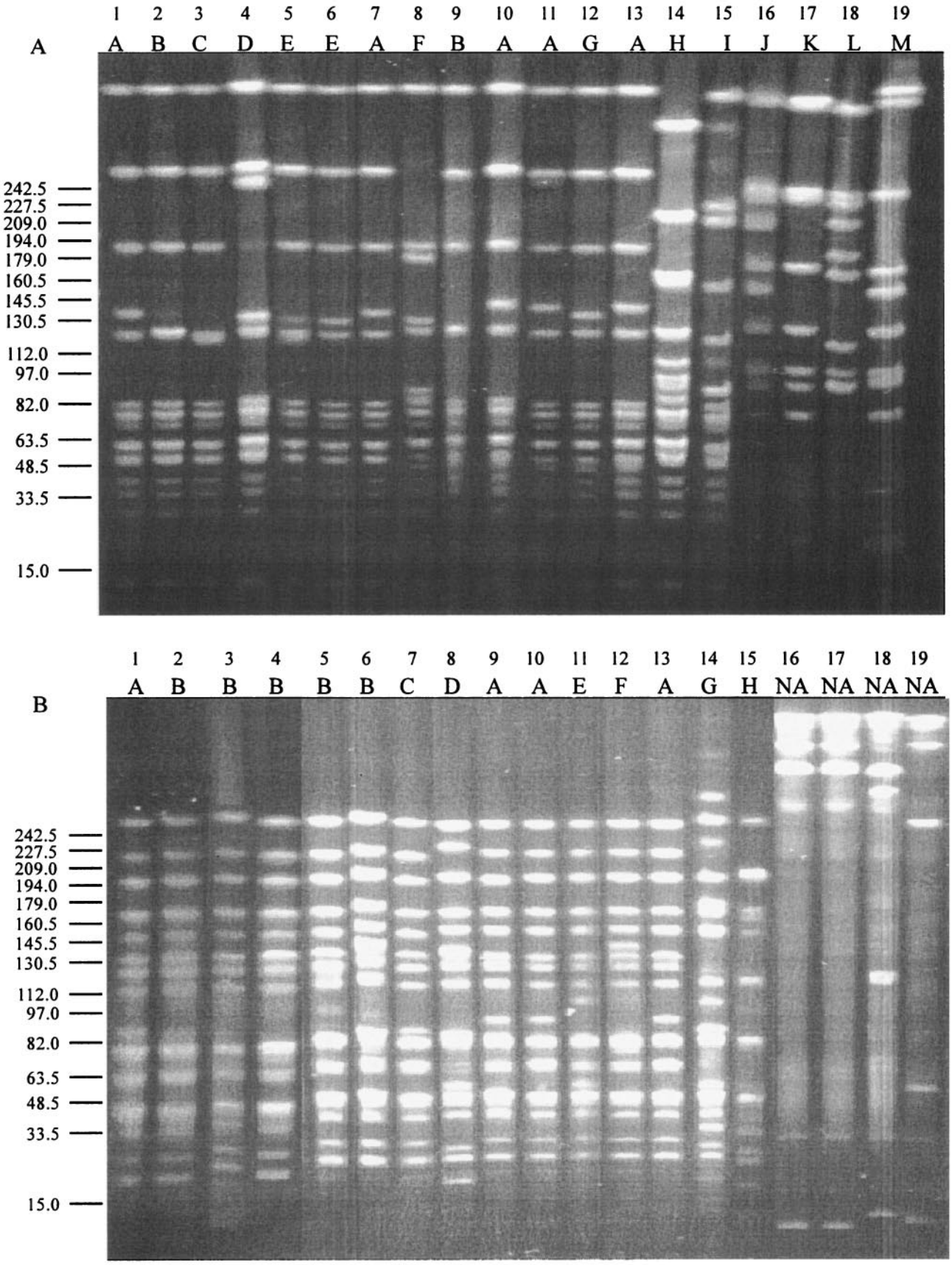

FIG. 2. PFGE profiles of 19 *C. fetus* strains digested with SmaI (A) and SalI (B). MidRange I PFG Marker was used. The lane numbers correspond to the strain numbers shown in Table 1.

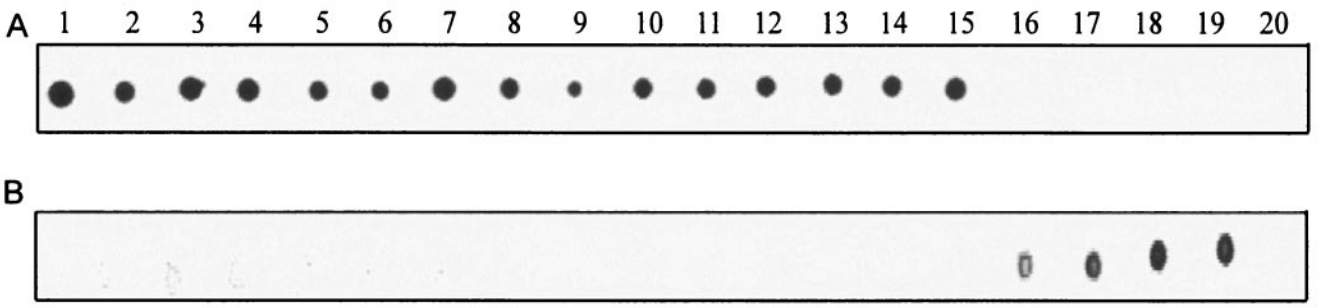

FIG. 3. Dot blot DNA-DNA hybridization with fluoresceinated genomic DNA probes prepared from DNAs of bovine (mammalian) strain 23D (A) and turtle (reptile) strain 85-388 (B). The lane numbers correspond to the strain numbers in Table 1. Lane 20 represents *C. jejuni* strain 111168 as a negative control. The homologous hybridizations are in lanes 4 and 19 for 23D (panel A) and 85-388 (panel B), respectively.

quence to *C. fetus* subsp. *fetus*. Based on the 16S rRNA sequence and on the present RAPD and PFGE studies, we believe that strain 99-256 should be considered *C. fetus* subsp. *fetus*. To better understand the genetic relationship between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, we sought to determine the 16S rRNA sequences of two other *C. fetus* subsp. *venerealis* strains. Within the 1,484-bp 16S rRNA determined, the two *C. fetus* subsp. *venerealis* strains (99-257 and 84-112) showed 100% sequence identity to the type A and type B *C. fetus* subsp. *fetus* strains from mammals (29). Our results showed that *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* had identical 16S rRNA sequences, which is consistent with reports using other determinants (21).

DISCUSSION

In the present study, we continued to investigate (6, 10, 12, 21, 25, 29, 35) the genetic differences within the species *C. fetus*, including isolates that were *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis*, type A or type B, and of mammal or reptile origin, using methods based on RAPD, PFGE, DNA-DNA hybridization, and 16S rRNA analyses. A schematic of the proposed ancestral relationships (Fig. 4) will be discussed below.

Discrimination between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* is usually based on the epidemiologic setting and whether an isolate is able to grow in the presence of 1% glycine (27, 28). *C. fetus* subsp. *fetus* usually causes sporadic abortion in cattle and sheep and also causes most human infections (4, 14). In contrast, *C. fetus* subsp. *venerealis* is mostly restricted to cattle, causing venereal campylobacteriosis, resulting in infertility; human infection is rare (25). The two *C. fetus* subspecies can be identified by specific PCR (17). The PFGE results for the two *C. fetus* subsp. *venerealis* strains studied showed patterns different from one another in both the SmaI and SalI digestions, which differed from those of the *C. fetus* subsp. *fetus* strains, and the RAPD results demonstrated that 5 of 10 primers were able to distinguish *C. fetus* subsp. *venerealis* strains from *C. fetus* subsp. *fetus* strains. Our results showed that *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* had identical 16S rRNA sequences, which is consistent with other reports (21). Comparing the present and prior (20, 29) studies, as well as the DNA-DNA hybridization results, indicates that these subspecies are very closely related. The close relatedness between the two subspecies is consistent with previous observations (21, 34). Strain 99-256 (ATCC 33561) had been classified as *C. fetus* subsp. *venerealis*, but our typing provides evidence that it is actually *C. fetus* subsp. *fetus*, illustrating the difficulty of separating the strains based on phenotype.

Differences between type A and type B *C. fetus* strains exist not only in lipopolysaccharide composition (19, 23), but also in the *sap* genes encoding the SLPs (7). Previous studies, based on partial *recA* and *sapD* gene sequence analyses, also suggested that type A and type B strains showed genetic differences greater than those between *C. fetus* subsp. *fetus* and *venerealis* (29). Similarly, the current RAPD analysis showed

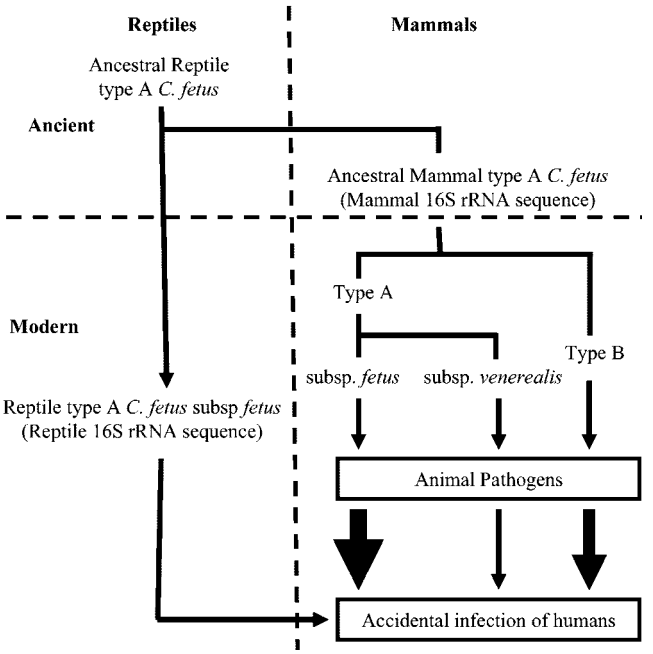

FIG. 4. Schematic of proposed ancestral relationships and pathogenicities for *C. fetus*, based on the prior (28) and present studies. *C. fetus* strains colonize a variety of animals and/or are pathogens of these species. Infection of humans usually reflects direct contact with such animals or exposure to *C. fetus*-contaminated foods. The widths of the arrows indicate the relative frequencies of human infection with these strains.

that use of 4 of the 10 primers studied identified differences between type A and type B strains. However, the SLPs of both types are antigenically cross-reactive (36), and there is 100% identity at the 16S rRNA level, consistent DNA-DNA hybridization, and similar SmaI and SalI digestion PFGE patterns. In total, these findings indicate that the mammalian-origin type A and type B *C. fetus* strains have shared ancestry and have not had major divergence at the genomic level. The data suggest that type A strains are most closely related to the distant *C. fetus* ancestors and that type B strains arose during their development in the mammalian niche, possibly in the gastrointestinal tract (29).

Of the four reptile strains studied, three were directly isolated from a reptile (15) and one (03-427), which was isolated from a human patient with bacteremia, has 16S rRNA and partial *sapD* sequences identical to those of the other three reptile strains (33). Although all four strains of reptile origin showed antigenic cross-reactivity with the antiserum raised against SLP of a strain of mammal origin, analyses of the 16S rRNA sequence, the housekeeping gene *recA*, and the *C. fetus*-specific gene *sapD* indicated substantial divergence from the mammalian *C. fetus* strains. In the present study, all 10 primers used for RAPD analyses showed differences between the reptile and mammalian strains. In the PFGE analyses, each of the four reptile strains showed a different SmaI digestion pattern, which differed from that of the mammalian strains. The results of the DNA-DNA hybridization indicated significant genomic divergence between the mammal and reptile isolates. In total, based on the multiple analyses described above, we conclude that reptile and mammal *C. fetus* strains have levels of genetic divergence higher than that between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* and between the type A and type B strains; the reptile strains may represent a new *C. fetus* subsp. *fetus*. Based on both the previous and current study, the reptilian *C. fetus* strains appear quite distinct from mammalian *C. fetus* strains. These might well represent a new subspecies, but to confirm that hypothesis, more studies are needed.

The results of these and prior studies are consistent with the hypothesis that *C. fetus* of a form related to type A strains was present in ancient reptiles and that this lineage has continued to the present (Fig. 4). Our previous studies indicating that all mammalian *C. fetus* strains (both *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* and both type A and type B strains) had identical 16S rRNA sequences and nearly identical *recA* and *sapD* sequences provided evidence that the divergence between type A and type B strains occurred prior to that between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (29). The 16S rRNA, *recA*, and *sapD* sequences all indicate that the reptilian strains are highly divergent from the mammalian strains. The present study confirms that reptilian *C. fetus* strains also show great divergence, based on the RAPD, PFGE, and DNA-DNA hybridization results. The data suggest that when mammals arose, *C. fetus* evolved with them into the present type A strains. The development of the *C. fetus* subsp. *venerealis* and type B *C. fetus* subsp. *fetus* strains likely represents more recent events. Analysis of full *C. fetus* genomes should permit improved exploration of these hypotheses.

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