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Identification of *Campylobacter fetus* Subspecies by Phenotypic Differentiation and PCR

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The species *Campylobacter fetus* is divided into the subspecies *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*, which differ in their epidemiologies and clinical importance. The differences between these subspecies make accurate distinction between the two essential. First, the value of seven key tests for the traditional differentiation of *C. fetus* was investigated. Afterwards, the results of the phenotypic differentiation and PCR were compared to address the question of the reliability of this PCR assay. Altogether, 103 *C. fetus* isolates were investigated, including the type strains of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*. Depending on the result of the glycine tolerance test, the isolates could be separated into 81 *C. fetus* subsp. *venerealis* isolates (glycine intolerant) and 22 *C. fetus* subsp. *fetus* isolates (glycine tolerant). For all *C. fetus* subsp. *venerealis* strains tested, the results of the selenite reduction assay and sensitivity to metronidazole and cefoperazone completely agreed with the results of the glycine tolerance test (correspondence, 100%). Seventy-three *C. fetus* subsp. *venerealis* isolates did not grow at 42°C (correspondence, 90.1%), but eight isolates showed a faintly discernible, flat, dark gray growth. For 22 *C. fetus* subsp. *fetus* isolates, the results of additional phenotypic tests only partly agreed with the results of the glycine tolerance test. For *C. fetus* subsp. *fetus* the results of the glycine tolerance test showed a relatively good correspondence with those of the selenite reduction assay (correspondence, 81.8%), assays for cefoperazone resistance (correspondence, 86.4%), and assays for growth at 42°C (correspondence, 81.8%). The results of the glycine tolerance test and PCR completely agreed for the 103 *C. fetus* isolates tested. We conclude that at present the traditional phenotypic characterization of *C. fetus* subspecies under strongly defined conditions remains indispensable, but this PCR assay constitutes a valuable adjunctive technique for the confirmation of phenotypic results.

The species *Campylobacter fetus* is divided into the subspecies *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*, which differ in their epidemiologies and clinical importance. *C. fetus* subsp. *venerealis* is the causative agent of bovine genital campylobacteriosis (bgC). This bacterium is characterized by a strong tropism for the genital tract of cattle and causes abortions (enzootic abortion). Infections caused by *C. fetus* subsp. *fetus* must be separated from those caused by *C. fetus* subsp. *venerealis*. The natural habitat of *C. fetus* subsp. *fetus* is the intestinal tract of cattle, but it can also cause abortions (sporadic abortion). The differences between these subspecies make accurate distinction essential (7). At present, the traditional differentiation between both subspecies is based on a single phenotypic test (tolerance to 1% glycine) in current phenotypic identification schemes, including the recommended international standard (1). Chang and Ogg (3) have shown that the glycine tolerance characteristic may not be reliable for separation of the two subspecies because glycine tolerance can be acquired by transduction or mutation. Additionally, glycine-tolerant variants of *C. fetus* subsp. *venerealis* (referred to as biovar "intermedius") have been described (14), although some evidence suggests that the strain characteristics are artifacts due to the inappropriate standardization of biochemical tests (13, 9). Furthermore, glycine-sensitive *C. fetus* subsp. *fetus* isolates have been observed (13). Recommendations for performing most phenotypic tests have been made but have not yet been widely adopted (8). The extent to which the divergent results in some laboratories may be due to the use of inappropriate methods is unknown (7). This situation emphasizes the need to base subspecies-level designations of *C. fetus* on more than one phenotypic reaction.

In recent years, molecular methods for distinguishing between the *C. fetus* subspecies (including PCR and pulsed-field gel electrophoresis [PFGE]) have been developed to substantiate the results of phenotypic differentiation and to avoid misidentification. In 2003, we reported on a specific PCR-based assay for identifying and differentiating the two *C. fetus* subspecies (6). This assay was originally described by Hum et al. (5) in 1997 and was adopted in our laboratory. In brief, a species-specific 764-bp amplicon is produced with primers MG3F and MG4R with the DNA of both subspecies of *C. fetus*. Afterwards, primers VenSF and VenSR are used for differentiation. The identification of *C. fetus* subsp. *venerealis* is based on the presence of a 142-bp amplicon, which is not formed by *C. fetus* subsp. *fetus*. Seventy-three field strains were investigated by this PCR assay (including 24 *C. fetus* subsp. *venerealis* strains and 26 *C. fetus* subsp. *fetus* strains). No discrepancies could be observed between the results of phenotypic differentiation and those of PCR. However, the reliability of this PCR was questioned (1). The subspecies of up to 10% of the strains were not correctly identified by this PCR assay, as described in the original paper (5), and this observation was confirmed in a later study (17). Therefore, we started an investigation to address this question. First, we investigated the value of seven key tests for the traditional differentiation of *C. fetus* isolates (sensitivity to gly-

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cine, metronidazole, cefoperazone, $KMnO_4$, basic fuchsin and selenite reduction, and growth at 42°C), as recommended by On and Harrington (13). Afterwards, we compared the results of the phenotypic differentiation and PCR assays to answer the question of the reliability of the PCR assay that is described.

MATERIALS AND METHODS

Bacterial isolates. Table 1 shows details of the origins of the isolates tested. All *C. fetus* subsp. *venerealis* strains were isolated from bovine specimens. The *C. fetus* subsp. *fetus* isolates came from different sources. The organism can cause sporadic abortions in cattle, but it is also one of the causative agents of ovine abortion. In our collection, 9 of 22 strains were isolated from ovine fetuses. Altogether, 103 *C. fetus* strains were investigated, including the type strains *C. fetus* subsp. *fetus* (DSMZ 5361) and *C. fetus* subsp. *venerealis* (NCTC 010354). Seventy-seven *C. fetus* field strains were isolated in the Federal Republic of Germany between 1999 and 2005 and were submitted to the National Reference Laboratory of bgC. Twenty-six *C. fetus* strains had been already isolated between 1985 and 1994 and derived from different collections. The primary phenotypic identifications of the strains were made by the laboratories submitting the strains.

Phenotypic differentiation. No standard methods for the phenotypic differentiation of *C. fetus* subspecies have been published. Most guidelines do not give explicit details on the minimum test requirements or test methodologies, and many workers use methods peculiar to their own laboratories. Differences in methods may lead to differences in the outcome of nominally the same test. Recommendations for performing the tests are given by On and Holmes (10, 11, 12). These recommendations were considered in our investigations.

Culture conditions. The basal media used were as follows: (i) Mueller-Hinton (MH) agar (TN 1162; Institut für Immunpräparate und Nährmedien, Berlin, Germany) supplemented with 10% calf blood and (ii) nutrient broth no. 2 (CM 67; Oxoid, Wesel, Germany).

The glycine medium (1%; 17-1323-01; Pharmacia Biotech, Uppsala, Sweden) was prepared by adding the test substance to MH agar before it was melted at 100°C. Metronidazole (4 mg/liter; M-1547; Sigma, Taufkirchen, Germany) as a stock solution of 4 mg per 10 ml *N*,*N*-dimethylacetamide (803235; Merck, Darmstadt, Germany), cefoperazone (64 mg/liter; C-4292; Sigma), and fuchsin (160 mg/liter; 1.15937; Merck) as a stock solution of 160 mg per 10 ml 96% ethanol (filter sterilized) were added to autoclaved MH agar after the agar was cooled to approximately 56°C.

Selenite (0.1%; sodium selenite pentahydrate; 1.06607; Merck,) and $KMnO_4$ (0.1% potassium permanganate; 1.59231; Merck) media were prepared by adding filter-sterilized solutions of these substances aseptically to nutrient broth no. 2.

The *Campylobacter* strains were grown microaerobically on MH agar plates for 72 h at 37°C. The bacteria were harvested from the plates with phosphate-buffered saline, and the suspensions were adjusted spectrophotometrically (CADAS 30 photometer; Lange GmbH, Berlin, Germany) to an optical density of 0.1. This density corresponded to 1.1×10^7 to 9.8×10^7 CFU per ml for *C. fetus* subsp. *venerealis* and 1.0×10^8 to 3.9×10^8 CFU per ml for *C. fetus* subsp. *fetus*. The suspensions of bacteria were diluted 1:10 for *C. fetus* subsp. *venerealis* and 1:100 for *C. fetus* subsp. *fetus*. The agar plates were inoculated with 20 μl of these dilutions in duplicate, and the spots were allowed to dry (for no more than 15 min). In this way, the number of culturable bacteria was used as a comparable basis for inoculation of the solid media.

In a similar way, liquid media (5 ml) were inoculated with 100 μl of undiluted suspension for *C. fetus* subsp. *venerealis* (optical density, 0.1 [see above]) and with 100 μl of a 1:10 dilution of suspension for *C. fetus* subsp. *fetus*.

All solid media supplemented with the test substances and the $KMnO_4$ medium were microaerobically incubated for 72 h (selenite medium was incubated for 120 h) at 37°C. In the same way, inoculated MH agar plates without test substances were incubated at 42°C and 37°C (control).

Evaluation. In all series of investigations, type strains of both *C. fetus* subspecies were used as controls. Each test was performed at least two times on separate occasions and on freshly prepared media in order to assess its reproducibility. Any growth on the test plates was compared with that on the accompanying unsupplemented basal medium (control plates). Table 1 shows all criteria of evaluation. The growth of the bacteria on the agar plates was evaluated with a stereomicroscope, and the growth was allocated to a scale of − to ++++. After inoculation of the MH agar plates (incubation for 48 h at 37°C), the inhibition of the bacteria in the liquid medium supplemented with $KMnO_4$ was assessed in the same way. Selenite reduction was evaluated on the basis of the reddening of the bouillon.

Identification of *C. fetus* subspecies by PCR. For DNA extraction, 2 ml of broth culture was centrifuged at 16,100 × *g* for 10 min. The pellet was resuspended in 200 μl of phosphate-buffered saline. DNA was extracted by using a High Pure PCR template preparation kit (Roche Diagnostics, Mannheim, Germany), according to the instructions of the manufacturer.

PCRs were carried out in two separate reactions. For detection of the *C. fetus* species, primer pair MG3F-MG4R (5) was used with a modified program. An initial denaturation (96°C for 60 s) was followed by 35 cycles of denaturation (96°C for 15 s), primer annealing (60°C for 60 s), and primer extension (72°C for 90 s). The reaction was terminated by a final extension step (72°C for 180 s). The same PCR program was used for the differentiation of *C. fetus* subsp. *venerealis* with primers VenSF and VenSR (5). Amplifications were carried out with a T3 thermocycler (Biometra, Göttingen, Germany) and the following conditions: a 50-μl PCR mixture contained 5 μl 10× PCR buffer (Roche Diagnostics), 2 μl deoxynucleoside triphosphate mixture (each deoxynucleoside triphosphate at 2 mM; Roche Diagnostics), 0.2 μl *Taq* DNA polymerase (Roche Diagnostics), 1 μl of the forward and reverse primers (10 pmol/μl; JenaBioScience, Jena, Germany), and 1 μl of DNA extract.

Analysis of the PCR products was carried out by agarose gel electrophoresis with 1.5% agarose gels. After ethidium bromide staining, visualization of the bands was done under UV light. A 100-bp DNA ladder (Genaxxon BioScience, Biberach, Germany) was used as a size marker.

RESULTS

Identification of *C. fetus* subspecies by phenotypic testing. Table 1 shows the details of the identification of the *C. fetus* subspecies isolates by phenotypic tests. In the course of the evaluation, the results of the phenotypic tests were summarized by the following classification: −, (+), and + reactions were classified as negative and ++, +++, and ++++ reactions were classified as positive. Depending on the result of the glycine tolerance test, the isolates were designated as *C. fetus* subsp. *venerealis* (glycine intolerant) and *C. fetus* subsp. *fetus* (glycine tolerant). In this way, the 103 *C. fetus* isolates could be separated into 81 *C. fetus* subsp. *venerealis* isolates and 22 *C. fetus* subsp. *fetus* isolates. Glycine-tolerant variants of *C. fetus* subsp. *venerealis* and glycine-sensitive variants of *C. fetus* subsp. *fetus* could not be observed.

Table 2 shows the expected reactions of the *C. fetus* subspecies in the key tests in comparison to our results. For all *C. fetus* subsp. *venerealis* field strains, the results of selenite reduction and sensitivity to metronidazole and cefoperazone completely agreed with glycine tolerance (correspondence, 100%) (Table 2). Seventy-three *C. fetus* subsp. *venerealis* isolates did not grow [−, (+), and + reactions] at 42°C (correspondence, 90.1%), but 8 isolates showed a faintly discernible, flat, dark gray growth, indicated by ++ in Table 1. Unlike the *C. fetus* subsp. *venerealis* isolates, the *C. fetus* subsp. *fetus* isolates showed a luxurious, light gray growth (with three exceptions), which enabled easy differentiation of the isolates.

For 22 *C. fetus* subsp. *fetus* isolates, the results of four additional phenotypic tests only partly agreed with the glycine tolerance test results. The numbers of isolates with the expected results were as follows: 18 for selenite reduction (correspondence, 81.8%), 9 for metronidazole resistance (correspondence, 40.9%), 19 for cefoperazone resistance (correspondence, 86.4%), and 18 for growth at 42°C (correspondence, 81.8%).

The inhibitory effects of basic fuchsin and $KMnO_4$ against the *C. fetus* isolates were not reproducible. Numerous modifications of these tests did not improve the reproducibility (results not shown).

TABLE 1. Origins of *C. fetus* isolates and results of phenotypic and PCR typing

Strain no.	Origin	Denomination	Subspecies-level identification by:						
			Phenotypic testing						PCR[d]
			1% Glycine[a]	Selenite reduction[c]	Metronidazole[a]	Cefoperazone[a]	Growth at 42°C[a]	Result[d]	
473/99	Heifer vaginal mucus, type strain	NCTC[e] 010354	−	−	−	+	+	V	V
208/99	Fetus	C 51/88	−	−	−	−	−	V	V
209/99	Fetus	C 50/88	−	−	(+)	(+)	−	V	V
210/99	Fetus	C 1/85	−	−	(+)	−	−	V	V
211/99	Fetus	C 93/87	−	−	(+)	(+)	−	V	V
212/99	Fetus	C 49/88	(+)	−	(+)	(+)	(+)	V	V
322/99	Unknown		−	−	−	−	−	V	V
327/99	Preputial washing	P 757/93	−	−	(+)	(+)	(+)	V	V
505/99	Preputial washing	P 783/93	(+)	−	(+)	−	−	V	V
147/00	Artificial vagina	D 71/106	−	−	(+)	−	(+)	V	V
149/00	Artificial vagina	D 71/03	−	−	(+)	−	−	V	V
150/00	Artificial vagina	D 71/96	−	−	−	−	(+)	V	V
151/00	Artificial vagina	D 71/100	−	−	−	−	(+)	V	V
152/00	Artificial vagina	D 71/103	+	−	−	−	(+)	V	V
153/00	Artificial vagina	D 71/84	(+)	−	(+)	−	+	V	V
154/00	Artificial vagina	D 71/115	−	−	−	(+)	−	V	V
18/01	Fetus	RD 15	−	−	(+)	(+)	(+)	V	V
51/01	Artifical vagina	D23/48	−	−	−	(+)	−	V	V
52/01	Artifical vagina	D23/59	−	−	−	−	(+)	V	V
53/01	Artifical vagina	D23/68	−	−	(+)	(+)	+	V	V
54/01	Artifical vagina	D23/154	(+)	−	−	−	(+)	V	V
55/01	Artifical vagina	D23/225	−	−	(+)	−	(+)	V	V
61/01	Artifical vagina	D38/6	(+)	−	(+)	−	+	V	V
135/02	Preputial washing	D66/17	−	−	(+)	−	+	V	V
201/02	Preputial washing	D80/325	−	−	+	(+)	(+)	V	V
202/02	Preputial washing	D80/340	−	−	(+)	(+)	+	V	V
206/02	Preputial washing	B4-670	−	−	−	(+)	+	V	V
209/02	Preputial washing	D80/191	−	−	+	−	+	V	V
210/02	Preputial washing	D80/392	(+)	−	+	(+)	(+)	V	V
212/02	Vaginal mucus	D88/3	−	−	(+)	(+)	(+)	V	V
213/02	Preputial washing	D80/245	(+)	−	(+)	(+)	+	V	V
38/03	Preputial washing	D37/2	−	−	(+)	(+)	(+)	V	V
47/03	Fetus	D61	−	−	−	(+)	−	V	V
57/03	Preputial washing	D77/13	−	−	−	−	−	V	V
61/03	Vaginal mucus	D71/2	−	−	−	−	−	V	V
63/03	Vaginal mucus	D71/9	−	−	−	−	−	V	V
99/03	Placenta	Bru141/N6	(+)	−	+	(+)	−	V	V
101/03	Preputial washing	D104/18	(+)	−	+	(+)	(+)	V	V
105/03	Preputial washing	D115	(+)	−	(+)	(+)	(+)	V	V
109/03	Vaginal mucus	D133/1-03	−	−	(+)	(+)	(+)	V	V
111/03	Vaginal mucus	D142/7	−	−	−	(+)	−	V	V
113/03	Vaginal mucus	D142/8	−	−	−	−	(+)	V	V
09/04	Fetus	B3-47	−	−	−	−	(+)	V	V
38/04	Fetus	B3-186	(+)	−	+	(+)	++[b]	V	V
74/04	Semen	B/Zu2424-9	(+)	−	(+)	(+)	++[b]	V	V
75/04	Preputial washing	B/Zu2424-5	(+)	−	(+)	(+)	++[b]	V	V
76/04	Fetus	B3-317	−	−	(+)	−	++[b]	V	V
84/04	Fetus	Bru42-04	(+)	−	+	(+)	+	V	V
85/04	Preputial washing	B/Zu3717-2	(+)	−	(+)	(+)	+	V	V
86/04	Preputial washing	B/Zu3717-4	(+)	−	(+)	(+)	(+)	V	V
87/04	Preputial washing	B/Zu3717-10	−	−	(+)	(+)	(+)	V	V
88/04	Preputial washing	B/Zu3717-11	(+)	−	(+)	(+)	−	V	V
90/04	Fetus	Bru51-04	(+)	−	(+)	(+)	+	V	V
91/04	Preputial washing	B4142/20	(+)	−	(+)	(+)	++[b]	V	V
136/04	Preputial washing	B4-434	−	−	(+)	(+)	++[b]	V	V
137/04	Preputial washing	B4-470	(+)	−	(+)	(+)	++[b]	V	V
138/04	Preputial washing	B4-473	(+)	−	(+)	(+)	++[b]	V	V
141/04	Preputial washing	B4-582	(+)	−	(+)	(+)	(+)	V	V
142/04	Preputial washing	B4-610	(+)	−	−	−	(+)	V	V
144/04	Fetus	Bru129-04	(+)	−	(+)	(+)	(+)	V	V
148/04	Unknown		(+)	−	+	(+)	(+)	V	V
149/04	Preputial washing	B4-1108	−	−	(+)	(+)	(+)	V	V
150/04	Preputial washing	B4-1109	(+)	−	(+)	(+)	(+)	V	V
205/04	Preputial washing	B4-1627	−	−	(+)	(+)	(+)	V	V

Continued on following page

TABLE 1—*Continued*

Strain no.	Origin	Denomination	Subspecies-level identification by:						
			Phenotypic testing						PCR[d]
			1% Glycine[a]	Selenite reduction[c]	Metronidazole[a]	Cefoperazone[a]	Growth at 42°C[a]	Result[d]	
214/04	Preputial washing	D108/130-04	(+)	−	(+)	(+)	(+)	V	V
11/05	Preputial washing	B4-2	(+)	−	(+)	(+)	(+)	V	V
62/05	Preputial washing	B4-183	(+)	−	(+)	(+)	(+)	V	V
63/05	Preputial washing	B4-187	(+)	−	(+)	(+)	(+)	V	V
64/05	Preputial washing	B4-189	(+)	−	(+)	(+)	(+)	V	V
68/05	Preputial washing	B4-277	−	−	−	−	−	V	V
69/05	Preputial washing	B4-279	−	−	−	−	−	V	V
70/05	Preputial washing	B4-280	−	−	−	−	+	V	V
71/05	Preputial washing	B4-281	−	−	−	−	−	V	V
100/05	Fetus	B3-425	−	−	−	−	−	V	V
122/05	Fetus	Bru76-05	−	−	+	++	−	V	V
134/05	Fetus	172aZ05056	−	−	(+)	−	(+)	V	V
145/05	Preputial washing	AZ178a-Z-05490/1	(+)	−	(+)	(+)	(+)	V	V
146/05	Preputial washing	AZ178a-Z-05490/3	(+)	−	(+)	(+)	(+)	V	V
147/05	Preputial washing	AZ178a-Z-05490/7	(+)	−	(+)	(+)	(+)	V	V
151/05	Preputial washing	AZ178a-Z-05842/1	(+)	−	(+)	(+)	−	V	V
152/05	Preputial washing	AZ178a-Z-05842/2	−	−	(+)	(+)	−	V	V
484/99	Ovine fetus, type strain	DSMZ[f] 5361	++++	++++	++++	++++	++++	F	F
325/99	Ovine fetus	F286/94	+++	++++	+++	++++	++++	F	F
452/99	Ovine fetus	C48/86	++++	++	+	+++	++++	F	F
453/99	Preputial washing	C56/88	++++	++	−	(+)	+++	F	F
454/99	Bovine uterus	C63/88	++++	++++	++++	++++	++++	F	F
455/99	Ovine fetus	C33/89	++++	−	++++	++++	++++	F	F
456/99	Ovine fetus	C293/89	++++	−	(+)	−	+++	F	F
457/99	Ovine fetus	C76/90	+++	++++	−	++++	−	F	F
458/99	Bovine fetus	C44/91	++++	(+)	+++	++++	++++	F	F
459/99	Ovine fetus	C1/92	++++	+++	++++	++++	+	F	F
460/99	Ovine fetus	C4/99	++++	++++	+++	++++	++++	F	F
155/00	Artificial vagina	D71/279	+++	+++	−	+	−	F	F
56/01	Artificial vagina	D23/193	++++	++	−	++++	++++	F	F
1/02	Ovine fetus	E159/01	++++	−	−	++++	++++	F	F
211/02	Preputial washing	D75/5	++++	++	++++	+++	++++	F	F
03/04	Bovine fetus	Bru141	++++	++++	+	++++	++++	F	F
222/04	Bovine sperm	Z3030	+++	++	(+)	++++	++	F	F
91/05	Preputial washing	ZU3854/24/1	++++	++	(+)	++++	++	F	F
93/05	Preputial washing	ZU3854/24/3	+++	++	(+)	++++	++	F	F
94/05	Preputial washing	ZU3930/3/1	++++	++	(+)	++++	+++	F	F
96/05	Preputial washing	ZU3930/3/2	+++	++	(+)	++++	++	F	F
101/05	Bovine fetus	B3-445	+++	++	++	++++	+	F	F

[a] Agar plates were used. −, no growth; (+), faintly discernible growth; +, some distinct colonies; ++, many distinct colonies; +++, dense bacterial lawn; ++++, confluent growth.
[b] ++, weak, flat, dark gray growth on agar plates.
[c] Reddening of selenite bouillon: −, no; +, weak; ++, light; +++, strong; ++++, very strong.
[d] V, *C. fetus* subsp. *venerealis*; F, *C. fetus* subsp. *fetus*.
[e] NCTC, National Collection of Type Cultures, London, United Kingdom.
[f] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Identification of *C. fetus* subspecies by PCR. All *C. fetus* strains were tested by PCR for their subspecies identity by using the primer systems described by Hum et al. (5). Eighty-one of 103 isolates were identified as *C. fetus* subsp. *venerealis*; the others were identified as *C. fetus* subsp. *fetus*. All PCR results confirmed the subspecies identities obtained by the glycine tolerance tests (Table 1).

It was remarkable that the amplicons obtained by the spe-

TABLE 2. Correspondence of glycine tolerance with other phenotypic tests and PCR in *C. fetus* isolates[a]

Subspecies	Glycine tolerance	Selenite reduction	Resistance to:		Growth at 42°C	PCR
			Metronidazole	Cefoperazone		
C. fetus subsp. *venerealis*	Intolerant	− (100)	− (100)	− (100)	− (90.1)	+ (100)
C. fetus subsp. *fetus*	Tolerant	+ (81.8)	+ (40.9)	+ (86.4)	+ (81.8)	+ (100)

[a] − and +, no growth and growth, respectively, on the basis of the expected results of the phenotypic tests according to the identification schemes of On and Harrington (13). Values in parentheses are the percent correspondence of the reactions in reference to the glycine tolerance test result.

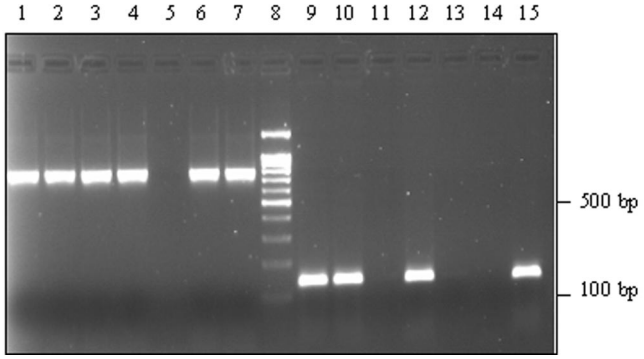

FIG. 1. Agarose gel electrophoresis of PCR products obtained by using primer pairs MG3F-MG4R (lanes 1 to 7) and VenSF-VenSR (lanes 9 to 15). Lanes: 1 and 9, *C. fetus* subsp. *venerealis* 69/05; 2 and 10, *C. fetus* subsp. *venerealis* 70/05; 3 and 11, *C. fetus* subsp. *fetus* 91/05; 4 and 12, *C. fetus* subsp. *venerealis* 122/05; 5 and 13, negative control; 6 and 14, *C. fetus* subsp. *fetus* type strain; 7 and 15, *C. fetus* subsp. *venerealis* type strain; 8, size marker.

cies-specific *C. fetus* PCR were about 750 bp (Fig. 1), nearly 200 bp smaller in fragment length, as originally described by Hum et al. (5).

DISCUSSION

Resistance to 1% glycine was used by Florent (4) as a criterion to separate *C. fetus* into the two subspecies *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (former designation, *Vibrio fetus* with the varieties *Vibrio fetus* var. *intestinalis* and *Vibrio fetus* var. *venerealis*). For decades the glycine tolerance test has been the only reaction ("gold standard") for the differentiation of *C. fetus* subspecies. Some months ago, the international standard for the diagnosis of bgC was revised (1), but this test remains the only reaction recommended for this purpose. However, the phenotypic differentiation of *C. fetus* isolates can be a cumbersome task. In some laboratories, variants of both *C. fetus* subspecies have been observed. At present, it cannot be excluded that these divergences are based on insufficiently standardized methods. In many aspects, the *C. fetus* subspecies are very similar (7). Therefore, the differences are more quantitative than qualitative in most reactions. For that reason, the standardization of phenotypic tests is a decisive requirement. It is well established in antimicrobial susceptibility testing that the inadequate or inappropriate standardization of the inocula can result in false-positive or -negative results. In studies on the phenotypic characterization of *Campylobacter* species, false-positive results were obtained by several key tests, generally when the inoculum size exceeded 10^7 to 10^8 CFU per ml (10). They indicated that for some commonly used tolerance tests, an inoculum size of approximately 10^6 CFU per ml is the most appropriate when blood agar is used as the basal medium. Therefore, we used a suspension of this density of bacteria to perform the tests. Furthermore, blood agar would appear to be the medium of choice for most tolerance tests used for the identification of *Campylobacter* isolates, provided that other important factors (such as the size of the inoculum and atmospheric conditions) are also considered (11). Whenever possible, we preferred blood agar to liquid medium to avoid misinterpretation of the phenotypic test results.

The tolerance to 1% glycine has been the only internationally accepted phenotypic test for the differentiation of *C. fetus* subspecies (1). Depending on the result of this test, the 103 field isolates investigated were classified as 81 *C. fetus* subsp. *venerealis* isolates (glycine intolerant) and 22 *C. fetus* subsp. *fetus* isolates (glycine tolerant). Glycine-tolerant variants of *C. fetus* subsp. *venerealis* and glycine-sensitive *C. fetus* subsp. *fetus* could not be observed.

The *C. fetus* subsp. *venerealis* strains were isolated over a long period of time and came from different sources. Despite this fact, these isolates showed relatively uniform reactions by the phenotypic tests. For all isolates of this subspecies, the results for selenite reduction and sensitivity to metronidazole and cefoperazone completely agreed with the results for glycine tolerance. Only in the parameter of growth at 42°C did the isolates reacted differently: 73 isolates had no growth and 8 isolates had faintly discernible, flat, dark gray growth. However, these eight isolates could be also separated from *C. fetus* subsp. *fetus*, because this subspecies (with three exceptions) showed a luxurious, light gray growth.

For *C. fetus* subsp. *fetus*, the four additional phenotypic tests only partly agreed with the glycine tolerance test results and a certain degree of nonuniformity was observed. *C. fetus* subsp. *fetus* showed a relatively good correspondence between the glycine tolerance test results with the results of selenite reduction, cefoperazone resistance, and growth at 42°C (correspondences, 81.8%, 86.4%, and 81.8%, respectively). In general, *C. fetus* is considered a nonthermophilic *Campylobacter*, but in our studies the majority of the *C. fetus* subsp. *fetus* isolates showed abundant growth at 42°C. In other investigations, the proportion of strains able to grow at 42°C varied from 0% (17) to 59% (2) and 62% (13).

In the last decade, progress has been made in the development of molecular methods for the identification of *C. fetus*. In 1997, Hum et al. (5) described a specific PCR-based assay for the identification and differentiation of the two *C. fetus* subspecies. This assay was evaluated, and 97 *C. fetus* field isolates were investigated. The initial identifications obtained by conventional phenotypic methods agreed with those suggested by PCR for 78 isolates, of which 56 were *C. fetus* subsp. *venerealis* isolates and 22 were *C. fetus* subsp. *fetus* isolates. The characterization of 19 isolates for which the results of conventional phenotyping and genotyping by PCR differed suggest that misidentification of *C. fetus* by phenotypic testing may be relatively common. Repeat testing of these 19 problem isolates revealed that for only 2 (2.1%) of 97 isolates were the identifications suggested by the PCR assay (as *C. fetus* subsp. *venerealis*) discordant with those made by macrorestriction profile analysis and/or conventional and probabilistic phenotypic methods. On and Harrington (13) examined 31 *C. fetus* strains by phenotypic, PCR-based, and PFGE-based methods; and the 16S rRNA gene sequences of 18 strains were compared. For 28 of the 31 strains, the results of the subspecies-level identifications by phenotypic, PCR-based, and PFGE-based methods concurred. Discrepancies between the results of the methods were observed for three strains. Wagenaar et al. (18) investigated 69 *C. fetus* isolates from three geographical regions by phenotypic testing, PCR, and amplified fragment length polymorphism

(AFLP) analysis. Depending on the result of the glycine tolerance test, 47 of the strains were typed as *C. fetus* subsp. *fetus* and 22 were typed as *C. fetus* subsp. *venerealis*. The investigation by PCR resulted in 54 *C. fetus* subsp. *fetus* strains, and 7 of these strains were negative by the glycine tolerance test. In these cases, the PCR results were supported by data from the AFLP analysis, suggesting that the strains were mistyped by phenotypic testing. These seven strains were part of a group of nine strains from South Africa, and the results may indicate some evolutionary distinction between *C. fetus* subsp. *fetus* strains from South Africa and those from other geographical regions. Conversely, 1 of the 15 *C. fetus* subsp. *venerealis* strains (strain 98/v445) identified by PCR was positive for growth on 1% glycine. Vargas et al. (16) investigated 31 bovine *C. fetus* isolates. Four isolates were tolerant to glycine and, therefore, were classified as *C. fetus* subsp. *fetus* but were considered *C. fetus* subsp. *venerealis* when molecular diagnostic methods (including PCR) were used. Willoughby et al. (19) reported that phenotyping of *C. fetus* subsp. *venerealis* isolates could not be confirmed reliably by multiplex PCR. A specific amplicon of 142 bp was obtained for only 14 of 32 *C. fetus* subsp. *venerealis* isolates. They suspected that the presence of an unusual clone of this subspecies in the United Kingdom could have been the reason for the failure of PCR genotyping. Despite these discrepancies, the PCR assay is recommended by Hum et al. (5), On and Harrington (13), Wagenaar et al. (18), and Vargas et al. (16) as a rapid adjunctive technique for the identification and differentiation of *C. fetus* subspecies.

In our investigations, 103 *C. fetus* isolates were typed by phenotypic testing and the results were confirmed by PCR. For evaluation of the PCR results, the glycine tolerance test was used as reference method. It is noteworthy that the phenotypic methods used in these investigations have been standardized as far as possible, especially concerning the number of culturable *C. fetus* organisms per ml of inoculum. In each investigation, the number of culturable bacteria per ml of inoculum was checked. Additionally, blood agar was preferred to liquid medium in most reactions. Under these stringent conditions, the results of the glycine tolerance test and PCR were compared, and there was a complete correspondence between the results of both methods. Some groups (5, 13, 16, 19) used a multiplex PCR assay to identify and differentiate the *C. fetus* isolates. In our experience, problems can occur because of the insufficient optimization of the assay, such as by the use of different primer concentrations. To avoid any complications from the use of a multiplex PCR system, two separate PCRs were used. Our investigations with the described PCR assay resulted in one remarkable observation. In other investigations (5, 13, 16), which used primers MG3F and MG4R, amplicons with fragment lengths of 960 bp were observed. In our work with these primers, as well as in the studies of Wagenaar et al. (18) and Willoughby et al. (19), the fragment length of the amplicon was approximately 750 bp. The differences in the amplicon lengths in different studies cannot be explained. The *C. fetus* strains used in these investigations were exclusively isolated in Germany, but bgC is of significant importance in countries that breed cattle all over the world. Consequently, the possibility of the existence of *C. fetus* variants in other countries cannot be excluded. For a further evaluation of phenotypic tests and PCR, these investigations should be extended and isolates from different countries should be included. Recently, the possibilities for the identification of *C. fetus* subspecies have been improved. Van Bergen et al. (15) used AFLP analysis to identify *C. fetus* subspecies-specific markers and designed a novel PCR primer set (primer Cf C05) for the identification of *C. fetus* subsp. *venerealis*.

We conclude that at present the traditional phenotypic characterization of *C. fetus* subspecies remains indispensable, but the PCR assay described by Hum et al. (5) constitutes a valuable method for confirmation of the results of phenotypic tests.

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