Comparison of Aerotolerant and Reference Strains of *Campylobacter* Species by Polyacrylamide Gel Electrophoresis

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Acid-phenol extracts of as-yet-unnamed aerotolerant *Campylobacter* sp. strains isolated from animal genitalia and fetuses were compared with extracts of reference strains of *Campylobacter* species by using polyacrylamide gel electrophoresis. The electrophoretic protein profiles confirmed the uniqueness of the aerotolerant isolates within the genus *Campylobacter* and provided a means of differentiation at the species level. The reproducibility of the results demonstrated the value of this technique in taxonomic studies.

Gram-negative helically curved bacteria have been associated with genital disease and abortion in animals since the early part of this century (19). These organisms were initially placed in the genus *Vibrio* (20), but subsequently they were placed in the genus *Campylobacter* (16). There are now two commonly used classifications of *Campylobacter* (18, 22), and this has contributed to much of the confusion that surrounds the terminology of these organisms in the medical and veterinary literature.

In our laboratory, helically curved gram-negative rods have been recovered from a high proportion of bovine, porcine (2, 3), ovine, and equine abortions (unpublished data). Initial attempts to isolate these organisms by using conventional methods for culturing *Campylobacter* species were unsuccessful (11). Characteristically, the isolates were aerotolerant after subculturing, and although they were assigned to the genus *Campylobacter* (12), they were serologically distinct from other species in this genus (3).

It is generally accepted that the tests currently used in classification studies of *Campylobacter* species have limitations and in some instances give unreliable results (1, 4). Electrophoretic studies carried out with acid-phenol (AP) extracts of campylobacters have indicated that this method is a more reliable taxonomic aid than the more commonly used biochemical tests (4, 10). In this paper we describe a study of the electrophoretic patterns of AP extracts of aerotolerant *Campylobacter* isolates and compare these patterns with those of reference strains of *Campylobacter* species.

MATERIALS AND METHODS

Organisms. The 41 aerotolerant *Campylobacter* isolates examined in this study included 25 strains from

cattle, 9 strains from pigs, 5 strains from sheep, and 2 strains from horses. The reference strains of *Campylobacter* included 9 strains of *Campylobacter fetus* subsp. *venerealis*, 10 strains of *C. fetus* subsp. *fetus* (including 5 strains recently isolated from bovine feces), 10 strains of *Campylobacter coli*, 10 strains of *Campylobacter jejuni*, 3 strains of *Campylobacter sputorum* subsp. *sputorum*, 3 strains of *C. sputorum* subsp. *mucosalis*, and 4 strains of *C. sputorum* subsp. *bubulus*. (The reference cultures were obtained from the following sources: National Collection of Type Cultures, London, England; Collection of the Institut Pasteur, Paris, France; B. D. Firehammer, Montana State University, Bozeman; and G. H. K. Lawson, University of Edinburgh, Edinburgh, Scotland.) The reference strains were stored at -70°C , and the aerotolerant *Campylobacter* isolates were held in liquid nitrogen (11). Details concerning the *campylobacter* reference strains used in electrophoretograms (see Fig. 1 and 4) are given in Table 1.

Bacterial cell production. The aerotolerant *Campylobacter* isolates were cultured at 30°C in air for 2 days in semisolid Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 0.75% agar, after which they were inoculated onto agar slopes (blood agar base no. 2 [Oxoid Ltd., London, England]) overlaid with Trypticase soy broth and incubated at 30°C in air for 3 days. The reference strains were cultured at 37°C in air containing 10% CO_2 in semisolid brucella broth (Difco Laboratories, Detroit, Mich.) containing 0.075% agar. After 2 days of growth, the broth cultures were inoculated onto agar slopes overlaid with brucella broth and incubated at 37°C in 10% CO_2 for 3 days. The cells were harvested and centrifuged at $20,000 \times g$ for 30 min. The resulting deposits were washed three times with sterile saline, and then each was suspended to a concentration of 250 mg/ml.

Extraction of protein fraction from bacterial cells. AP extracts were prepared by the method of Morris and Park (10) and stored at -20°C in 0.5-ml portions.

Effect of growth conditions and age of culture on electrophoretic patterns. The effect of growth conditions on the electrophoretic patterns of AP extracts

TABLE 1. *Campylobacter* reference cultures used^a

Strain	Source ^b	Origin
<i>C. fetus</i> subsp. <i>fetus</i> 12351	M.S.U.	Ovine fetus
<i>C. fetus</i> subsp. <i>fetus</i> B/1672	V.R.L.	Bovine feces
<i>C. fetus</i> subsp. <i>venerealis</i> 10354	N.C.T.C.	Bovine vaginal mucus
<i>C. coli</i> 70.80	C.I.P.	Porcine feces
<i>C. jejuni</i> 70.2	C.I.P.	Bovine feces
<i>C. sputorum</i> subsp. <i>sputorum</i> 53.103	C.I.P.	Bull semen
<i>C. sputorum</i> subsp. <i>bubulus</i> 10355	N.C.T.C.	Bull semen
<i>C. sputorum</i> subsp. <i>mucosalis</i> 982	E.U.	Porcine intestine

^a See Fig. 1 and 4.

^b N.C.T.C., National Collection of Type Cultures, London, England; C.I.P., Collection of the Institut Pasteur, Paris, France; M.S.U., B. D. Firehammer, Montana State University, Bozeman; E.U., G. H. K. Lawson, Edinburgh University, Edinburgh, Scotland.

was studied by using aerotolerant *Campylobacter* strain B/600, which grew under the conditions used for both the aerotolerant and reference *campylobacter* strains. Cells were produced under the regime used for the aerotolerant and reference strains, as described above. The effect of age of culture on the electrophoretic patterns was established by using cells of a reference strain and a recent isolate of *C. fetus* subsp. *fetus* prepared as described above.

Vertical slab gel preparation. Slab gels (16 cm by 18 cm by 1.5 mm) were prepared by the method of Morris and Park (10), but they were modified by increasing the concentration of acrylamide from 7.5 to 10%. The gels were made by adding 36 ml of solution A (10 g of acrylamide, 0.275 g of *N,N'*-methylenebisacrylamide, 30 g of urea, 35% [vol/vol] aqueous acetic acid to 100 ml) to 10 mg of ammonium persulfate and 0.12 ml of *N,N,N',N'*-tetramethylethylenediamine and then pouring this preparation into a vertical slab gel mold (LKB, Uppsala, Sweden). A 75% acetic acid solution was layered on top of the gel to ensure that a straight edge was formed during polymerization, which was carried out at 37°C for 90 min. A stacking gel was prepared by adding 9 ml of solution B (4.5 ml of solution A, 4.5 ml of 35% acetic acid) to 7 mg of ammonium persulfate and 0.1 ml of *N,N,N',N'*-*m*-tetramethylethylenediamine. A slot former was inserted, and polymerization was carried out at 37°C for 90 min, after which the slot former was carefully removed and the sample wells were thoroughly washed with 10% acetic acid.

Electrophoresis. A 10% acetic acid solution was added to the upper and lower reservoirs of a vertical electrophoresis unit (LKB). AP extracts containing 100 to 150 µg of protein were applied to the wells through the reservoir fluid with a microsyringe. The upper electrode was used as the anode, and the gels were run at a constant current of 50 mA/gel for 2.5 h at 8°C. The gels were then stained with Coomassie blue for 30 min at 22°C and destained with a methanol-acetic acid-water mixture (7). Photographs of each gel were made as permanent records.

RESULTS

Effect of growth conditions and age of culture on electrophoretic protein profiles. Figure 1 shows that AP extracts of *Campylobacter* strain B/600 grown under different conditions gave identical electrophoretic protein profiles (Fig. 1, lanes a to d). The extracts prepared from the reference strain and the recent isolate of *C. fetus* subsp. *fetus* demonstrated identical profiles (Fig. 1, lanes e and f).

Polyacrylamide gel protein profiles of the aerotolerant *Campylobacter* isolates. Of 41 aerotolerant *Campylobacter* isolates, 38 (92.5%) demonstrated very similar electrophoretic protein profiles, and therefore only representative results are shown in Fig. 2. Figure 2, lanes a to d show the patterns of aerotolerant *Campylobacter* strains isolated from aborted fetuses; lane e shows the pattern of a strain isolated from the placenta of an aborted cow, and lanes f and g show the patterns of strains isolated from a normal bovine placenta and fetus, respectively. The patterns usually consisted of 20 bands, and there were 2 major components. There was some variation in the staining intensities of the various bands, including bands with identical electrophoretic mobilities. Figure 3 shows the patterns of the three aerotolerant *Campylobacter* isolates (Fig. 3, lanes a to c) which differed from the majority of the aerotolerant strains (lane d). Isolates A/49 and A/22 (Fig. 3, lanes a and b, respectively) were quite similar, although not identical. Strain B/600 (Fig. 3, lane c) produced a pattern very similar to the patterns of

FIG. 1. Comparison of AP extracts in 10% vertical slab gels. Lane a, Strain B/600 grown aerobically at 30°C on blood agar base no. 2 with a brucella broth overlay; lane b, strain B/600 grown aerobically on blood agar base no. 2 with a Trypticase soy broth overlay; lane c, strain B/600 grown in 10% CO₂ in air at 37°C on blood agar base no. 2 with a brucella broth overlay; lane d, strain B/600 grown in 10% CO₂ in air on blood agar base no. 2 with a Trypticase soy broth overlay; lane e, reference strain *C. fetus* subsp. *fetus* 12351; lane f, strain B/1672, a recent isolate of *C. fetus* subsp. *fetus*. The gel was stained with Coomassie blue.

FIG. 2. Comparison in 10% vertical slab gels of AP extracts of aerotolerant *Campylobacter* isolates from aborted and nonaborted material. Isolates were obtained from an aborted bovine fetus (lane a), an aborted porcine fetus (lane b), an aborted ovine fetus (lane c), an aborted equine fetus (lane d), the placenta of an aborted bovine (lane e), the placenta of a nonaborted bovine (lane f), and a nonaborted bovine fetus (lane g). The gel was stained with Coomassie blue.

the *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* strains examined (Fig. 4, lanes g and h).

Comparison of aerotolerant *Campylobacter* isolates with reference *Campylobacter* strains. As Fig. 4 shows, a representative sample of the aerotolerant *Campylobacter* isolates studied (Fig. 4, lane a) appears to be quite distinct from all of the reference strains examined. *C. sputorum* subsp. *sputorum* (Fig. 4, lane b) and *C. sputorum* subsp. *bubulus* (lane c) had identical

FIG. 3. Comparison in 10% vertical slab gels of AP extracts of those aerotolerant *Campylobacter* isolates which differed from the main group. Lane a, Strain A/49 from an aborted porcine fetus; lane b, strain A/22 from an aborted porcine fetus; lane c, strain B/600 from an aborted bovine fetus; lane d, reference sample from the main group of aerotolerant *Campylobacter* isolates. The gel was stained with Coomassie blue.

patterns, which differed from that of *C. sputorum* subsp. *mucosalis* (lane d). *C. sputorum* subsp. *mucosalis*, *C. coli* (lane e), and *C. jejuni* (lane f) generally produced individually distinct patterns. *C. fetus* subsp. *venerealis* (lane g) and *C. fetus* subsp. *fetus* (lane h) had identical patterns, which differed from the patterns of *C. jejuni* and the other strains examined. It was obvious that although there were differences between the patterns of the aerotolerant *Campylobacter* isolates and those of the reference strains, there were several bands with common electrophoretic mobilities.

DISCUSSION

In agreement with other workers (9, 14), we found that the different growth conditions used to produce bacterial cells did not affect the composition of the electrophoretic protein profiles (Fig. 1), thus permitting a valid comparison between the aerotolerant *Campylobacter* isolates and reference strains. The electrophoretic patterns obtained by using recent isolates of *C. fetus* subsp. *fetus* were identical to those of the corresponding reference strains. This indicated that the age of a culture and storage apparently did not affect the reproducibility of *Campylobacter* electrophoretic patterns.

It has been reported previously (10) that marked differences demonstrated by electrophoresis among bovine, porcine, and ovine isolates of *Campylobacter* indicate that pigs are unlikely to serve as a source of infection for cattle and sheep and vice versa. We believe that this does not apply to the aerotolerant *Campylobacter* isolates examined in this study, as the electrophoretic patterns of these isolates were very similar irrespective of origin. Like other workers (10), we were unable to differentiate between

FIG. 4. Comparison in 10% vertical slab gels of AP extracts of an aerotolerant *Campylobacter* isolate and *Campylobacter* reference strains. Lane a, Aerotolerant *Campylobacter* isolate; lane b, *C. sputorum* subsp. *sputorum* 53.103; lane c, *C. sputorum* subsp. *bubulus* 10355; lane d, *C. sputorum* subsp. *mucosalis* 982; lane e, *C. coli* 70.80; lane f, *C. jejuni* 70.2; lane g, *C. fetus* subsp. *venerealis* 10354; lane h, *C. fetus* subsp. *fetus* 12351. The gel was stained with Coomassie blue.

isolates recovered from aborted and nonaborted animals. Variation in the intensity of staining within the patterns of the aerotolerant *Campylobacter* isolates (Fig. 2) provides evidence of a quantitative difference in the various proteins present (13). The three aerotolerant *Campylobacter* isolates giving electrophoretic protein patterns that were atypical of the main group (Fig. 3) had other different characteristics. Strains A/49 and A/22 were more tolerant of a wide range of inhibitory substances than the main group of aerotolerant *Campylobacter* isolates (unpublished data). Strain B/600 also differed from the remainder of the group in that it grew in the presence of 1% glycine and 4% nalidixic acid, as did *C. fetus* subsp. *fetus* (unpublished data). It is interesting that strain B/600 gave an electrophoretic protein profile identical to the profiles of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (Fig. 4, lanes g and h).

Although the aerotolerant isolates which we examined are assigned to the genus *Campylobacter*, the typical electrophoretic pattern for these isolates is quite distinct from the pattern of any of the reference strains examined. These findings, combined with the cultural (2), biochemical (11, 12), and serological (3) characteristics of the aerotolerant isolates, confirm the uniqueness of this hitherto unrecognized species within the genus *Campylobacter*.

The results shown in Fig. 4 demonstrate that by using the gel patterns of AP extracts, we can readily differentiate *Campylobacter* to at least species level. Other workers have successfully applied this technique to the classification of *Mycoplasma* species (14), *Corynebacterium* species (6), *Brucella* species (8), and bacterial isolates of gingival crevice floras (8). AP extraction is thought to solubilize the hydrophobic portions of membrane proteins (21), which may be used as a measurement of genetic relatedness (15). The marked differences in the electrophoretic patterns of *C. jejuni* and *C. fetus* extracts support the classification of these organisms as two distinct species (17, 22). It is interesting that *C. sputorum* subsp. *mucosalis* has an electrophoretic pattern which differs from the patterns of the other two subspecies of *C. sputorum* examined. Further studies of intestinal campylobacters in which we are using electrophoresis of AP extracts are currently underway.

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