Isolation of *Campylobacter fetus* subsp. *fetus* from a Patient with Cellulitis

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*Campylobacter fetus* subsp. *fetus* is a gram-negative, slender, spirally curved bacterial pathogen. It has been isolated from human blood, spinal fluid, and abscesses, but cellulitis associated with bacteremia is rare. We report its isolation from a blood culture of a human patient with cellulitis as well as difficulties encountered in determining the identity of the subspecies of *C. fetus*.

**CASE REPORT**

A 78-year-old female came to the emergency department of the Royal Victoria Hospital in Montreal with a 5-day history of left leg pain and swelling and a 1-day history of fever and shaking chills. She had been diagnosed with diabetes mellitus type II 9 years previously, cryptogenic liver cirrhosis with ascites 2 years previously, and hepatocellular carcinoma 3 months previously. Her medications at admission included insulin, furosemide, spironolactone, famotidine, hydroxyzine, and lactulose. She had recently been undergoing repeated paracentesis to relieve symptoms of abdominal distension. She had had no recent travel or any exposure to animals or unpasteurized food products. Physical examination revealed an oral temperature of 38.1°C, a distended but nontender abdomen, and diffuse tender, red swelling of her left lower extremity from her toes to just below the knee.

Her laboratory tests returned results for levels of hemoglobin (105 g/liter), platelet count (121 × 10^9/liter), white blood cell count (9.47 × 10^9/liter), neutrophils (8 × 10^9/liter), serum creatinine (144 μmol/liter), serum alkaline phosphatase (148 U/liter), serum alanine transferase (23 U/liter), and total bilirubin (19.60 μmol/liter). Except for the slightly elevated neutrophil count, these results were not significantly different from her most recent previous results. Chest X rays revealed no infiltrate, and abdominal ultrasound test results were unchanged from previous results. A clinical diagnosis of cellulitis was made, and an intravenously administered clindamycin regimen for the patient was begun. Culture of ascitic fluid was negative for spirochete-like cells, but on the second hospital day, the blood cultures drawn at admission were reported to be positive for spirochete-like cells. Although the patient was clinically improving, her treatment was changed to intravenously administered ticarcillin-clavulanate. Another set of blood cultures drawn on the second hospital day were again positive for spirochete-like cells, but blood cultures drawn on the fourth hospital day gave negative results. The patient’s leg improved steadily, and treatment was changed to oral amoxicillin-clavulanate on the tenth hospital day and was continued for an additional week. The patient was discharged from the hospital on the fourteenth hospital day, and her state of health 1 year after discharge has remained stable without a recurrence of leg infection.

The two blood cultures drawn at admission and on the second hospital day were reported positive for spirochete-like cells, since the bacterial cells seen by dark-field microscopic examination of the cultures resembled spirochetes (Fig. 1). No other morphological forms of microorganisms were seen in scans of the wet mounts, nor did the spirochete-like cells exhibit any rapid darting-type motility. Small volumes of the blood cultures were inoculated into New Oral Spirochete broth (1) and Brewer’s meat broth (BMB). At the same time, the blood cultures were streak inoculated with a bacteriological loop on the surface of blood agar plates. All cultures were incubated anaerobically in an anaerobic chamber (5% CO_2, 10% H_2, 85% N_2) at 37°C for 48 h. No growth occurred in New Oral Spirochete broth, but a surface pellicle of bacterial cells formed in BMB. Confluent growth was found in the primary streaks of inoculation on the blood agar plates, but small, smooth, round, and slightly raised colorless (translucent) colonies (about 0.5 mm in diameter) developed at the diluted sites of streaking. All colonies showed similar morphological characteristics. No hemolysis or hemolytic zones were noted. Single colonies were picked and inoculated into BMB; a pellicle of growth occurred in all tubes incubated aerobically. One culture was chosen and maintained by weekly transfers in BMB under aerobic conditions at 37°C.

The pellicle in BMB, when shaken gently, dispersed as a turbid culture in the broth. Gram staining of the cells revealed gram-negative slender single cells of a spiral form and long forms that resembled spirochetes. Similar cell morphology was observed after basic fuchsin staining. However, negative stain-
ing with nigrosin revealed not spirochete-like cells but campylobacter-like cells, i.e., S- and gull-shaped cells. This appearance probably occurred because no harsh treatment, such as heat fixation, was imposed on the cells to distort or shrink them. But the most characteristic revelation of the identity of the culture was obtained by dark-field observation of the dispersed cells from the pellicle in BMB. This was rendered possible by the scattering of light by the cells, thereby increasing contrast as well as the apparent diameters of the cells. Furthermore, corkscrew-like darting motility of the cells was seen (since the sample was observed live); a typical morphology of the individual cells (slender spiral rods that appear as comma, S, and gull shaped when two cells form a short chain) was also evident. It could be surmised that the adhesion of individual cells and the consequent formation of long filaments gave the impression of spirochete shapes when seen microscopically after the application of staining techniques that shrank the cell images and did not enhance their diameters (such as occurs by light scattering in dark-field microscopy). The motility and cellular morphology from images of nigrosin staining and dark-field illumination suggested that the culture could be that of a species of Campylobacter.

Further cell morphology characterization showed that the cells were flagellated at one or both poles. Such flagellation was noted by examination of the cells by light microscopy after Ziehl’s carbol fuchsin staining with use of Gray’s mordant. After negative staining with 2% phosphotungstic acid, electron microscopy confirmed the presence of flagella. The gram-negative nature of the cells was also confirmed with thin-section electron micrographs revealing typical membranous cell wall structure (Fig. 2).

The classification of the genus Campylobacter is primarily phylogeny based, and phenotypic Campylobacter fetus subspecies determination methods (such as growth in the presence of 1% glycine, selenite reduction, and cefoperazone resistance) for the genus are unreliable (4). Some phenotypic cultural characteristics of the isolate strain revealed by assays that were carried out by us are summarized as follows. Under aerobic incubation conditions at 35°C, there was pellicle growth of the isolate strain in BMB (as before) as well as in thioglycolate broth but not in nutrient broth; no growth occurred on blood agar plates or nutrient agar plates. Using a CampyPak envelope (Becton Dickinson, Sparks, Md.) under microaerophilic conditions of incubation (35°C) in an anaerobic jar, growth of the culture was also seen to occur as a pellicle in BMB and thioglycolate broth but not in nutrient broth; small colorless colonies developed on blood agar plates (no hemolysis) and on nutrient agar plates. No growth of the subspecies occurred at 42°C. A comparison of the phenotypic properties of C. fetus subsp. fetus and C. fetus subsp. venerealis in Bergey’s Manual of Systematic Bacteriology revealed that sensitivity to 1% glycine is the only differentiating characteristic between the two subspecies (8). All other phenotypic properties, such as catalase reaction, oxidase reaction, inhibition by 2% NaCl and by 2% ox bile, and H2S production, are similar. Indeed, the strain harbored by our patient was determined by us to be inhibited by 1% glycine in brucella broth, suggesting that it was C. fetus subsp. venerealis and differentiating it phenotypically from C. fetus subsp. fetus, which is not inhibited. However, glycine-tolerant variants of C. fetus subsp. venerealis have been described previously (5). Such observations made our single differing test result suspect and mitigated our molecular approaches to subspeciation.

For speciation of the isolate strain, a centrifuged pellet of the cells grown in BMB was submitted to MIDI Labs Inc., Newark, Del., for 16S rRNA gene sequence analysis. The protocol used to generate the 16S rRNA gene sequence was as follows. The 16S rRNA gene was PCR amplified from genomic DNA isolated from the pelleted bacterial cells. The primers used for the amplification correspond to Escherichia coli positions 905 and 1540. Amplification products were purified from excess primers and dNTPs by using Microcon 100 (Amicon) molecular weight cutoff membranes and were checked for quality and quantity by running a portion of the products on an agarose gel.

Cycle sequencing of the 16S rRNA amplification products was carried out by using AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Excess dye-labeled terminators were removed from the sequencing reaction mixtures by using a Sephadex G-50 spin column. The products were collected by centrifugation, dried under vacuum, and frozen at −20°C until ready to load. Samples were resuspended in a solution of formamide-blue dextran-EDTA and denatured prior to loading. The samples were electrophoresed on an ABI Prism 377 DNA sequencer. Data were analyzed using PE/Applied Biosystems DNA editing and assembly software. Shown is a neighbor-joining phylogenetic tree (7) constructed by using the top ten alignment matches (Fig. 3). The data presented indicate that the unknown isolate strain is C. fetus subsp. venerealis, since there was complete alignment of 1,510 bp with 0% genetic difference between the sequence of the unknown isolate strain and that of C. fetus subsp. venerealis in the Applied Biosystems MicroSeq database of MIDI Labs.

Identification of the unknown isolate strain based on the 16S rRNA gene sequence (1,510 bp) was made by MIDI Labs, Inc., using MicroSeq software and validated sequence libraries. The phylogenetic data generated suggested that the unknown isolate strain is C. fetus subsp. venerealis. We have supplemented
FIG. 2. Electron micrograph of a thin section of *C. fetus* subsp. *venerealis* cells reveals membranous cell walls typical of gram-negative prokaryotes as well as typical *Campylobacter* cell morphology (shape). Bar, 200 nm.
this information by aligning the gene sequence of our isolate strain as obtained by MIDI Labs with the 16S rRNA gene sequences of two C. fetus subsp. venerealis ATCC 19438 genes deposited in the GenBank database (accession nos. M65011 and L14633). Alignment was also made with the 16S rRNA gene sequence of C. fetus subsp. fetus ATCC 27374 gene (accession no. M65012). There was 100% alignment of the MIDI Labs gene sequence with the available GenBank gene sequences (1,448 bp) of C. fetus subsp. venerealis but 0.1% difference in alignment with the gene sequence of C. fetus subsp. fetus (the same as that obtained by MIDI Labs).

However, a 0.1% difference in sequence alignment between the sequence of C. fetus subsp. venerealis (AF482990, M65011, and L14633) and C. fetus subsp. fetus (M65012) represents no more than 2 base pairs of T and C at positions 525 and 725 of our isolate strain. Besides, it has been documented in various taxonomic studies (2, 5, 6) that the 16S rRNA gene sequence of C. fetus is too conserved for subspecies-level differentiation. Our initial assumption that our isolate strain was C. fetus subsp. venerealis was not tenable, and an alternate test was required. For example, genotyping of C. fetus subsp. fetus and C. fetus subsp. venerealis by PCR and amplified fragment length polymorphism showed a clear discrimination between them (5, 9).

Consequently, for identification to the subspecies level, a C. fetus subspecies-specific multiplex PCR assay was performed as previously described by other workers (3, 5). Genomic DNA was isolated from the pelleted bacterial cells by using the DNeasy Tissue kit (Qiagen Inc., Mississauga, Ontario, Canada). The PCR assay was performed in 50 μl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.0 mM MgCl2, 0.1 mM each deoxynucleoside triphosphate, 250 ng of DNA isolates as the template, 2.5 U of Taq DNA polymerase, and 25 pmol of each primer. For C. fetus subsp. fetus the primers used were CFF (5′GGTAGCGCCGACGTGCTAAAG T3′) and CFR (5′TAGCATCAATAACGACAACCT3′), and for C. fetus subsp. venerealis the primers were VenF (5′CTTA GCGATTGCATTTGCGATT3′) and VenR (5′GCTTTT GAGATAACATAAGGCTT3′). In addition, each PCR mixture contained a reagent negative control that consisted of all of the reagents used in the PCR except the DNA template. Amplification was performed in a GeneAmp thermal cycler (Applied Biosystems), with an initial denaturation step (95°C, 4 min) followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 20 s, and primer extension at 72°C for 1 min. The cycling was terminated by a final extension step at 72°C for 10 min. Samples of 10 μl were separated on a 2% agarose gel containing 0.5 μg of ethidium bromide (Sigma)/ml in Tris-borate-EDTA buffer (90 mM Tris-borate, 2 mM EDTA) and run at 80 V. Amplicons were detected and photographed under a UV transilluminator to visualize the amplified DNA pattern. C. fetus subsp. fetus is characterized by the observation of a single amplicon of approximately 960 bp, whereas C. fetus subsp. venerealis identification is based on an additional amplicon of 142 bp. Observation of a single amplicon of 960 bp for our isolate strain as well as for C. fetus subsp. fetus (ATCC 25936) indicates that our isolate strain was C. fetus subsp. fetus.

As has been observed previously, the campylobacter cells in primary blood cultures resembled spirochetes (Fig. 1). The cells appeared slender and helical, with no exhibition of any type of darting motility. After transfer and subsequent Gram staining and other aniline dye staining requiring prior heat fixation, cells of the pure cultures of the organism continued to resemble spirochetes. Typical characteristics of comma-, S- and gull-shaped cell morphologies were seen microscopically only after mild staining procedures (e.g., negative staining) and dark-field examination; additionally, corkscrew-like darting motility was seen only in microscopic dark-field illumination because of the presence of live cells in the specimen. The scattering of light reflected by the cells with the use of this latter microscopic technique, and the consequent increase of cell image diameters, enabled the visualization of typical campylobacter cell morphology. Such typical cells may also be observed after flagellum staining by Gray’s method, since the cells as well as the flagella are thickened by the mordant dye used. Of course, electron micrographs, both negatively stained and thin sectioned, reinforced the determination of the campylobacter cell morphology of the unknown isolate strain (Fig. 2). An appreciation of such observational difficulties (which can introduce an artifact of apparent spirochetoid presence) can facilitate the recognition of C. fetus in more cases of human infection.

The fact that clinical and epidemiological differences exist between C. fetus subsp. fetus and C. fetus subsp. venerealis confirms that accurate subspecies identification is essential. For instance, the latter subspecies causes venereal campylobacteriosis in animals and is rarely isolated from human blood. Human infections caused by C. fetus subsp. fetus include enteritis, abortion, bacteremia, endocarditis, and meningitis. The phenotypic differentiation between the two subspecies based on 1% glycine tolerance or susceptibility is suspect, as mentioned above. Further subspecies identification carried out based on 16S rRNA sequencing alone indicated that our isolate strain was C. fetus subsp. venerealis. However, additional analyses using multiplex PCR assays confirmed that our isolate strain was indeed C. fetus subsp. fetus. Such a finding demonstrates that multipronged molecular approaches are necessary for definitive strain identification of the campylobacters.

**Nucleotide sequence accession number.** The complete nucleotide sequence of the 16S rRNA gene from the isolated bacterium was deposited in GenBank with the accession number AF482990.
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REFERENCES