Campylobacter fetus subsp. testudinum subsp. nov., isolated from humans and reptiles

Collette Fitzgerald,1‡ Zheng chao Tu,2 Mary Patrick,1 Tracy Stiles,3 Andy J. Lawson,4 Monica Santovenia,1 Maarten J. Gilbert,5,6 Marcel van Bergen,5,7 Kevin Joyce,1 Janet Pruckler,1 Steven Stroika,1 Birgitta Duim,5,6 William G. Miller,8 Vladimir Loparev,1 Jan C. Sinnige,9 Patricia I. Fields,1 Robert V. Tauxe,1 Martin J. Blaser2 and Jaap A. Wagenaar5,6,7

1Biotechnology Core Facility Branch, Division of Scientific Resources, National Center for Emerging and Zoonotic Infectious Diseases, CDC, Atlanta, GA, USA
2Department of Medicine, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA
3Massachusetts Department of Public Health, Jamaica Plain, MA, USA
4Public Health England, London, UK
5Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
6WHO Collaborating Center for Campylobacter/OIE Reference Laboratory for Campylobacteriosis, Utrecht, The Netherlands
7Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands
8USDA, ARS, WRRC, Produce Safety and Microbiology Research Unit, Albany, CA, USA
9Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands

A polyphasic study was undertaken to determine the taxonomic position of 13 Campylobacter fetus-like strains from humans (n=8) and reptiles (n=5). The results of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS and genomic data from sap analysis, 16S rRNA gene and hsp60 sequence comparison, pulsed-field gel electrophoresis, amplified fragment length polymorphism analysis, DNA–DNA hybridization and whole genome sequencing demonstrated that these strains are closely related to C. fetus but clearly differentiated from recognized subspecies of C. fetus. Therefore, this unique cluster of 13 strains represents a novel subspecies within the species C. fetus, for which the name Campylobacter fetus subsp. testudinum subsp. nov. is proposed, with strain 03-427T (=ATCC BAA-2539T=LMG 27499T) as the type strain. Although this novel taxon could not be differentiated from C. fetus subsp. fetus and C. fetus subsp. venerealis using conventional phenotypic tests, MALDI-TOF MS revealed the presence of multiple phenotypic biomarkers which distinguish Campylobacter fetus subsp. testudinum subsp. nov. from recognized subspecies of C. fetus.

1Present address: National Campylobacter and Helicobacter Reference Laboratory, Enteric Diseases Laboratory Branch, Division of Foodborne, Waterborne and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road NE, Mailstop C-03, Atlanta, GA 30329, USA.

Abbreviations: AFLP, amplified fragment length polymorphism; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PFGE, pulsed-field gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains ATCC 49616T, CCUG 56292T, ATCC 33559T, ATCC 33237T, ATCC 35224T and ATCC 27374T are JX912503–JX912508, respectively, and of strains D4355, D6659, D6856, D6683, ATCC 19438T, ATCC 33236T, ATCC 51209T, ATCC 35217T, CCUG 48653T, ATCC 33560T, NCTC 13004T, CCUG 55786T, ATCC 35221T, ATCC 43264T, ATCC 33238T, ATCC 51146T, ATCC 35980T and NCTC 11541T are JX912510–JX912527, respectively.

Disclaimer: Use of trade names is for identification only and does not imply endorsement by the CDC or by the US Department of Health and Human Services.

Two supplementary tables and six supplementary figures are available with the online version of this paper.
The type species of the genus Campylobacter, Campylobacter fetus, has two subspecies: Campylobacter fetus subsp. fetus and Campylobacter fetus subsp. venerealis (Sebald & Véron, 1963; Véron & Chatelain, 1973). C. fetus subsp. fetus, which was originally described as a species of the genus Vibrio associated with disease in cattle (Smith & Taylor, 1919), has been isolated from a wide variety of animal hosts and human source sites. It is considered to be of both public health and veterinary significance, where it is most commonly associated with systemic infection in immunocompromised patients and abortion in cattle and sheep. In contrast, C. fetus subsp. venerealis is considered to be of primarily veterinary importance, where it is the cause of bovine genital campylobacteriosis.

While more commonly isolated from ruminant production animals such as cattle and sheep, C. fetus has also been isolated from various reptile species (Wang et al., 2013), including an asymptomatic box turtle (Terrapene carolina) from California (Harvey & Greenwood, 1985), a blotched blue-tongued skink (Tiliqua nigrolutea) and a western hog nose snake (Heterodon nasicus) from the UK (Dingle et al., 2010). Strains of C. fetus isolated from reptiles have previously been shown to be genetically divergent from strains of C. fetus isolated from humans, with the suggestion that they may represent a distinct taxonomic group, based on the results from 16S rRNA, recA and sapD gene sequence analyses and multi-locus sequence typing (Dingle et al., 2010; Tu et al., 2005); molecular methods to differentiate them have been developed (Tu et al., 2001). The first confirmed isolation of a human of strains of C. fetus with markers of reptile origin occurred in New York in 2003 (Tu et al., 2004b), with two isolates (strains 03-427 and 03-445) recovered 37 days apart from a patient symptomatic due to recurrent C. fetus bacteraemia.

As part of ongoing national surveillance efforts, presumptive campylobacters are sent voluntarily from state and local health departments and collaborating partners to the National Campylobacter and Helicobacter Reference Laboratory at the Centers for Disease Control and Prevention (CDC) for further characterization. Between December 2004 and March 2007 the CDC identified four C. fetus-like strains that also had markers of reptile origin. Screening of additional strains of C. fetus in the CDC and New York University Campylobacter culture collections, using a sap insertion PCR (Tu et al., 2004a), identified two additional human strains of C. fetus with markers of reptile origin (strains D4335 and 91-2). Epidemiological information from these seven case-patients described above is summarized elsewhere (Patrick et al., 2013).

Using a polyphasic approach, our aim was to characterize these eight human strains, along with five isolates of C. fetus from reptiles, to determine their taxonomic position. Strain details are provided in Table S1 (available in the online Supplementary Material).

For initial phenotypic and molecular characterization, strains were cultured on heart infusion agar (HIA) with 5% rabbit blood and incubated under microaerobic conditions at 37 ºC for 48 h. The phenotypic characteristics of these strains were determined as described by Barrett et al. (1988). The biochemical characteristics tested included a Gram-stain reaction, analysis of catalase, indoxyl acetate and oxidase activities, as well as acid production from glucose, hydrolysis of hippurate and urea, H2S production in triple-sugar iron agar, and reduction of nitrate, nitrite and triphenyltetrazolium chloride. Growth tests included temperature tolerance at 25 ºC, 37 ºC and 42 ºC under microaerobic conditions, growth under aerobic and anaerobic conditions on 5% blood agar at 37 ºC as well as NaCl and glycine tolerance and growth on MacConkey agar; results are shown in the novel taxon description below. All strains shared the C. fetus-specific phenotype of growing at 25 ºC under microaerobic conditions at 48 h, a characteristic that allows differentiation of this species from other recognized species of the genus Campylobacter.

Genus- and species-specific PCR assays (Hum et al., 1997; Linton et al., 1996) as well as a serotype-specific PCR based on the sap locus (Tu et al., 2004b) and a sap insertion PCR, previously reported to be specific for strains of C. fetus of reptile origin (Tu et al., 2004a), were also performed. Antimicrobial susceptibilities were determined by broth microdilution (Sensititre; Trek Diagnostics) according to the manufacturer’s instructions and interpreted using Clinical and Laboratory Standards Institute (CLSI) criteria when available. Phenotypic characterization, and genus- and sap-PCR identified all human strains and three of five strains from reptiles as serotype A C. fetus subsp. fetus with sapA homologues, which encode C. fetus-specific surface layer proteins that together with lipopolysaccharide are associated with this serotype (Tu et al., 2004a). The remaining two C. fetus strains from a turtle and a skink, possessed both sapA and sapB homologs as previously described for the turtle strain 85-387 (Tu et al., 2001). All 13 strains were negative in the two C. fetus-PCR assays (Hum et al., 1997; Linton et al., 1996) but positive for Campylobacter hyointestinalis in a C. fetus/C. hyointestinalis multiplex PCR (Linton et al., 1996), which differentiates them from recognized subspecies of C. fetus. All but one (85-387) of the strains was positive in the sap insertion PCR, with three different amplicon sizes observed. All isolates were resistant to nalidixic acid.

To determine the phylogenetic position of this taxon group, genomic DNA was extracted using the ArchivePure DNA Cell/Tissue kit (5Prime) and 16S rRNA gene sequences were generated as previously described (Weisburg et al., 1991). Sequence assembly and alignment of the 16S RNA gene sequences of the C. fetus group and selected Campylobacter and other epsilonproteobacterial taxa were performed using Lasergene version 9.0 (DNASTAR). The alignment was edited to remove ambiguous bases. The resulting 1399 nt alignment was analysed using BioNumerics version 5.1 (Applied Maths). Distances were corrected using the Jukes–Cantor algorithm; the tree was built by the neighbour-joining method, with Escherichia coli K-12 (GenBank accession number U00096) as the outgroup. Bootstrap
values were determined by using 500 repetitions (Fig. S1). The 16S rRNA gene sequence similarity between all 13 strains was 100% The isolates formed a distinct clade with the two subspecies of C. fetus (>99% 16S rRNA gene sequence similarity), followed by C. hyointestinalis (98.5%) as the next closest phylogenetic neighbour.

Additionally, the partial Hsp60 (GroEL) protein sequences of C. fetus subsp. fetus and C. fetus subsp. venerealis (GenBank accession numbers AAZ94791.1 and AAZ94779.1, respectively) and partial Hsp60 protein sequences extracted from the predicted proteomes (see below) of two strains of the novel 16S taxon (03-427T and SP3), C. fetus subsp. fetus (strain 82-40) and other species of the genus Campylobacter were aligned using ClustalX. A neighbour-joining phylogenetic tree was reconstructed using MEGA software version 5.1 (Tamura, Peterson, Peterson et al., 2011); bootstrap values were determined using 500 repetitions (Fig. S2). Here also the two novel strains of C. fetus form a clade distinct from both the two subspecies of C. fetus and other species of the genus Campylobacter.

This divergence was further supported by pulsed-field gel electrophoresis (PFGE) using Smal and KpnI, and amplified fragment length polymorphism (AFLP) analysis using HindIII and HhaI, both performed as previously described (Ribot et al., 2001; van Bergen et al., 2005). Numerical analysis of both the PFGE and the AFLP profiles of the 13 strains representing this distinct taxon group were divergent from both subspecies of C. fetus (Figs S3 and S4).

To support the designation of this new taxon, selected strains were subsequently examined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS, whole genome sequencing and DNA--DNA hybridization. Proteins from two strains of the novel taxon [C. fetus strain SP3, originating from an asymptomatic captive-held western hognose snake (Heterodon nasicus) and strain 03-427T, originating from a human], and three strains of C. fetus subsp. fetus and one strain of C. fetus subsp. venerealis were extracted with ethanol and formic acid according to the standard protocol supplied by Bruker. Spectra were acquired with a MicroFlex LT mass spectrometer (Bruker Daltonics) and recorded in a mass range from 2000 to 20 000 Da. For each strain, eight technical replicates were spotted on the target plate. Three spectra were acquired per spot, resulting in 24 spectra per strain. In addition, for each strain, one mixed Campylobacter/Bruker Test Standard (BTS) spot was included for calibration. Each spectrum was exported and visually inspected with BioNumerics version 7.0 software (Applied Maths). After processing (baseline subtraction and smoothing) 24 spectra were summarized in a newly generated spectrum. Peak-based clustering analysis confirmed phenotypic differentiation of these strains from recognized subspecies of C. fetus and other closely related species of the genus Campylobacter (Fig. 1). A large number of peaks are present in both C. fetus and the novel subspecies patterns and indicate the close relationship of the novel taxon to C. fetus (Fig. S5).

Whole genome sequences of two strains of the novel subspecies were obtained with a Roche 454 FLX Genome sequencer and titanium chemistry. A minimum of 221 000 mate-paired and shotgun reads were assembled for each strain to provide draft genome sequences with a mean coverage of 44–62 x. Using Perl scripts, the draft contigs were assembled into a single predicted contiguous sequence. Sequences across the contig junctions and sequences of putative split genes, sap loci and homopolymeric GC tracts were confirmed with Sanger sequencing and bacterial optical mapping (Gilbert et al., 2013). Both genome sequencing projects have been deposited at the NCBI (Genome Bioprojects PRJNA177177 and 177181). For genome alignments, the genome sequence of C. fetus subsp. fetus 82-40 in the GenBank database (accession number NC_008599) was used.

The predicted proteomes of strains 03-427T and SP3 were determined and compared with other members of the C. fetus group and related species of the genus Campylobacter using pair-wise BLASTP analysis. The core proteomes (i.e. proteins conserved across all tested taxa) for these Campylobacter taxa were identified and the average amino acid sequence identity of these core proteins between any two taxa was used as a determinant of genetic divergence. This is based on Lan and Reeves who proposed that the core genome is the principal genomic unit defining bacterial species (Murate et al., 2001). Analyses of the 03-427T and SP3 genomes indicated a high degree of both synteny and similarity in core proteins, when compared with the genomes of C. fetus subsp. fetus and C. fetus subsp. venerealis, however, although the core protein sequences of strains 03-427T and SP3 were >99% identical on average, only 95–96% average amino acid sequence identity was observed between the core protein sequences of these two strains and those of either C. fetus subsp. fetus and C. fetus subsp. venerealis (Fig. S6). Average amino acid sequence identity between C. fetus and the most closely related species C. hyointestinalis and Campylobacter lanienae was <80%. As in all known strains of C. fetus, S-layer (sap) coding regions were present in both genomes; these loci were not identified in the genomes of the closely related species C. hyointestinalis and C. lanienae. These results indicate that the reptile-associated strains of C. fetus form a distinct genetic cluster within the C. fetus taxonomic group, which is clearly separated from the most closely related, recognized species of the genus Campylobacter.

DNA–DNA relatedness was determined by relative binding ratios, using the free-solution hydroxyapatite method performed at both the optimal (55 °C) and the stringent (70 °C) temperatures, as previously described (Brenner et al., 1982). In vitro labelling of DNA was performed by using [32P]dCTP provided in a nick translation kit (Bethesda Research Laboratories), as directed by the manufacturer. Under the optimal reassociation conditions, strains assigned to the same taxon exhibited mean DNA binding values ranging from 85 to 99% (novel taxon group of strains) and 90–100% (both subspecies of C. fetus) (Table S2). The DNA–DNA relatedness values between
members of these three taxa were lower, ranging from 73 to 80 %, although still above the threshold suggested for species delineation (Stackebrandt & Goebel, 1994). Relatedness was less than 44 % to DNAs from two other species of the genus *Campylobacter*.

The results of the MALDI-TOF MS and the genomic data from *sap* analysis, 16S rRNA and hsp60 gene sequence comparisons, PFGE, AFLP, DNA–DNA hybridization and whole genome sequencing demonstrated that the 13 *C. fetus*-like strains were closely related to *C. fetus* but were differentiated from the recognized subspecies of *C. fetus*. This novel taxon cannot be differentiated from *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* using conventional phenotypic tests, yet MALDI-TOF MS revealed the presence of multiple phenotypic biomarkers that distinguish this novel taxon from both the recognized subspecies of *C. fetus* and other nearest phylogenetic neighbours.

The unique phenotypic and genotypic characteristics justify and warrant the formal classification of this taxon as a novel subspecies of *C. fetus*, for which we propose the name *Campylobacter fetus* subsp. *testudinum* subsp. nov. Strains from this novel taxon group are opportunistic, given clinical isolates were most commonly isolated from immunocompromised patients, and appear to have a particular epidemiological setting (Patrick et al., 2013). A strong host association with reptiles is observed to date; this host range characteristic differentiates this taxon group from recognized subspecies of *C. fetus*. Further work is needed to investigate the clinical outcomes, associated exposures and public health significance of this new taxon group, especially in Asian–American communities.

**Description of Campylobacter fetus subsp. testudinum subsp. nov.**

*Campylobacter fetus* subsp. *testudinum* (tes.tu’di.num. N.L. pl. n. *Testudines* scientific name of an order to which turtles belong; N.L. gen. pl. n. *testudinum* of *Testudines*).

Strains have phenotypic properties typical of the species (Barrett, Patton and Morris, 1988). Oxidase- and catalase-positive. Hippurate and indoxyl acetate are not hydrolysed. Urease-negative. Nitrate and triphenyltetrazolium chloride are reduced but not nitrite. Under microaerobic conditions, growth occurs at 25 °C and 37 °C and the majority of strains (69 %) grow at 42 °C. Growth on 5 % blood agar under anaerobic conditions is observed. A majority of strains (69 %) do not grow in air at 37 °C. Acid from glucose is not detected. H₂S negative in TSI agar. Growth is observed on medium containing 1 % glycopeptid. but not on a medium containing 3.5 % NaCl under microaerobic conditions. A majority of strains (85 %) grow microaerobically on MacConkey agar. Strains of this subspecies can be distinguished phenotypically from *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* by MALDI-TOF MS analysis.

The type strain is 03-427T (=ATCC BAA-2539T=LMG 27499T), isolated from human blood culture in 2003 (Tu et al., 2004b).

**Acknowledgements**

We thank Jean Lee and Jennifer Huang (CDC) for interviewing case-patients, and Emily Harvey (Massachusetts Department of Public Health) and Erin Delaune (Louisiana Office of Public Health) for gathering historical epidemiological information on case-patients. We also thank Lexie Vaughn (CDC) for assisting with the susceptibility testing, Jean Euzéby (École Nationale Vétérinaire, France) for his assistance with naming the novel subspecies, and Hans Kusters (University Medical Center, Utrecht) and Olga Stuchlik (CDC) for assistance with the MALDI-TOF MS analysis.

**References**


