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

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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 03-427^T (=ATCC BAA-2539^T=LMG 27499^T) 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*.

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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 49616^T, CCUG 56292^T, ATCC 33559^T, ATCC 33237^T, ATCC 35224^T and ATCC 27374^T are JX912503–JX912508, respectively, and of strains D4355, D6659, D6856, D6683, ATCC 19438^T, ATCC 33236^T, ATCC 51209^T, ATCC 35217^T, CCUG 48653^T, ATCC 33560^T, NCTC 13004^T, CCUG 55786^T, ATCC 35221^T, ATCC 43264^T, ATCC 33238^T, ATCC 51146^T, ATCC 35980^T and NCTC 11541^T are JX912510–JX912527, respectively.

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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 from 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^T 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 °C 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, H_2S production in triple-sugar iron agar, and reduction of nitrate, nitrite and triphenyltetrazolium chloride. Growth tests included temperature tolerance at 25 °C, 37 °C and 42 °C under microaerobic conditions, growth under aerobic and anaerobic conditions on 5 % blood agar at 37 °C 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 °C 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 *Camplyobacter 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 rRNA 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 (03-427^T 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 *Sma*I and *Kpn*I, and amplified fragment length polymorphism (AFLP) analysis using *Hin*dIII and *Hha*I, 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 03-427^T, 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 kDa. 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 ×. 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 03-427^T 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 03-427^T 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 03-427^T 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 [^{32}P]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

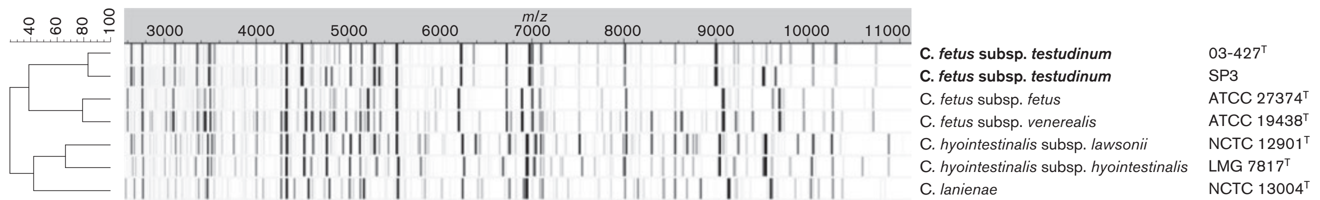

Fig. 1. Dendrogram of the MS peaks of *C. fetus* and most closely related species of the genus *Campylobacter* using Pearson correlation with UPGMA.

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. H_2S negative in TSI agar. Growth is observed on medium containing 1.0 % glycine, 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 03-427^T (=ATCC BAA-2539^T=LMG 27499^T), isolated from human blood culture in 2003 (Tu *et al.*, 2004b).

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