Inconsistency of phenotypic and genomic characteristics of *Campylobacter fetus* subspecies requires re-evaluation of current diagnostics

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Running Head: Molecular characteristics of *C. fetus* subspecies

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Abstract

Classification of the *Campylobacter fetus* subspecies *fetus* and *venerealis* was first described in 1959 and was based on the source of isolation (intestinal versus genital) and the ability of the strains to proliferate in the genital tract of cows. Two phenotypic assays (1% glycine tolerance and H$_2$S production) were described to differentiate the subspecies. Multiple molecular assays have been applied to differentiate the *C. fetus* subspecies, but none of these tests are consistent with the phenotypic identification. In this study, we defined the core genome and accessory genes of *C. fetus*, based on the closed genomes of five *C. fetus* strains. Phylogenetic analysis using the core genomes of 23 *C. fetus* strains of both subspecies showed a division into two clusters. The phylogenetic core genome clusters were not consistent with the phenotypic classification of the *C. fetus* subspecies. However, they were consistent with the molecular characteristics of the strains, determined by multilocus sequence typing, *sap*-typing, and the presence/absence of insertion sequences and a type I restriction-modification system. The fact that three of the phenotypically-defined *C. fetus* subsp. *fetus* strains have the same genomic characteristics as *C. fetus* subsp. *venerealis* strains, when considering the core genome and accessory genes, requires a critical evaluation of the clinical relevance of *C. fetus* subspecies identification by phenotypic assays.
Introduction

Campylobacter fetus is an important veterinary pathogen, associated with genital infections in cattle and sheep, resulting in abortion and infertility (1). Based on clinical and phenotypic observations, the species C. fetus was subdivided into two subspecies: C. fetus subsp. venerealis (Cfv) and C. fetus subsp. fetus (Cff) (2). Cfv was of venereal origin, had a high ability to cause abortions and could persist in the genital tracts, whereas Cff was of intestinal origin, caused only sporadic abortions and was cleared from the genital tract of the cow following the abortion. The two subspecies were phenotypically differentiated with the 1% glycine tolerance and H₂S production tests: Cff is positive in both tests (glycine tolerant and H₂S production) and Cfv is negative in both tests (no growth in presence of 1% glycine and no H₂S production) (2). C. fetus strains were isolated that were able to establish themselves in the genital tract of a non-pregnant cow, like the venereal Cfv strains (3); however, the glycine resistance of these strains was reduced, compared to most Cff strains, and they were positive in the H₂S test, like the intestinal Cff strains (4). They were classified in an intermediate group (4) and designated Campylobacter fetus subsp. venerealis biovar intermedius (1).

For molecular subspecies identification, several PCR-assays have been described, but these lack specificity (5). The subspecies can be genetically differentiated with multilocus sequence typing (MLST) (6) and Amplified Fragment Length Polymorphism (AFLP) analyses (7), but these methods are laborious and therefore not useful for routine diagnostic methods. The rationale behind the differentiation between the C. fetus subspecies is the supposed difference in pathogenicity and disease epidemiology. Cfv is described as the causative agent of Bovine Genital Campylobacteriosis (BGC), a statutory disease in many countries of
the world and listed by the OIE (World Organisation for Animal Health), in contrast
with Cff, which is associated with sporadic abortions (8).

Comparative genomics of two *C. fetus* strains revealed several unique regions
for both subspecies, as shown by Kienesberger et al. (9): *C. fetus* subsp. *venerealis*
contained multiple unique regions representing insertion sequences and genomic
islands with Type IV secretion system components and phage-related/hypothetical
proteins (9); and *C. fetus* subsp. *fetus* contained CRISPR-cas loci and unique genes
involved in LPS biosynthesis (9). These data suggested that *C. fetus* subspecies can be
distinguished on genomic features, but comparative genomics of a larger set of *C.
fetus* strains is lacking. The aim of this study is to characterize *C. fetus* strains of both
subspecies based on whole genome sequencing data and to compare the results of
classification, based on core and accessory genome analysis, with the current *C. fetus*
subspecies identification based on phenotypic assays.
Materials and methods

Bacterial strains; phenotyping and genotyping

*C. fetus* strains (listed in Table 1) were grown on heart-infusion agar supplemented with 5% sheep blood (Biotrading, Mijdrecht, the Netherlands) for two days under microaerobic conditions (6% O$_2$, 7% CO$_2$, 7% H$_2$, 80% N$_2$, (Anoxomat, Mart Microbiology, Lichtenvoorde, the Netherlands)). The subspecies of the strains were phenotypically identified with the 1% glycine tolerance test and hydrogen sulphide (H$_2$S) production in medium with 0.02% cysteine-HCl test, as described (1). Molecular identification was performed using MLST (6) and AFLP analysis (7).

Whole genome sequencing

Whole genome sequence data of 21 *C. fetus* strains was obtained using Roche GS-FLX Titanium sequencing. Roche 454 reads were assembled into contigs using the Newbler assembler (v2.6). The genomes of two *C. fetus* strains, 04/554 and 97/608 were closed through assembly of the Roche 454 contigs into scaffolds by using Perl scripts. To validate the assembly of the contigs and to determine the orientation and order of the scaffolds, a circular, high resolution *Afl* II restriction map of the genome was generated by optical mapping (Argus Optical Mapper, OpGen Inc, Gaithersburg, MD). Assembly of the *sap* locus, genomic islands, regions with insertion sequences and repeats were confirmed with PacBio Continuous Long Reads (Keygene N.V., Wageningen, the Netherlands). All base calls and polymeric tracts were validated using the high-depth Illumina MiSeq reads. The genomes of strain 04/554 and 97/608 were annotated as described (10).

Phylogenetic analysis of core and accessory genomes
Three available closed *C. fetus* genomes were used as a reference: strain 82-40 (GenBank accession number CP000487), strain 84-112 (GenBank accession numbers HG004426-HG004427) and strain 03/293 (GenBank accession numbers CP006999-CP007002). The amino acid sequences of the open reading frames (ORFs) encoded by five genomes (the three reference genomes plus two genomes sequenced in this study (04/554 and 97/608)) were used as input for an all-versus-all sequence similarity search using BLASTp (-e 0.0001, >80% similarity cutoff). ORFs that exist in each of the five strains (>80% identity over at least 80% of the protein length) were considered to be part of the *C. fetus* core genome. The ORF sequences of strain 82-40 were used as reference sequences of the core genes. Regions encoding the *sap* locus, genomic islands, restriction-modification systems, prophages and insertion sequences were considered as accessory genes.

The accessory genes in the Roche 454 contigs of 21 *C. fetus* strain were identified with a local BLASTn analysis (-e 0.0001, >80% similarity cutoff) against the identified accessory genes of the five closed *C. fetus* strains. The strains were considered positive for the specific accessory regions if the BLASTn match was >80% over at least 80% of the region.

The phylogenetic analysis of the core genomes was performed as follows: nucleotide sequences of the predicted genes of the Roche 454 contigs were generated using GeneMark (v2.8) (11). For each core gene, the corresponding nucleotide sequence of each strain was extracted and aligned on a gene-by-gene basis using MUSCLE (12). The alignments were concatenated into a contiguous sequence for each *C. fetus* strain. From this concatenated alignment, a phylogenetic maximum likelihood tree was built using RAxML (v7.2.8) under the GTRCAT model.
Results

Genome Features

The genome of *C. fetus* subsp. *fetus* 04/554 is a circular chromosome of 1,800,764 bp with an average G+C content of 33.2%, and one megaplasmid of 25,862 bp. The genome of *C. fetus* subsp. *venerealis* 97/608 has a circular chromosome of 1,935,028 bp with an average G+C content of 33.3% and contains two megaplasmids of 38,272 bp and 27,124 bp. The general features of the assembled genomes are shown in Table 2. The genome features of strains 03/293, 82-40 and 84-112 have been described previously (9, 10) and are summarized in Table 2.

Nucleotide sequence accession numbers

In GenBank, the genome sequence of *C. fetus* subsp. *fetus* 04/554 has been deposited under accession numbers CP008808-CP008809 and *C. fetus* subsp. *venerealis* 97/608 under accession numbers CP008810-CP008812.

Phylogeny of the *C. fetus* core genome

Comparison of five closed *C. fetus* genomes (82-40, 84-112, 04/554, 97/608 and 03/293) revealed highly syntenic genomes, sharing >90% sequence identity. The core genome of *C. fetus* was defined on the ORFs present in the closed genomes of five *C. fetus* strains, and was determined to consist of 1,409,454 bp and 1509 ORFs. The core genome was then identified in an additional 18 *C. fetus* strains and the genetic distance of all 23 core genomes was visualized with a phylogenetic maximum likelihood tree (Figure 1). The phylogenetic tree is arranged in two clusters of strains,
designated Cluster A and B. The majority (n=18) of strains were located in Cluster A, whereas Cluster B consisted of five strains with a higher genetic distance.

Accessory genes of C. fetus strains

Major differences between the five closed C. fetus genomes were found in the accessory genes. Table 1 shows the identified genes belonging to the sap loci, insertion sequences, genomic islands, type I restriction-modification systems, prophages and CRISPR-cas systems of the 23 analyzed C. fetus strains.

*Sap locus.* All analyzed C. fetus strains contained a sap locus, but the composition of this region differed between strains. Sap-type B strains belonged to the core genome Cluster B, whereas sap-type A strains were found in both genome Clusters (Table 1). The sap locus of strain 84-112 and strain 97/608 contained transposable elements and a set of phage-related genes and hypothetical genes, indicating the presence of a prophage. These prophage sequences shared 100% identity, but were inserted at different positions in the sap-locus.

*Genomic islands.* Two genomic islands (GI) encoding a Type IV secretion system (T4SS), as defined by Kienesberger et al. (9, 13), were identified in the chromosomes of the closed C. fetus genomes. These two chromosomally-located T4SS regions were present in strains that were distributed over the two core genome clusters (Table 1).

*Insertion Sequences.* Insertion sequences (IS) were found in the chromosomes as well as in plasmids of the closed genomes of strains 84-112, 97/608 and 03/293. The identified IS belonged to the IS605, IS607 and IS200 families. The IS are only found in C. fetus strains belonging to core genome Cluster A (Table 2). Each IS positive strain contained all of the identified IS families.
Restriction-modification system. Three of the closed *C. fetus* genomes, 84-112, 97/608 and 03/293 contained a type I restriction-modification (RM) system. This type I RM system consists of *hsd* genes with intervening ORFs, similar to the described type I RM systems in *C. jejuni* (14). The complete type I RM system is only found in *C. fetus* strains belonging to core genome Cluster A (Table 2). The three other *C. fetus* strains of *sap*-type A, 82-40, 110800-21-2 and BT 10/98, contained a remnant of this type as the type I RM system of these strains lacked the *hsdS2* gene. The genomes of the *sap*-type B strains did not contain any type I RM-encoding genes.

CRISPR-cas system. CRISPR repeats were present in all *C. fetus* strains, but only two *C. fetus* subsp. *fetus* strains, 82-40 and 98/v445, contained *cas* genes. These strains were not linked with the same core genome cluster.

Core genome clusters compared with accessory genes

The presence of specific components of the accessory genes encoding prophages, genomic islands and CRISPR-cas system were not associated with a specific core genome cluster. The IS elements and complete type I RM system were exclusively found in the strains of Cluster A. Strains of Cluster B did not contain IS elements or a complete type I RM system and have different *sap* types.

Core genome clusters compared to subspecies identification

The subspecies were not consistently phenotypically- and genotypically-subdivided (Table 1). The genotypic method MLST was consistent with the obtained core genome clustering: strains of Cluster A are all MLST ST-4, whereas Cluster B consisted of strains with other MLST STs. Cluster B included two strains with a similar MLST ST-2, and these strains were determined to have a lower genetic
distance than the other strains in Cluster B. AFLP is able to distinguish the strains within Cluster A with a minor difference in fingerprint as CfV and Cfvi(6), but this discrimination is not observed with the phylogenetic analysis of the core genomes. Strains that were phenotypically classified as CfV and Cfvi belonged to core genome Cluster A, but the eight phenotypically-classified Cff strains were dispersed among both genome clusters. This is represented in Figure 1, where all phenotypically identified Cff strains are marked with an asterisk.

Discussion

The original classification of the *C. fetus* subspecies is based on differences in the colonization of different niches and phenotypic characteristics (2-4). The two *C. fetus* subspecies are highly syntenic, sharing 92.9% sequence identity(9), and the subspecies cannot be distinguished by DNA-DNA hybridization(15), which questions the validity of subspecies differentiation and hampers an adequate taxonomic positioning of the subspecies. Furthermore, the reliability of the 1% glycine tolerance test can be influenced by the fact that glycine tolerance can be transduced by phages (16). Several molecular assays for the identification of the *C. fetus* subspecies have been published(5), however, none of the molecular assays corresponded fully to the phenotypic identification of the *C. fetus* subspecies (5, 6).

In this study, phylogenetic analysis of the core genomes subdivided the *C. fetus* strains in two clusters. All strains that are phenotypically identified as CfV (including biovar intermedius) clustered in one core genome cluster, containing only strains with MLST ST-4 and harboring IS elements and a type I RM system. The strains phenotypically identified as Cff were assigned to both clusters. Three Cff strains, 03/293, Zaf 65 and ADRI 1362 were assigned to the core genome cluster with CfV
and Cfvi strains, despite their phenotypic identification of Cff. The similarity of the MLST identification and core genome clusters can be explained by the fact that MLST is a small-scale reflection of the core genome. The phylogenetic analysis showed an obvious resemblance with the MLST STs of the strains; the genetic distances between strains with the same STs are very low as shown for strains of ST4 and ST2, and the genetic distances increased for strains with different STs.

*Campylobacter fetus venerealis* biovar intermedius is described as a phenotypic variant of *C. fetus* subsp. *venerealis* (1). The phenotypically-identified Cfvi strains are all positioned together with Cfv strains in cluster A of the phylogenetic tree. The accessory genes of Cfv and Cfvi strains showed no consistent presence of a Cfv or Cfvi specific region. However, it is remarkable that, except strain ADRI 513, all of the with AFLP identified Cfv strains contain a prophage in the *sap*-locus and that this prophage is absent in the majority of Cfvi strains. Almost all proteins of this prophage are hypothetical with unknown function, but one may speculate that the presence of this prophage influences the phenotypic difference, such as the H$_2$S production between the Cfv and Cfvi strains.

The differentiation between *C. fetus* subspecies goes beyond only taxonomic interest. Clinically, the subspecies have been described to be different. Cfv (including biovar intermedius) is described as the causative agent of Bovine Genital Campylobacteriosis (BGC). There is a generally accepted association between the *C. fetus* subspecies and their specific clinical features, epidemiological characteristics and host niche specificity. Bovine products for trade must be checked for the absence of Cfv as stated in the Terrestrial Animal Health Code by the World Organisation of Animal Health (OIE) (17). When in such a screening *C. fetus* is detected, subspecies identification is generally done by phenotypic assays as described in the OIE Manual.
of Diagnostic Tests and Vaccines for Terrestrial Animals (18). However, there is no evidence that the phenotypic markers (glycine tolerance and H$_2$S production) are linked to virulence characteristics of *C. fetus* subspecies. Future diagnostics of *C. fetus* should preferably detect genomic characteristics associated with virulence and different host niches. The virulence genes of genomic islands present in *C. fetus* strains were described as Cfv-specific and proposed as targets for diagnostic assays (19, 20). However these genes are not consistently present in Cfv genomes (13, 21) and therefore not useful as a diagnostic assay. Pending the identification of virulence-associated genes, one should be aware that the current association between phenotype and virulence is questionable, since several phenotypically-defined Cff strains have the same genomic characteristics as Cfv strains, on the basis of core genome and accessory gene similarity, as shown in this study. The inconsistency of phenotypes and genomic characteristics of *C. fetus* strains encourages a critical evaluation of the clinical relevance of *C. fetus* subspecies identification by phenotypic assays.

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References


Figure 1. Phylogenetic tree of C. fetus strains based on the core genomes. Strains that are phenotypically identified as Cff are marked with an asterisk (*). Bootstrap supports are indicated on the branches. The scale represents the mean number of nucleotide substitutions per site.
Table 1. General characteristics of *C. fetus* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Country</th>
<th>Source</th>
<th>Phenotype</th>
<th>Genotype</th>
<th>Accessory genes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1% Glycine tolerance</td>
<td>H₂S production</td>
<td>Phenotypic ID</td>
</tr>
<tr>
<td>82-40</td>
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<td>Human</td>
<td>+</td>
<td>+</td>
<td>Cff Cff</td>
</tr>
<tr>
<td>110800-21-2</td>
<td>NL</td>
<td>Bovine (bull)</td>
<td>+</td>
<td>+</td>
<td>Cff Cff</td>
</tr>
<tr>
<td>BT 10/98</td>
<td>UK</td>
<td>Ovine</td>
<td>+</td>
<td>+</td>
<td>Cff Cff</td>
</tr>
<tr>
<td>04/554</td>
<td>AR</td>
<td>Bovine (foetus)</td>
<td>+</td>
<td>+</td>
<td>Cff Cff</td>
</tr>
<tr>
<td>98/v445</td>
<td>UK</td>
<td>Bovine (bull)</td>
<td>+</td>
<td>+</td>
<td>Cff Cff</td>
</tr>
<tr>
<td>03/293</td>
<td>AR</td>
<td>Bovine (foetus)</td>
<td>+</td>
<td>+</td>
<td>Cff Cfvi</td>
</tr>
<tr>
<td>ADRI 1362</td>
<td>AR</td>
<td>Bovine</td>
<td>+</td>
<td>+</td>
<td>Cff Cfvi</td>
</tr>
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<td>SA</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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</tr>
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<td>+</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>-</td>
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</tr>
<tr>
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<td>-</td>
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</tr>
<tr>
<td>CCGU 33900</td>
<td>F</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>Cfvi Cfvi</td>
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Abbreviations: MLST (ST): Multi Locus Sequence Typing (Sequence Type)  
GI with T4SS: Genomic Island with type IV secretion system  
IS: Insertion Sequence  
RM system: Restriction-Modification system  
CRISPR: Clustered regularly interspaced short palindromic repeats  
Country code:  AR Argentina, AU Australia, BE Belgium, CZ Czech Republic, F France, NL Netherlands, SA South Africa, UK United Kingdom, US United States  
Phenotype: + positive in assay, - negative in assay  
Accessory: + genes are present, - genes are absent  
Taxon: Cff *C. fetus fetus*, Cfvi *C. fetus venerealis*, Cfvi *C. fetus venerealis* biovar intermedius
Table 2. Features of assembled *C. fetus* genomes

<table>
<thead>
<tr>
<th>Strain ref</th>
<th>04/554 this study</th>
<th>97/608 this study</th>
<th>03/293 (10)</th>
<th>82-40 (9)</th>
<th>84-112 (9)</th>
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<td>1,935,028</td>
<td>1,866,009</td>
<td>1,773,615</td>
<td>1,926,886</td>
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<td>33.3%</td>
<td>33.3%</td>
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<tr>
<td>tRNA genes</td>
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<td>Homopolymeric GC tracts (≥ 8bp)</td>
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<td>Open reading frames (Pseudogenes)</td>
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<td>1,879 (60)</td>
<td>1,773 (48)</td>
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<td>91,440 / 35,326 / 3,993</td>
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<td>1</td>
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<td>- G+C content</td>
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<td>31.3% / 28.1%</td>
<td>29.4% / 33.0% / 31.4%</td>
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* with modifications from original publication