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Campylobacter fetus subspecies specific PCR assays inferred from comparative genomic analysis for accurate subspecies identification

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ABSTRACT

Bovine Genital Campylobacteriosis (BGC) is caused by *Campylobacter fetus* subsp. *venerealis* and is a notifiable disease to the WOAH (World Organisation for Animal Health). For an effective BGC control program, the reliable differentiation of *Campylobacter fetus* subsp. *venerealis* (Cfv) from the closely related *Campylobacter fetus* subsp. *fetus* (Cff) is required. However, the available molecular *C. fetus* subspecies identification assays lack sensitivity and specificity to differentiate *C. fetus* isolates based on their phenotypic or genotypic differences. Furthermore, the current biochemical subspecies identification is not fully congruent with the genomic differentiation of *C. fetus* strains.

In this study, the genome sequences of 41*C. fetus* strains with well identified subspecies, were analyzed with the large-scale BLAST score ratio (LS-BSR) pipeline to identify Cff and Cfv specific sequences. With this analysis, the *asd* gene encoding an aspartate-semialdehyde dehydrogenase was identified, which contained a 6-bp Cff-specific sequence, and this 6-bp sequence was absent in the *asd* gene of Cfv strains. This sequence was used for the development of PCR assays to differentiate Cff and Cfv strains. The *C. fetus* subspecies identification of the developed *asd* PCR assays was in full congruence with the genomic classification of strains and are recommended for molecular identification of *C. fetus* subspecies in BGC control programs.

The *asd* PCR can be assessed on sequenced genomes using a web interface containing the Cfvcatch tool, which includes placement of the tested genome in a phylogenetic tree with reference *C. fetus* genomes to distinguish the two subspecies and to detect antimicrobial resistance genes.

1. Introduction

Bovine Genital Campylobacteriosis (BGC) is characterized by abortion and infertility in cattle and caused by *Campylobacter fetus* subsp. *venerealis* (Cfv) (Thompson and Blaser, 2000). *Campylobacter fetus* comprises two other subspecies: *Campylobacter fetus* subsp. *testudinum* (Cft) and *Campylobacter fetus* subsp. *fetus* (Cff). The *C. fetus* subspecies show a host and niche association: Cft is reptile-associated (Fitzgerald et al., 2014) and both Cff and Cfv are mammal-associated (Thompson and Blaser, 2000). Cfv is restricted to the genital tract of cattle and includes a biochemical variant, designated Cfv biovar intermedius (Cfvi) (Véron and Chatelain, 1973). Cff can be isolated from a variety of different hosts (Thompson and Blaser, 2000) and cause sporadic abortion in cattle and sheep (Garcia et al., 1983) and infections in humans where it can cause diarrheal intestinal illness as well as severe systemic infections (Wagenaar et al., 2014).

BGC is notifiable to the WOAH (World Organisation for Animal Health) and an effective BGC control program requires the reliable differentiation of Cfv from Cff when using bacteriological culturing. The original described phenotypic methods to identify Cff, Cfv and Cfvi strains are the 1 % glycine tolerance test and H_2S production test; Cff is tolerant to 1 % glycine and able to produce H_2S, Cfv is not tolerant to 1 % glycine and not able to produce H_2S and Cfvi is not 1 % glycine tolerant (like Cfv), but able to produce H_2S (like Cff) (Florent, 1959, 1963; Véron and Chatelain, 1973). A PCR assay based on the L-cysteine transporter genes was developed for identification of hydrogen sulfide-

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producing *C. fetus* isolates, that discriminates Cfv from Cff/Cfvi (Farace et al., 2019). Multiple molecular methods for *C. fetus* subspecies identification have been described, but several of these methods are laborious and not suitable for standard routine diagnostic laboratories (Van der Graaf-van Bloois et al., 2013), or they are not consistent with the phenotypes (Van Bergen et al., 2005a, 2005b; Van der Graaf-van Bloois et al., 2013) or genotypes (Iraola et al., 2015; Van Bergen et al., 2005a, 2005b) of the *C. fetus* strains.

Concerns on the reliability of biochemical tests have arisen as these assays have shown a poor reproducibility (Van Bergen et al., 2005a, 2005b) and the phenotypes are not consistent with the genotypes of the strains (Van der Graaf-van Bloois et al., 2014a, 2014b). This emphasizes the need for a reliable molecular *C. fetus* subspecies identification method, which can be implemented in the BGC control programs.

The currently published *C. fetus* genome sequences (Van der Graaf-van Bloois et al., 2014a, 2014b; Gorkiewicz et al., 2010; Van der Graaf-van Bloois et al., 2023) allow comparative genomics to identify Cff and Cfv specific sequences. In this study, we analyzed the mammal-associated *C. fetus* genomes to find specific Cff and Cfv sequences, which were used for the development of Cff- and Cfv-specific conventional and real-time PCR assays. Furthermore, a web interface with *C. fetus* determination tools including the developed PCR assay was released, to analyse *C. fetus* genomes to subspecies level and detect antimicrobial resistance genes.

2. Materials and methods

2.1. Genome analysis using LS-BSR pipeline

The genome sequences of 41 well characterized reference *C. fetus* strains from our previously study (Van der Graaf-van Bloois et al., 2016a) were analyzed with the large-scale BLAST score ratio (LS-BSR) pipeline (Sahl et al., 2014) to search for Cff and Cfv specific sequences. Strain information and accession numbers are presented in Supplemental Table 1. The genome sequences are online available from NCBI GenBank and the European Nucleotide Archive (ENA).

2.2. Development of PCR assays for C. fetus subspecies identification

With Primer3 (Untergasser et al., 2012), conventional Cff and Cfv specific primers were developed on the sequences of a aspartate-semialdehyde dehydrogenase (*asd*) (CFF8240_1458) encoding gene and its intergenic region, and real-time primers and Cff and Cfv specific probes were developed on the *asd* gene and a flanking hypothetical protein (CFF8240_1457). The sequences of the developed primers and probes were checked for specificity with a BLAST against the NCBI GenBank database and with a local BLAST against the available sequence reads of 141*C. fetus* genomes in project PRJEB8721 (European Nucleotide Archive).

For the conventional *asd* PCR assay, a single forward primer for both subspecies was designed, and two reverse primers; one specific for Cff and one specific for Cfv (Table 1). The *asd* PCR assay consists of two separate PCR reactions; one for Cff identification with primers ASD_forw and ASD_Cff_rev, and one for Cfv identification with primers ASD_forw and ASD_Cfv_rev. PCR assays were performed in volumes of 20 µl in an Abi 2720 Thermo Cycler (Applied Biosystems), using the following thermal cycler protocol: 95 °C for 3 min, followed by 35 cycli of 95 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s and extended with 72 °C for 7 min. Each PCR reaction contained 10 µl GoTaq Green Master Mix (Promega), 1 µl of 10 pmol/µl forward primer, 1 µl of 10 pmol/µl reverse primer, 2 µl of DNA template, adjusted to 20 µl with nuclease free-water. PCR fragments were visualized on a 2 % agarose gel stained with Midori Green Advanced DNA staining (GC Tech) and positive results were indicated by a fragment of 335 bp for Cff and 329 bp for Cfv.

Table 1
Sequences of *asd* PCR primers and probes.

Primer name	Sequence 5′-3′	Specificity
Conventional PCR		
ASD_forw	CACTTGTGTTCGTGTTC	Cff/Cfv
ASD_Cff_rev	TTTTTAGCTAATGGTTTAATTC	Cff-specific
ASD_Cfv_rev	TTTTTAGCTAATGGTCTTG	Cfv-specific
Real-time PCR		
ASD_RT_forw	TCTTTGGTGCGTTGCTGATCA	Cff/Cfv
ASD_RT_rev	GATGACGGGCAAAATAGTCACGT	Cff/Cfv
ASD_Cff_P	*FAM*-TGGTGTCAAATACAAGAATTAAACC-*BHQ*	Cff-specific
ASD_Cfv_P	*HEX*-AAATGGTGTCAAATACAAGACCA-*BHQ*	Cfv-specific

A real-time *asd* PCR assay was developed in a multiplex format, with forward primer ASD_RT_forw and reverse primer ASD_RT_rev, and two probes; ASD_Cff_P specific for Cff and ASD_Cfv_P specific for Cfv, which can be combined in one reaction if different fluorescent labels are used (Table 1). The *asd* real-time PCR assays were performed in volumes of 20 µl on a LightCycler 480 System (Roche), using the following thermal cycler protocol: 95 °C for 6 min, followed by 45 cycli of 95 °C for 10 s and 58 °C for 30 s and extended with one step of 40 °C for 10 s. Each qPCR reaction contained 10 µl 2× LightCycler 480 Probes Master Mix (Roche), 1 µl of 12 pmol/µl for each primer, 1 µl of 4 pmol/µl for each probe, 2 µl of DNA template, adjusted to 20 µl with nuclease free-water.

2.3. Development of web interface to identify C. fetus genomes

A web interface to identify *C. fetus* genomes to subspecies level was developed and can be accessed at https://klif.uu.nl/cfvcatch/. This web interface included the tool Cfvcatch (Abdel-Glil et al., 2020), the developed *asd* PCR, mashtree (Katz et al., 2019) to generate phylogenetic trees and abricate with resfinder (Florensa et al., 2022) to search for antimicrobial resistance genes. A set of 19 Cff and 22 Cfv/Cfvi genomes from a previous publication (Van der Graaf-van Bloois et al., 2016a) were used as reference genomes for the phylogenetic tree.

2.4. Validation of asd PCR assays

- Bacterial strains

Both conventional and real-time *asd* PCR assays were tested with a set of 142*C. fetus* strains consisting of 104 Cff, 38 Cfv, and 38 non-fetus *Campylobacter* spp. strains (Supplemental Table 2). The strains 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)) and chromosomal DNA was isolated with the Gentra PureGene DNA isolation Kit (Qiagen). The *C. fetus* strains were phenotypically identified with the 1 % glycine tolerance test and H_2S production in medium with 0.02 % cysteine-HCl test as described before (Véron and Chatelain, 1973). All strains were genotypically identified with AFLP, MLST and WGS as described previously (Van der Graaf-van Bloois et al., 2016a).

- In silico validation

Online available mammal *C. fetus* genomes were downloaded (accession date 25 May 2023) and were checked for their completeness (cutoff ≥95 %) and contamination (≤5 %) with Checkm v1.1.2 (Parks et al., 2015). This resulted in a set of 285*C. fetus* genomes, consisting of 151 Cff and 134 Cfv genomes (Supplemental Table 1). All genomes were classified with the web interface Cfvcatch using default settings.

3. Results

3.1. LS-BRS analysis for Cff and Cfv specific sequences

The LS-BSR analysis of 41*C. fetus* genomes revealed several sequences that were specific for the subspecies. These sequences were located in a gene encoding an aspartate-semialdehyde dehydrogenase (*asd*) (CFF8240_1458) and in multiple type IV secretion system (T4SS) genes.

The *C. fetus asd* gene contained a 6 bp insertion/deletion, which was inserted in all genotypic identified Cff strains and was lacking in Cfv strains. Due to this 6 bp deletion in Cfv strains, there is no shift in the codon reading frame, but the strains are missing two amino acids, aspartate and glutamate, in the final protein. It is unknown if this deletion will hamper the functionality of the ASD protein.

Multiple T4SS encoding genes were specifically present in Cfv strains. However, a previous study has shown that T4SS encoding genes are not Cfv-specific as these genes are carried by a mobile chromosomal element that can be transferred between subspecies (Van der Graaf-van Bloois et al., 2016b). Such targets are unsuitable for *C. fetus* subspecies differentiation.

The specificity of the *asd* primer and probe sequences (Table 1) were checked against a large set of 285 online available *C. fetus* genomes consisting of 151 Cff and 134 Cfv genomes were analyzed (Supplemental Table 1) and three *C. testudium* genomes, strain 03–427 (NCBI accession number CP006833.1), strain sp3 (NCBI accession number CP010951.1 and strain 85–387 (NCBI accession number GCA_001699345.1). The *asd* Cfv-specific primers and probe fitted 100 % on all 151 Cfv genomes, and the *asd* Cff-specific primers and probe fitted 100 % on all 134 Cff genomes. The *C. fetus* subspecies *testudinum* genomes shared the *asd* gene with the Cff sequence, having the 6 bp insertion, however, the PCR primers did not match 100 % with the Cft genomes. The conventional PCR ASD_forw primer contains two mismatches and the real-time ASD_rt_rev primer contains two mismatches for all three Cft genomes, whereas strains 03–427 and 85–387 contained an additional mismatch in the real-time ASD_rt_forw primer sequence.

With a general NCBI Genbank BLAST, the specificity of the *asd* PCR was checked for other microorganisms. Aspartate-semialdehyde dehydrogenase is an essential enzyme in the biosynthesis of amino acids in bacteria and the *asd* gene is present in all *C. fetus* genomes and most other bacterial species. Therefore, the in this study developed primers and probes had multiple matches with other bacteria species. However, there was never a match with both forward and reverse primer on the same bacteria species, except *C.fetus*, showing that the primer-pairs are specific for *C. fetus.*

3.2. Validation of the asd PCR assays with bacterial strains

Both conventional and real-time *asd* PCR assays were validated with 104 Cff, 38 Cfv strains, and 38 strains of other *Campylobacter* spp. as shown in Supplemental Table 2. The non-*C. fetus* species that are tested included *C. coli, C. hyointestinalis, C. lari, C. peloridis,* and *C. sputorum* isolates. The *asd* PCR assays, both conventional and real-time, showed 100 % consistency with the previously genomically classified genotypes of the Cff and Cfv strains and showed 100 % specificity for the non-*C. fetus* strains.

3.3. Construction of a web-interface for rapid assessment of genome sequences

The web-page https://klif.uu.nl/cfvcatch was developed as an online tool for identification of *C. fetus* genomes to subspecies level, to perform phylogenetic analysis with reference genomes, and to identify antimicrobial resistance genes. The developed *asd* PCR was incorporated into the web interface and used in this study for in silico *asd* PCR validation.

The web interface of the Cfvcatch tool was validated using the set of 285*C. fetus* genomes (Supplemental Table 1). This resulted in 100 % sensitivity for subspecies detection showing that the *asd* PCR is a reliable tool to distinguish Cff and Cfv genomes.

4. Discussion

To prevent spread of BGC in cattle between countries and improve animal health, strict trade regulations have been prescribed by the WOAH (World Organisation for Animal Health, 2024a). Crucial in this approach is the establishment of reliable diagnostics that could be used in BGC control programs, also in resource poor countries. Two biochemical assays, tolerance to 1 % glycine and production of H_2S in cysteine-rich medium, are still the prescribed methods to identify Cff, Cfv and Cfvi strains (World Organisation for Animal Health, 2024b). However, the 1 % glycine tolerance test has poor reproducibility, and cannot always make a reliable differentiation between Cff and Cfv strains (Van Bergen et al., 2005a, 2005b). Cff and Cfv strains can be genotypically differentiated with whole genome sequence analysis based on their core genes and core genome SNPs (Van der Graaf-van Bloois et al., 2014a, 2014b; Van der Graaf-van Bloois et al., 2016a), but WGS requires bioinformatic expertise to analyse *C. fetus* genomes and perform phylogenetic analysis. To support labs with the WGS analysis, we developed the web interface Cfvcatch with online tools for the classification of *C. fetus* genomes and identification of antimicrobial resistance genes without bioinformatic expertise.

Both conventional and real-time *asd* PCR assays developed in this study performed perfectly to identify Cff and Cfv in full congruence with the genotypes of the tested strains and can be used in BGC control programs. Because *asd* is an essential gene, we avoid problems that have plagued other PCR approaches that use accessory genes, which can result in false positives or negatives because of gene incompleteness and/or accidental gene loss. The validation of the novel PCRs was done with 38 non-*C. fetus* strains. Since *C. hyointestinalis* and *C. sputorum* can be present in the bovine genital tract, we included a large number of clinical isolates of these species to test the specificity of the PCR assays. The genomic validation was done with a limited number of non-*C. fetus* genomes and since a *C. hyointestinalis* strain has been described to give a cross-reaction in a Cfv real-time PCR (Spence et al., 2011), it is recommended to identify an isolate first with the *nahE* PCR (Abril et al., 2007; Van der Graaf-van Bloois et al., 2013) to confirm that the isolate is *C. fetus*, and use the novel ASD PCR assays to identify the *C. fetus* isolates to the subspecies level.

The *C. fetus* subspecies identification of the *asd* PCR assays is congruent with the genotypic identification of Cff and Cfv strains, but it is currently unknown if this differentiation is also congruent with the potential pathogenicity of *C. fetus* strains (Van der Graaf-van Bloois et al., 2016b). For the control of *C. fetus*-induced fertility problems, one would prefer to differentiate strains based on their potential pathogenicity. The factors that have been suggested to be associated with *C. fetus* virulence are the lipopolysaccharide determinants of strains (Thompson and Blaser, 2000) or the presence of T4SSs (Van der Graaf-van Bloois et al., 2016b). To confirm how pathogenicity of *C. fetus* is regulated and to pinpoint the genes driving virulence of *C. fetus*, more research is needed. As both Cff and Cfv have been isolated from bovine abortions, it seems conceivable that both Cff and Cfv strains can be pathogenic (Van der Graaf-van Bloois et al., 2016b) (20). As the virulence of *C. fetus* is poorly understood, we preferred to differentiate strains based on their genomic differences as we assume a stronger association between WGS genotype and virulence than biochemical phenotype and virulence. Once differences in virulence of *C. fetus* strains have been identified, a diagnostic PCR can be developed to differentiate virulent *C. fetus* strains from non-virulent *C. fetus* strains.

5. Conclusion

The conventional and real-time PCR assays developed in this study,

targeting the essential *asd* gene coding for an aspartate-semialdehyde dehydrogenase, showed 100 % specificity for differentiation of *C. fetus* subspecies, that was in full congruence with the genomic classification of the strains. The web interface https://klif.uu.nl/cfvcatch is available to identify *C. fetus* genomes to subspecies level.

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Author contributions

LG, AZ, and JW designed and directed the study; LG, BD, AZ, performed the experiments and analysis; JW supervised the findings of the experiments and analysis; All authors discussed the results and commented on the manuscript.

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CRediT authorship contribution statement

Linda van der Graaf - van Bloois: Writing – original draft, Methodology, Formal analysis, Data curation. **Aldert L. Zomer:** Writing – review & editing, Validation, Supervision, Software, Investigation. **Birgitta Duim:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Jaap A. Wagenaar:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The author(s) declare that there are no conflicts of interest.

Data availability

No data was used for the research described in the article.

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