UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

BOVINE GENITAL CAMPYLOBACTERIOSIS: NEW INSIGHTS INTO THE MOLECULAR DIAGNOSIS AND PATHOGENESIS

MARTA FILIPA SERRA DA SILVA

Orientador(es): Professora Doutora Luísa Maria Freire Leal Mateus

Doutora Maria Elisabete Tomé Sousa Silva

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Ciências Biológicas e Biomédicas

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"The greatest enemy of knowledge is not ignorance,

it is the illusion of knowledge."

Daniel J. Boorstin

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Campilobacteriose Genital Bovina: novas abordagens no diagnóstico molecular e na patogenia

RESUMO

Campylobacter fetus subespécie *venerealis* (*Cfv*) é o agente etiológico da Campilobacteriose Genital Bovina (CGB), uma doença venérea cujo diagnóstico depende da correta identificação de *Cfv*. Apesar de relevante para o controlo e maneio da CGB, a utilidade dos métodos moleculares no diagnóstico da doença é controversa. Por outro lado, os mecanismos subjacentes à patogenicidade da CGB não estão esclarecidos. Os objetivos deste trabalho foram avaliar a adequação de diferentes ensaios de PCR em tempo real na deteção de *Cfv* em amostras prepuciais, assim como avaliar o potencial de virulência de isolados de campo. Os resultados demonstraram que os alvos moleculares atualmente utilizados na identificação de *Cfv*, nomeadamente o gene *parA* e a sequência de inserção ISCfe1, estão associados à obtenção de uma elevada taxa de falsos-positivos (até 50 %), quando testados em amostras clínicas. Esta falha de especificidade foi também evidenciada pelo isolamento e identificação de uma nova espécie de *Campylobacter* presente no prepúcio, a espécie *Campylobacter portucalensis*, a qual possui no genoma sequências com elevada identidade com o gene *parA* (98 %) e ISCfe1 (94 %) de *Cfv*. A ausência deste gene numa elevada proporção (77 %) dos isolados de *Cfv* testados é também um resultado que invalida a utilização deste gene como alvo de diagnóstico. Estes dados são de extrema relevância, uma vez têm um impacto negativo no diagnóstico da CGB. A identificação de três novas *Sequências Tipo* (ST) coloca em causa a utilização do *Multilocus Sequence Typing* (MLST) na identificação da subespécie *venerealis*. A análise comparativa de genomas de isolados de campo permitiu identificar características que distinguem *Cfv* e o seu biovar *intermedius*, nomeadamente no que se refere aos perfis de *Single Nucleotide Polymorphisms* (SNPs) e a proteínas específicas do género *Campylobacter*. Os resultados da análise do genoma sugerem ainda que o sistema de secreção tipo IV, anteriormente considerado um fator de virulência de *Cfv*, não deverá desempenhar um papel crucial na patogenia da doença. Adicionalmente, os isolados de campo testados foram sensíveis à estreptomicina, penicilina, tetraciclina e enrofloxacina, ainda que apresentem no genoma genes que codificam para bombas de efluxo do tipo CmeABC e YkkCD. Este dado sugere que a presença destes genes isoladamente não deverá ser suficiente para conferir resistência *in vitro*, podendo estar envolvidos mecanismos adicionais. Os resultados têm um impacto significativo no diagnóstico da BGC e evidenciam novos dados relativos à diversidade genética e potencial de virulência de *Cfv*.

Palavras-chave: *Campylobacter fetus* subespécie *venerealis*, Campilobacteriose Genital Bovina, diagnóstico molecular, potencial de virulência, *Campylobacter portucalensis*.

Bovine Genital Campylobacteriosis: new insights into the molecular diagnosis and pathogenesis

ABSTRACT

C. fetus subsp. *venerealis* is the pathogen responsible for the Bovine Genital Campylobacteriosis (BGC), a cattle's venereal disease whose diagnosis relies on the accurate identification of *Cfv*. Despite its relevance for BCG control and management, the applicability of the molecular methods on the disease diagnosis is controversial whereas the mechanisms underlying the pathogenesis of BGC remain unclear. Therefore, the main objectives of this work were to assess the suitability of different real-time PCR assays to be used in bovine preputial samples for *Cfv* detection and to further elucidate the virulence potential of *Cfv* through the genomic characterization of *Cfv* field isolates.

The results showed that the currently used molecular targets for *Cfv* identification, the *parA* gene and the insertion sequence ISCfe1, originate a high rate of false-positive results (up to 50 %) in clinical samples. This lack of specificity was also evidenced by the identification of a novel bacterial species in the bovine prepuce, *Campylobacter portucalensis*, with sequences homologous to *parA* (98 % identity) and ISCfe1 (94 % identity) sequences of *Cfv,* hindering the molecular diagnosis of BGC. On the other hand, the absence of the *parA* gene in a high proportion of *Cfv* strains (77 %), also invalidate the use of this gene as a diagnostic target for *Cfv* identification. The identification of three novel Sequence Types (ST) in *venerealis* subspecies, previously characterized by a high genetic stability, questions the use of Multilocus Sequence Typing (MLST) for subspecies identification. This work additionally evidenced differential features between *Cfv* and its biovar intermedius, namely in whole-genome Single Nucleotide Polymorphisms (SNPs) and genes encoding genus-specific proteins families. Moreover, the results suggest that a type IV secretion system, previously indicated as involved in *Cfv* virulence, does not play a crucial role in the pathogenesis. *In vitro* antimicrobial resistance to streptomycin, penicillin, tetracycline and enrofloxacin was not detected, even in strains encoding two multidrug efflux pumps, CmeABC and YkkCD, revealing that its sole presence in the genome is not enough to provide *in vitro* antimicrobial resistance.

Overall, these results have a significant impact on the molecular diagnosis of BGC and revealed new insights into the *Cfv* genetic diversity and virulence potential.

Keywords: *Campylobacter fetus* subsp. *venerealis*, Bovine Genital Campylobacteriosis,

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LIST OF ABREVIATIONS

AAI	Average amino acid identity
AFLP	Amplified fragment-length polymorphism
ANI	Average nucleotide identity
APHA	Animal and Plant Health Agency
BA	Blood agar
BGC	Bovine genital campylobacteriosis
bp	Base pairs
BHI	Brain heart infusion
cAMP	Cyclic adenosine monophosphate
Cas	CRISPR-associated systems
CDS	Coding sequences
CDT	Cytolethal distending toxin
Cff	*Campylobacter fetus* subsp. *fetus*
Cft	*Campylobacter fetus* subsp. *testudinum*
CFU	Colony forming units
Cfv	*Campylobacter fetus* subsp. *venerealis*
Cfvi	*Campylobacter fetus* subsp. *venerealis* biovar intermedius

CiaB	*Campylobacter* invasion antigen
CRISPR	Clustered regularly interspaced short palindromic repeats
CSA	Campylobacter skirrow agar
Ct	Cycle threshold
CV	Coefficient of variation
DIF	Direct immunofluorescence
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	Amplification efficiency
ELISA	Enzyme-linked immunosorbent assay
EUCAST	European Commitee on Antimicrobial Susceptibility Testing
FIC	Filamentation induced by cAMP
FITC	Fluorescein isothiocyanate
GI	Genomic island
H_2	Hydrogen
H_2S	Hydrogen sulphide
IACUC	Institutional Animal Care and Use Committee
INT-407	Intestine-407
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MDBK	Madin-Darby bovine kidney
MIC	Minimum inhibitory concentration
MLST	Multilocus Sequence Typing
NaCl	Sodium chloride
NARMS	National Antimicrobial Resistance Monitoring System for Enteric Bacteria
OIE	Office International des Epizooties - World Organization for Animal Health
PATRIC	Pathosystems Resource Integration Center
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PldA	Phospholipase A
RAST	Rapid annotation using subsystem technology
RPP	Ribosomal protection proteins
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
Sap	Surface array proteins

S-Layer	Surface layer
SLP	Surface-layer proteins
SNP	Single-nucleotide polymorphism
ST	Sequence type
T4SS	Type IV secretion system
TEM	Transport enrichment medium
TSI	Triple sugar iron
TTC	Tetrazolium chloride
UK	United Kingdom
UV	Ultraviolet
VF	Virulence factor
WGS	Whole-genome sequencing

CHAPTER 1 – GENERAL INTRODUCTION

The species *C. fetus* includes two subspecies with particular medical and veterinary relevance, *C. fetus* subsp. *fetus (Cff)* and *C. fetus* subsp. *venerealis (Cfv)* (Wagenaar et al. 2014; OIE 2018). Additionally, *Cfv* includes a phenotypic variant named biovar intermedius (*Cfvi*) (Silveira et al. 2018). The subspecies *fetus* has a tropism for the intestinal tract of several mammals and humans, whereas the subspecies *venerealis* is restricted to the bovine genital tract and is responsible for a venereal disease designated Bovine Genital Campylobacteriosis (BGC) (Wagenaar et al. 2014; Sahin et al. 2017). This disease manifests in the herd through a decline in the reproductive performance as a result of temporary infertility, embryonic mortality and abortion (Silveira et al. 2018). BGC is a notifiable disease to the World Organisation for Animal Health (OIE), responsible for a significant economic burden to the cattle industry worldwide (More et al. 2017; OIE 2018). Therefore, an accurate diagnosis is indispensable for the implementation of BGC control programs and international trade of bulls and semen (More et al. 2017; OIE 2019). The diagnosis relies on the identification of *Cfv* and the recommended method by the OIE is bacterial culture followed by phenotypic characterization (OIE 2018; OIE 2019). Nevertheless, *Cfv* is a fastidious and slow-growing microorganism, which is often undetected due to the overgrowth of other microorganisms present in the sample (Mshelia et al. 2010; Seid 2019). This has driven the development of molecular diagnostic assays to identify *Cfv* through the detection of *Cfv*-specific nucleotide sequences (van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). Although *Cfv* and *Cff* exhibit different epidemiology, their genomes have more than 90 % sequence identity, which has hindered the selection of sensitive and specific molecular markers of *Cfv* (van der Graaf-Van Bloois et al. 2014; Kienesberger et al. 2014). Furthermore, even though several polymerase chain reaction (PCR) assays were described for *Cfv* identification, their performance in clinical samples has been poorly assessed.

The high genomic and phenotypic similarity of the two subspecies hinders the understanding of its ecological and pathogenic specificities (van der Graaf-Van Bloois et al. 2014; Sahin et al. 2017). In fact, the molecular mechanisms behind *Cfv* pathogenicity are not yet fully understood. Both *C. fetus* subspecies harbour genes encoding several well-characterized virulence factors common to other *Campylobacter* species (Ali et al. 2012). However, its prevalence in *Cfv* and role in the development of BGC has not yet been addressed. Interestingly, a particular genomic island encoding a type IV secretion system (T4SS) and filamentation induced by cyclic adenosine monophosphate (cAMP) (FIC)-domain proteins was primarily associated with the subspecies venerealis (Gorkiewicz et al. 2010).

The work here presented focused on two major knowledge gaps in the management of BGC: (1) the assessment of the diagnostic value of the current molecular diagnostic tools and (2) the elucidation of the pathogenic potential of *Cfv*. To address the first issue, this work aimed

to validate the adequacy of molecular diagnostic assays based on the targets currently used when applied directly in clinical samples, ultimately to develop a diagnostic assay with high specificity and sensitivity. This achievement will enable the secure integration of molecular diagnostics into clinical practice. Concerning the second issue, the work here presented aimed to characterize *Cfv* strains, at the phenotypic and genomic level, to disclose features relevant for the diagnosis and pathogenicity of this microorganism.

These studies resulted in three manuscripts, submitted to international peer-reviewed and indexed journals, which were converted in the three chapters of the experimental work in the thesis.

1. ***Campylobacter portucalensis* sp. nov., a new species of *Campylobacter* isolated from the preputial mucosa of bulls**

   Silva MF[1], Pereira G[1], Carneiro C, Hemphill A., Mateus L., Lopes-da-Costa L, Silva E. 2020. *PLoS ONE 15*(1): e0227500. DOI: 10.1371/journal.pone.0227500

   [1]These authors contributed equally to this work

2. **Assessment of *Campylobacter fetus* subsp. *venerealis* molecular diagnosis using clinical samples of bulls**

   Silva MF, Duarte A, Pereira G, Mateus L, Lopes-da-Costa L, Silva E. 2020. *BMC Veterinary Research, 16*, 410. DOI: 10.1186/s12917-020-02634-7

3. **Genomic and phenotypic characterization of *Campylobacter fetus* subsp. *venerealis* strains**

   Silva MF, Pereira AL, Fraqueza MJ, Pereira G, Mateus L, Lopes-da-Costa L, Silva E. 2021. *Microorganisms, 9,* 340. DOI: 10.3390/microorganisms9020340

CHAPTER 2 – LITERATURE REVIEW

2.1 The genus *Campylobacter*

Campylobacter, from the Greek *Kampylos* (curved) and *Baktron* (rod), is the type genus of the family *Campylobacteraceae*, which also includes the genera *Arcobacter* and *Sulfurospirrilium* (Miller and Parker 2011; Vandamme et al. 2015). It was first proposed in 1963 by Sebald and Véron to encompass taxa formerly classified as *Vibrio* spp. clearly distinct from other members of the *Vibrio* genus (On 2001; Lastovica et al. 2014). Classification within the *Campylobacter* genus is supported primarily by phylogenetic criteria, unifying a large and diverse group of bacteria, which currently comprises 34 species with validly published names (Fitzgerald 2015; Vandamme et al. 2015). Despite their biological diversity, *Campylobacter* species have several features in common (Fitzgerald 2015). Cells of most *Campylobacter* species are small, slender (0.2-0.8 x 0.5-5 μm), curved-to-spiral, Gram-negative rods (Lastovica et al. 2014; Vandamme et al. 2015; Facciolà et al. 2017). However, degenerative forms, with a coccoid appearance, may develop under unfavourable conditions and in old cultures (Smibert 1978; Lastovica et al. 2014; Fitzgerald 2015; Vandamme et al. 2015). Most *Campylobacter* species are motile and exhibit a characteristic corkscrew-like movement by using a single polar flagellum at one or both ends of the cell (Smibert 1978; Vandamme et al. 2015). Yet, some members of the genus do not fit in this general description. The cells of *Campylobacter gracilis*, *Campylobacter hominis*, *Campylobacter ureolyticus,* and *Campylobacter blaseri* are aflagellated and, consequently, non-motile (Vandamme et al. 1995; Lawson et al. 2001; Vandamme et al. 2010; Gilbert et al. 2018), and the cells of *Campylobacter showae* have a unipolar bundle of flagella (Lastovica et al. 2014). The straight rod shape exhibited by some species, such as *Campylobacter rectus*, *C. showae*, *C. blaseri, C. gracilis,* and *C. hominis*, is another atypical characteristic of the genus *Campylobacter* (Vandamme et al. 2015; Gilbert et al. 2018).

Members of the genus *Campylobacter* predominantly exhibit oxidase activity and reduce nitrate (Vandamme et al. 2015). Carbohydrates are not fermented neither oxidized by *Campylobacter* species, which obtain energy from tricarboxylic acid intermediates and amino acids (Vandamme et al. 2015; Facciolà et al. 2017; Sahin et al. 2017). *Campylobacter* species are generally microaerophilic and require microaerobic conditions to grow (*i.e.* approximately 5 % O_2, 10 % CO_2, and 85 % N_2), although some species have optimal growth in anaerobic atmosphere conditions (Miller and Parker 2011; Sahin et al. 2017). Other species (e.g. *Campylobacter concisus*, *Campylobacter curvus*, *C. gracilis*) require supplementation of hydrogen or formate as an electron donor for microaerobic growth (Vandamme et al. 2015).

At the *genomic* level, *Campylobacter* species are characterized as low G+C organisms, with a G+C content ranging from 28 to 40 % (Liu et al. 2018). These microorganisms have a host-associated lifestyle and naturally inhabit a wide range of mammals, birds, reptiles, and humans (Miller and Parker 2011; Vandamme et al. 2015; Liu et al. 2018).

Several *Campylobacter* species are pathogenic to humans and/or animals (Vandamme et al. 2005). In fact, *Campylobacter* species cluster in five phylogenetic groups and all contain pathogenic species to humans or animals, as shown in Figure 1 (Costa and Iraola 2019). This underscores the medical and veterinary importance of this genus.
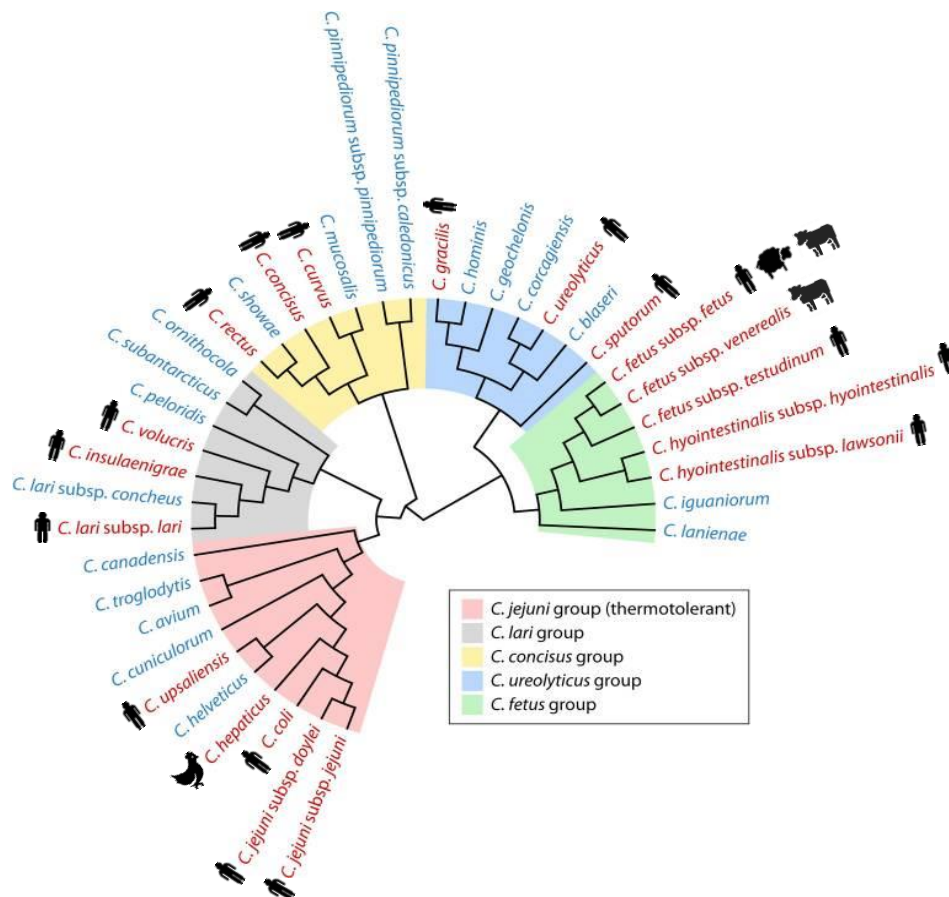
Figure 1. Phylogenetic tree of Campylobacter species with division in five distinct groups. Red coloured designations represent the most clinically relevant species for humans and/or animals, as illustrated. Adapted from Costa and Iraola (2019).

Some species of this genus colonize animals as harmless commensals, namely *Campylobacter jejuni* and *Campylobacter coli*, and exhibit pathogenicity to humans (Liu et al. 2018). Other members of the genus were not yet associated with disease and appear to be commensals (Sahin et al. 2017). Notably, several new species have been described in the

last years (e.g. *Campylobacter armoricus, Campylobacter blaseri, Campylobacter novaezeelandiae, Campylobacter ornithocola*) and, therefore, their possible involvement in disease has not yet been addressed (Cáceres et al. 2017; Gilbert et al. 2018; Boukerb et al. 2019; Bloomfield et al. 2020).

2.1.1 *Campylobacter* infections in humans and animals

Campylobacteriosis is the most commonly reported zoonosis in the European Union (EFSA and ECDC 2019). In fact, *Campylobacter* species cause more than 400-500 million infections per year worldwide (Igwaran and Okoh 2019). In the European Union, Campylobacteriosis has been the most reported gastrointestinal disease in humans (EFSA and ECDC 2019). *C. jejuni* is the leading cause of bacterial diarrheal disease, although other species including *C. coli*, *C. upsaliensis*, *C. lari, C. fetus, C. hyointestinalis, and C. ureolyticus* may also cause gastroenteritis in humans (Fitzgerald 2015; Vandamme et al. 2015; Casey et al. 2017; O'Brien 2017). Furthermore, *Campylobacter* species are also involved in a wide range of extraintestinal infections (*e.g.* periodontal disease, abscesses, meningitis, bacteremia) in humans, which are more common in immunocompromised or elderly patients (Fitzgerald 2015). *C. fetus* is the species most commonly isolated from patients with bacteremia and extraintestinal infections (Wagenaar et al. 2014). Apart from its importance in human medicine, *C. fetus* is the *Campylobacter* species with the highest economic importance in veterinary medicine, as a causative agent of reproductive failure in ruminants (Sahin et al. 2017). In cattle, the main *C. fetus* pathogen responsible for infertility and abortion is *Cfv*, the etiologic agent of BGC (Sahin et al. 2017). Likewise, *Cff* and *C. jejuni* are leading causes of abortion in sheep (Sahin et al. 2017)*.*

2.1.2 The species *Campylobacter fetus*

The first known description of a *Campylobacter* species was made in 1913, by McFaydean and Stockman, that identified a vibrioid-shaped organism as the pathogen responsible for abortions in sheep and cattle (Lovell 1963; Smibert 1978). Later, in 1919, Smith and Taylor isolated a similar microorganism from aborted bovine foetal fluids, which was named *Vibrio fetus* (Smibert 1978). Only three decades later this species was linked to reduced breeding efficiency in cattle (Lovell 1963). The species *Vibrio fetus* was then divided into two subspecies, *V. fetus intestinalis* and *V. fetus venerealis* (Lovell 1963)*,* which, after reclassification, are currently known as *C. fetus subsp. fetus and C. fetus subsp. venerealis*,

respectively. More recently, a new subspecies isolated from reptiles and humans was described and designated as *C. fetus* subsp. *testudinum* (*Cft*) (Fitzgerald et al. 2014).

C. fetus cells are slender and curved rods (0.2-0.3 x 1.5-5 µm) that may appear as comma-, s- or gull-shaped (Vandamme et al. 2005). Colonies on blood agar are non-haemolytic, round, with approximately 1-1.5 mm in diameter, smooth, convex, raised and greyish white after 24-72h of incubation (Smibert 1978; Vandamme et al. 2015). Differentiation between *Cff* and *Cfv* subspecies can be achieved by phenotypic tests, as detailed in chapter 2.2.2.2. However, *Cft* can only be identified by proteotyping (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF-MS) or using genotyping methods, since this subspecies exhibit similar phenotypic properties to *Cff* (Fitzgerald et al. 2014; Emele et al. 2019)*.*

These three *C. fetus* subspecies have different host preferences, being *Cft* isolated primarily from reptiles, *Cfv* from cattle, and *Cff* from cattle, sheep, and humans (Wagenaar et al. 2014; Costa and Iraola 2019). Recent studies revealed that *Cft* is genetically distinct from *Cfv* and *Cff*, which suggests a divergent evolution of the reptile and mammal-associated subspecies (Gilbert et al. 2016). Studies focused on the mammal-associated subspecies indicate that *C. fetus* may have appeared in humans 10.500 years ago and a distinct lineage adapted to cattle emerged during the livestock domestication period (Iraola et al. 2017). According to phylogenetic analyses, *Cfv* evolved from a *Cff* ancestor, being usually referred to as a defective clone of *Cff*, restricted to the bovine genital tract (Van Bergen et al. 2005a; van der Graaf–van Bloois et al. 2016).

The mammal-associated subspecies *Cff* and *Cfv*, exhibit different pathogenicity and epidemiology (Costa and Iraola 2019). The subspecies *Cff* is responsible for endemic abortion in sheep and sporadic abortion in cattle (Vandamme et al. 2015). This pathogen is a natural inhabitant of the gastrointestinal tract of healthy ruminants and is transmitted to pregnant ewes orally, from the environment, or aborted foetuses and placentas (Sahin et al. 2017). In susceptible pregnant sheep, *Cff* translocates across the intestinal mucosa and reaches the gravid uterus through the bloodstream, causing abortion (Sahin et al. 2017). Usually, *Cff* is isolated from the placentas and stomach of aborted foetuses, or blood and intestinal content of ewes and cattle (Vandamme et al. 2015). *Cff* also inhabits the intestinal tract of humans with the potential to cause bacteremia, gastroenteritis, and extra-intestinal infection in this host (Vandamme et al. 2015). In contrast, *Cfv* is unable to colonize the intestinal tract of humans and animals (Vandamme et al. 2015). This pathogen is specific of the bovine genital tract and is responsible for the BGC (Sahin et al. 2017).

2.2 Bovine Genital Campylobacteriosis

BGC is a venereal disease of cattle caused by *Cfv* (OIE 2018). Bulls are asymptomatic carriers, carrying *Cfv* in the glans penis, distal urethra, and prepuce (Noakes et al. 2001). More precisely, *Cfv* inhabits the preputial crypts resultant from invaginations of the preputial mucosa (Sahin et al. 2017). These microscopic structures provide a suitable environment for *Cfv* colonization with low oxygen tension, becoming more extensive and deeper with aging, which might contribute to a prolonged carrier status in older bulls (Noakes et al. 2001; Sahin et al. 2017). Typically, bulls do not exhibit clinical signs of disease neither altered semen quality (Sahin et al. 2017). However, a recent *in vitro* study showed that *Cfv* is capable to attach to bovine spermatozoa, affecting its viability and inducing sperm alterations (Cagnoli et al. 2020).

Females are infected by bulls during natural breeding or through artificial insemination with infected semen or materials (Mshelia et al. 2010; Michi et al. 2016). *Cfv* colonizes the anterior vagina and cervix, and in 12 to 14 days ascends to the uterus and oviducts, causing endometritis and salpingitis (Yaeger and Holler 2007). Although *Cfv* apparently does not interfere directly with the normal embryo development, the uterine inflammatory response generated by the infection may lead to embryonic death (Balzan et al. 2020). However, in approximately 10 % of females, the disease manifests as a mid-term abortion, between the 4th and 7th month of gestation (Noakes et al. 2001; Yaeger and Holler 2007). Clinically, the disease is manifested by delayed return to estrus, an increase in services per conception, and low pregnancy rates (Sahin et al. 2017). In opposition to bulls that may remain persistently infected, females are able to clear the infection from the uterus and oviducts and develop protective immunity generally within 3 to 6 months after infection (Balzan et al. 2020). Since the period to clear the infection is variable between animals, at pregnancy examination a wide variety of gestational ages can be found (Sahin et al. 2017). Nevertheless, *Cfv* may persist in the vagina and cervix of some animals for long periods, perpetuating the disease within the herd (Sahin et al. 2017).

The herd may manifest an acute or choric form of the disease, depending on the time passed since the introduction of *Cfv*. In a newly infected herd, the disease appears in the acute form with conception rates at first service ranging from 15 to 45 % (Lovell 1963). If mating is continued, most females become pregnant 90 days after infection or longer and the disease becomes chronic with less impact on fertility (Lovell 1963). In the chronic phase, symptoms of infertility are manifested mainly by heifers (Noakes et al. 2001). Some of the herds remain in this phase, undiagnosed, although with a sub-optimal reproductive performance (Lovell 1963).

2.2.1. Global distribution and economic impact

BGC is a notifiable disease to the World Animal Organization (OIE) with worldwide distribution (Mshelia et al. 2010). BGC has been identified in several countries, including Argentina, Australia, Bangladesh, Brazil, Canada, Costa Rica, France, Ireland, United Kingdom, Uruguay, and Venezuela (OIE [2019]) (Figure 2). This disease was also identified in several regions of the United States of America and Namibia. Additionally, in 2018, it was detected in Spain, New Caledonia, Germany, and Colombia. Malta and South Africa reported the last occurrence of BGC in 2017 (OIE [2019]).

The absence of monitoring programs in many countries and the use of diagnostic tests with low specificity or sensitivity are key limiting factors for the diagnosis and control of BGC (Silveira et al. 2018). The incidence of this disease is higher in developing countries, where natural breeding is commonly used (Mshelia et al. 2010). The common practice of artificial insemination in dairy herds reduced the incidence of BGC in developed countries as a result of testing programmes for bulls at artificial insemination stations and the use of antibiotics in semen extenders (Noakes et al. 2001)
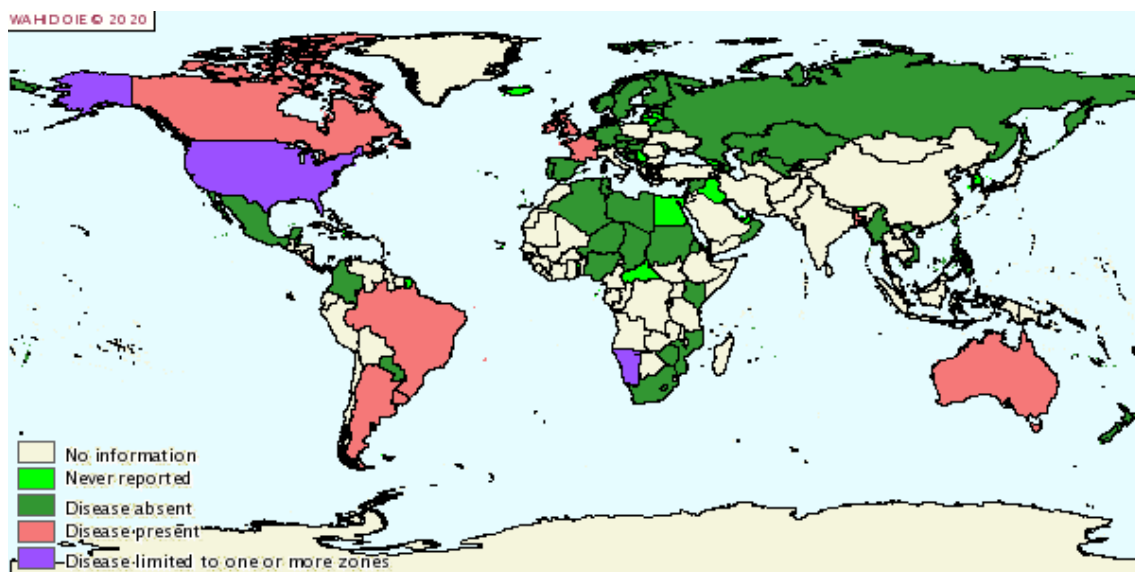
Figure 2 Bovine genital campylobacteriosis distribution map based on data collected between July and December 2019. From OIE [2019].

The profitability of beef suckling herds is dependent on the calves reared per cow annually and BGC can have a significant economic impact on infected herds (Truyers et al. 2014). The subclinical presentation of BGC, generally not suspected until low calving rates are detected, gave the label of "the quiet profit taker" to this disease (Blaser et al. 2008). During the first and following years after BGC introduction, the decline in gross margins can achieve

up to 66 % and 33 %, respectively (Hum 2007). In addition to the losses in production, herds with BGC occurrence have to support the costs associated with the diagnosis and management of the disease (Truyers et al. 2014).

2.2.2 Diagnosis

2.2.2.1 <u>Samples</u>

Diagnosis of BGC requires the identification of the pathogen *Cfv.* Samples used for diagnostic purposes include preputial samples, cervico-vaginal mucus samples, and placental or foetal tissues (OIE 2018). Preputial smegma samples may be collected from bulls using aspiration, scraping or washing techniques (Silveira et al. 2018). According to McMillen et al. (2006), the scraping technique, using a bull rasper to scarify the foreskin and penile mucosa, provides the highest recovery of *Cfv* cells, when compared with the other techniques. Alternatively, smegma may be obtained by aspiration using an artificial insemination pipette or after washing with 20-30 mL of phosphate buffered saline (PBS), followed by a massage and fluid recovery (Silveira et al. 2018). Since the infection is transient in females, their samples are not routinely used for screening purposes. However, when there is clinical suspicion, cervico-vaginal mucus samples may be collected using artificial insemination pipettes through gentle suction or washing with PBS, followed by fluid recovery (OIE 2018). The microorganism detection may also be performed on abortion products, including placenta, foetal stomach contents, liver, and lungs (Michi et al. 2016; OIE 2018).

2.2.2.2 <u>Microbiological culture</u>

The recommended diagnostic method by the OIE is microbiological culture followed by phenotypic identification (OIE 2018). However, routinely used culture techniques are laborious, time-consuming and, particularly in preputial samples, can have low sensitivity (Michi et al. 2016). These challenges are a consequence of the limited survival of *Cfv* under aerobic conditions and its slow and fastidious growth, which can be overtaken by the rapid overgrowth of other preputial microorganisms (Mshelia et al. 2010; Seid 2019). Fast-growing or swarming contaminants, including *Pseudomonas* spp., *Proteus* spp. and fungi adversely affect the detection of *Cfv* by covering large areas on the surface of culture plates (Monke et al. 2002; Chaban et al. 2013). This is a problem that occurs not only with preputial samples but also with cervico-vaginal mucus samples, limiting the identification of *Cfv* (Hum et al.

1994; McMillen et al. 2006). Therefore, the inherent objectives for successful isolation of *Cfv* are minimizing contaminant growth and maximizing *Cfv* recoverability (Monke et al. 2002). Additionally, the use of a transport enrichment medium (TEM) is recommended when samples are not processed on the same day as the collection, to support the survival of *Cfv* (OIE 2018).

Several TEM have been described, of which the most studied are the Clarke's, Weybridge's, and Thomann's TEM (Clark et al. 1972; Lander 1990; Harwood et al. 2009; OIE 2018). These media maintain the viability and support the multiplication of *Cfv*, as well as restrict the growth of competing microorganism, namely *Pseudomonas* spp. and *Proteus* spp, through the use of selective agents (Clark et al. 1972; Garcia et al. 1983; Lander 1990; Monke et al. 2002). The modified Weybridge's TEM (Lander 1990) is considered more effective than Clark's medium, with *Cfv* recovery rates of 74 % and 25 %, respectively. This medium is also more accessible to prepare than Clark's TEM which is a semi-solid medium that requires large quantities of bovine fresh serum (Hum et al. 1994). Recently, Thomann's TEM was developed without blood and toxic compounds as an alternative to allow the enrichment of *Cfv* and its direct use for *Cfv* detection by molecular biology techniques (Harwood et al. 2009). Nevertheless, the recommendation of TEM incubation before culture on solid media is not consensual since the increase in contaminants growth may originate poor recovery rates (Monke et al. 2002; Chaban et al. 2013). A shorter transport time, even when using a TEM, is preferred to obtain better yields of *Cfv* (Lander 1990; Monke et al. 2002). Furthermore, whenever it is possible to transport samples in a short period, direct culture is preferred, avoiding the use of TEM (Hum et al. 1994).

The recommended solid selective culture medium by the OIE for isolation of *Cfv* is Skirrow's medium, a blood-based medium with selective agents. The selective medium presents the advantage of increasing the diagnostic accuracy by supporting the growth of *Cfv*, while inhibiting the growth of other microorganisms (Monke et al. 2002). However, polymyxin B susceptible strains were reported and this antibiotic is commonly used in selective media such as Skirrow's agar and most TEM (Hum et al. 1994). Thus, only polymyxin B resistant strains are isolated, which decreases the sensitivity of these methods (Hum et al. 1994). An alternative technique consists in the use of blood agar plates associated with a passive filtration technique, which may lead to higher recovery rates than direct culture on Skirrow's Agar (Chaban et al. 2013). Larger microorganisms get retained in the filter and only small and motile cells such as *Campylobacter* spp. pass through the small pores of the filter (Gharst et al. 2013). This technique is also very effective in reducing the growth of contaminant colonies, as *Pseudomonas* spp. and fungi (Hum et al. 1994; Chaban et al. 2013). Furthermore, it allows the detection of strains sensitive to antibiotics used in selective

culture media (Casey et al. 2017), despite reducing the number of *Cfv* colonies yielded (Hum et al. 1994).

Overall, the best method to yield better *Cfv* recovery rates appears to be the passive filtration of a recently collected sample, avoiding the use of TEM (Chaban et al. 2013). Other techniques may lead to a high rate of false negative results (Chaban et al. 2013), but sensitivity may be improved with repeated sampling (Sahin et al. 2017). For optimal growth, cultures should be incubated under a microaerophilic atmosphere containing approximately 5 % oxygen, 10 % carbon dioxide, and 85 % nitrogen at 37 ºC, for 48 to 72 hours (OIE 2018; Seid 2019).

Phenotypic Identification

The macroscopic visualization of *Campylobacter*-like colonies together with the microscopic observation of spiral-shaped Gram-negative bacilli is not enough to determine the identification of *C. fetus*. The identification at the species and subspecies level requires a panel of phenotypic tests, to distinguish *C. fetus* from other cattle-associated *Campylobacter* species (Vandamme et al. 2015). For instance, *C. sputorum* is also an inhabitant of the bovine genital tract and is distinguished from *C. fetus* through its ability to produce H_2S in Triple Sugar Iron (TSI) medium and its tolerance to 2 % sodium chloride (NaCl) (Vandamme et al. 2015). *C. jejuni* and *C. coli* also exhibit differentiating characteristics from *C. fetus*, namely the capability to hydrolyse indoxyl acetate (Vandamme et al. 2005).

Differentiation of *C. fetus* subspecies is not straight forward due to their genotypic and phenotypic similarities (Sahin et al. 2017). This distinction may be achieved by the tolerance to 1 % glycine and hydrogen sulfide (H_2S) production in cysteine enriched medium (OIE 2018). Differently from *Cfv, Cff* growths in media containing 1 % glycine and produces H_2S in Brucella broth medium supplemented with 0.02 % cysteine. *Cfv* besides being intolerant to 1 % glycine, is unable to produce H_2S, except *Cfvi* which can produce H_2S such as *Cff* (van der Graaf-Van Bloois et al. 2014). A recent study revealed that the phenotype characterized by the inability to produce H_2S is associated with the partial deletion of a putative cysteine transporter operon (van der Graaf–van Bloois, Duim, et al. 2016; Farace et al. 2019).

Nevertheless, the phenotypic tests have poor reproducibility, and, in some cases, the phenotype is not consistent with the genotypic characteristics (van der Graaf-Van Bloois et al. 2014). Indeed, *Cfv* can acquire tolerance to glycine either by transduction or by spontaneous or induced mutations (Chang and Ogg 1971). Thus, the sole use of the glycine tolerance test may not be a reliable characteristic to differentiate *Cff* and *Cfv* (Chang and Ogg 1971). Recently, van der Graaf et al. (2014) found inconsistencies between subspecies

identification by biochemical tests and the phylogeny of core genome single-nucleotide polymorphisms (SNP), questioning the current phenotypic subspecies differentiation.

2.2.2.3. Direct Immunofluorescence and Enzyme-linked Immunosorbent Assay

Direct immunofluorescence (DIF) constitutes an easy and affordable methodology to detect *C. fetus*, widely practiced in several countries (Silveira et al. 2018). This can be performed either in *Campylobacter* isolates or directly in samples conserved in PBS with 1 % formalin. Succinctly, samples are incubated with a fluorescein isothiocyanate (FITC) conjugated (FITC) antiserum and observed under ultraviolet (UV) light. Samples with fluorescent bacteria with morphology typical of *C. fetus* are considered positive. This method has a sensitivity of 92.59 % and specificity of 88.89 %, with a limit of detection ranging between 10^2 and 10^4 colony forming units (CFU)/mL according to Figueiredo et al. (2002).

Enzyme-linked immunosorbent assays (ELISA) also can be used to identify *C. fetus* at the species level in enriched samples incubated in TEM for 4 to 5 days (Brooks et al. 2004). In the study carried out by Brooks et al. (2004), ELISA offered a higher sensitivity (98 %) than microbiological culture (74 %) to detect *C. fetus* in cervico-vaginal samples. Nevertheless, neither ELISA nor DIF are enough to diagnose BGC, due to their inability to differentiate *C. fetus* subspecies (Silveira et al. 2018).

2.2.2.4 Molecular diagnosis

The accurate differentiation of *C. fetus* at the subspecies level is crucial for BGC control and eradication programs (Abril et al. 2007). Thus, the inconsistencies in phenotypic tests for *Cfv* identification, described above, encouraged the development of PCR-based diagnostic methods (McMillen et al. 2006; van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013; Iraola et al. 2016). Several PCR assays have been described to perform the identification of *C. fetus* at two different taxonomic levels, the species and the subspecies level.

The multiplex-PCR described by Hum et al. (1997) targeting a sequence specific of *C. fetus* species (*cstA* gene) and a sequence of the subspecies *venerealis* (*parA* gene) is among the first and best characterized PCR assays. Amplification of *cstA* gene confirms the presence of the *C. fetus* species, while the detection of *parA* gene identified the subspecies *venerealis* (Hum et al. 1997). This species molecular marker has also been introduced in other multiplex-PCR assays for *C. fetus* identification (Yamazaki-Matsune et al. 2007; Iraola et al. 2012). However, according to a recent study, the primers directed towards the *cstA* gene failed to detect several *C. fetus* subsp*. testudinum* strains (Iraola et al. 2016).

Thereafter, several real-time PCR assays have been developed for subspecies differentiation (McMillen et al. 2006; van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). This approach offers promising results by detecting approximately one single cell, with sensitivity at least 10 times higher than conventional PCR and 250 times higher than selective microbiological culture (McMillen et al. 2006). The first probe-based real-time assay for *Cfv* detection was directed toward the *parA* gene, a target considered specific of the subspecies *venerealis* (McMillen et al. 2006). Later, in 2011, two *C. hyointestinalis* isolates from New Zealand cross-reacted in the real-time described by McMillen et al. (2006), producing a false positive result (Spence et al. 2011). Specificity failures were also suggested when another study revealed the absence of association between herds with *parA* positive bulls and reduced pregnancy rates in New Zealand (Sanhueza et al., 2014). However, these results could also be related with low/non-virulent strains (Sanhueza et al. 2014).

The primers described by Hum et al. (1997) to detect the *parA* gene were also adapted to a real-time PCR assay with SYBR Green chemistry (Chaban et al. 2012). This newly developed assay was tested in bovine preputial samples, and the specificity of positive results was confirmed by nucleotide sequencing of PCR products (Chaban et al. 2012). In these samples, the limit of detection for bacterial culture ranged between $1.4x10^4$ CFU/mL and $1.4x10^6$ CFU/mL (Chaban et al. 2012), whereas the PCR assay reliably detected 10^3 CFU/mL, which corresponds approximately to 1 copy per reaction (Chaban et al. 2012). This study also enabled the identification of two *parA* gene sequences, diverging in 8 nucleotide positions in 91 base pairs (bp) (Chaban et al. 2012). These nucleotide variations include 4 mismatches with the reverse primer described by McMillen et al. (2006), leading possibly to false negative results. In fact, more recent studies revealed that *parA*-based assays are associated with low sensitivity. A study involving 143 *C. fetus* strains, of which 60 *Cfv*, revealed that the assays described by McMillen et al. (2006) and Hum et al. (1997), failed to identify 47 % and 42 % of *Cfv* strains, respectively (van der Graaf-van Bloois et al. 2013). In accordance, several studies have been dedicated to the evaluation of *parA* detection assays (Table 1).

At the same time that *parA* was used as a molecular marker for *Cfv* detection, another sequence present only in *Cfv* strains was identified, the insertion sequence ISCfe1 (Abril et al. 2007). This insertion element is composed of two open reading frames, the *tnpA* and *tnpB* genes, flanked by 12-bp inverted repeats and by 3-bp direct repeats (Abril et al. 2007). The *Cfv* strains harbour at least one copy of ISCfe1, although the number of copies of this element among *Cfv* strains is variable, which suggest their spread in the genome by transposition (Abril et al. 2007). In addition to this subspecies specific target, the gene *nahE*

was identified by the same authors as a *C. fetus* specific gene, suggesting that it could be a suitable target for the identification of *C. fetus* at the species level (Abril et al. 2007).

Table 1 Evaluation of *parA* gene detection assays for *Cfv* identification

Primer Set	Study	Samples/Strains	Performance
VenSF/VenSR (Hum et al. 1997)	(Hum et al. 1997)	59 *Cfv* and 40 *Cff* strains	Only 2.02 % of results discordant with PFGE and/or conventional and probabilistic phenotyping methods
	(Wagenaar et al. 2001)	69 *C. fetus* strains	98.6 % agreement with AFLP 88 % agreement with phenotypic identification
	(On and Harrington 2001)	31 *C. fetus* strains	100 % agreement with PFGE 96.8 % agreement with phenotypic identification
	(Willoughby et al. 2005)	44 *Cff*, 32 *Cfv*, 16 non-*C. fetus* strains	100 % specificity 45 % sensitivity
	(Van Bergen et al. 2005a)	140 *C. fetus* strains	72.1 % agreement with AFLP 72.9 % agreement with phenotypic identification
	(Schulze et al. 2006)	81 *Cfv*, 22 *Cff* strains	100 % agreement with phenotypic identification
	(Abril et al. 2007)	26 *Cfv*, 27 *Cff* strains	96 % specificity 96 % sensitivity
	(Chaban et al. 2012)	401 preputial samples from 377 animals	Reliably detected 10^3 CFU/mL; Identification of two different *parA* gene sequences
	(van der Graaf-van Bloois et al. 2013)	83 *Cff*, 60 *Cfv*, 12 non-*C. fetus* strains	58 % sensitivity; 83 % specificity
	(Guerra et al. 2014)	300 preputial samples from virgin bulls, 260 repeated samples from 13 infected bulls	85 % specificity; 85 % sensitivity
CFVF/CFVR (McMillen et al. 2006)	(McMillen et al. 2006)	249 preputial samples and 120 cervico-vaginal samples with unknown status	39 positive results using the PCR assay vs. 9 positive results detected by culture
	(van der Graaf-van Bloois et al. 2013)	83 *Cff*, 60 *Cfv*, 12 non-*C. fetus* strains	100 % specificity 53 % sensitivity
	(Sanhueza et al. 2014)	222 preputial samples from 31 herds	28.8 % of positive samples; absence of association between PCR results and pregnancy rates

AFLP – Amplified fragment-length polymorphism; PFGE – Pulsed-field gel electrophoresis.

In 2013, McGoldrick et al. (2013) developed two SYBR Green real-time PCR assays for detection of ISCfe1 and *nahE* gene, tested in 1071 *Campylobacter* isolates, including 485 *Cff,* 223 *Cfv* isolates, and other *Campylobacter* species (Table 2). These assays revealed high specificity and sensitivity, with the *C. fetus* detection assay targeting *nahE* showing 100 % sensitivity and 99.6 % specificity, whereas the *Cfv* detection assay directed towards ISCfe1 was 98.7 % sensitive and 99.8 % specific (McGoldrick et al. 2013). These promising molecular targets were also used for the development of Taqman probe-based real-time PCR assays. In accordance, van der Graaf *et al.* (2013) developed a real-time PCR assay directed to *nahE* that was 100 % specific and sensitive for *C. fetus* identification. However, the identification of two different ISCfe1 sequences, although sharing 98.7 % sequence identity, was enough to decrease the sensitivity of one assay (ISC1) that accuratly identified 97 % of the *Cfv* isolates (van der Graaf-van Bloois et al. 2013). Additionally, a second assay directed to ISCfe1 (ISC2), designed to target a conserved region in both sequence types of ISCfe1 misidentified one *C. hyointestinalis* isolate as *Cfv* (98 % specificity). All of these assays have been tested on isolates and there are no data available about their performance on field samples. The development and evaluation of PCR assays targeting ISCfe1 is summarized in Table 2.

Table 2 Evaluation of ISCfe1 detection assays for *Cfv* identification

Primer Set	Study	Strains	Performance
CVEN-L/CVEN-R2 (Abril et al. 2007)	(Abril et al. 2007)	26 *Cfv*, 27 *Cff* strains, 23 non-*C. fetus* strains	100 % specificity 96 % sensitivity
	(van der Graaf-van Bloois et al. 2013)	83 *Cff*, 60 *Cfv*, 12 non-*C. fetus* strains	100 % specificity 97 % sensitivity
ISC1-F/ ISC1-R (van der Graaf-van Bloois et al. 2013)	(van der Graaf-van Bloois et al. 2013)	83 *Cff*, 60 *Cfv*, 12 non-*C. fetus* strains	100 % specificity 97 % sensitivity
ISC2-F/ISC2-R (van der Graaf-van Bloois et al. 2013)	(van der Graaf-van Bloois et al. 2013)	83 *Cff*, 60 *Cfv,* 12 non-*C. fetus* strains	98 % specificity 100 % sensitivity
CampF7/CampR7 (McGoldrick et al. 2013)	(McGoldrick et al. 2013)	485 *Cff*, 223 *Cfv* and 363 non-*C. fetus* strains	99.8 % specificity 98.7 % sensitivity

Recently, a phylogenomic study showed that most cattle-associated *C. fetus* strains clustered in a clade of strains harbouring ISCfe1, almost exclusively of ST-4 (Abdel-glil et al. 2020). These genomes were proposed as belonging to the subspecies *venerealis*, based

only on their genomic features, ignoring their phenotypic classification (Abdel-glil et al. 2020). Although ISCfe1 was present in all genomes, the *parA* gene was found only in 55 % of the genomes (Abdel-glil et al. 2020).

Other molecular targets were also described for identification of *Cfv*, although with limited success. Indeed, the *virB11* gene located within a genomic island considered specific of *Cfv* was used as a diagnostic molecular marker (Moolhuijzen et al. 2009; Iraola et al. 2012). However, later studies revealed that this genomic island is not universally present in *Cfv* and is also found in *Cff*, invalidating the use of this gene as a molecular diagnostic target (van der Graaf–van Bloois et al. 2016). Recently, a PCR assay was developed targeting a putative L-cysteine transporter coding operon, associated with the H_2S production phenotype of *C. fetus*. However, this assay only differentiates *Cfv*, which has a partial deletion of the operon, from *Cfvi* and *Cff* which harbour the intact gene (Farace et al. 2019), having no effective value for diagnosis of BGC.

Although the molecular differentiation between the two *C. fetus* subspecies is challenging, PCR assays developed to detect *C. fetus* at the species level have high sensitivity and specificity. In addition to the already stated molecular markers *cstA* and *nahE* genes, several PCRs were developed to identify *C. fetus*, namely directed to sequences of the 16S and 23S rRNA, *cpn60,* and *cdtABC* genes (Inglis and Kalischuk 2003; Asakura et al. 2008; Chaban et al. 2009; Iraola et al. 2016).

Other more laborious techniques and therefore not routinely used for *Cfv* identification were developed such as Amplified Fragment-Length Polymorphism (AFLP) and Multilocus Sequence Typing (MLST) (Duim et al. 2001; Wagenaar et al. 2001; Van Bergen et al. 2005a; van der Graaf-van Bloois et al. 2013). The AFLP fingerprinting reliably differentiates both *C. fetus* subspecies but is a time-consuming method and therefore is not routinely used for diagnostic purposes (Duim et al. 2001; Wagenaar et al. 2001). The clonal structure of *Cfv,* was used for subspecies identification using MLST. A MLST scheme described by van Bergen et al. (2005a) classified 55 out of the 57 isolates analysed as ST-4, with the two remaining isolates belonging to ST-7 and ST-12. These sequence types were not found in *Cff* isolates, and therefore, MLST was considered an effective technique for *C. fetus* subspecies differentiation (Van Bergen et al. 2005a; van der Graaf-van Bloois et al. 2013). However, recently, a *Cff* strain belonging to ST-4 isolated from a rural worker was identified (Iraola et al. 2015), putting into question the use of this technique for diagnostic purposes.

2.2.3 Treatment and control

In herds in which the BGC was diagnosed, control of the disease is based on the elimination of chronic infection sources and the prevention of transmission of *Cfv* (Yaeger and Holler 2007). Artificial insemination of cows is a highly effective measure to control BGC (Noakes et al. 2001; More et al. 2017). Since *Cfv* may persist in the female genital tract for extended periods, artificial insemination should be performed for at least two breeding seasons (Yaeger and Holler 2007). This strategy prevents disease transmission, while the infection is eliminated in cows with the development of protective immunity (Balzan et al. 2020). Heifers that have never been exposed to *Cfv* may be mated naturally with virgin bulls, in a group segregated from the infected herd (Noakes et al. 2001). In this situation, the segregation of infected and non-infected animals must be effective (Noakes et al. 2001). An alternative strategy consisting of extensive culling of infected bulls and barren females may substitute a whole herd artificial insemination program (Truyers et al. 2014). Infected bulls should be culled, but particularly valuable animals may be treated (Truyers et al. 2014), although the treatment with antimicrobials has revealed limited success (Michi et al. 2016). Streptomycin alone or combined with penicillin may be used to eliminate the carrier state in bulls, with higher efficacy in younger animals. This antibiotic combination proved to be effective by systemic administration (subcutaneous or intramuscular route) combined with topical application of streptomycin and/or penicillin in the penis and prepuce (Hum et al. 1993; Noakes et al. 2001; Truyers et al. 2014).

Although not available in all countries, vaccination with *Cfv* bacterin may control the disease where artificial insemination is not an option (Noakes et al. 2001). Vaccination creates immunization against *Cfv* infection and may cure the carrier state of bulls and cows (Yaeger and Holler 2007; Michi et al. 2016). The initial vaccination in infected herds should be performed with two administrations separated by two to four weeks and at least 30 days before the breeding season, followed by annual vaccination (Yaeger and Holler 2007). Several clinical trials indicate the effectiveness of vaccines (Clark et al. 1968; Clark et al. 1975; Clark et al. 1979; Vasquez et al. 1983), but this is not consensual, since failures in therapeutic immunization of bulls and protective immunity in females have been reported (Hum et al. 1993; Cobo et al. 2003; Fóscolo et al. 2005; Erickson et al. 2017). The main reason pointed for vaccination failure is the surface antigenic variation of *Cfv* and insufficient or poor quality of antigens (Hum et al. 1993; Cobo et al. 2003). Additionally, regional strains may present different surface antigens from vaccinal strains (Cobo et al. 2003).

Since *Cfv* can be transmitted to females through artificial insemination with frozen semen, it is crucial to ensure their sanitary quality, which can be achieved through semen processing with antibiotics (Morrell et al. 2014). Indeed, *Cfv* contamination was the initial concern that

motivated the use of antibiotics in the processing of bovine semen, in order to eliminate the risk of transmission of this pathogen (Shisong et al. 1990). The incubation of semen with a combination of penicillin (500 IU), streptomycin (500 μg), lincomycin (160 μg) and spectinomycin (300 μg) have proved to eliminate *Cfv* without compromising the semen viability (Shisong et al. 1990). Of these, penicillin and streptomycin are the most effective antibiotics against *C. fetus*, but the potential transmission of *Ureaplasma* and *Mycoplasma* spp. led to the addition of spectinomycin and lincomycin for semen treatment (Hänel et al. 2011). In the European Union, semen processing with antibiotics is mandatory for intra-community trade of bovine semen according to the EU Directive 88/407/CEE.

2.3   Virulence traits of *C. fetus* and *C. fetus* subsp. *venerealis*

2.3.1 Virulence factors

The *C. fetus* subspecies *Cff* and *Cfv* have highly syntenic genomes, with more than 99 % of average amino acid identity (AAI) between the core proteomes (Sprenger et al. 2012). These similarities are also reflected by the presence of putative virulence genes common to both subspecies, potentially involved in motility, adherence, invasion, and cytotoxicity (Ali et al. 2012).

A comparative genomic analysis identified several genes associated with flagella, namely genes encoding the flagellar hook associated proteins (FlgK, FlgE, FlgL), flagellar body basal protein, and flagellin B protein (Ali et al. 2012), which may contribute to host colonization, by their influence in chemotaxis and motility processes as demonstrated for other *Campylobacter* species (Guerry 2007; Bolton 2015). In fact, flagellar motility is considered an important determinant for *Campylobacter* host colonization and virulence (Lertsethtakarn et al. 2011; Miller and Parker 2011). The motility driven by the flagellum is regulated by a chemotactic signalling system, following favourable chemical gradients (Lertsethtakarn et al. 2011; Bolton 2015). Studies on *C. jejuni* demonstrated that mutations in flagellar genes inhibited colonization, and these genes were upregulated during adhesion and invasion of cell cultures (Bolton 2015). Additionally, the base of the flagellum includes a flagellar type III secretion system (Lertsethtakarn et al. 2011). Thus, besides having a role in motility, the flagellar apparatus acts as a transporter system, transporting, for instance, campylobacter invasion antigens into host cells (Bolton 2015). Although the *C. fetus* flagellum has not been comprehensively investigated (Liu et al. 2018), its involvement in *Cfv* adhesion to Madin-Darby bovine kidney (MDBK) cells was already demonstrated by *in vitro* studies (Chiapparrone et al. 2014). Scanning electron micrographs revealed that attachment to host

cells was mediated by the apical portion of the flagellum, which could suggest the presence of adhesins in it (Chiapparrone et al. 2014).

One of the most important *C. fetus* virulence factors are the surface-layer proteins (SLPs), which form a layer on the bacterial surface external to the outer membrane (Lastovica et al. 2014). This layer confers protection, evading the host immune system by preventing the binding of complement C3b. Moreover, the high-frequency antigenic variation in the proteins of the surface layer (S-layer) avoids antibody-mediated responses (Lastovica et al. 2014). The S-layer is encoded by the surface array protein (*sap)* locus, with conserved *sapCDEF* genes and multiple copies of either *sapA*, *sapB* genes or *sapAB* recombinants (Gilbert et al. 2016). In some *Cfv* strains, the *sapCDEF* locus is absent, suggesting that some *Cfv* strains are unable to synthetize a S-layer (Gilbert et al. 2016). The expression of sap proteins type A or B correlates with *C. fetus* serotypes: *Cfv* is serotype A, *Cff* can be serotype A, B and rarely AB, while *Cft* can be serotype A, B or AB (Gilbert et al. 2016). *Cff* mutant strains with deletion of SLPs were unable to cause abortion in sheep, underlining its importance as a virulence factor (Grogono-Thomas et al. 2000).

Other surface proteins involved in *Campylobacter* spp. adhesion to host cells are also encoded in the genomes of *C. fetus*, namely the fibronectin-binding protein CadF, the protein PEB1 and the phospholipase A (Ali et al. 2012; Bolton 2015). The protein CadF mediates adhesion of *C. jejuni* and *C. coli* to fibronectin in host tissues (Monteville et al. 2003; Konkel et al. 2005; Krause-gruszczynska et al. 2007). Mutational studies also confirmed its involvement in cecal colonization of chicks, evidenced by the inability of colonization by the isogenic mutant (Ziprin et al. 1999). The protein PEB1 is also involved in adhesion and invasion of epithelial cells, demonstrated by *in vitro* and *in vivo* studies using a mouse model (Pei et al. 1998). Recently, it was demonstrated that this adhesin is an aspartate/glutamate-binding protein of an ABC transporter involved in the utilization of aspartate and glutamate as carbon sources (Leon-kempis et al. 2006). Additionally, the mutation of the gene encoding the membrane phospholipase A (PldA) also interferes with *C. jejuni* cecal colonization of chicks (Ziprin et al. 2001) and in *C. concisus* this protein is a recognized virulence factor responsible for cytolytic effects (Liu et al. 2018).

C. fetus also encodes proteins putatively involved in invasion processes, including the *Campylobacter* invasion antigen (CiaB) (Ali et al. 2012). Indeed, the invasion properties of *Campylobacter* spp. are strongly correlated with the presence of CiaB (Iraola et al. 2014; Bolton 2015). Nevertheless, this gene is also found even in *Campylobacter* species with an apparently null invasion capacity (Iraola et al. 2014). Therefore, the CiaB alone is not enough to accomplish a successful invasion or the sequence variations in the *ciaB* gene may have an effect in switching and/or specialization of this protein (Iraola et al. 2014). Interestingly, *C.*

fetus and *C. sputorum* have several conserved nucleotide positions in the *ciaB* gene, which are absent in other *Campylobacter* spp., suggesting a probable specialization of CiaB to invade genital tissues resultant of co-evolution (Iraola et al. 2014).

A well-known exotoxin of *Campylobacter* spp., the cytolethal distending toxin (CDT) is also encoded in the genome of *C. fetus* (Ali et al. 2012). This is a tripartite toxin encoded by three genes (*cdtA*, *cdtB* and *cdtC*) to be functionally active (Bolton 2015). The genes *cdtA* and *cdtC* encode two heterodimeric subunits responsible for the binding of the enzymatically active subunit – *cdtB*- to the cell membrane and delivery (Bolton 2015). This toxin causes an irreversible block of the cell cycle in G2 phase and, subsequently, cell death (Miller and Parker 2011). Iraola *et al.* (2014) through a comparative genomic approach, found that genes encoding CDT were one of the most important virulence factors differentiating pathogenic from non-pathogenic *Campylobacter* species. The two other main differentiating factors were the capsular and *sap* genes (Iraola et al. 2014). Additionally, pathogenic *Campylobacter* species had more genes encoding lipopolysaccharides, adhesion and motility proteins (Iraola et al. 2014). These observations suggest that the most recent common ancestor of *Campylobacter* was a non-pathogenic or putative pathogen and virulence genes were then acquired horizontally (Iraola et al. 2014). Particularly in *C. fetus*, Iraola et al. (2014) suggested that this species acquired its virulence factors horizontally from other pathogenic *Campylobacter* species.

The importance of the above mentioned virulence factors in the pathogenesis of BGC is poorly understood. This is also hindered by a lack of suitable animal models for the study of *Cfv* virulence. Koya et al. (2015) attempted to compare the virulence of three *Cfv* strains using a guinea pig model. However, the intravaginal route of infection failed for this purpose, since none of the three strains caused abortion (Koya et al. 2015). Only through an intraperitoneal route of inoculation, *Cfv* was able to cause abortion and neutrophil infiltration in the uterus and placenta (Koya et al. 2015).

Although the *C. fetus* subspecies are highly similar at the genomic level, these microorganisms have different ecology and are responsible for different clinical presentations of disease, as detailed in the previous chapter. Indeed, the two *C. fetus* subspecies, *Cff* and *Cfv*, are highly similar at the genomic level with 92.9 % overall sequence identity and 99.8 % identity in homologous regions (Kienesberger et al. 2014). The sequences differentiating both subspecies exhibit features indicative of acquisition by horizontal transfer (Ali et al. 2012; Kienesberger et al. 2014). Comparative genomic analysis revealed several genomic islands in the genomes of *Cff* and *Cfv*, and a particular genomic island was suggested as involved in *Cfv* virulence (Gorkiewicz et al. 2010; Kienesberger et al. 2011; Sprenger et al.

2017). This genomic island contains genes encoding FIC-domain proteins coding genes and a T4SS, analogous to the *Agrobacterium tumefaciens virB/virD4* (Ali et al. 2012). T4SSs are secretion apparatus present in several bacterial species, which play important roles in DNA conjugative transfer and secretion of toxic molecules to eukaryotic target cells (Grohmann et al. 2018). These features assume particular importance in bacterial delivery of virulence determinants to colonize and infect host cells, and to disseminate antibiotic-resistant genes and other fitness traits by conjugative DNA transfer (Grohmann et al. 2018). T4SS are generally composed of eleven VirB proteins, VirB1-VirB11, and one VirD4 protein (Figure 3). These include three cytoplasmic ATPases (VirB4, VirB11, and VirD4) that participate in the assembly of the system and secretion of substrates (Fronzes et al. 2009). The proteins VirB3, VirB6, and VirB8 form an inner membrane platform, and VirB7, VirB9 and VirB10 establish the membrane core complex (Grohmann et al. 2018). VirB2 and VirB5 constitute the major and minor subunits, respectively, of an extracellular pilus, and VirB1 is a lytic transglycosylase (Fronzes et al. 2009).
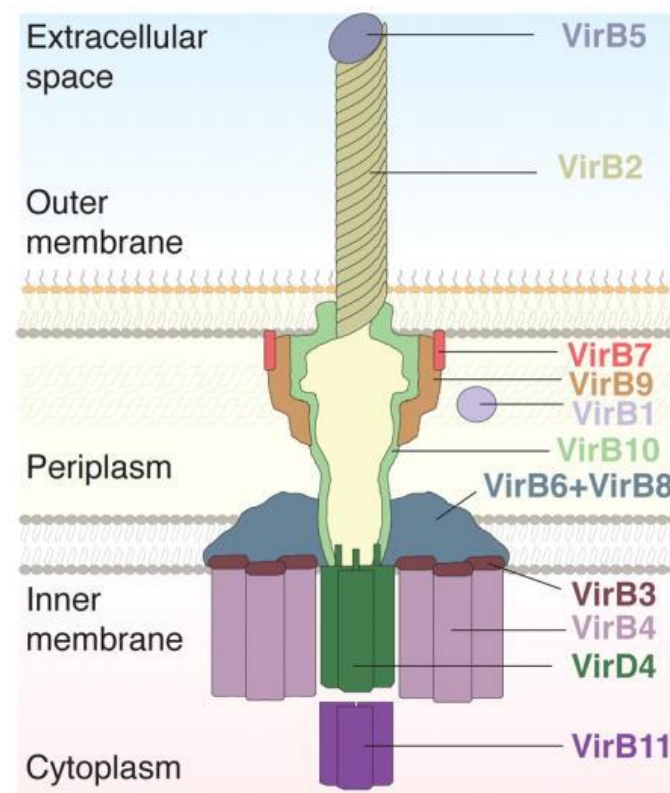
Figure 3 Schematic representation of a VirB/VirD type IV secretion system.
From Grohmann et al. (2018)

The most studied T4SS of *C. fetus* lacks the *virB1* gene, whose function is probably replaced by a different lytic transglycosylase present in the genomic island. Functional studies with mutational analysis confirmed their role in the invasion and cytotoxicity to eukaryotic cells, supporting its contribution to *Cfv* virulence (Gorkiewicz et al. 2010). On the other hand, this secretion apparatus is involved in conjugative DNA transfer, intra and interspecies, which may enable the sharing of fitness or virulence-related traits (Kienesberger et al. 2011). The genomic island also harbours FIC protein-encoding genes, which are part of a toxin-antitoxin system network in *Cfv* (Sprenger et al. 2017). Briefly, the toxin-antitoxin systems are composed of a toxin module that interferes with cellular processes forcing the bacterial cell into a transient dormant state, and an antitoxin module that inactivates or regulates the toxin expression. In normal circumstances, the toxins and antitoxins counteract, but under unfavourable environmental conditions, the antitoxin is depleted or degraded leaving the toxin available to inhibit cell growth (Wen et al. 2014). Toxin-antitoxin systems have been implicated in the formation of persister cells (dormant cells resistant to unfavourable conditions and antibiotics), abortive infection (altruistic suicide after bacteriophage infection), and post-segregational killing (cell death after the loss of a mobile element) (Harms et al. 2018). Interestingly, the FIC-domain proteins encoded by this region may be translocated to the host cells by the T4SS, similarly with what occurs in other species, such as *Legionella pneumophila*, *Coxiella burnetti* and *Bartonella henselae*, causing cytoskeleton damage in the host cells (Harms et al. 2016). Therefore, the genomic island, in which the T4SS and FIC-domain protein encoding genes are located, has been suggested as contributing to *Cfv* virulence properties and adaptation to the genital tract (Gorkiewicz et al. 2010; Kienesberger et al. 2011; Kienesberger et al. 2014). More recently, this genomic island was found also in *Cff* isolates, harbouring also two genes responsible for antimicrobial resistance to tetracycline and streptomycin (Abril et al. 2010; Escher et al. 2016). *Cfv* strains also have other T4SS encoding regions, with a different structural organization and sequence homology, suggesting that they were acquired from different donor species (van der Graaf–van Bloois et al. 2016). However, these poorly studied T4SS coding regions lack several *virB* genes (van der Graaf-van Bloois et al. 2016), which may impact on their functionality.

2.3.2 Antimicrobial resistance

The available studies on *in vitro* antimicrobial susceptibility testing in *Cfv* isolates were performed only using disk diffusion susceptibility testing in field isolates from Germany and Brazil (Vargas et al. 2005; Hänel et al. 2011). Among 50 *Cfv* isolates from Germany, all revealed susceptible to gentamicin, although only 14 % exhibited reduced susceptibility to

antimicrobials, predominantly to lincomycin and spectinomycin (Hänel et al. 2011). Reduced antibiotic susceptibility by comparison with the reference strain *Cfv* NCTC 10354 was also found to streptomycin, ciprofloxacin, erythromycin and tetracycline (Hänel et al. 2011). Another study, involving 21 *Cfv* isolates from Brazil, reported resistances to lincomycin and enrofloxacin, besides intermediate susceptibility to enrofloxacin, polymyxin B, neomycin and lincomycin (Vargas et al. 2005). However, all isolates were susceptible to penicillin and streptomycin used in semen and carrier bulls' treatment (Vargas et al. 2005).

Aminoglycosides, which include streptomycin and spectinomycin, interfere with the bacterial protein synthesis by binding to the ribosomal 30S subunit (Wieczorek and Osek 2013). The mechanism of resistance in *Campylobacter* spp. is mediated by an enzymatic modification that leads to a decrease in the affinity of aminoglycosides to the target site (Wieczorek and Osek 2013). *Cfv* may acquire resistance to streptomycin by spontaneous mutation, without previous contact with this antimicrobial (Morgan 1958), but also through the gene *ant-6-Ib* present in a genomic island specific of *C. fetus* (Abril et al. 2010). The gene *tet(44)* is also present in the same genomic island, conferring resistance to tetracycline (Abril et al. 2010). Tetracycline binds to the 30S ribosomal unit, inhibiting the peptide elongation and resistance to this antibiotic is mediated by genes encoding ribosomal protection proteins (RPPs) capable of inducing conformational changes in the ribosome, thus leading to the release of the tetracycline molecule (Wieczorek and Osek 2013). Macrolide antibiotics (e.g. erythromycin) also act by binding to the 50S ribosomal subunit targeting the 23S rRNA and interfering with protein synthesis (Vester and Douthwaite 2001). Mutations in the macrolide target site of the 23S rRNA are associated with resistance to macrolides and cross-resistance with drugs from the lincosamide group, which include lincomycin (Vester and Douthwaite 2001). Resistance to quinolones in *Campylobacter* spp. (*e.g.* ciprofloxacin, enrofloxacin) is acquired mainly by mutations that result in amino acid substitutions in the DNA gyrase and topoisomerase IV (Wieczorek and Osek 2013). These bacterial enzymes are the targets of quinolones and are involved in DNA replication, transcription, recombination and repair (Wieczorek and Osek 2013). There are several amino acid substitutions in the GyrA subunit of DNA gyrase described in *Campylobacter* that provide resistance to quinolones (Wieczorek and Osek 2013).

CHAPTER 3 – EXPERIMENTAL WORK

3.1. *Campylobacter portucalensis* sp. nov., a new species of *Campylobacter* isolated from the preputial mucosa of bulls

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3.1.1 Abstract

A new species of the *Campylobacter* genus is described, isolated from the preputial mucosa of bulls (*Bos taurus*). The five isolates obtained exhibit characteristics of *Campylobacter*, being Gram-negative non-motile straight rods, oxidase positive, catalase negative and microaerophilic. Phenotypic characteristics and nucleotide sequence analysis of 16S rRNA and *hsp60* genes demonstrated that these isolates belong to a novel species within the genus *Campylobacter*. Based on *hsp60* gene phylogenetic analysis, the most related species are *C. ureolyticus*, *C. blaseri* and *C. corcagiensis*. The whole genome sequence analysis of isolate FMV-PI01 revealed that the average nucleotide identity (ANI) with other *Campylobacter* species was less than 75 %, which is far below the cut-off for isolates of the same species. However, whole genome sequence analysis identified coding sequences highly homologous with other *Campylobacter* spp. These included several virulence factor coding genes related with host cell adhesion and invasion, transporters involved in resistance to antimicrobials, and a T4SS, containing *virB2-virB11*/*virD4* genes, highly homologous to the *C. fetus* subsp. *venerealis*. The genomic G+C content of isolate FMV-PI01 was 28.3 %, which is one of the lowest values reported for species of the genus *Campylobacter*. For this species the name *Campylobacter portucalensis* sp. nov. is proposed, with FMV-PI01[T] (= LMG 31504, = CCUG 73856) as the type strain.

3.1.2 Introduction

The *Campylobacteraceae* family of the order Campylobacterales is the largest and most diverse family in class Epsilonproteobacteria of the phylum Proteobacteria (Vandamme et al. 2005). *Campylobacter*, the type genus of the family, contains species known to be pathogenic to humans (Gölz et al. 2014) and other animals (Sahin et al. 2017) as well as non-pathogenic species that colonize a large range of molluscs, reptiles, birds and mammals

(Lastovica et al. 2014). Cells of most *Campylobacter* species are motile, microaerophilic, Gram-negative, slender, spirally curved rods and 0.5–5 µm long by 0.2–0.8 µm wide. However, some species exhibit straight rod morphology (Vandamme et al. 2005) and *C. gracilis*, *C. hominis*, *C. ureolyticus* and *C. blaseri* are non-motile (Lawson et al. 2001; Vandamme et al. 2010; Shinha 2015; Gilbert et al. 2018). Seven species colonize cattle (Lastovica et al. 2014), of which *C. coli, C. hyointestinalis*, *C. jejuni, C. lanienae* and *C. ureolyticus* are found in faeces (Inglis et al. 2005; Hakkinen et al. 2007; Koziel et al. 2012), *C. sputorum* is a commensal of the penile and preputial mucosae (Quinn et al. 2011) and *C. fetus* includes two subspecies with clinical relevance in cattle. The *C. fetus* subspecies *fetus* colonizes the bovine intestinal tract, causing sporadic abortion, whereas the subspecies venerealis inhabits exclusively the genital tract of cattle, and is the etiologic agent of BGC (Michi et al. 2016). Herein, we describe a new species of *Campylobacter* isolated from a beef herd with history of reproductive failure compatible with BGC, in the Alentejo province of Portugal.

3.1.3 Material and Methods

3.1.3.1 Ethics statement

The *in vivo* samples used in this study were obtained from a bull that performed natural mating in a herd with clinical signs of BGC. The samples were collected by a certified veterinarian, using the recommended OIE sampling methods for diagnostic purposes. No ethical approval was required as this was part of a routine veterinarian evaluation of beef herd reproductive failure. *Ex vivo* samples were obtained from animals slaughtered for human consumption. As sampling was performed *post mortem*, in a certified slaughterhouse, no ethical approval from an Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board was required.

3.1.3.2 Sampling, isolation procedures and culture conditions

Samples were obtained *in vivo* and *post-mortem*. The *in vivo* sample was collected from one mature Charolais bull, for laboratory diagnostic purposes. This sample was obtained from the preputial fornix using a technique combining scraping and small volume fluid washing (PBS) of the mucosa (Monke et al. 2002), and transported to the laboratory within 4 hours in two aliquots, one in Weybridge TEM and one in PBS. The bull had a normal routine breeding soundness evaluation and performed natural mating in a beef herd in the Alentejo province of Portugal. This herd showed fertility features compatible with BGC, namely a low breeding season fertility, and late and spread calvings within the calving season. *Post-*

mortem samples were also collected from the preputial fornix at two slaughterhouses and transported to the laboratory in PBS within 4 hours. The *in vivo* collected sample transported in Weybridge TEM was incubated at 37 °C in a microaerobic atmosphere (Genbox Microaer, Biomérieux, France) for 48 hours, as an enrichment step. Enriched samples were plated through two different approaches: i) passively filtered onto blood agar (BA) and ii) spread in *Campylobacter* Skirrow Agar (CSA) (Chaban et al. 2013). In the BA approach, 0.65-µm mixed cellulose ester membrane filters (Advantec, Japan) were applied to the surface of BA plates supplemented with 5 % sheep blood (Columbia agar + 5 % sheep blood, Biomérieux) and inoculated with 100 µL of enriched sample for 30 minutes in aerobic conditions at room temperature; filters were then removed. In the CSA approach, 100 µL of enriched sample was spread on CSA plates. The BA and CSA plates were then incubated in microaerobic conditions at 37 °C for 72 hours. The samples transported in PBS (*in vivo* and *post-mortem* collected) were diluted (ten-fold dilutions; 10^{-1}, 10^{-2} and 10^{-3}), streaked onto BA plates and incubated in a microaerobic atmosphere at 37 °C for 48 hours. Colonies with *Campylobacter*-like morphology (small, smooth, translucent) were streaked onto BA and returned to a microaerobic atmosphere for a further incubation at 37 °C for 48 hours. Before phenotypic and genotypic characterization, cells were microscopically examined using Gram staining.

3.1.3.3. Molecular identification and phylogenetic analysis

Genomic DNA from bacterial isolates was extracted using DNeasy Blood and Tissue kit (Qiagen, Germany), according to the manufacturer's instructions. The 16S rRNA gene was amplified and sequenced using a universal set of primers–fD1 and rP1 (Table 3). Additionally, the flanking regions were amplified and sequenced with the primer sets FrAF/Vc1-2 and FrBF/FrBR (Table 3). Primers (FrAF, FrBF and FrBR) were designed using Primer-BLAST (Ye et al. 2012), based on the whole genome sequencing data. Primers FrAF and FrBR were designed to target a neighbour sequence of the 16S rRNA gene in order to obtain a full-length sequence. PCR reactions were carried out in a 50 µL mixture containing 0.3 µM of each primer, 200 µM of deoxynucleotide-triphosphates (4you4 dNTP Mix, Bioron, Germany), 1x reaction buffer (Complete NH4 reaction Buffer, 10x, Bioron), 2 units of DFS-Taq Polymerase (Bioron) and 3 µL of DNA. The thermal cycle conditions were as follows: 94 °C for 2 min, followed by 30 cycles of denaturation (94 °C for 30 s), annealing (30 s), and extension (72 °C for 60 s), with a terminal extension step of 72 °C for 5 min. The annealing temperature set for each primer pair is shown in Table 3. The amplified sequences were aligned and trimmed to create a full-length 16S rRNA gene sequence. The nucleotide sequence of FMV-PI01 isolate (GenBank accession no: MN417497) was compared with

other 16S rRNA gene sequences deposited in the NCBI database, using BLASTN algorithm. To investigate the taxonomic position of the bacterial isolates, a phylogenetic tree based on 1513 nucleotide positions of 16S rRNA gene sequences was reconstructed. Available sequences of 16S rRNA gene of other *Campylobacter* species were retrieved from the GenBank database for phylogenetic analysis with Molecular Evolutionary Genetic Analysis (MEGA) X software (Kumar et al. 2018). Sequences were aligned with Clustal W algorithm (Thompson et al. 1994) and positions with missing data were trimmed. The phylogenetic tree was reconstructed by the neighbour-joining method and stability of grouping was estimated by bootstrap analysis, set for 1000 replications. To further refine the phylogenetic analysis, a phylogenetic tree based on *hsp60* gene (also known as *cpn60* and *groEL)* sequences was also reconstructed, as described above. The amplification and sequencing of *hsp60* gene were carried out using two primer pairs (hsp60_AF/hsp60_AR and hsp60_BF/hsp60_BR, Table 3) to obtain a 1472 bp long sequence.

Table 3 Primer sequences used for 16S rRNA and *hsp60* genes amplification

Gene	Designation	Primers (5´-3´)	Annealing temperature	Amplicon size (bp)	Reference
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	52 ºC	1475	(Weisburg et al. 1991)
	rP1	ACGGTTACCTTGTTACGACTT			
	FrAF	CGATTGAGCCAAGGGCTTTA	52 ºC	461	This study
	Vc1-2R	ACTTAACCCAACATCTCACG			(Gorkiewicz et al. 2003)
	FrBF	ACACGTGCTACAATGGCATA	53 ºC	451	This study
	FrBR	TCTCTGAAAACTAAACAAGGATGA			
hsp60	hsp60_AF	AACTTTATGGTGGCGTTAAAA	52 ºC	1118	
	hsp60_AR	AGTTTCTGTTGCAGCACCTA			
	hsp60_BF	AGCTTAATGTTGTTGAGGGA	51 ºC	1085	
	hsp60_BR	TTACATCATACCACCCATAC			

3.1.3.4 Biochemical characterization and growth conditions

For biochemical characterization, bacterial cultures grown in a microaerobic atmosphere at 37 °C for 48 hours were used. Oxidase activity was determined with oxidase test sticks (Liofilchem, Italy) and catalase activity was evaluated by observation of bubbling formation

on a 3 % peroxide hydrogen solution within 5 seconds. Urease and H_2S production were assessed on Christensen Urea Agar (Liofilchem) and TSI Agar (Liofilchem), respectively. Additionally, commercial tests were used to evaluate nitrate reduction, hippurate hydrolysis (Liofilchem) and indoxyl acetate hydrolysis (Indoxyl strips, Sigma-Aldrich), following the manufacturer's instructions. The growth on BA supplemented with 1 % glycine, 2 % NaCl, 3.5 % NaCl, 0.04 % Tetrazolium chloride (TTC) and on MacConkey agar was determined according to standardized procedures, previously described (On and Holmes 1991a; On and Holmes 1991b). The evaluation of growth on anaerobic and aerobic atmospheres at 37 °C, and microaerobic growth at 25 °C, 37 °C or 42 °C, after 48 to 96 hours was also performed. The bacterial motility was assessed by the hanging drop technique (Public Health England [2018]), using bacterial suspensions in PBS after 48 hours of growth on BA.

Reference strains *Cff* NCTC 10842, *Cfv* NCTC 10354, *C. coli* CNET 068, *C. jejuni* subsp. *jejuni* NCTC 11168, and isolates identified as *C. sputorum* bv. *sputorum*, and *Proteus* sp. were used as controls in the tests described above.

3.1.3.5 Electron microscopy

Electron micrographs were taken from a pure culture of isolate FMV-PI01. Preparations for electron microscopy were performed as previously described (Hemphil and Croft 1997; Hemphill et al. 2004; Basto et al. 2017), followed by post-fixation in 2 % osmium tetroxide and stepwise dehydration (30/50/70/90/100 %) in ethanol. For transmission electron microscopy, samples were embedded in EPON812. Ultrathin sections (80 nm) were placed onto 300-mesh formvar-carbon-coated nickel grids (Plano, Wetzlar, Germany) and stained with Uranyless and lead citrate (Basto et al. 2017). Specimens were viewed on a CM12 transmission electron microscope operating at 60 kV.

For scanning electron microscopy, fixed and dehydrated samples were resuspended in two changes of hexamethyl-disilazane (Sigma), sputter coated with gold, and inspected on a JEOL 840 scanning electron microscope operating at 25 kV.

3.1.3.6 Whole genome sequencing and comparative genomic analysis

The genomic DNA was extracted from a pure culture of the isolate FMV-PI01, grown on BA over 48 hours, using the DNeasy Blood and Tissue kit (Qiagen, Germany). After the genomic library preparation, the generated DNA fragments were sequenced using the HiSeq 4000 System (Illumina), with 150 bp paired-end reading sequences and assembled using CLC Genomics Workbench version 11.0.1 (CLC bio, Denmark), at Stabvida (Caparica, Portugal). The assembled genome was annotated with the Rapid Annotation Using Subsystem Technology (RAST) 2.0 pipeline (Aziz et al. 2008; Overbeek et al. 2014).

The ANI was calculated with the webserver JspeciesWS (Richter et al. 2016) for the isolate FMV-PI01 and other *Campylobacter* species. The G+C content was determined based on the whole genome sequence of isolate FMV-PI01.

The identification of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) / CRISPR-associated systems (Cas) was performed using the CRISPRCasFinder webserver (Couvin et al. 2018). Additionally, the presence of putative virulence factor coding genes was evaluated based on the homology of translated sequences using BLASTP. Only sequences with query coverage >95 % and identity >50 % with known protein sequences of virulence factors were considered.

To estimate the pathogenic potential of this novel species, the assembled genome of isolate FMV-PI01 was analysed with the PathogenFinder Web server (Cosentino et al. 2013), using the automatic model option.

3.1.4 Results and discussion

3.1.4.1 <u>Morphological characterization</u>

The aliquot of the *in vivo* collected preputial sample transported in Weybridge TEM produced no *Campylobacter*-like morphology colonies on either approach (BA and CSA). In contrast, the *in vivo* collected preputial sample aliquot transported in PBS and streaked onto BA produced *Campylobacter*-like morphology colonies (isolate FMV-PI01). *Post-mortem* preputial samples, collected in PBS, produced four isolates (isolates FMV-PI02 to FMV-PI05) from beef bulls arising from four geographically distinct herds.

Colony morphology in BA was common to all isolates, colonies being punctiform, circular, with convex elevation, smooth margins, without pigmentation and no haemolysis observable. Bacterial cells from all isolates were Gram-negative and exhibited a straight rod shape. Electron microscopy revealed that isolate FMV-PI01 bacterial cells had an average length of 1.68±0.07 µm (n = 30) and an average width of 0.44±0.01 µm (n = 30), and were devoid of a flagellum. Occasional filamentous cells were observed displaying lengths up to 18 µm (Figure 4).
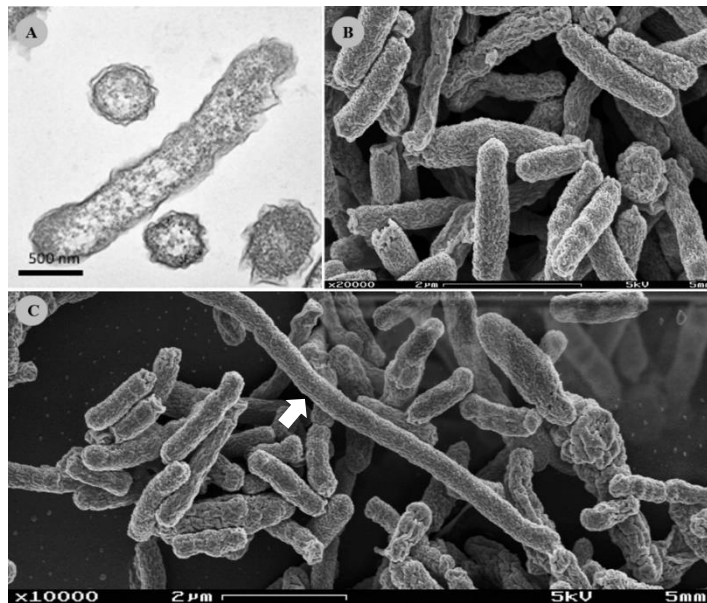
Figure 4 Electron micrographs of isolate FMV-PI01. (A) Transmission electron microscope micrograph in longitudinal and transverse views. Scale bar, 500 nm. (B) Scanning electron microscope micrograph in detail. Scale bar, 2 μm (C) Scanning electron microscope micrograph with evident long filamentous cells (white arrow). Scale bar, 2 μm.

3.1.4.2. Phenotypic characterization

The phenotypic tests were validated by control *Campylobacter* species strains, whose results were all in agreement with those reported in the literature (On et al. 2017). All five isolates presented identical phenotypic characteristics (Table 4). Isolates were positive for oxidase activity, a feature present in all *Campylobacter* species except *C. gracilis* and sporadic isolates of *C. concisus* and *C. showae* (Vandamme et al. 2005). Isolates were negative for catalase activity and were unable to hydrolyse urea, hippurate and indoxyl acetate. From the seven *Campylobacter* species that colonize cattle, only *C. jejuni* is able to hydrolase hippurate (Vandamme et al. 2010; Lastovica et al. 2014). Contrary to the majority of *Campylobacter* species, the isolates were unable to reduce nitrate. In fact, only three other species of the *Campylobacter* genus fail to reduce nitrate: *C. hominis, C. mucosalis* and *C. concisus* (Vandamme et al. 2005). Production of H_2S was not observed in TSI agar. The growth tests showed that the isolates were microaerophilic, although they could tolerate anaerobic conditions with weaker growth. The microbial growth in microaerobic conditions was similar to the majority of the *Campylobacter* species (Vandamme et al. 2005), observable at 37 °C and 42 °C, but not at 25 °C. Further testing showed that four of the five isolates grew in the presence of 1 % glycine, but none grew on BA supplemented with 2 % or 3.5 % NaCl. Growth was not observed either on MacConkey agar or on Muller Hinton Agar

supplemented with 5 % sheep blood. Unlike other *Campylobacter* species (*e.g. C. hominis and C. ureolyticus*) (Kaakoush et al. 2015), the isolates did not require hydrogen (H_2) to grow since the employed gas-generating sachet Genbox does not release hydrogen. No motility was observed on the hanging drop method preparation, which can justify the absence of colonies with *Campylobacter*-like morphology in the BA approach since that technique was developed to isolate motile *Campylobacter* spp. that can cross the 0.65-µm mixed cellulose ester membrane filters. To summarize, the five isolates were Gram-negative rods, microaerophilic and oxidase positive, which are phenotypic traits common to the genus *Campylobacter* (Quinn et al. 2011). However, the isolates were distinguishable from the most related species *C. sputorum*, *C. corcagiensis, C. blaseri, C. ureolyticus* and *C. geochelonis* (see genomic characterization), since unlike these species, the isolates could not reduce nitrate (Table 4).

Table 4 Phenotypic characteristics differentiating FMV-PI isolates from the other *Campylobacter* species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Motility	-	-	-	+	-	+	+	+	nd	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	nd	+	nd	-	nd	nd
Oxidase	+	+	-	+	+	+	+	v	+	+	+	v	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	(-)	v*	v	+	-	-	+	-	(-)	+	+	+	+	+	(+)	+	w	v	+	+	-	+	+	+	+	-	+	+	+	-
α-haemolysis	-	-	-	+	v	-	-	(-)	+	(-)	+	+	(-)	v	+	+	v	-	-	-	+	-	+	nd	+	nd	+	+	nd	-	+	-
Urease	-	-	-	v*	+	+	-	-	+	-	-	-	-	-	-	-	-	-	v	-	-	-	-	-	-	nd	nd	-	nd	+	-	+
Hippurate hydrolysis	-	-	-	-	-	-	-	-	-	(-)	-	-	-	-	+	-	-	-	(+)	+	-	-	+	-	-	-	-	-	-	-	-	-
Indoxyl acetate hydrolysis	-	-	(+)	-	v	v	-	-	-	v	+	v	+	-	+	-	-	+	+	-	+	-	+	-	-	nd	-	+	-	+	-	-
Nitrate reduction	-	-	(+)	(+)	+	(+)	(-)	(-)	+	+	+	+	+	+	+	+	+	(+)	v	+	v	+	+	+	+	nd	nd	+	+	+	+	+
H₂S production	-	-	-	+	-	+	+	-	+	(-)	-	v	-	+	-	-	-	-	v	-	-	-	-	+	-	nd	-	-	-	-	+	+
Growth in/at/on:																																
1% glycine	v	+	+	+	+	+	v	(-)	v	+	+	v	(+)	+	+	-	(-)	+	-	v	-	+	v	+	+	+	(+)	+	-	+	+	-
2% NaCl	-	nd	+	+	+	+	-	(-)	nd	v	v	+	-	-	-	nd	-	-	-	nd	-	+	-	-	+	(+)	+	-	-	nd	nd	nd
3.5% NaCl	-	nd	-	v	+	nd	-	-	nd	-	-	-	-	-	-	nd	-	-	nd	-	nd	nd	-	-	-	nd	-	-	nd	nd	nd	nd
MacConkey Agar	-	-	(+)	v	v	-	(+)	-	nd	(+)	-	+	v	v	-	+	v	-	-	+	-	-	-	nd	-	nd	(-)	-	-	w	+	nd
TTC 0.04%	-	-	-	-	-	-	-	-	nd	v	-	-	+	-	+	nd	-	+	-	nd	v	-	-	nd	+	nd	nd	v	-	nd	nd	nd
25ºC, microaerobic	-	-	-	-	-	nd	-	-	+	-	-	-	-	(-)	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+
37ºC, microaerobic	+	+	-	+	+	+	+	+	+	v	-	v	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42ºC, microaerobic	+	(-)	v	v	v	+	+	(+)	-	v	(-)	v	+	+	+	+	-	+	+	+	(+)	-	+	-	+	+	+	+	+	+	-	-
37ºC, anaerobic	w	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	V	-	-	+	-	+	-	-	nd	+	-	+	+	+	+	-
37ºC, aerobic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H₂ requirement	-	+	+	-	+	-	+	+	-	+	+	+	-	v	-	-	-	-	v	-	-	-	-	nd	-	nd	-	-	nd	-	-	-

*test results differ between biovars.

Taxa: 1—FMV-PI isolates (n = 5); 2—*C. hominis*; 3—*C. gracilis*; 4—*C. sputorum*; 5—*C. ureolyticus*; 6—*C. corcagiensis*; 7—*C. mucosalis*; 8—*C. concisus*; 9—*C. pinnipediorum* subsp. *pinnipediorum*; 10—*C. curvus*; 11—*C. rectus*; 12—*C. showae*; 13—*C. coli*; 14—*C. hyointestinalis* subsp. *hyointestinalis*; 15—*C. jejuni* subsp. *jejuni*; 16—*C. lanienae*; 17- *C. fetus* subsp. *venerealis*;; 18—*C. hepaticus*; 19—*C. avium*; 20—*C. canadensis*; 21—*C. cuniculorum*; 22—*C. geochelonis*; 23—*C. helveticus*; 24—*C. insulaenigrae*; 25—*C. lari* subsp. *lari*; 26—*C. peloridis*; 27—*C. subantarcticus*; 28—*C. upsaliensis*; 29—*C. volucris*; 30—*C. blaseri*; 31—*C. iguaniorum*; 32—*C. ornithocola*. Data for reference taxa were obtained from previous species descriptions (Logan et al. 2000; Lawson et al. 2001; Foster et al. 2004; Inglis et al. 2007; Rossi et al. 2009; Zanoni et al. 2009; Debruyne et al. 2010b; Debruyne et al. 2010a; Vandamme et al. 2010; Koziel, O'Doherty, et al. 2014; Gilbert et al. 2015; Piccirillo et al. 2016; Van et al. 2016; Cáceres et al. 2017; Gilbert et al. 2017; On et al. 2017; Gilbert et al. 2018). + 90–100 %; (+) 75–89 %; v 26–74 %; (-) 11–25 %; - 0–10 %; nd–not determined; w—weakly positive.

3.1.4.3 <u>Phylogenetic analysis</u>

The 16S rRNA gene sequence alignment revealed that the five bacterial isolates shared 99.87 % sequence similarity. This homology, associated to their similar phenotypic characteristics, confirms that they belong to the same species. The comparative analysis of the 16S rRNA gene sequence of isolate FMV-PI01, using the BLASTN algorithm, confirmed that this isolate is closely related to the genus *Campylobacter*. The highest identities were obtained with *C. concisus* and *C. gracilis* (100 % coverage and 94.7 % identity) and *C. hominis* (100 % coverage and 94.1 % identity). These 16S rRNA sequence identities are below the threshold of 97 %, defined for bacteria belonging to the same species (Stackebrandt and Goebel 1994), which supports the identification of a novel species within the *Campylobacter* genus.

The phylogenetic analysis using 16S rRNA and *hsp60* genes (Figures 5 and 6) demonstrated that these isolates form a robust cluster. However, the phylogenetic position of the five isolates was not clearly established based on the phylogenetic analysis. The low bootstrap values observed (< 70 %) indicate that the 16S rRNA and *hsp60* genes have a weak discriminatory power relatively to the group of phylogenetically related species.

The 16S rRNA phylogenetic analysis identified *C. gracilis*, *C. hominis*, *C. sputorum*, *C. geochelonis*, *C. blaseri*, *C. corcagiensis* and *C. ureolyticus* as the most related taxa. This was also observed in the phylogenetic analysis based on *hsp60* gene, except for *C. gracilis*, which was not grouped with FMV-PI isolates. The *hsp60* gene analysis provided a better phylogenetic resolution, with a higher number of branches with high bootstrap values. Although the 16S rRNA gene is the most commonly accepted for use in taxonomic studies, phylogenetic analysis based on *hsp60* gene is more discriminative for some bacterial taxa as the family *Campylobacteraceae* (Kärenlampi et al. 2004; Sakamoto and Ohkuma 2010; On et al. 2017). A greater interspecies variation in the nucleotide sequence of hsp60 gene than in 16S rRNA gene may explain these findings (Kärenlampi et al. 2004).
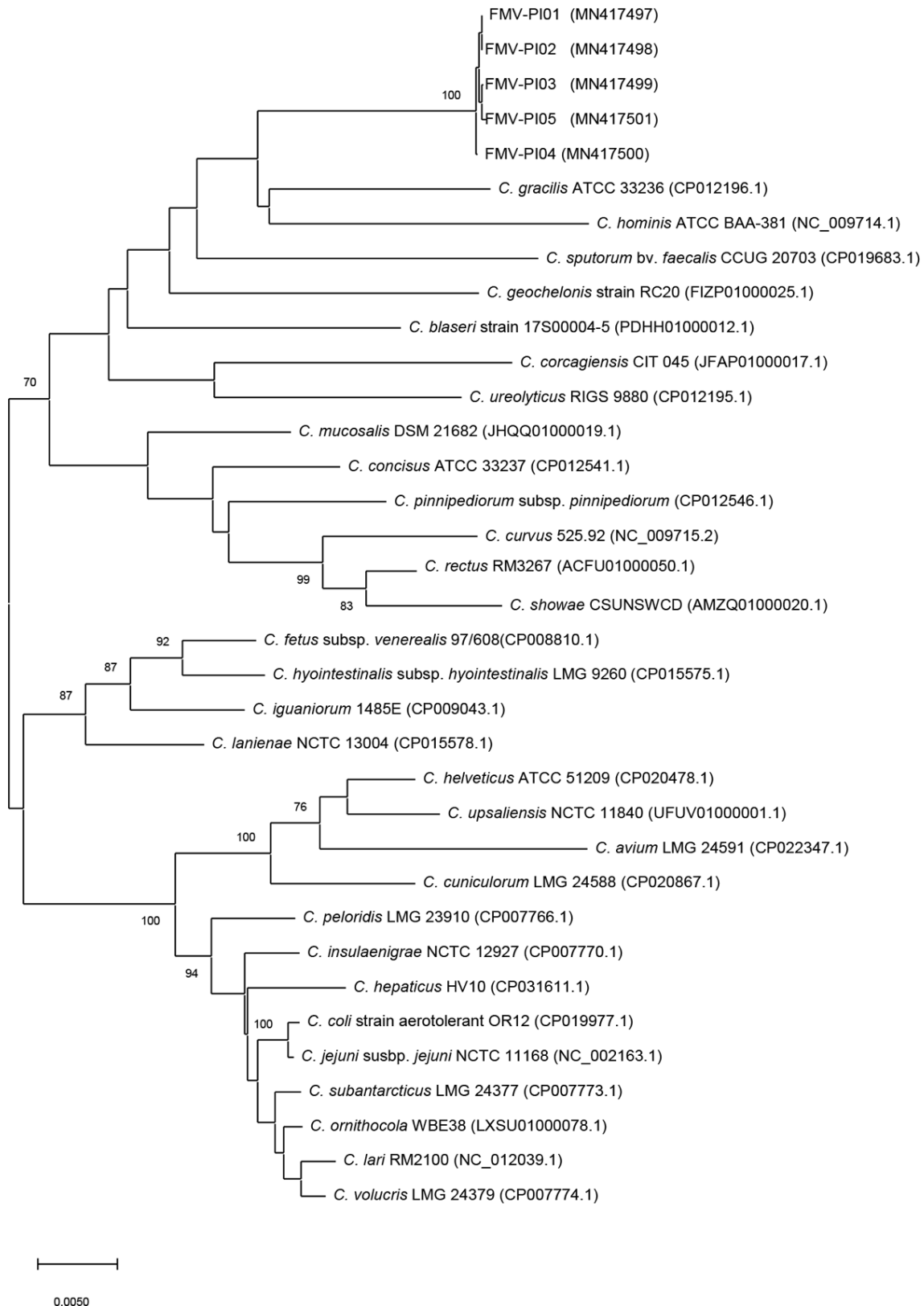
Figure 5. Phylogenetic tree based on 16S rRNA gene sequences of *Campylobacter* species, reconstructed by the neighbour-joining method. Bootstrap values (%) obtained from 1000 simulations are indicated at the nodes. Bootstrap values lower than 70 % are not shown. Bar: 0.050 substitutions per site.
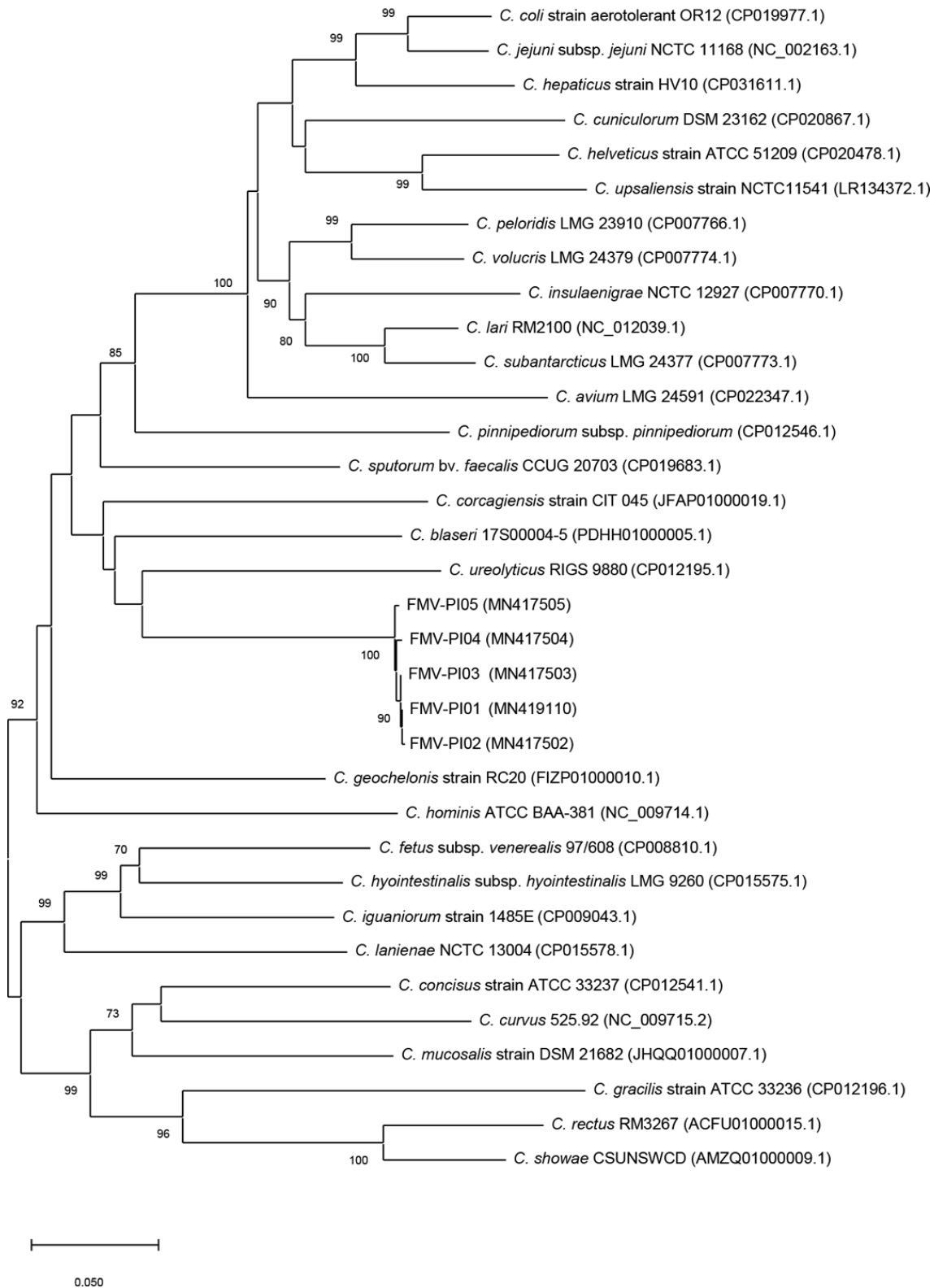
Figure 6 Phylogenetic tree based on *hsp60* gene sequences of *Campylobacter* species, reconstructed by the neighbour-joining method. Bootstrap values (%) obtained from 1000 simulations are indicated at the nodes. Bootstrap values lower than 70% are not shown. Bar: 0.050 substitutions per site.

3.1.4.4. <u>Genomic characterization</u>

The Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the accession VWSJ00000000. The version described in this paper is version VWSJ01000000. The genome of isolate FMV-PI01 is 1 767 933 bp long, composed of 98 contigs, with 28.3 % G+C content. This G+C content is one of the lowest reported for species of the genus *Campylobacter*. The G+C content of a bacterial species may reflect adaptation to environmental niches and lifestyles, since non-free-living bacteria generally have shorter genomes with lower G+C content, promoting energy conservation in environments with scarcity of nutrients (Rocha and Danchin 2002; Mann and Chen 2010). Interestingly, *C. sputorum*, also with one of the lowest G+C contents within the genus *Campylobacter* (29 %) (Iraola et al. 2014), colonizes the bull's preputial mucosa, exhibiting niche preferences similar to herein described isolates.

The ANI analysis revealed that homology with other *Campylobacter* species was less than 75 %, which is far below the 95–96 % cut-off for isolates of the same species (Richter and Rosselló-Móra 2009). These nucleotide similarities are in accordance with the *hsp60* and 16S rRNA genes phylogenetic analysis results, showing that *C. ureolyticus*, *C. corcagiensis* and *C. blaseri* are the most closely related taxa with ANI values of 74.3 %, 73.3 % and 73.0 %, respectively, followed by *C. geochelonis* (72.3 %), *C. sputorum* (72.2 %), C. hominis (70.1 %) and *C. gracilis* (65.9 %). The ANI values of these two latter species (*C. gracilis* and *C. hominis*) support the results of the analysis based on *hsp60* gene rather than the 16S rRNA gene. Overall, these findings support that the herein described isolates belong to the *Campylobacter* genus, representing a novel species, for which the designation *Campylobacter portucalensis* sp. nov. is proposed. The ANI analysis homology between *Campylobacter portucalensis* sp. nov. and its most related *Campylobacter* species is shown in Table 5.

Table 5 Average nucleotide identity (ANI) values (%) based on BLAST for _C. portucalensis_ sp. nov. and the most related _Campylobacter_ species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*	70.1	69.8	69.7	69.6	69.6	69.3	69.3	73.3	73.0	74.7	72.2	65.9	68.4	72.3
2	70.2	*	69.2	68.3	68.2	69.1	68.2	68.3	69.8	69.7	71.6	70.2	68.2	68.3	70.2
3	69.4	68.5	*	68.2	67.9	78.5	67.6	67.8	68.8	69.4	69.3	69.5	66.6	68.2	69.7
4	70.0	68.5	68.9	*	81.9	68.8	74.3	74.5	69.0	69.8	69.8	69.8	65.5	68.0	69.0
5	69.7	68.3	68.4	81.9	*	68.4	74.1	74.0	68.6	69.5	69.6	69.3	65.4	67.5	68.5
6	69.1	68.4	78.6	67.9	67.7	*	67.4	67.8	68.7	69.0	69.2	69.5	66.7	68.6	69.7
7	69.4	68.2	68.5	74.5	74.5	68.2	*	84.4	68.6	69.1	69.3	69.2	65.1	67.0	68.1
8	69.1	68.1	68.1	74.4	73.9	68.3	84.4	*	68.5	68.8	69.1	68.9	65.6	67.6	68.4
9	72.3	68.9	68.6	67.8	67.8	68.5	67.5	67.7	*	71.2	73.0	70.3	65.4	67.7	71.1
10	72.5	69.3	69.2	68.9	68.6	69.2	68.3	68.3	71.8	*	72.8	71.2	65.4	67.9	72.6
11	74.8	71.6	69.8	69.7	69.4	69.8	69.1	69.2	73.9	73.1	*	72.0	65.8	68.6	72.3
12	71.9	70.2	69.8	69.4	69.1	69.8	68.8	68.8	71.2	71.5	71.8	*	66.2	68.8	70.9
13	65.5	67.2	66.2	64.6	64.4	66.4	64.4	64.8	65.2	64.7	64.8	65.4	*	67.7	67.0
14	67.7	67.4	68.3	67.0	66.6	68.6	66.5	66.9	67.7	67.8	67.81	68.2	68.0	*	69.0
15	71.4	69.2	69.3	67.9	67.2	69.3	67.1	67.5	71.0	72.1	71.33	70.2	67.3	68.8	*

Strains: 1 –_C. portucalensis_ sp. nov. FMV-PI01; 2 –_C. hominis_ ATCC BAA-381; 3 –_C. fetus_ subsp. _fetus_ 82–40; 4 –_C. lari_ RM2100; 5 –_C. insulaenigrae_ NCTC 12927; 6 –_C. hyointestinalis_ subsp. _hyointestinalis_ LMG 9260; 7 – _C. hepaticus_ HV10; 8 –_C. jejuni_ subsp. _jejuni_ LMG 9872; 9 –_C. corcagiensis_ CIT 045; 10 –_C. blaseri_ 17500004–5; 11 –_C. ureolyticus_ DSM 20703; 12 –_C. sputorum_ bv. _faecalis_ CCUG 20703; 13 –_C. gracilis_ ATCC 33236; 14 – _C. concisus_ ATCC 33237; 15 –_C. geochelonis_ RC20.

A total of 1877 coding sequences (CDS) and 41 RNAs (36 transfer RNA and 5 ribosomal RNA genes) were identified in FMV-PI01's genome. Of the identified CDS, 497 were assigned to 191 subsystems. Subsystems with higher number of genes are related to metabolic processes, and include "protein metabolism" (n = 126), "amino acids and derivatives" (n = 136) and "cofactors, vitamins, prosthetic groups and pigments" (n = 65) (Figure 7). Nineteen genes involved in "Virulence, disease and defence" and 16 genes related to "stress response" were identified (Figure 7).
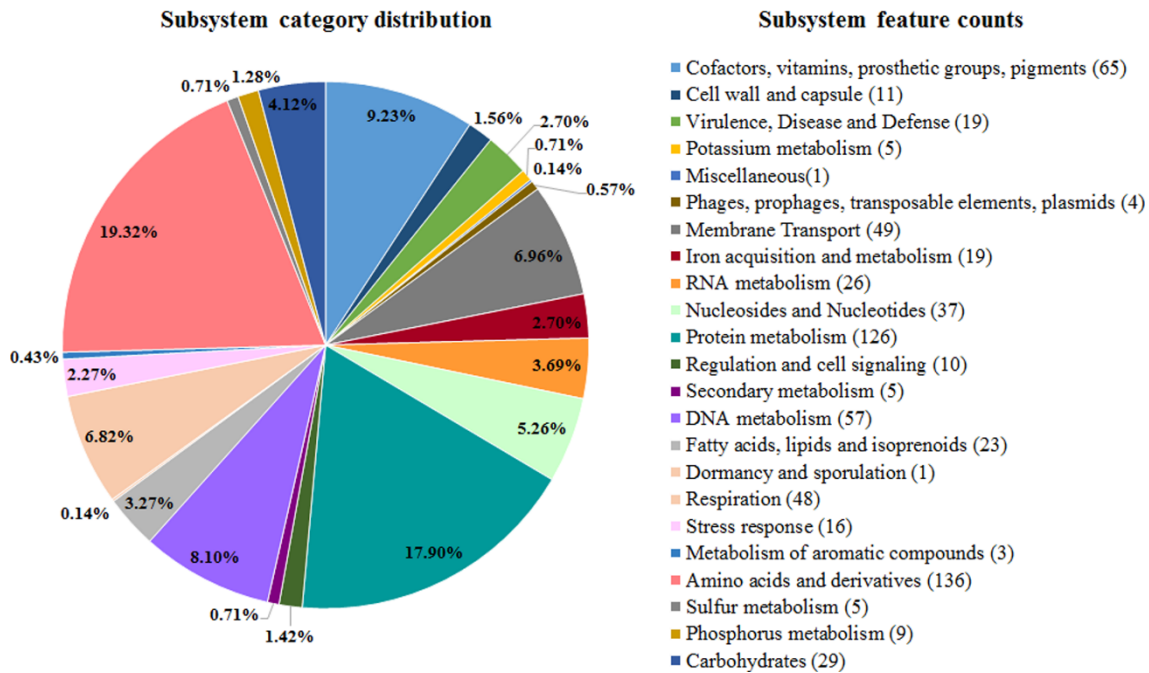
Figure 7 Subsystem category distribution in the genome of isolate FMV-PI01, based on the RAST server.

The CRISPRCasFinder identified one CRISPR-Cas system in FMV-PI01's genome, which contains 15 CRISPR repeats with 13 CRISPR spacers and a type III-D Cas system. Despite CRISPR-Cas systems being associated with phage defence mechanisms, these systems may also play a role in bacterial virulence and host immune evasion (Louwen et al. 2014). The diversity of CRISPR-Cas systems within the *Campylobacter* genus is wide, being the Cas systems type I and II the most common among *Campylobacter* species (Louwen et al. 2014). However, the type III-D, which was found in the genome of *C. portucalensis* sp. nov., was also identified in *C. fetus*. The mechanisms behind the selection of each system in different bacterial species (Westra et al. 2019), as well as the role of Cas system type III-D in *C. fetus*, remain unclear.

A more detailed analysis using BLASTP algorithm allowed the identification of genes potentially involved in adhesion and invasion to host cells. These genes encode homologous of the fibronectin/fibrinogen binding protein (98 % query cover, 62 % identity), collagenase like peptidase of U32 family (100 % query cover, 81 % identity) and *Campylobacter* invasion antigen B, CiaB (100 % query cover, 64 % identity). These virulence factor coding genes are present in several *Campylobacter* species, namely in *C. fetus* and *C. jejuni* (Ali et al. 2012). Adhesion of *C. jejuni* to host cells is mediated by the fibronectin binding protein (Ziprin et al. 1999; Monteville et al. 2003; Krause-gruszczynska et al. 2007), and the mutational inactivation of the *ciaB* gene reduced the invasion in Intestine-407 (INT-407) cells, revealing that CiaB is involved in the internalization of *C. jejuni* (Konkel et al. 1999a; Konkel et al.

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1999b). Therefore, the presence of these genes in the genome of *C. portucalensis* is potentially related to host cell adhesion and invasion in the bovine reproductive tract. In addition, genes encoding multidrug efflux pumps of the resistance-nodulation-cell division family were found, namely a CmeABC efflux pump. These transporters are present in several species of *Campylobacter* (e.g. *C. jejuni*, *C. coli*, *C. fetus* and *C. lari*) and contribute to multidrug resistance (Guo et al. 2010). For instance, this efflux pump in *C. jejuni* is involved in resistance to bile salts (Lin et al. 2003), macrolides, tetracycline (Gibreel et al. 2007), ciprofloxacin and other antimicrobials (Lin et al. 2002).

The *C. portucalensis* FMV-PI01's genome encodes a T4SS, containing *virB2-virB11/virD4* genes, highly homologous to the *Cfv* T4SS (99 % coverage and 97.2 % identity). This T4SS was confirmed to contribute to *Cfv* virulence properties, namely invasive and cytotoxic potential (Gorkiewicz et al. 2010; Kienesberger et al. 2011). The fact that *C. portucalensis* sp. nov. and *Cfv*, the causative agent of BGC, share virulence factor coding genes and are both inhabitants of the bull preputial mucosa, may suggest that this novel *Campylobacter* species has the potential to cause disease in cattle. The herd from which the isolate FMV-PI01 was obtained presented signs of reproductive failure compatible with BGC, the reason for the disease investigation. As in the above disease, where the bull acts as an asymptomatic carrier and signs of disease are only reflected on the female (embryonic and fetal mortalities) and herd (fertility rate, calving pattern and calving interval) sides (Michi et al. 2016), the bull from which the samples were taken was clinically sound. However, since several interacting factors may contribute to beef cattle herd's infertility, one cannot conclude that the observed reproductive failure was the result of the infection with *C. portucalensis* sp. nov. To further investigate the pathogenic potential of *C. portucalensis* sp. nov in cattle fertility, research in the female reproductive tract needs to be addressed.

The evaluation of the pathogenic potential based on the PathogenFinder analysis showed that the probability of isolate FMV-PI01 being a human pathogen was 82.6 %, indicating that this isolate may have the potential to cause disease in humans.

3.1.5 Conclusion

The distinct phenotypic and genotypic characteristics of the bacterial isolates confirm the identification of a novel species within the *Campylobacter* genus, for which the name *Campylobacter portucalensis* sp. nov. is proposed. *C. portucalensis* sp. nov. is an inhabitant of bulls' preputial mucosa with unknown pathogenic potential.

3.1.6 Description of *Campylobacter portucalensis* sp. nov

Campylobacter portucalensis sp. nov. (por.tu.cal.en'sis. N.L. masc. adj. *portucalensis* referring to Portugal, from where the type strain was originally isolated). In Columbia agar (supplemented with 5 % sheep blood), after 48 hours in microaerobic atmosphere at 37 °C, colonies are punctiform (1 mm in diameter), convex, circular with smooth margins and without any pigments. Colonies are non-haemolytic. Cells are Gram-negative, straight rods (length 1.68±0.07 μm and width 0.44±0.01 μm). A flagellum is absent and cells are non-motile. Occasional longer filamentous cells are observable.

Growth is observed on blood agar at 37°C under microaerobic and anaerobic (weak growth), but not aerobic conditions. Does not require H_2 supplementation to grow. Isolates grow in microaerobic conditions at 37°C and 42°C but not at 25°C. Strains may differ in their ability to grow on blood agar medium supplemented with 1 % glycine. Growth is not observed on blood agar medium supplemented with 2 % and 3.5 % NaCl. Unable to grow on Macconkey agar or Mueller-Hinton agar. Phenotypically, *C. portucalensis* sp. nov. is oxidase positive and catalase negative. Negative for urease activity. Unable to hydrolyse hippurate and indoxyl acetate. Does not reduce nitrate. Does not produce hydrogen sulfide in TSI medium.

The genomic G+C content of the type strain is 28.3 %.

The type strain FMV-PI01^T (= LMG 31504, = CCUG 73856) was isolated from the reproductive tract of a bull (*Bos taurus*) sampled in the Alentejo province of Portugal in 2018.

3.2 Assessment of *Campylobacter fetus* subsp. *venerealis* molecular diagnosis using clinical samples of bulls

3.2.1 Abstract

Campylobacter fetus subsp. *venerealis* (*Cfv*) is the pathogen responsible for BGC, a venereal disease of cattle associated with impaired reproductive performance. Although several PCR assays were developed to identify this pathogen, most of them are still poorly evaluated in clinical samples. This study evaluated real-time PCR assays for *Cfv* detection in preputial samples of bulls ($n = 308$).

The detection at the subspecies level (*Cfv*) compared four assays: two targeting ISCfe1 and two targeting *parA* gene. The detection at the species level (*C. fetus*) considered an assay targeting the *nahE* gene and a commercial kit for *C. fetus* identification. At the subspecies level, assays directed either to different targets (*parA* and ISCfe1), or to the same target (ISCfe1 or *parA*), showed a high percentage of disagreeing results. All samples positive at the subspecies level ($n = 169$) were negative in *C. fetus* detection assays, which strongly suggests the horizontal gene transfer of ISCfe1 and *parA* to other bacterial species. This was confirmed by microbiological isolation of three *Campylobacter portucalensis* strains responsible for false positive results. Sequences with a high level of identity with ISCfe1 and *parA* gene of *Cfv* were identified in *C. portucalensis* genome.

Overall, this study reveals that PCR assays solely directed to a subspecies target originate a high rate of false positive results, due to the presence of *parA* and ISCfe1 homologous sequences in other bacterial species, namely of the *genus Campylobacter*. Although the specificity of these methods may be higher if applied to bulls from herds with clinical features of BGC or in other geographical regions, current PCR diagnosis should couple subspecies and species targets, and further research must be envisaged to identify *Cfv* specific molecular targets.

3.2.2 Introduction

BGC is a venereal disease of cattle, caused by the bacterial pathogen *Cfv*, responsible for reproductive failure and significant economic losses, mainly in beef herds where natural breeding prevails (Michi et al. 2016; OIE 2018). Bulls are asymptomatic carriers, harbouring *Cfv* in the preputial crypts, and infect females during breeding (Cobo et al. 2011). By contrast, *Cfv* infection in females induces endometritis, early embryonic death and abortion (OIE 2018). An accurate diagnosis of BGC is essential for implementation of disease control programs and international trade of bulls and semen (van Bergen et al. 2005b). However, BGC diagnosis is hindered by the two cattle-associated *C. fetus* subspecies, *Cff* and *Cfv,* with distinct niche preferences but with similar genotypic and phenotypic characteristics (Van Bergen et al. 2005b; van der Graaf-Van Bloois et al. 2014). Although *Cff* inhabits cattle intestinal tract, it can be occasionally recovered from bovine preputial samples and it is responsible for sporadic cases of abortion (Mai et al. 2013; Michi et al. 2016). The OIE recommended method for BGC diagnosis is microbiological isolation of *C. fetus*, followed by the 1 % glycine tolerance test, for which *Cfv* is intolerant and *Cff* is tolerant (OIE 2018). Due to the fastidious growth of *Cfv* and potential overgrowth of other microorganisms, microbiologic techniques are laborious, time-consuming, and associated to poor sensitivity (Michi et al. 2016). Additionally, *Cfv* can acquire glycine tolerance by mutation or transduction mechanisms being misidentified as *Cff* (Chang and Ogg 1971). Therefore, molecular biology techniques have evolved as attractive tools for diagnosis of BGC. At the species level, real-time PCR assays to detect the *nahE* gene of *C. fetus* were developed and validated, showing high specificity and sensitivity (van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). However, the identification at the subspecies level is hampered by the high genetic similarity of *Cfv* and *Cff* (Moolhuijzen et al. 2009). Nevertheless, several PCR assays have been described to identify *Cfv* (Hum et al. 1997; McMillen et al. 2006; Abril et al. 2007; Iraola et al. 2012). The most used molecular targets for *Cfv* identification are the *parA* gene and the ISCfe1 insertion element (McMillen et al. 2006; Abril et al. 2007; van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). Assays based on ISCfe1 detection are described as more sensitive than *parA*-based assays to identify *Cfv* isolates (van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). Although real-time PCR assays targeting the *parA* gene were evaluated in clinical samples (McMillen et al. 2006; Chaban et al. 2012; Guerra et al. 2014; Waldner et al. 2017), available data is insufficient to allow its routine use for diagnosis of BGC. Assays based on ISCfe1 detection were not yet assessed in clinical samples despite showing promising results on *Cfv* isolates (van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). Therefore, the diagnostic value of PCR assays for bull clinical samples has not been elucidated. This is a relevant

clinical issue because bull testing is the most appropriate prophylactic measure for BGC control. This study evaluated different real-time PCR assays for *Cfv* identification on bull preputial samples in order to assess their suitability for BGC diagnosis.

3.2.3 Material and Methods

3.2.3.1 Samples and DNA extraction

This observational study considered samples collected by certified veterinarians, within a bull breeding soundness examination, for diagnostic purposes. The number of tested bulls ($n = 308$) and herds ($n = 61$) is a representative sampling subset of the province of Alentejo, which represents the main beef cattle production area in Portugal, accounting over 10.000 beef cows of several breeds, in natural mating, distributed in the different regions of the province. Herds had unknown sanitary status for BGC, although some showed clinical features compatible with the disease (low breeding season fertility, extended time from bull introduction to conception depicted from calving records, embryo-fetal mortality observed at pregnancy diagnosis). Preputial samples were collected using a scraping/washing technique (Silveira et al. 2018) and were sent to the laboratory under refrigeration conditions. Total DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. Extracted DNA was quantified in a Nanodrop 2000C spectrophotometer (Thermo Scientific) and stored at − 20 °C until use.

3.2.3.2. Real-time PCR assays

Samples were tested by real-time PCR assays directed to subspecies (*Cfv*) and species (*C. fetus*) molecular targets. Detection at the subspecies level (*Cfv*) was performed using four assays, of which two targeting the *parA* gene (parA-A and parA-B assays) and the other two targeting the insertion element ISCfe1 (ISC-A and ISC-B assays). These pairs of assays, although directed to the same molecular target, detect different nucleotide regions as shown in Table 6. Additionally, two assays were performed for *C. fetus* detection, including an assay targeting the *nahE* gene and a commercial real-time PCR Kit (VetMAX *C. fetus* kit, Life Technologies). These two assays are designed to amplify sequences common to both *C. fetus* subspecies (*Cfv* and *Cff*), and, consequently, do not allow the identification at the subspecies level.

Table 6 Real-time PCR assays used for *Cfv* detection

Assay	Target	Nucleotide Region [a]	Reference	*n*
ISC-A	ISCfe1: *tnpB* gene	764-843	This study	308
ISC-B	ISCfe1: *tnpA* gene	567-626	van der Graaf-van Bloois et al. (2013)	141
parA-A	*parA* gene	321-406	McMillen et al. (2006), with modifications	308
parA-B	*parA* gene	84-161	This study	141

n: number of samples tested; [a] Nucleotide region of ISCfe1 and *parA* sequences with NCBI accession numbers AM260752.1 and CP043435.1: c1229121-1228459, respectively.

The flow chart of the sampling procedure for *Cfv* and *C. fetus* detection assays is schematically represented in Figure 8. Briefly, all samples (*n* = 308) were tested with ISC-A and parA-A assays. In addition, a subset of these samples (*n* = 141) was blindly selected to be tested with ISC-B and parA-B assays. Detection of *C. fetus* specific targets was performed in all ISCfe1 and/or *parA* gene positive samples (*n* = 169).
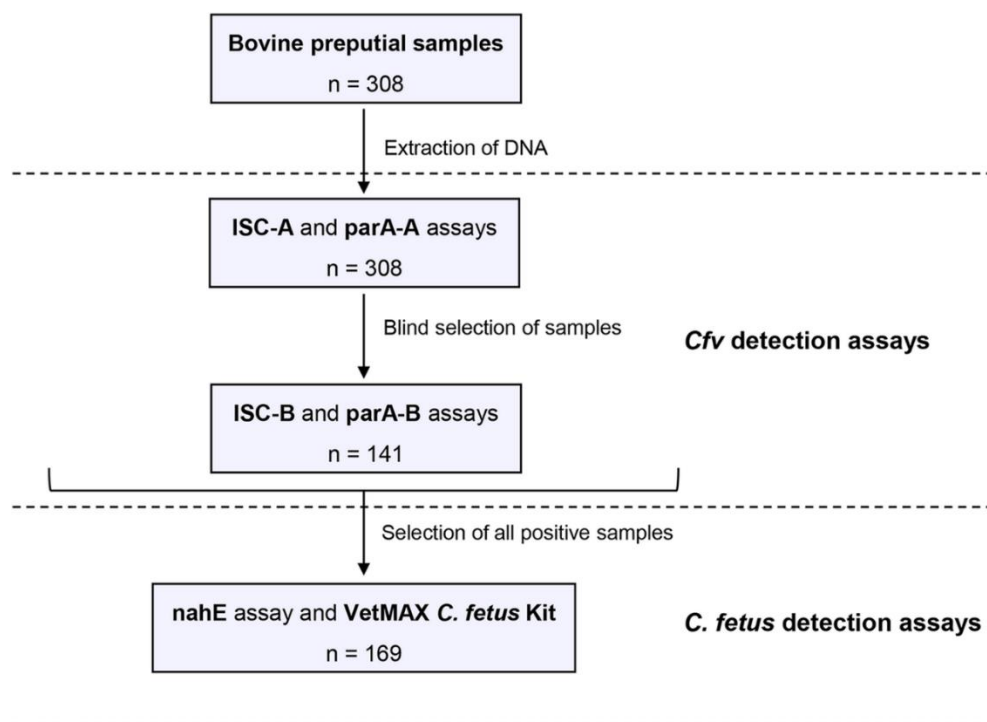
Figure 8 Flow chart of the sampling procedure for *Cfv* and *C. fetus* detection assays

For all assays, a PCR positive control was used in every experiment, which was prepared by adding 100 copies of *Cfv* genomic DNA from strain NCTC 10354 to 25 ng of DNA extracted from a *Cfv*-negative preputial sample to simulate a *Cfv*-positive preputial sample.

Detection of ISCfe1 insertion sequence: ISC-A and ISC-B assays

For the detection of the insertion element ISCfe1 two different Taqman MGB probe-based assays were used (ISC-A and ISC-B assays; Table 6). The ISC-A assay was developed to detect the *tnpB* gene of ISCfe1 (Table 6). The primers and probe (Annex I) were designed to target a 109 bp sequence using Primer Express software v2.0 and specificity was assessed using BLAST search in the NCBI database. PCR reactions were performed in duplicate, in 20 µL mixtures containing 1x SensiFAST probe Hi-ROX mastermix (Bioline Reagents Ltd), 400 nM of each primer, 100 nM of Taqman MGB probe and 25 ng of total DNA. Amplifications were performed on a StepOnePlus system (Applied Biosystems), using the following thermal cycle conditions: 2 min incubation step at 50 °C, denaturation for 4 min at 95 °C, followed by 35 cycles of 10 s at 95 °C and 30 s at 60 °C. The ISC-B assay targets the *tnpA* gene of ISCfe1, using previously published primers and probe (van der Graaf-van Bloois et al. 2013). PCR reaction mixtures and thermal cycle conditions were as previously described for ISC-A. Samples with a Ct < 35 were considered positive.

Detection of parA gene: parA-A and parA-B assays

The detection of *parA* gene also used two different Taqman MGB probe assays (parA-A and parA-B assays; Table 6). The parA-A assay is a real-time PCR assay previously described (McMillen et al. 2006), with minor modifications (Annex I). The reverse primer was modified according to sequencing data of PCR products amplified with primers VENSF/VENSR (Hum et al. 1997) in the bovine preputial samples. All reactions were carried out in 20 µL reaction volume, with 900 nM of each primer, 250 nM of Taqman MGB probe, 1x SensiFAST probe Hi-ROX mastermix and 25 ng of DNA. Amplifications were performed on a Step One Plus System, using the thermal cycle conditions previously described (McMillen et al. 2006). The parA-B assay was designed to amplify a different sequence of the *parA* gene with 78 bp (Table 6). The primers and probe (Annex I) were designed using Primer Express software v2.0 and specificity was assessed using BLAST search in the NCBI database. PCR reaction mixtures and thermal cycle conditions were the same as described for ISC-A assay. Samples with a Ct < 35 in parA-A or parA-B assays were considered positive.

Detection of C. fetus species-specific targets: nahE and VetMAX C. fetus kit

C. fetus detection was performed using a real-time PCR assay targeting the *nahE* gene and a commercial diagnostic kit (VetMAX *C. fetus* kit, Life Technologies) in all samples positive to ISCfe1 (ISC-A and/or ISC-B assays) and/or *parA* gene (parA-A and/or parA-B assay). The *nahE* gene was detected using previously described primers and probe (van der Graaf-van Bloois et al. 2013), with the conditions described above for ISCfe1 amplification. Samples with a Ct < 35 were considered positive. Samples were also tested with VetMAX *C. fetus* kit following the instructions and validation criteria recommended by the manufacturer.

Real-time PCR assays' performance: linearity, amplification efficiency and reproducibility

The analytical performance of the real-time PCR assays was assessed in DNA extracted from preputial samples, spiked with genomic DNA from *Cfv* strain NCTC 10354. Standards were prepared using DNA from *Cfv* NCTC 10354, previously extracted with Qiagen DNeasy Blood and Tissue kit (Qiagen). The genomic DNA was quantified using a Nanodrop 2000C spectrophotometer and the number of *Cfv* genome copies was determined based on the strain's genome size (1,874,244 bp), according to the formula: Number of copies = (DNA concentration (ng/µL) x [6.022×10^{23}]) / (length of template (bp) x [1×10^9] × 650). Ten-fold dilutions were performed from 1.25×10^6 to 1.25×10^2 genome copies/µL. Then, DNA preputial samples of three bulls, previously tested as negative in the PCR assays under study, were spiked with ten-fold dilutions of *Cfv* NCTC 10354, representing 10^1 to 10^5 genome copies per 25 ng of preputial sample DNA in 2 µL of template (PCR reaction). These three sets of standards were aliquoted and stored at − 20 °C until use. Three independent experiments were performed for each real-time PCR assay, one for each set of standards, all tested with two replicate wells per dilution. The performance of the real-time PCR assays was evaluated based on the linearity (r^2), amplification efficiency (E) and reproducibility. The amplification efficiency was estimated based on the slope of the standard curve, using the formula: E = 10 $^{(- 1/slope)}$ -1. The reproducibility was evaluated using the inter-assay and intra-assay coefficients of variation (CV) for each dilution.

3.2.3.3. <u>Detection of genomic island genes in bovine preputial samples</u>

The presence of GI genes formerly considered *Cfv*-specific, which include type 4 secretion system-coding genes (*virB2-virB11*/*virD4* genes), was assessed by conventional PCR. This *Cfv*-associated genomic island may also include the *parA* gene and *fic* genes. Amplification of *virB9* and *virB11* was performed with previously described primers (Moolhuijzen et al. 2009). Primers for amplification of *fic1* and *fic2* (Annex I) were designed

with Primer-BLAST (Ye et al. 2012) using the sequence of *Cfv* strain NCTC 10354 as a reference. These assays for *fic1*, *fic2*, *virB9* and *virB11* gene detection were performed in 49 samples, from which 30 *parA* negative and 19 *parA* positive in the parA-A assay. PCR reactions were carried out in 25 µL mixtures containing 0.4 µM of each primer, 400 µM of each dNTP (4you4 dNTP Mix, Bioron), 1 x reaction buffer (Complete reaction buffer, Bioron), 2 units of DFS-Taq DNA polymerase (Bioron) and 150 ng of DNA. Amplifications were performed in a Doppio thermal cycler (VWR) using the following conditions: 3 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 1 min, with a final extension step of 5 min at 72 °C. The annealing temperatures selected for amplification of *fic1/fic2*, *virB9* and *virB11* were 57 °C, 53 °C and 56 °C, respectively. Amplification products were detected by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide and bands were visualized in a ChemiDoc XRS+ System (Biorad).

3.2.3.4. Statistical analysis

Statistical data analysis was performed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corporation). To evaluate inter and intra-assay reproducibility of real-time PCR assays, the coefficient of variation of the Ct was calculated as follows: % CV = (standard deviation Ct / mean Ct) × 100. Agreement between results of different real-time PCR assays was evaluated using the Cohen's Kappa coefficient. Additionally, results were analysed using McNemar's test for testing the null hypothesis that methods were equally likely to identify samples as positive or negative. The presence of association between the detection of GI-associated genes and the detection of the *parA* gene was assessed using the phi (φ) coefficient. Values of $P < 0.05$ were considered statistically significant.

3.2.4 Results

3.2.4.1 Performance of the real-time PCR assays

The performance of the real-time PCR assays was evaluated using DNA from preputial samples spiked with *Cfv* genome copies. All assays were able to detect 10 to 10^5 *Cfv* genome copies within 40 cycles of amplification and considering a positivity threshold set to Ct ≤ 35, were able to detect 100 *Cfv* genome copies. The efficiency (Table 7) was within the acceptable range of 90–110 % (Broeders et al. 2014) and a linear relationship between the cycle threshold (Ct) value and the log of copy number was evidenced by an $r^2 \geq 0.98$. The intra- and inter-assay coefficients of variation were less than 5 %, as shown in Table 7.

Table 7 Performance parameters of the real-time PCR assays used in this study

Assay	Slope	Y-Intercept	r^2	E (%)	Intra-assay CV (%)	Inter-assay CV (%)
ISC-A	-3.3732	36.472	0.99	97.90	≤ 1.22	≤ 0.71
ISC-B	-3.3193	37.285	0.99	100.1	≤ 1.41	≤ 0.98
parA-A	-3.1270	38.134	0.98	108.8	≤ 0.81	≤ 1.37
parA-B	-3.3723	40.008	0.99	97.94	≤ 2.01	≤ 1.05
16S rRNA	-3.5070	34.899	0.99	92.82	≤ 2.86	≤ 1.74
nahE	-3.4440	37.773	0.99	95.20	≤ 1.88	≤ 0.61

E - Efficiency of amplification; CV - coefficient of variation.

3.2.4.2 Detection of *Cfv* and *C. fetus* molecular targets in preputial samples

Four different assays for *Cfv* identification were tested in clinical samples with unknown BGC sanitary status, targeting the ISCfe1 sequence (ISC-A and ISC-B assays) and the *parA* gene (parA-A and parA-B assays). The agreement between parA-A and ISC-A assays was evaluated in all the 308 bovine preputial samples. The parA-A assay gave a lower number of positive results ($n = 78$, 25.3 %) than the ISC-A assay ($n = 155$, 50.3 %) ($P < 0.001$). This originated a high percentage of disagreeing results (34.1 %, Kappa = 0.32), mainly represented by ISCfe1 positive and *parA* negative samples (Table 8).

Table 8 Agreement between parA-A and ISC-A assays

parA-A	ISC-A		Total
	Positive	**Negative**	
Positive	64 (20.8%)	14 (4.5%)	78 (25.3%)
Negative	91 (29.5%)	139 (45.1%)	230 (74.7%)
Total	155 (50.3%)	153 (49.7%)	308

The conservation of *parA* and ISCfe1 sequences was evaluated on a subset of 141 samples, comparing results obtained with assays targeting two different regions of each nucleotide sequence (ISC-A/ISC-B and parA-A/parA-B assays). The parA-A and parA-B assays provided 28.4 and 7.8 % positive results, respectively, resulting in a high percentage of disagreeing results (20.6 %; Kappa = 0.35) (Annex II). Likewise, the ISC-A and ISC-B assays

originated 58.9 and 17.0 % positive results, respectively, also resulting in a high percentage of disagreeing results (41.8 %; Kappa = 0.25) (Annex II). These differences observed between assays directed towards the same target are statistically significant ($P < 0.01$). The number and percentage of positive samples detected by each assay, or double combination is shown in Figure 9, for all samples tested by the four assays ($n = 141$). Results obtained by different assays were highly inconsistent. ParA-B assay provided the lowest number of positive samples ($n = 11$), while ISC-A assay detected the highest number of positive samples ($n = 83$). In contrast, there were no samples tested simultaneously positive by ISC-B and *parA* detection assays.
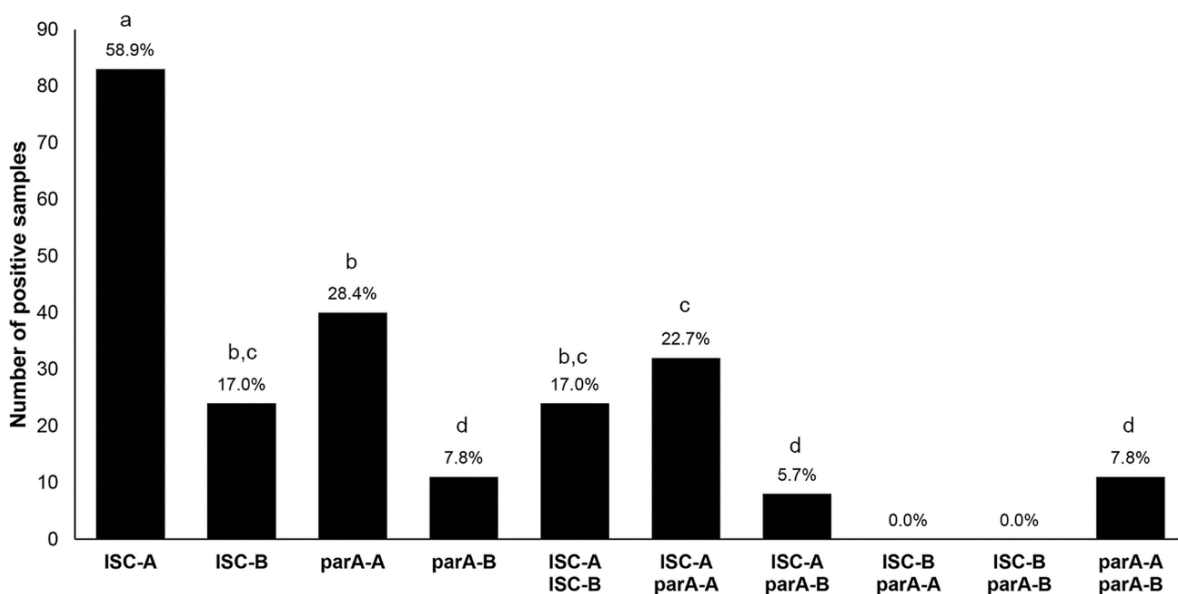
Figure 9 Distribution of positive results identified by each assay and double combinations of assays (n = 141). The percentage of positive results is displayed above the columns. Different letters above columns indicate statistically significant differences (P < 0.05)

The presence of genes from the *Cfv*-associated genomic island (GI), which may contain the *parA-A* gene, was evaluated by PCR. Results showed that genes of this GI (*fic1*, *fic2*, *virB9*, *virB11*) were detected in 95 % ($n = 18$) of the parA-A positive samples (Table 9), and a significant association was found between the presence of the *parA* gene and *C. fetus* GI genes ($\varphi = 0.873$, $P < 0.001$).

Table 9 Comparison of results obtained with the parA-A assay and amplification of *C. fetus* specific genomic island genes

parA-A	GI-associated genes		Total
	Positive	**Negative**	
Positive	18 (36.7%)	1 (2.0%)	19 (38.8%)
Negative	2 (4.1%)	28 (57.1%)	30 (61.2%)
Total	20 (40.8%)	29 (59.1%)	49

To evaluate the specificity of the *parA* gene and ISCfe1 as *Cfv* diagnostic targets, samples tested positive for at least one of these targets were also tested using a real-time PCR assay to detect the *C. fetus* specific *nahE* gene and a commercial diagnostic kit (VetMAX C. fetus kit) approved *for C. fetus* detection in DNA isolated from clinical samples. These assays detect targets common to both *C. fetus* subspecies (*Cff* and *Cfv*). All samples (*n* = 169) that tested positive to *Cfv* specific targets (*parA* gene and/or ISCfe1) were negative in *C. fetus* detection assays.

3.2.4.3 Impairment of Cfv detection in clinical samples by *Campylobacter portucalensis*

To investigate the cause of false positive results, microbiological culture and isolation of *Campylobacter* spp. was performed in three samples positive to *parA* and/or ISCfe1 and negative in *C. fetus* detection assays. Three strains identified biochemically and by 16S rRNA gene sequencing as *Campylobacter portucalensis* (Silva et al. 2020a) were isolated from these samples. These strains were confirmed to be responsible for false positive results in the above *Cfv* detection assays. Whole genome sequencing (WGS) data of the type strain *C. portucalensis* FMV-PI01[T] (NCBI accession no: VWSJ00000000) allowed the identification of the *parA* gene (98.1 % identity with sequence from *Cfv* strain WBT011/99) and of an insertion sequence highly similar to ISCfe1 (93.5 % identity with sequence from *Cfv* strain zaf3). The WGS of FMV-PI01 also showed that *parA* gene is located in a GI, which is highly homologous to the *Cfv*-associated GI with 97.42 % of identity in 80 % of the sequence. The arrangement of *parA*, *fic* and T4SS genes in the GI is schematically represented in Figure 10.
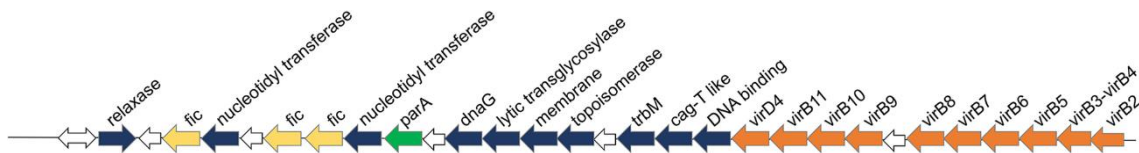
Figure 10 Schematic representation of the genomic island identified in *C. portucalensis* FMV-PI01. Orange arrows represent T4SS genes. White arrows indicate genes with unknown function.

Two of the *C. portucalensis* strains harbouring *Cfv* molecular markers, tested positive for both ISCfe1 and *parA* gene by ISC-A and parA-A assays, and GI-associated genes, whereas one strain tested positive for ISC-A and negative for parA-A and GI-associated genes.

3.2.5 Discussion

The analytical performance of the assays used in this study was evaluated using preputial samples spiked with *Cfv* DNA. Assays directed to species and subspecies targets showed similar analytical sensitivities, detecting 10^1 to 10^2 genome copies of *Cfv* NCTC 10354. All assays provided reproducible results in the same experiment and between experiments, as evidenced by the coefficient of variation (CV) below 5 %. Overall, the results showed a good analytical performance of real-time PCR assays and indicate their suitability for detection of *Cfv* in the bovine preputial sample matrix.

A considerable disagreement was observed between results obtained with parA-A and ISC-A assays. This was also observed for assays targeting the same molecular target (ISC-A/ISC-B and parA-A/parA-B), which suggests divergences in the nucleotide sequences of *parA* and ISCfe1, specifically in primer and probe binding sites. Moreover, in a considerable number of samples only one of the molecular targets was detected. This finding evidences a sensitivity and/or specificity failure of one or both detection assays. Previous studies showed that the *parA*-based PCR assays have a lower sensitivity when compared with assays targeting ISCfe1 for the identification of *Cfv* isolates (van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). In fact, *parA* sequence variation may potentially hinder *parA*-based diagnostic methods (Willoughby et al. 2005; Chaban et al. 2012). On the other hand, at least two different ISCfe1 sequences were found in *Cfv* strains, sharing 98.7 % sequence homology (van der Graaf-van Bloois et al. 2013). Therefore, inconsistent results obtained by assays directed towards the same target may result from divergences in the nucleotide sequences.

Samples positive for subspecies specific targets revealed to be negative in the *C. fetus* detection assays. These samples were expected to be positive for *C. fetus* detection, since the species-specific assays are based on targets common to both *C. fetus* subspecies (*Cff* and *Cfv*). The *nahE* detection assay showed an analytical sensitivity comparable to that obtained with the subspecies detection assays, indicating that the absence of amplification is

not related with differences in the analytical sensitivity. Moreover, although all results obtained with the VetMAX *C. fetus* kit were negative, the amplification of an internal positive control allowed to validate results. These findings strongly suggest that subspecies targets were horizontally transferred to other bacterial species. Although the *parA* gene is still used for *Cfv* identification, the horizontal gene transfer of *parA* to *C. hyointestinalis* isolates and *Cff* strains was reported (Abril et al. 2010; Spence et al. 2011). Also, *parA* detection assays identified putative positive *Cfv* bulls in herds without impairment of the reproductive performance (Sanhueza et al. 2014) and in virgin bulls (Guerra et al. 2014; García-Guerra et al. 2016). Indeed, the *parA* gene can be found in mobile genetic elements, namely in a GI almost exclusive of *Cfv* (Gorkiewicz et al. 2010; van der Graaf–van Bloois et al. 2016), in the chromosome and in extra-chromosomal plasmids (Willoughby et al. 2005; van der Graaf–van Bloois et al. 2016). On the other hand, genome sequencing data recently deposited in the NCBI database revealed that ISCfe1 can be found in plasmids (e.g. acc. no. CP043436.1), which may facilitate the horizontal transfer of this sequence to non-*Cfv* microorganisms. As a transposable element, ISCfe1 may spread in the genome, thus justifying the variable number of ISCfe1 copies in different *Cfv* strains (Abril et al. 2007).

Although the possibility of the above targets being transferred horizontally has already been described or suggested, there is no indication in the literature that assays based on these targets can lead to such a high rate of false positive results as those found in this study. In fact, these targets are still used as sole molecular targets for *Cfv* detection, as evidenced in recent studies (de Oliveira et al. 2015; Filho et al. 2018; Lúcio et al. 2019). The rate of false positive results may depend on the geographical region, since previous studies in Canada (Waldner et al. 2017) or Brazil (de Oliveira et al. 2015; Filho et al. 2018) reported lower rates of positive results with *parA*-based assays.

C. portucalensis, which is an inhabitant of the bull's prepuce (Silva et al. 2020a), was identified as a cause of false positive results. The high identity between the sequences found in *C. portucalensis* and ISCfe1 and *parA* sequences of *Cfv* suggests the horizontal transfer of these molecular targets. Although *C. portucalensis* and *Cfv* are not phylogenetically close species (Silva et al. 2020a), the sharing of the same niche by *Cfv* and *C. portucalensis* may have facilitated the horizontal gene transfer of *parA* and ISCfe1. The genomic island associated to *Cfv,* in which the *parA* gene is included, was found in the genome of *C. portucalensis*, supporting the results of *parA* positive samples, with amplification of GI genes (*fic1, fic2, virB11, virB9*) and without amplification of *C. fetus* specific molecular targets. One of the *C. portucalensis* strains is positive for ISCfe1, without amplification of GI genes or *parA*. This finding suggests an independent horizontal gene transfer of *parA* and ISCfe1 and may justify the high percentage of samples positive in ISC-A assay and negative in parA-A assay ($n = 91$, 29.5 %). The high similarity between the sequences found in *C.*

portucalensis and ISCfe1 and *parA* gene sequences of *Cfv* is enough to impair the accuracy of molecular diagnostic methods based on these targets' detection.

Overall, this study evidenced that ISCfe1 and *parA* gene-based assays are associated with considerable specificity failures and, consequently, these targets are unsuitable for *Cfv* identification when used solely. However, the detection of ISCfe1 or *parA* may be used as part of a diagnostic strategy, with validation of positive results to *parA* or ISCfe1 with previous or subsequent *C. fetus* detection assays. This strategy overcome the specificity failures associated to ISCfe1 and *parA* detection assays found in this study. Still, as described in previous studies (Abril et al. 2010; Spence et al. 2011; Van Der Graaf-Van Bloois 2016), these molecular targets were sporadically found in *Cff* isolates, leading to false positive results even with the above diagnostic strategy. However, the specificity of these methods is expected to be higher if applied to bulls from farms with reproductive failure, where other causes were ruled out, as described previously (Waldner et al. 2017).

3.2.6 Conclusion

The results of this study have major implications in the molecular diagnosis of BGC, invalidating the use of ISCfe1 and *parA* as sole targets for *Cfv* identification. Diagnosis of BGC based solely on these genomic targets originates false positive results, which may lead to the unnecessary treatment or culling of animals. The combined use of *Cfv* and *C. fetus* detection assays should be encouraged as part of the BGC diagnostic strategy, in order to improve the specificity of molecular diagnostic methods. This study also described, for the first time, the presence of the *C. fetus* GI in another *Campylobacter* species inhabitant of the bull's preputial mucosa, evidencing that this genomic element can be transferred to other C*ampylobacter* species. A highly homologous sequence of ISCfe1, previously considered a promising *Cfv* molecular marker, and the *parA* gene, were also found in another *Campylobacter* species. This evidences the horizontal gene transfer of current diagnostic *Cfv* molecular targets to other *Campylobacter* species and prompts for the development of reliable molecular diagnostic tools for BGC diagnosis.

3.3. Genomic and phenotypic characterization of *Campylobacter fetus* subsp. *venerealis* strains

Silva MF, Pereira AL, Fraqueza MJ, Pereira G, Mateus L, Lopes-da-Costa L, Silva E. 2021. *Microorganisms, 9,* 340. DOI: 10.3390/microorganisms9020340

3.3.1 Abstract

The pathogenesis mechanisms of *Cfv*, the etiologic agent of BGC remain elusive. This study evaluated the virulence potential and biovar characteristics of *Cfv* isolates (n = 13), by PCR screening of putative virulence-factor (VF) genes, Multilocus Sequence Typing (MLST) analysis, antimicrobial susceptibility to tetracycline, penicillin, enrofloxacin and streptomycin testing and whole-genome sequencing (WGS; n = 5), also comparing the latter with 26 other whole-genome sequences of *Cfv* strains. The putative VF genes encoding the T4SS of *Cfv* (*virB2-virB11/virD4*) were absent in 92 % of isolates, including isolates from aborted foetuses, evidencing that these VF genes are not essential for *Cfv* pathogenicity. The *parA* gene, used as a *Cfv* diagnostic molecular target, was detected in only 3 of 13 isolates, invalidating its use for diagnosis purposes. Three novel sequence types were identified by MLST. Although no *in vitro* antimicrobial resistance was detected, WGS identified antimicrobial resistance-related genes, including those encoding the multidrug efflux pumps CmeABC and YkkCD, indicating that their presence is not enough to provide antimicrobial resistance. The SNP and accessory protein families analysis segregated the *Cfv* and *Cfvi* strains into different clusters. In conclusion, this study evidenced virulence potential and biovar characteristics of *Cfv* and *Cfvi*, which are of relevance for the control of BGC.

3.1.2 Introduction

Cfv is the etiological agent of BGC, a notifiable venereal disease of cattle responsible for low herd reproductive efficiency and significant economic losses worldwide (Mshelia et al. 2010; OIE 2018). Infected bulls asymptomatically carry *Cfv* in the preputial and penile mucosa and infect females during natural breeding or through semen, causing embryo loss or early fetal abortion (Michi et al. 2016). For that reason, BGC control is based on bull preputial testing

and culling of infected bulls, which requires an accurate identification of *Cfv* (McGoldrick et al. 2013; Michi et al. 2016).

The MLST identified a clonal structure with lower genetic diversity among *C. fetus* isolates than among other *Campylobacter* species (Van Bergen et al. 2005a). In fact, *Cff* which colonizes the intestinal tract and occasionally the preputial cavity, causing sporadic abortion in cattle, and *Cfv* have more than 90 % genome similarity (Kienesberger et al. 2014). This hampers the selection of suitable molecular targets for subspecies identification. The subspecies *Cfv* includes the biovar intermedius, which is differentiated by its ability to produce H_2S from L-cysteine (Veron and Chatelain 1973; Sprenger et al. 2012). However, this is also common to *Cff,* hindering the subspecies differentiation by phenotypic tests. The determinants behind *Cfv* pathogenicity and niche restriction are still unclear. Several putative virulence factor (VF) genes were identified in both subspecies, including those encoding proteins involved in bacterial adhesion, invasion and cytotoxicity, which are common to other *Campylobacter* species, namely the fibronectin-binding protein, the campylobacter invasion antigen (CiaB) and the cytolethal distending toxin (CDT), among others (Ali et al. 2012). Nevertheless, comparative genomic analyses revealed the presence of a genomic island almost exclusive of *Cfv* and highly prevalent in this subspecies (Abril et al. 2010; Gorkiewicz et al. 2010; Ali et al. 2012), which harbours one of the most well studied genes for *Cfv* identification, the *parA* gene (Hum et al. 1997; McMillen et al. 2006). This genomic island harbours genes encoding FIC-domain proteins (*fic* genes) and a bacterial T4SS (*virB-virD4* genes) (Gorkiewicz et al. 2010). *In vitro* studies demonstrated that the T4SS contributes to cytotoxicity and invasiveness of *Cfv*, besides being involved in interbacterial DNA transfer by conjugation (Gorkiewicz et al. 2010; Kienesberger et al. 2011). Additionally, FIC proteins form a toxin-antitoxin network in *Cfv* that may favour its survival under adverse conditions (Sprenger et al. 2017). These findings prompt for a role of this genomic island in *Cfv* pathogenicity and/or adaptation to the genital tract (Gorkiewicz et al. 2010; Sprenger et al. 2017). A recent study revealed that *C. fetus* strains commonly harbour multiple T4SS encoding regions, which are phylogenetically different and were possibly acquired from different *Campylobacter* species (van der Graaf–van Bloois et al. 2016). Nevertheless, some T4SS encoding regions lack several *virB* genes and their function is still unclear.

Bulls may be treated with antibiotics, namely penicillin and streptomycin, although with limited efficacy, particularly in mature bulls (Hum et al. 1993; Truyers et al. 2014; Michi et al. 2016). These antibiotics are also routinely used in semen processing and their use is mandatory for intra-community trade of bovine semen according to the EU Directive 88/407/CEE. However, the prevalence of antimicrobial resistance among *Cfv* isolates has been poorly investigated. Indeed, a genomic island with two genes involved in tetracycline

and streptomycin resistance was identified in *Cff* isolates (Abril et al. 2010; Escher et al. 2016), but its occurrence in *Cfv* is unknown.

This study aimed to characterize *Cfv* and *Cfvi* isolates, assessing their genomic characteristics, genetic diversity, load of virulence-related genes and *in vitro* antimicrobial susceptibility.

3.3.3 Materials and Methods

3.3.3.1 *Campylobacter fetus* subsp. *venerealis* isolates

The *Cfv* isolates (n =13) were kindly provided by the Starcross Veterinary Investigation Centre from Animal and Plant Health Agency (APHA), United Kingdom, where they were phenotypically identified as *Cfv* (Table 10). The *Cfv* isolates were grown in Columbia agar plates with 5 % sheep blood (COS, Biomerieux) for 48h, under microaerobic conditions (GENbox Microaer, Biomerieux). The subspecies identification (*Cfv*) was further confirmed by the amplification of *nahE* and ISCfe1 sequences, as described by van der Graaf et al. (2013).

Table 10 *C. fetus* subsp. venerealis isolates used in this study

Isolate	Source of isolation	Year	Herd
IS26-04236	Aborted fetus	2014	A
IS26-07793	Aborted fetus	2016	B
IS16-01257	Aborted fetus	2013	C
IS14-13272	Aborted fetus	2015	D
IS21-05213	Unknown	2018	E
IS12-08947	Aborted fetus	2019	F
IS21-08727	Aborted fetus	2019	G
IS21-08528	Aborted fetus	2019	H
IS21-08525	Aborted fetus	2019	H
SA21-221439	Bull sheath wash	2019	I
SA21-221825	Bull sheath wash	2019	I
SA21-217832	Bull sheath wash	2019	J
SA21-217833	Bull sheath wash	2019	J

3.3.3.2 <u>DNA isolation</u>

Total DNA was extracted by a rapid boiling method. Briefly, bacterial cells were suspended in 1.5 mL PBS, centrifuged (17000 g, 8 min), the supernatant discarded, and the cellular pellet resuspended in 500 µL of sterile water. After a second centrifugation (17000 g, 5 min), the pellet was resuspended in 100 µL of sterile water and incubated at 95 ºC for 15 min. Finally, the lysate was centrifuged (17000 g, 8 min), and the DNA containing supernatant collected. The DNA was quantified using a Nanodrop 2000C spectrophotometer (Thermo Scientific) and diluted to 50 ng/µL.

3.3.3.3 <u>Multilocus sequence typing (MLST)</u>

The MLST analysis was performed according to a previously described scheme, based on seven housekeeping genes: *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt,* and *uncA* (van Bergen et al. 2005a). The sequence types (STs) were assigned using the *Campylobacter* MLST database (https://pubmlst.org/campylobacter/) sited at the University of Oxford (Jolley et al. 2018). New alleles and profiles were submitted to this database.

3.3.3.4 <u>Surface array protein and L-cysteine transporter typing</u>

The isolates were classified as *Cfv* or *Cfvi* using a multiplex-PCR for detection of an L-cysteine transporter operon previously described (Farace et al. 2019). DNA from *Cfv* strain NCTC 10354 and *Cff* strain NCTC 10842 were used as positive controls.
The sap serotype (*sapA* and *sapB*) was identified as described before (Dworkin et al., 1995), using primers ACF/ACR and BCF/BCR. DNA from *Cfv* strain NCTC 10354 and *Cff* strain NCTC 10842 were used as positive controls in *sapA* and *sapB* PCRs, respectively.

3.3.3.5 <u>Detection of putative virulence factor genes using PCR</u>

The presence of putative VF genes involved in adhesion (*cadF*), invasion (*invA* and *ciaB*) and cytotoxicity (*cdt and pldA*) of host cells (Ali et al. 2012) was assessed by PCR. For genes *cadF, invA, ciaB and pldA,* primers were designed with Primer-BLAST (Ye et al. 2012) using gene sequences of *Cfv* NCTC 10354 as template (Annex III). PCR reactions were carried out in 25 µL mixtures, containing 200 µM of each dNTP (4you4 dNTP Mix, Bioron), 400 nM of each primer, 1X reaction buffer (Complete reaction buffer, Bioron), 2 units of DFS-Taq DNA polymerase (Bioron) and 100 ng of DNA. Amplifications were performed in a Doppio thermal cycler (VWR) with the following cycling conditions: initial denaturation step at 94 ºC for 2 min, followed by 30 cycles of denaturation at 94 ºC for 30 sec, annealing

temperature for 30 sec, extension at 72 ⁰C for 1 min, with a final extension step at 72 ⁰C for 5 min. Detection of *cdtA*, *cdtB* and *cdtC* genes was carried out by PCR as described previously (2008).

The presence of genes encoding the most studied T4SS of *Cfv* (Gorkiewicz et al. 2010; Kienesberger et al. 2011), which includes *virB2-virB11*, *virD4* and *fic1* and *fic2* genes were also screened by PCR. Primers were designed with primer-BLAST (Ye et al. 2012) to target *virB2*, *virB3-virB4*, *virB5*, *virB6, virB7*, *virB8*, *virB10* and *virD4* genes, using the sequences of *Cfv* NCTC 10354 (GenBank accession no. CP043435.1, loci CFVT_1262 – 1267, CFVT_1258 and CFVT_1256) as template (Annex III). Genes *fic1*, *fic2*, *virB9* and *virB11* were detected as recently described (Silva et al. 2020b). PCR mixtures and thermal cycling conditions were performed as described above. The amplification products were separated in a 1.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized using a ChemiDoc XRS + System (Bio-Rad).

3.3.3.6 Detection of *parA* gene

The parA gene was detected by three PCR assays directed towards distinct nucleotide regions, comprising a conventional PCR with VenSF/VenSR primers (Hum et al. 1997) and two real-time PCR assays, (McMillen et al. 2006) and parA-B assay (Silva et al. 2020b).

3.3.3.7 Antibiotic susceptibility testing

The minimum inhibitory concentrations (MICs) of streptomycin, tetracycline, enrofloxacin and penicillin G were *in vitro* determined using Etest gradient strips (Biomerieux). *Cfv* colonies grown on COS plates for 48 hours were suspended in Brain Heart Infusion (BHI) broth to a turbidity of 1.0 McFarland measured with a Densimat densitometer (Biomerieux). The inoculum was spread on Mueller Hinton agar plates supplemented with 5 % horse blood and 20 mg/L of β-NAD (MHF, Biomerieux) and one strip was applied on each agar plate. The concentration gradients of antibiotics in Etest strips used were 0.064 -1024 µg/mL for streptomycin, 0.016-256 µg/mL for tetracycline, and 0.002-32 µg/mL for enrofloxacin and penicillin G. Plates were incubated for 48 h at 35⁰C in a microaerobic atmosphere, and MICs were read at the point where the zone of inhibition intersected the MIC scale on the Etest strip. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control, as recommended by the manufacturer.

In the absence of specific interpretative criteria for *Cfv*, the MIC breakpoints of enrofloxacin and tetracycline were defined according to the ciprofloxacin and tetracycline breakpoints defined for *Campylobacter jejuni* and *Campylobacter coli* by the European Commitee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST 2020) (Table 11). Results of penicillin G were interpreted according to the criteria defined for Gram-negative anaerobes by the EUCAST (EUCAST 2020) (Table 11). For streptomycin MIC breakpoints, due to the absence of EUCAST breakpoints, results were interpreted according to criteria of The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) for *Escherichia coli* (NARMS 2019) (Table 11).

Table 11 MIC breakpoints for antibiotic susceptibility testing

Antibiotic	MIC Breakpoints (µg/mL)		Reference
	S ≤	R >	
Penicillin G	0.25	0.5	(EUCAST 2020)
Enrofloxacin	0.5	0.5	
Tetracycline	2	2	
Streptomycin	16	32	(NARMS 2019)

MIC – minimum inhibitory concentration; S – susceptible, standard dose regimen; R- resistant.

3.3.3.8 Whole Genome Sequencing of *Cfv* strains

Strains IS26-07793, IS16-01257, SA21-221439, SA21-217832 and SA21-217833 were selected for whole-genome sequencing (WGS) analysis, as they have unique genomic traits, namely represent a novel ST or harbour the screened T4SS-encoding genes. Strains were grown on blood agar plates supplemented with 5 % sheep blood at 37 ºC for 48 hours and genomic DNA isolated using the DNeasy Blood and Tissue Kit (Qiagen, Germany), according to manufacturer's instructions. Following preparation of DNA libraries, the genomes were sequenced with the Illumina Novaseq Platform at Stabvida (Caparica, Portugal), using 150-bp paired end reads. The reads were *de novo* assembled in the Pathosystems Resource Integration Center (PATRIC) version 3.6.7 web platform (Davis et al. 2020), using SPAdes version 3.12.0 (Bankevich et al. 2012). Assembled genomes were submitted to the Comprehensive Genome Analysis service of PATRIC (Davis et al. 2020), which includes an annotation service using RAST tool kit (RASTtk) (Brettin et al. 2015). The whole genome shotgun projects of strains IS26-07793, IS16-01257, SA21-221439, SA21-

217832 and SA21-217833 were deposited at DDBJ/ENA/GenBank under the accession numbers JAENPS000000000, JAENPT000000000, JAENPU000000000, JAENPV000000000 and JAENPW000000000, respectively.

The genomes were visualized by comparison against reference genomes (*Cfv* strain NCTC 10354 and *Cfv* strain 01/165) using the BLAST Ring Image Generator (BRIG) version 0.95 (Alikhan et al. 2011), with an upper identity threshold of 90 % and a lower identity threshold of 70 %. Genomic islands and T4SS encoding regions VG III (Kienesberger et al. 2011), PICFV8/T4SS region 1A (Ali et al. 2012; van der Graaf–van Bloois et al. 2016), T4SS region 2A and 1F (van der Graaf–van Bloois et al. 2016) were included in the BRIG analysis.

The MLST allele sequences were extracted from WGS data using the MLST software version 2.0.4 (Larsen et al. 2012) to confirm results of MLST analysis.

3.3.3.9 Comparative genomic analysis

The genomes of the five sequenced strains were compared to 26 whole genome sequences of *Cfv* strains retrieved from the GenBank (Table 12). Genomes were analysed by the Comprehensive Genome analysis service of PATRIC (Davis et al. 2020), which includes a k-mer based detection method for antimicrobial resistance genes. Genes assigned to mechanisms of antibiotic inactivation and efflux pumps, were considered for this analysis. Aditionally, genes *ant(6)-Ib* and *tet*(44) conferring resistance to streptomycin and tetracycline (Abril et al. 2010) and putative VF encoding genes (*cadF, pldA, invA, ciaB, cdtA, cdtB* and *cdtC*) were searched by the BLAST tool in the genomes.

The PATRIC's Family Protein Sorter service (Davis et al. 2020) was used to evaluate the distribution of protein families across the analysed genomes, and genus-specific families (PLfams) represented in more than 5 % and less than 95 % of the genomes (2 to 29 genomes), considered accessory protein families, were selected for further analysis. These data were used for the construction of a HeatMap using Next-Generation Clustered Heat Map (NG-CHM) Builder (Ryan et al. 2020) with hierarchical clustering using the Euclidean distance metric with the complete agglomeration method.

Single nucleotide polymorphisms (SNP) detection and analysis were performed with CSI Phylogeny version 1.4 (Kaas et al. 2014) to reconstruct a phylogenetic tree using the genome of strain NCTC 10354 as reference, with a minimum distance between SNPs set for 10 bp. The tree was illustrated using the Molecular Evolutionary Genetics Analysis (MEGA) X software version 10.1.7 (Kumar et al. 2018) and bootstrap values lower than 70 % were hidden.

Table 12 Genomes of *Campylobacter fetus* subsp. *venerealis* strains used for the comparative genomic analysis

Cfv strain	GenBank accession	Country	Source of isolation (bovine)	Assembly level
NCTC 10354	CP043435.1	United Kingdom	Vagina	complete
WBT011/09	LMBI00000000.1	United Kingdom	Unknown	contig
CCUG 33900	LREV00000000.1	France	Aborted fetus	contig
CCUG 33972	LREU00000000.1	Czech Republic	Unknown	contig
cfvi03/293	CP006999.2	Argentina	Aborted fetus	complete
cfvi9825	LRES00000000.1	Argentina	Aborted fetus	contig
cfvi97/532	LRER00000000.1	Argentina	Vagina	contig
cfvi92/203	LRVL00000000.1	Argentina	Vagina	contig
cfvi03/596	LRAM00000000.1	Argentina	Aborted fetus	contig
cfvi02/298	LRVK00000000.1	Argentina	Aborted fetus	contig
01/165	CP014568.1	Argentina	Mucus	complete
97/608	CP008810.1	Argentina	Unknown	complete
99541	ASTK00000000.1	Argentina	Preputial sample	contig
ADRI1362	LREX00000000.1	Argentina	Unknown	contig
06/341	SOYW00000000.1	Argentina	Aborted fetus	contig
cfvB10	LRET00000000.1	USA	Unknown	contig
84-112	HG004426.1	USA	Unknown	complete
TD	JPPC00000000.1	Canada	Preputial sample	contig
66Y	JPQC00000000.1	Canada	Preputial sample	contig
B6	AJMC00000000.1	Australia	Vagina	scaffold
642-21	AJSG00000000.1	Australia	Uterus	scaffold
ADRI513	LRFA00000000.1	Australia	Unknown	contig
zaf3	LREZ00000000.1	South Africa	Aborted fetus	contig
zaf65	LREY00000000.1	South Africa	Unknown	contig
NW_ME2	JAATTN000000000.1	South Africa	Unknown	contig

3.3.4 Results

3.3.4.1 Multilocus sequence typing of *Cfv* isolates

A total of 4 STs were identified among the 13 *Cfv* isolates (Table 13). The allelic profile of isolates IS16-01257 and SA21-221439 was not listed in the PubMLST database and after its submission, the isolates were assigned to ST-71. Two new alleles of *gltA* and *tkt*, assigned respectively to alleles 12 and 14, were deposited in the PubMLST database. The alleles 7 and 12 of *gltA* differ from allele 2, which is the most common, in one nucleotide position. Also, alleles 2 and 14 of *tkt* are distinguished by a single nucleotide. Overall, nine isolates were assigned to ST-4 (69.2 %), two to ST-71 (15.4 %), one to ST-72 (7.7 %) and one to ST-73 (7.7 %). Interestingly, the isolates from herds I and J were assigned to different STs (herd I - SA21-221825 to ST-4 and SA21-221439 to ST-71; herd J - SA21-217832 to ST-72 and SA21-217833 to ST-73).

Table 13 Sequence types and corresponding allelic profiles

ST	Isolates	Alleles						
		aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*
4	IS26-04236 IS26-07793 IS14-13272 IS21-0513 IS21-08528 IS12-08947 IS21-08525 SA21-221825 IS21-08727	1	2	2	2	1	2	1
71	IS16-01257 SA21-221439	1	2	7	2	1	2	1
72	SA21-217832	1	2	12	2	1	14	1
73	SA21-217833	1	2	2	2	1	14	1

3.3.4.2 Genomic characterization of *Cfv* isolates: Surface array protein and L-cysteine transporter typing, virulence factor genes and *parA* gene

All the 13 *Cfv* isolates were classified as serotype A (*sapA* positive and *sapB* negative). Most isolates (n = 9) revealed a *Cfvi* pattern in L-cysteine transporter PCR (Table 14). The remaining 4 isolates harbour the L-cysteine transporter encoding operon partially deleted and, consequently, were classified as *Cfv*.

All isolates harbour the CDT operon genes (*cdtABC*), which encode the cytolethal distending toxin, and genes *cadF, ciaB, invA, pldA*, which encode the fibronectin-binding protein, *Campylobacter* invasion antigen B, invasin A and phospholipase A, respectively. These genes were also found in the 26 whole genome sequences of *Cfv* strains, using BLAST search.

Only 3 isolates (23.1 %) were *parA* positive and this result was consistent using the three different assays. Genes *fic1* and *fic2* were found in all *Cfv* isolates, whereas T4SS encoding genes (*virB2-virB11* and *virD4*) were present only in *Cfv* isolate IS26-07793 (Table 14).

Table 14 Genomic characteristics of the *Cfv* isolates

Isolate	Sap type	L-cysteine transporter profile	*parA* gene	*fic* genes	T4SS enconding genes
IS26-04236	A	*Cfv*	+	+	-
IS26-07793	A	*Cfv*	+	+	+
IS16-01257	A	*Cfvi*	-	+	-
IS14-13272	A	*Cfv*	+	+	-
IS21-05213	A	*Cfv*	-	+	-
IS21-08528	A	*Cfvi*	-	+	-
IS12-08947	A	*Cfvi*	-	+	-
IS21-08525	A	*Cfvi*	-	+	-
SA21-221439	A	*Cfvi*	-	+	-
SA21-221825	A	*Cfvi*	-	+	-
IS21-08727	A	*Cfvi*	-	+	-
SA21-217832	A	*Cfvi*	-	+	-
SA21-217833	A	*Cfvi*	-	+	-

3.3.4.3 Antibiotic susceptibility testing of *Cfv* isolates

Antibiotic resistances were not found among the *Cfv* isolates. All the 13 isolates were susceptible to tetracycline, streptomycin and enrofloxacin. Eight isolates were categorized as susceptible to penicillin G with standard dose regimen and the remaining 5 isolates (38.5 %) were considered susceptible with increased exposure. The MIC values of tetracycline, streptomycin and enrofloxacin for all isolates were bellow the susceptibility breakpoints.The range of MIC values for each antibiotic and the MIC ihnibiting 50 % (MIC50) and 90 % of the isolates (MIC90) are shown in Table 15.

Table 15 Minimum inhibitory concentrations of selected antibiotics for *Cfv* isolates

Antibiotic	MIC (µg/mL)		
	Range	50 %	90 %
Tetracycline	0.047 - 0.064	0.064	0.064
Streptomycin	0.5 – 3.0	2.0	2.0
Enrofloxacin	0.032 – 0.125	0.064	0.094
Penicillin	0.047 – 0.38	0.25	0.38

3.3.3.4 <u>Whole genome sequencing of 5 *Cfv* strains</u>

The WGS analysis confirmed the PCR result of absence of genes *parA, virB2-virB11* and *virD4* of T4SS encoding region 1A, in four of the five sequenced strains. Only *Cfv* strain IS26-07793 harbours the genomic island with the T4SS encoding region 1A (van der Graaf–van Bloois et al. 2016). However, all the five strains have other T4SS clusters, with gene sequences and gene composition distinct from region 1A (Table 16).

Table 16 T4SS encoding genes found in different genomic regions

Region	virD4	virB11	virB10	virB9	virB8	virB7	virB6	virB5	virB4	virB3	virB2	virB1
1A	■	■	■	■	■	■	■	■	■	■	■	
1B	■	■	■	■	■	■		■	■	■		
1F	■	■	■	■	■		■	■	■	■		
2A	■	■	■	■	■	■			■			

Blue or white coloured cells represent presence or absence of the gene, respectively. T4SS encoding regions according with a previous classification (van der Graaf–van Bloois et al. 2016).

Plotting the sequenced genomes against the genome of *Cfv* NCTC 10354 as reference showed that all 5 strains harbour the T4SS encoding region 2A (Figure 11), whereas the region 1A is present only in strain IS16-07793. An additional comparison using the genome of *Cfv* strain 01/165 as reference showed the presence of a T4SS encoding region 1F, in strains SA21-217832 and SA21-217833.

All except strain IS26-07793 harbour a T4SS encoding region 1B, and strains SA21-217832 and SA21-217833 also present T4SS tra/trb encoding regions.

The comparison with the genome of *Cfv* NCTC 10354 revealed the absence of a prophage in VGI III (Kienesberger et al. 2014) in strains IS16-01257, SA21-221439, SA21-217832 and SA21-217833 within the sap locus.
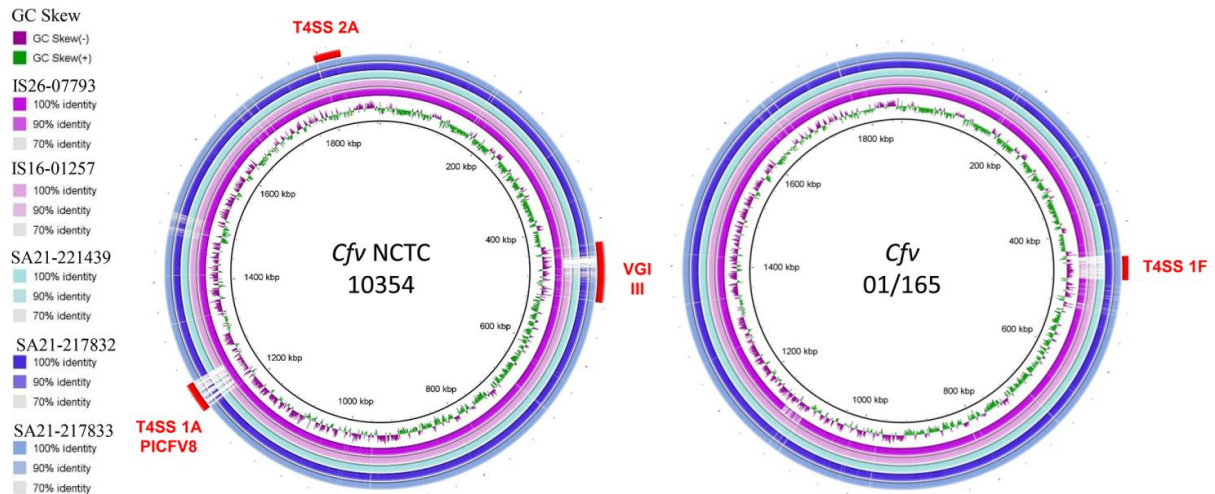
Figure 11 Comparative genomic analysis of *Cfv* strains with reference strains NCTC 10354 and 01/165. Image created using Blast Ring Image Generator version 0.95. The inner ring represents the GC Skew and the remaining rings represent a BLASTN comparison of genomes of IS26-07793, IS16-01257, SA21-221439, SA21-217832 and SA21-217833 with the reference strains NCTC 10354 (left) and 01/165 (right). Red curved bars indicate chromosomal genomic islands previously identified by other authors (T4SS encoding regions 1A, 1F, 2A) (van der Graaf–van Bloois, Miller, et al. 2016)

3.3.4.5 Comparative genomic analysis of *Cfv* strains

The SNP analysis using the strain NCTC 10354 as reference was based on 1,630,344 nucleotide positions that were common to all genomes. As shown in Figure 12, the strain IS26-07793 is phylogenetically related to strains CCUG 33900, B6, and NCTC 10354. The remaining four sequenced strains are phylogenetically distant from IS26-07793. Strains SA21-217832 and SA21-217833, isolated from the same herd, are highly related although having different STs. These strains typed as ST-72 and ST-73 are phylogenetically close to IS16-01257 and SA21-221439 typed as ST-71, and the ST-4 WBT011/09.
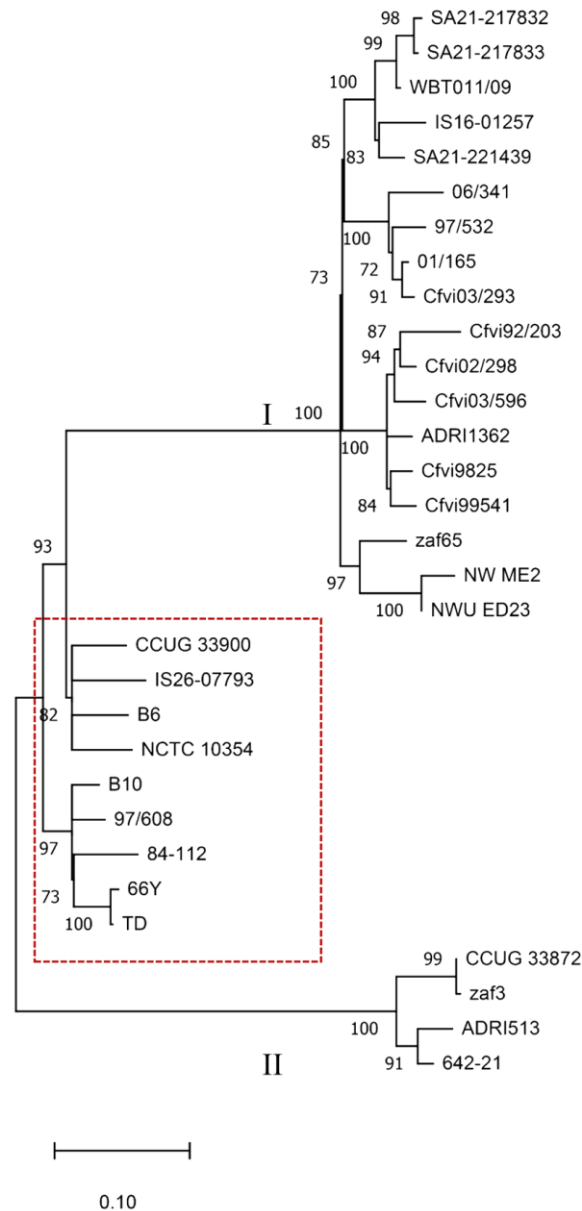
Figure 12 Phylogenetic tree based on single nucleotide polymorphisms (SNPs) of 31 *C. fetus* subsp. *venerealis* strains. Numbers at the nodes represent bootstrap values and values lower than 70 % were hidden. The red border rectangle separates *Cfv* strains (inside) from strains biotyped as *Cfv* biovar intermedius (outside).

The phylogenetic tree also shows a clear distinction between strains typed as *Cfvi* or *Cfv* in previous studies (Van Bergen et al. 2005a; van der Graaf–van Bloois et al. 2016; Farace et al. 2019). Although NWU_ED23 and NW_ME2 strains were not typed in these studies, the BLAST search identified the complete L-cysteine transporter encoding gene in NWU_ED23 (contig 72) and the sequence divided into two contigs (contigs 26 and 417) in NW_ME2, which is compatible with a *Cfvi* classification. Overall, nine strains are classified as *Cfv* and 22 as *Cfvi. Cfvi* strains are divided into two distant groups (Clusters I and II), with strains

CCUG 33872, zaf3, ADRI513, and 642-21 segregated from the remaining *Cfvi* strains.This comparative genomic analysis of *Cfv* strains evidences the presence of similar SNP patterns in isolates from the same geographic region. For instance, strains zaf65, NW_ME2, and NWU_ED23 were isolated from different regions of South Africa. Morevover, *Cfvi* strains sequenced in this study cluster with the strain WBT011/09 strain from the UK. The results also showed that *Cfvi* strains from Argentina are included in two related clusters grouped with a bootstrap value of 100 %.

Regarding the antimicrobial resistance genes, those encoding the multidrug efflux system CmeABC, the broad-specificity multidrug efflux pump YkkCD, the Macrolide-specific efflux protein MacA, the Macrolide export ATP-binding/permease protein MacB and the nitroimidazole resistance protein were found in the genome of the 31 strains under study, whereas the genes *tet*(44) and *ant(6)-Ib* searched using BLAST were not identified in the genomes under analysis.

The analysis of protein families (PLfams) identified 2425 genus-specific protein families, from which 1641 were represented in all the 31 genomes. A total of 1693 proteins are encoded in the genome of 30 or more isolates (≥96.8 %), which represent the core gene families considering the commonly accepted cut-off value of 95 %. The accessory protein families, found in less than 95 % of the strains, are represented in a heatmap with hierarchical clustering (Figure 13). A total of 540 accessory protein families were found encoded in the 31 analysed genomes, of which 461 have an unknown function (hypothetical proteins). The groups formed based on the accessory protein families almost match those formed based on the SNP phylogenetic tree, with exception of strain *Cfvi* 06/341, 97/532 and UK *Cfvi* strains that were split into two groups. The *Cfvi* strains 642-21, ADRI 513, zaf3 and CCUG33872 are closely related and segregated from the remaining *Cfvi* strains, which is in accordance with SNP analysis. There were no exclusive *Cfv* protein families common to all strains. As expected, two protein families (PLF_194_00014554 and PLF_194_00049089) related to L-cysteine transporters were exclusively found in *Cfvi* strains, with exception of strain cfvi9925. Excluding this strain, 180 protein families were unique to *Cfvi* and 10 protein families were exclusively represented in the nine *Cfv* genomes.
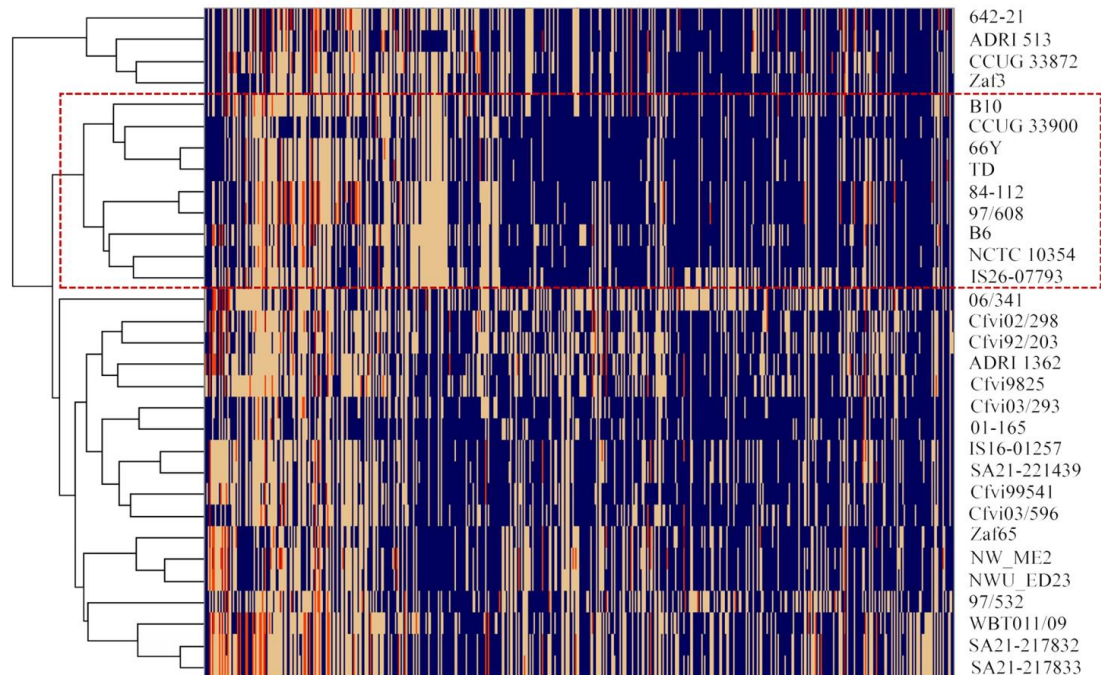
Figure 13 Heat map representing the distribution of accessory protein families (n=540) in the genomes of 31 *Cfv* strains. The absence of the protein family is represented in blue and the number of proteins per family is represented in yellow (n=1), orange (n=2) and red (n=3). *Cfv* strains are grouped by hierarchical clustering, using the Euclidean distance and the complete agglomeration method. The rectangle with red border separates *Cfv* strains (inside) from *Cfv* biovar intermedius strains (outside).

3.3.5   Discussion

This study evaluated the virulence potential of *Cfv* strains through genomic and phenotypic approaches and uncovered characteristics of this subspecies that are relevant for BGC control. MLST genotyping showed that most *Cfv* isolates were clustered in ST-4, which is reported to be the most prevalent ST among *Cfv* (van Bergen et al. 2005a). Thus, this genotyping method could be considered an effective method for typing *C. fetus* at the subspecies level (van der Graaf-van Bloois et al. 2013). Interestingly, this study revealed a considerable ST diversity and identified three novel STs. These STs differ from ST-4 in one to two nucleotide positions, which denotes the genetic stability of *Cfv*. Nevertheless, the ST variability was higher than expected and the use of MLST for subspecies identification should be further evaluated. In fact, the suitability of MLST for subspecies typing was questionable since the description of one *Cff* strain belonging to ST-4 (Iraola et al. 2015).

The pathogenicity mechanisms of *Cfv* are still unclear. All *Cfv* isolates and 26 genomes from different geographic regions harbour genes encoding the fibronectin-binding protein (*cadF)*, Campylobacter invasion antigen B (*ciaB*), invasin A (*invA*), phospholipase A (*pldA*), and

cytolethal distending toxin (*cdtABC*), which contribute to the virulence potential of other *Campylobacter* species, playing roles in adhesion, invasion, and/or cytotoxicity of host cells (Sprenger et al. 2012; Bolton 2015). The presence of these genes in all genomes of *Cfv* suggests their relevance for host colonization and/or pathogenicity. Further research is needed to understand the contribution of these genes to *Cfv* virulence.

A T4SS encoded by *virB2-virB11* and *virD4* genes, within a genomic island formerly considered unique to *Cfv* (Gorkiewicz et al. 2010; Ali et al. 2012) was suggested as being involved in *Cfv* virulence, namely in cell invasion, cytotoxicity and conjugative DNA transfer (Gorkiewicz et al. 2010; Kienesberger et al. 2011). Genes encoding this T4SS, corresponding to region 1A (van der Graaf–van Bloois et al. 2016), were detected in 91 % of 67 *Cfv* strains (Gorkiewicz et al. 2010). However, in this study only 1 out of 13 isolates harbour genes encoding this T4SS, and 7 of the 12 negative strains were isolated from aborted foetuses, which still evidences their pathogenicity even in the absence of this genomic island. The WGS of the five sequenced isolates identified other loci with T4SS encoding genes, with distinct gene sequences from encoding region 1A, whose putative role in *Cfv* pathogenicity or niche specialization have so far not been addressed. These T4SS encoding regions 1B, 1F and 2A lack some *virB/virD4* genes, which require further studies to evaluate their functionality. Other genes that were not analysed in this study may also contribute to the pathogenicity of these isolates and should be evaluated in a larger sample.

Tested by three different PCR assays, only 3 out of 13 isolates harbour the *parA* gene, and this negative status was confirmed in the WGS of four strains. A previous study reported that most *Cfv* isolates from the UK were negative for *parA* gene (Willoughby et al. 2005). The doubt remained whether this resulted from sequence variations in primer-binding sites or from absence of the gene. The present study confirms the absence of the gene in a high proportion of UK strains and clarifies the reason of sensitivity failures of *parA* detection methods for *Cfv* identification (van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). In accordance, a recent study (Abdel-glil et al. 2020) reported the absence of this gene in 45 % of *C. fetus* genomes proposed as belonging to subspecies *venerealis*, including some of the strains analysed in the present study. The *parA* gene is located in a genomic island, which was already described in *Cff* (Abril et al. 2010; van der Graaf–van Bloois et al. 2016) and other *Campylobacter* species (Silva et al. 2020b). Therefore, the sole use of this molecular marker for *Cfv* identification should be avoided due to its lack of specificity and sensitivity.

To author's best knowledge, this is the first report on antibiotic minimum inhibitory concentrations in *Cfv* field isolates. No antimicrobial resistance to streptomycin, penicillin, tetracycline, and enrofloxacin was found in the 13 *Cfv* isolates, and streptomycin and tetracycline resistance genes were not detected in the 31 *Cfv* analysed genomes. These

latter genes were identified in *Cff* strains harbouring the *Cfv*-associated genomic island with T4SS encoding genes (Abril et al. 2010; Escher et al. 2016). The antimicrobial susceptibility results of this study are in accordance with a previous study, in which all isolates were susceptible to penicillin, streptomycin, tetracycline and only 5 % of the isolates were susceptible to enrofloxacin (Vargas et al. 2005). Similarly, in another study with *Cfv* isolates from Germany, only 4 % of the isolates revealed decreased susceptibility to streptomycin and 2 % for ciprofloxacin and tetracycline (Hänel et al. 2011). Antimicrobial resistance data from this study must be reagarded with caution, as they refer to a very limited number of isolates from a narrow world geographical region. Nevertheless, they provide proof of concept for the simultaneous presence of *in vitro* susceptibility to antimicrobials and genes encoding for its resistance. A wide geographical survey with a large sampling is needed to ascertain the presence of antimicrobial resistance in different scenarios.

In contrast, genes encoding two multidrug efflux pumps were detected in all 31 analysed genomes. The CmeABC efflux pump, well-studied in *C. jejuni*, provides resistance to bile salts, heavy metals, and antibiotics (Lin et al. 2002; Lin et al. 2003). Mutational analysis of the *cmeB* gene in several *Campylobacter* species, including *C. fetus*, revealed its involvement in antimicrobial resistance (Guo et al. 2010). However, results showed that all 5 sequenced isolates harbour genes encoding this efflux pump and those encoding the ykkCD efflux pump without exhibiting phenotypic resistance to antimicrobials. The role of these efflux pumps in *C. fetus* antimicrobial resistance deserve further research with other antimicrobials, as these systems may act synergistically with other genes conferring antimicrobial resistance.

The resolution provided by MLST to differentiate *Cfv* strains was weak, compared with SNP or accessory protein family analysis. The housekeeping genes used in MLST are very stable among *Cfv* strains, which makes this method very limited for genetic diversity analysis. Although a *Cfv* clonal nature was reported (van Bergen et al. 2005a), this study identified genomic features that consistently grouped most strains by their SNPs or accessory protein families. Both methods segregated *Cfvi*s in two distant clusters, which is indicative of genetic diversity within this biovar. *Cfvi* were also segregated from *Cfv* strains, indicating a higher variability between biovars than the described by the L-cysteine transporter encoding operon. Analysis of protein families revealed 2 proteins (PLF_194_00014554 and PLF_194_00049089) present in *Cfvi* that are absent in *Cfv*. Genes encoding these proteins in *Cfvi* were described as responsible for the phenotypic differences found between biovars (van der Graaf–van Bloois, Duim, et al. 2016). Strain 9825 was the exception, as, while not exhibiting the above 2 protein families, it was still clustered by SNP and MLST as *Cfvi*. Strain 9825 was initially classified as *Cfvi* in a first study (Van Bergen et al. 2005a), although the isolate failed to produce H$_2$S in two other reports (van der Graaf–van Bloois et al. 2016;

Farace et al. 2019). This study indicates that this strain is closer to *Cfvi* than to *Cfv*, even lacking genes encoding the L-cysteine transporter.

3.3.6 Conclusions

This study combined MLST genotyping, VF genes PCR testing, antimicrobial resistance phenotyping, WGS and comparative genomic analysis to evaluate the virulence potential of *Cfv* isolates and strains. Three novel STs were identified by MLST (ST-71, ST-72 and ST-73). Most VF genes common to *Campylobacter* genus were detected, but genes encoding the T4SS, previously regarded as involved in *Cfv* virulence or niche adaptation, were absent in most *Cfv* strains. This indicates that T4SS is not essential for *Cfv* pathogenicity, as strains were isolated from aborted foetuses. The *parA* gene, still used for *Cfv* identification was absent in most *Cfv* strains, which precludes the sole use of this marker for BGC molecular diagnosis. As genes encoding the CmeABC and YkkCD efflux pumps were detected and *in vitro* antimicrobial resistance towards streptomycin, penicillin, tetracycline and enrofloxacin was not detected, it is demonstrated that the sole presence of those genes is not enough to provide antimicrobial resistance to tested antimicrobials. Genetic diversity was found in isolates from different geographic regions, and WGS and comparative genomic analysis of SNPs and accessory protein families allowed to differentiate biovars *Cfv* from *Cfvi*. Results of this study provided novel knowledge directed to *Cfv* virulence potential evaluation and BGC control.

CHAPTER 4 – GENERAL DISCUSSION

In recent years the discovery of novel *Campylobacter* species has increased, probably due to an improvement in culturing conditions and the general use of 16S rRNA amplicon sequencing for bacterial identification (Costa and Iraola 2019). In this work, the identification of *C. portucalensis* was motivated by the investigation of inconsistencies in the molecular diagnosis of *Cfv*. Curiously, the species *C. fetus* and *C. sputorum* are among the first *Campylobacter* species described, known for several decades to inhabit the preputial mucosa (Veron and Chatelain 1973), but somehow *C. portucalensis* has remained unnoticed until recently. The reasons behind this late discovery are possibly related to the methods used for *Campylobacter* isolation and the atypical morphology of this *Campylobacter* species. One of the most used methods for *Cfv* isolation employs a passive filtration of the sample through membrane filters with 0.45-0.65 μm pore size, onto blood agar plates (Chaban et al. 2013; Guerra et al. 2014; OIE 2018). This method relies on the principle that the small and highly motile cells of *Campylobacter* spp. cross the filters, whereas other microorganisms present in the sample are retained in the membrane (Chaban et al. 2013; Fitzgerald 2015; Vandamme et al. 2015). However, in opposition to most *Campylobacter* species, *C. portucalensis* cells do not have a flagellum and are non-motile, not passing through these filters. An alternative method for *Cfv* isolation is based on the use of selective media that support the growth of *Cfv* and minimizes the growth of other microorganisms (Truyers et al. 2014; OIE 2018). These selective media commonly include several antibiotics, such as polymyxin B, vancomycin, and trimethoprim (Harwood et al. 2009; OIE 2018). In preliminary experiments we failed in isolating *C. portucalensis* using Skirrow agar selective media, and real-time PCR experiments suggested the absence of multiplication of this microorganism in modified Weybridge's TEM (data not shown), both media containing the antibiotics described above. Probably *C. portucalensis* strains are susceptible to one or more of the antibiotics commonly used in selective media for *Campylobacter* isolation, remaining undetected in clinical investigations of BGC. Furthermore, despite *C. portucalensis* colonies resembling those of *Campylobacter* spp., the straight rod morphology might avoid its prompt identification as a species of *Campylobacter*. In fact, some *Campylobacter* species exhibit a straight rod shape, including *C. gracilis*, *C. hominis, C. geochelonis,* and *C. corcagiensis* (Vandamme et al. 1995; Lawson et al. 2001; Koziel, Doherty, et al. 2014; Piccirillo et al. 2016), which are among the closest phylogenetic neighbours of *C. portucalensis*, but these species are not found in cattle. Another atypical characteristic common to some of the most related species with *C. portucalensis* is the absence of motility, found in *C. gracilis, C. hominis, C. ureolyticus*, and *C. blaseri* (Vandamme et al. 2010; Vandamme et al. 2015; Gilbert et al. 2018)*.* In fact, *C. gracilis* and *C. ureolyticus* were initially included in the genus *Bacteroides* and later reclassified as *Campylobacter* species, although *C. ureolyticus* was considered a species *incertae sedis* for 15 years, pending the isolation of other similar

species (Vandamme et al. 1995; Vandamme et al. 2010). Overall, this group of species contributes to the heterogeneity found in the *Campylobacter* genus.

The type strain of *C. portucalensis* was isolated from a herd with decreased reproductive performance, although the pathogenic potential of this species remains to be clarified. Indeed, a T4SS encoding region, which is highly homologous to the T4SS encoding region of *Cfv* (99 % coverage and 97.2 % identity) was identified in the genome of *C. portucalensis*. Previous *in vitro* studies showed that this T4SS contributes to the invasiveness and cytotoxicity of *Cfv* (Gorkiewicz et al. 2010). Nevertheless, the T4SS are specialized systems in the delivery of effector molecules into the host cells and, ultimately, its contribution to the virulence depends on the effector protein repertoire specific of the microorganism (Voth et al. 2012). In addition, C. *portucalensis* encodes FIC-domain proteins in the T4SS-harbouring genomic island. These proteins contribute to the virulence of other bacterial pathogens by modifying host proteins important to signaling, interfering with host cellular processes (Harms et al. 2016). Even so, the genomic data is insufficient to conclude about the pathogenicity of *C. portucalensis* and this should be the subject of further investigation.

The foremost importance of this species is perhaps related to their interference with the molecular diagnostic assays directed to *Cfv* detection. Indeed, *C. portucalensis* harbours sequences with high identity with the *Cfv* molecular targets, which were probably acquired by horizontal transfer from *Cfv* or a common ancestor. In fact, the molecular identification of *Cfv* has been challenging, since *Cfv* an*d Cff* have a high genomic similarity and the differentiating sequences exhibit features indicative of horizontal acquisition, comprising genomic islands, prophage-related sequences and insertion sequences (Ali et al. 2012; Kienesberger et al. 2014). In accordance, this difficulty in finding specific targets of the subspecies venerealis led to the selection of horizontally acquired sequences as molecular diagnostic targets (Hum et al. 1997; McMillen et al. 2006; Abril et al. 2007; Abril et al. 2010; van der Graaf–van Bloois et al. 2016). Among these, the *parA* gene and the insertion element ISCfe1 are the most studied molecular targets for *Cfv* identification (Hum et al. 1997; McMillen et al. 2006; Abril et al. 2007; van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). In the present work, four subspecies detection assays were evaluated in bovine preputial samples and the positive results were further evaluated with *C. fetus* detection assays. A high percentage of samples tested positive in the molecular assays directed to ISCfe1 and/or *parA* gene, and all revealed to be negative for *C. fetus* detection. Also, a commercial *Cfv* detection kit based on detection of one of these sequences also provided positive results in samples negative in *C. fetus* detection assays (data not shown). These results are highly suggestive of a lack of specificity in the subspecies detection methods targeting *parA* and ISCfe1, which was confirmed by the identification of highly similar sequences in *C. portucalensis*. Unfortunately,

it was not possible to understand whether all false positive results were related with *C. portucalensis*. A preliminary study (data not included in this thesis) revealed the presence of *C. portucalensis* in all of subset of *parA* and ISCfe1 positive samples screened by a PCR assay targeting the *cpn60* sequence of this species. However, *C. portucalensis* was also detected in samples negative for these targets, which is in agreement with the observation that several isolates of this species do not harbour *parA* and ISCfe1.

Previous studies have identified cross-reactions in *Cfv* detection methods, either based on *parA* or ISCfe1, with *C. hyointestinalis* strains (Spence et al. 2011; van der Graaf-van Bloois et al. 2013). Although being a matter of concern, *C. hyointestinalis* is not an inhabitant of the preputial mucosa, in contrast with the newly discovered species, *C. portucalensis*. Interestingly, a study in New Zealand identified positive *Cfv* bulls, using a *parA* detection assay, in herds without a decline in the pregnancy rates, raising the hypothesis of specificity failures in the molecular diagnostic methods (Sanhueza et al. 2014). Nevertheless, the hypothesis of the presence of less virulent strains in those herds cannot be excluded, as suggested by the authors (Sanhueza et al. 2014). Despite all these pieces of evidence, the molecular diagnostic assays targeting ISCfe1 and *parA* gene are still used.

Our results showed that the molecular diagnosis based solely on ISCfe1 or *parA* detection might lead to misdiagnoses and a subsequent overestimation of the disease prevalence. It can be partially overcome by the association of species targets to subspecies specific targets, where *Cfv* positive samples must amplify both targets. Nevertheless, the identification of the genomic island carrying the *parA* gene in *Cff* (Abril et al. 2010), together with the presence of the insertion element ISCfe1 in plasmids (Abdel-glil et al. 2020), may lead to misidentification of *Cff* harbouring ISCfe1 or the *parA* gene as *Cfv*, even using this combined strategy.

Moreover, our study revealed the absence of *parA* in a high proportion of *Cfv* strains. Another study also failed to identify this gene in several *C. fetus* strains isolated from cattle, which were proposed as *Cfv* considering their genomic analysis (Abdel-glil et al. 2020). Also, 22 % of these strains were considered negative for detection of ISCfe1 by two *in silico* PCR assays using the primers from Abril et al. (2007) and one primer set (ISC1) from van der Graaf-van Bloois et al. (2013) (Abdel-glil et al. 2020). Therefore, neither methods based on the detection of *parA* nor the above assays targeting ISCfe1 would identify all strains as *Cfv* (Abdel-glil et al. 2020). In fact, the occurrence of two sequence types of ISCfe1 in *Cfv* has previously been noted in the study of van der Graaf-van Bloois et al. (2013), responsible for reducing the ISC1 assay sensitivity in 3 %, which was largely underestimated considering the study of Abdel-glil et al. (2020). Nevertheless, the second ISCfe1 assay (ISC2) developed by van der Graaf-van Bloois et al. (2013) targets a conserved region common to both sequence

types, increasing the sensitivity with a loss in specificity. Most studies evaluating PCR assays for *C. fetus* detection are only assessed in bacterial isolates (Hum et al. 1997; Abril et al. 2007; van der Graaf-van Bloois et al. 2013; McGoldrick et al. 2013). Although this approach has been valuable to detect sensitivity failures due to the absence or sequence variations of the targets in the isolates, it limits the accurate specificity assessment. Several of these studies analysed a limited number of isolates of other species (Hum et al. 1997; Abril et al. 2007; van der Graaf-van Bloois et al. 2013) and/or used isolates not representative of the bacterial species found in the preputial niche (McGoldrick et al. 2013). Thus, issues similar to the one found in this study with *C. portucalensis* are easily overlooked. Overall, there is still a long way towards the implementation of molecular diagnostic methods to be used on the identification of *Cfv* directly in clinical samples.

Although molecular diagnostic tests have the advantage of providing fast results with high analytical sensitivity, their use with inappropriate evaluation may conduct to undesirable outcomes. Therefore, to better evaluate their suitability, these methods should be coupled with traditional culturing approaches. This would allow the discovery of variations in the targeted sequences and the identification of homologous sequences in other microorganisms.

Since the first whole-genome sequencing of a *Cfv* strain in 2009 (Moolhuijzen et al. 2009), much progress has been made to understand the virulence potential of this microorganism. However, there is still a lack of in-depth genotypic, phenotypic, and comparative genomic analyses focused on the subspecies venerealis. Therefore, we aimed to contribute to fill these knowledge gaps through the genotypic and phenotypic characterization of thirteen *Cfv* strains from the United Kingdom, accompanied by a comparative whole-genome analysis.

Previous studies showed that the mammalian-associated *C. fetus* are genetically stable with a clonal structure, and *Cfv* was suggested as a bovine clone of *C. fetus* (Van Bergen et al. 2005a; Gilbert et al. 2016; Iraola et al. 2017). Only three different STs had been identified by MLST so far, and a vast majority of *Cfv* isolates belong to ST-4 (Van Bergen et al. 2005a; van der Graaf-van Bloois et al. 2013; van der Graaf-Van Bloois et al. 2014). Thus, MLST has also been used to differentiate *C. fetus* subspecies since ST-4 was considered exclusive to *Cfv* (van der Graaf-van Bloois et al. 2013), until the recent identification of a *Cff* strain ST-4 (Iraola et al. 2015). Nevertheless, in our study three novel STs were identified, suggesting a greater diversity of STs in this subspecies than expected. Even so, these STs are characterized by differences in only one or two single nucleotide positions compared to ST-4, corroborating the high genetic stability evidenced by other authors. These findings create a demand for further studies using a higher number of strains from different geographic regions to assess the genetic diversity within *Cfv*.

The subspecies *venerealis* also includes a biovar designated intermedius, distinguished by its phenotype (Silveira et al. 2018). This biovar is identified through the H_2S production test in culture medium supplemented with 0.02 % cysteine, for which *Cfvi* shows a positive reaction, in opposition to the remaining *Cfv* strains which are unable to produce H_2S (Farace et al. 2019). This feature is associated with a partial deletion of a putative L-cysteine transporter coding gene in *Cfv* that remains intact in *Cfvi*, as in *Cff* (Gilbert et al. 2016; Farace et al. 2019). Interestingly, in this work, some variations were observed at the level of accessory protein families encoded in the genomes and SNP analyses that allow a differential grouping between *Cfv* and *Cfvi*. A total of 180 genus-specific protein families (PLfams) were found only in *Cfvi* strains and 10 PLfams were exclusive of *Cfv*, although not consistently present in both cases. Despite possible differences in the pathogenicity of both biovars have not yet been addressed, the results of this work suggest that it would be interesting to investigate this hypothesis since the dissimilarities found are not only associated with the putative L-cysteine transporter gene.

Several putative virulence factors coding genes were previously identified in the genome of both *C. fetus* subspecies, playing potential roles in adhesion, invasion, and cytotoxicity (Ali et al. 2012). Among these, genes encoding the fibronectin-binding protein (*cadF*), invasin A (*invA*), phospholipase A (*pldA*), and cytolethal distending toxin (*cdtABC*) were identified in the 13 *Cfv* strains and in all the available *Cfv* genomes in GeneBank. This may reflect the high genetic stability and clonal structure of the subspecies *venerealis* and suggest that these genes are important for host colonization and/or pathogenicity. Although these genes were subject of study in other *Campylobacter* species, their involvement in the infectious process triggered by *C. fetus* is still unclear. In fact, *C jejuni* and *C. coli* experimental isogenic mutants, with inactivation of the gene of interest, have been tested with *in vitro* and *in vivo* models, contributing to elucidate the role of these virulence factors in the pathogenesis of the disease (Bolton 2015). Hence, this will be the way forward to understand the role played by these proteins in *Cfv*.

The first sequenced *Cff* and *Cfv* genomes were compared to disclose possible genomic differences responsible for the distinct ecology and pathogenicity between both subspecies (Moolhuijzen et al. 2009; Ali et al. 2012; Kienesberger et al. 2014). Early studies identified genomic islands considered specific to *Cfv* and *Cff*, but due to the small number of genomes available, these studies failed to contemplate differences within each subspecies (Moolhuijzen et al. 2009; Ali et al. 2012; Kienesberger et al. 2014). A genomic island with a T4SS was initially described in 2010 in the subspecies venerealis (Gorkiewicz et al. 2010). This genomic island holds genes coding for a T4SS (*virB2-virB11* and *virD4* genes) and two FIC-domain protein-coding genes (Gorkiewicz et al. 2010; van der Graaf–van Bloois, et al.

2016). Genes of this T4SS were described in 91 % of 67 *Cfv* strains analysed and *in vitro* studies with *virB9* and *virB11* gene inactivation by mutagenesis revealed its involvement in cell invasion, cytotoxicity, and DNA transfer by conjugation (Gorkiewicz et al. 2010; Kienesberger et al. 2011). Indeed, this genomic island previously considered absent in *Cff* was indicated as responsible for the specific virulence features of the subspecies venerealis (Gorkiewicz et al. 2010). In this work, only one of the thirteen isolates from the United Kingdom harboured this genomic island, and seven out of twelve strains without this T4SS were isolated from aborted fetuses. Therefore, this finding demonstrates that this genomic island is not essential for the pathogenicity of *Cfv,* although it may contribute to its virulence. Recently, additional T4SS-coding regions were described in both subspecies, with chromosomal and plasmid locations (van der Graaf–van Bloois et al. 2016). Herein, the five sequenced strains also exhibited multiple T4SS coding regions, but it is unclear whether they are functional despite the absence of some *virB* genes in the operons. Assuming their functionality, the presence of multiple T4SS coding regions in the same strain may compensate for the loss of a T4SS coding region through functional redundancy.

The control of BGC involves the elimination of chronic sources of infection through the culling of infected bulls or the treatment of animals with valuable genetic potential (Noakes et al. 2001). Penicillin and streptomycin are the most used antibiotics for treatment and are also commonly used in semen processing to prevent *Cfv* transmission (Hum et al. 1993; Noakes et al. 2001; Truyers et al. 2014). Two genes conferring resistance to tetracycline and streptomycin were described on a genomic island in *Cff* (Abril et al. 2010; Escher et al. 2016), although their prevalence in *Cfv* is still unknown. The resistance to fluoroquinolones is also a major concern in *Campylobacter* spp. isolated from cattle (Tang et al. 2017). To the best of our knowledge, there are no recent studies on antimicrobial susceptibility testing in *Cfv* isolates (Vargas et al. 2005; Hänel et al. 2011). In the current study, resistant phenotypes to enrofloxacin, penicillin, streptomycin, and tetracycline were not found. These findings are in accordance with the study of Vargas et al. (2005), in which all isolates were susceptible to penicillin, streptomycin, tetracycline and only 5 % of the isolates were resistant to enrofloxacin. Similarly, in the study of Hänel et al. (2001) involving 50 *Cfv* isolates, only two isolates revealed resistance to streptomycin and one isolate to ciprofloxacin and tetracycline, respectively. The genes *tet*(44) and *ant*(6)-Ib, associated to resistant phenotypes to tetracycline and streptomycin (Abril et al. 2010), were also not found in the genomes analysed in this study. Overall, data from our work and from previous studies indicate that the prevalence of antimicrobial resistance in *Cfv* is considerably low, contrasting with *C. jejuni* and *C. coli* (Yang et al. 2019). These results continue to support the current use of penicillin and streptomycin in treatment protocols and semen processing. Nevertheless,

we may hypothesize that the prevalence of resistant phenotypes may be higher in strains from countries where antibiotics are overused, as a response to selective pressure under antibiotic exposure.

Conversely, the genomes of the sequenced *Cfv* isolates encoded a multidrug efflux pump CmeABC, despite being susceptible to the tested antimicrobials. These findings contradict a previous study, in which the mutational inactivation of the *cmeB* gene in *C. fetus* proved its involvement in resistance to several antimicrobials, including tetracycline (Guo et al. 2010). However, in the former study, it was not clear whether the wild-type strain had additional genes conferring resistance to tetracycline that were acting synergistically with the multidrug efflux pump. Hence, the CmeABC multidrug efflux pump deserves further research to elucidate its role in *C. fetus* antimicrobial resistance.

Ultimately, the whole-genome sequencing has become an affordable and accessible technique to many laboratories, with a promising potential to improve our knowledge about the virulence and pathogenicity of several bacterial pathogens. Nevertheless, to achieve more significant data it is essential to correlate the genomic analysis outputs with the clinical manifestations of the disease. This type of approach has the potential to clarify whether there are strains with distinct virulence traits responsible for disease with different severity and whether the distinction between *Cfv* biovars has any clinical significance.

CHAPTER 5 – CONCLUSION

The work presented herein contributed to the clarification of ambiguous aspects of BGC molecular diagnosis and pathogenesis. More specifically these results show that the current molecular diagnosis based solely on PCR assays targeting ISCfe1 or *parA* may originate a high rate of false-positive results. Indeed, the specificity failures of these molecular diagnostic methods conducted to the discovery of a novel species in the bovine prepuce, named *Campylobacter portucalensis*, which harbours sequences homologous to ISCfe1 and *parA* gene of *Cfv*, hindering the molecular diagnosis of BGC. Moreover, the sensitivity failures of the molecular assays based on *parA* detection, also reported by others, were confirmed to be associated with the absence of the *parA* gene in a high proportion of *Cfv* strains. The identification of three novel *Cfv* STs by MLST revealed a higher diversity of STs than previously reported, questioning the use of MLST for subspecies identification. Overall, several molecular assays for *Cfv* detection were evaluated in field samples and we showed that the molecular BGC diagnosis can be improved by coupling a species-specific target, preferably to an assay targeting ISCfe1. Despite the differences in pathogenesis between *Cfv* and *Cfvi* are not documented, the identification of differential *Cfv* and *Cfvi* biovar intermedius genomic traits, namely in the whole-genome single nucleotide polymorphisms and in genes encoding genus-specific protein families, highlights the need of further research in order to provide information regarding the biovar specificities. Regarding the elucidation of the *Cfv* virulence mechanisms, this work demonstrated that the presence of a T4SS, which was previously indicated as contributing to *Cfv* virulence, is not essential for *Cfv* pathogenicity. The results also suggested that antimicrobial resistance in *Cfv* does not appear to be of major concern once resistance phenotypes to tetracycline, penicillin, streptomycin and enrofloxacin were not observed, even in strains harbouring genes encoding multidrug efflux pumps.

CHAPTER 6 – FUTURE PERSPECTIVES

The present work elucidated several aspects related to the molecular diagnosis, genetic diversity, and virulence potential of *Cfv*, however, it raised several questions that would be interesting subjects for further research.

One of the most relevant achievements of this work was the discovery of a new species of *Campylobacter*. The strains of *C. portucalensis* were isolated from preputial samples obtained from bulls and the genomic analysis suggested that this species may have pathogenic potential. However, it is still unclear whether this microorganism is capable of causing disease in the same way as *Cfv*. This issue deserves further investigation in order to elucidate several aspects. Firstly, it is important to assess the presence of *C. portucalensis* in the genital tract of females, for instance through isolation attempts in samples from herds with impairment of the reproductive performance, either from the cervicovaginal mucus or aborted fetuses. Overall, there is still a long way until the elucidation of this issue.

Another issue raised by this work was the current absence of real-time PCR assays sufficiently specific and sensitive for *Cfv* detection in clinical samples. In the future, more efforts should be directed to the WGS of *C. fetus* field isolates, in order to identify *Cfv*-specific sequences, highly conserved and without the potential to be transferred horizontally. In order to guarantee a higher specificity of the molecular tests, a duplex real-time PCR should be developed, associating the detection of a *Cfv-specific* target to a conserved target in the *C. fetus* species. Moreover, for its use in clinical samples, a third target should be included, to be amplified in all reactions and thus ensure the absence of PCR reaction inhibitors in the sample.

Finally, there is a lack of knowledge on the mechanisms underlying *Cfv* pathogenesis. Indeed, many *in vitro* virulence studies have been carried out with pathogenic species of *Campylobacter*, enlightening important aspects related to their pathogenic mechanisms. Thus, the development of *in vitro Cfv* infection models using endometrial and placental cells or tissue explants should be encouraged. In addition to their relevance on the identification of the *Cfv* virulence traits transcribed, for example through RNA-seq, this type of approach allows also the evaluation of host cells' immune response to infection. Moreover, these *in vitro* models have the potential to disclose possible differences in the virulence of *Cfv* and *Cfvi* strains. Hopefully, in the coming years, the pathogenic mechanisms behind *Cfv* pathogenicity will be clarified and will contribute to future research envisaging the development of effective vaccines.

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Annex I

Primers and probes used in real time PCR assays for *C. fetus* susbp. *venerealis* detection

Target (Assay)	Designation	Function	Sequence (5'-3')	Reference
ISCfe1 (ISC-A)	ISC-A_F	Forward primer	AAACCAAACAATAAAGCAATCACTCA	This study
	ISC-A_R	Reverse primer	ACACCTTGCTTATAATACTCTTGCCATT	
	ISC-A_P	Probe	**FAM**-TTGGCTGTTCTCGTTTAG-**MGB-NFQ**	
ISCfe1 (ISC-B)	ISC1-F	Forward primer	AGGCGAAGAGAATGTTAAATTTGAA	van der Graaf-van Bloois et al. (2013)
	ISC1-R	Reverse primer	CCATAAAGCCTAGCTGAAAAAACTG	
	ISC_P	Probe	**FAM**-CCAAAGATGTCTTAGAAATA-**MGB-NFQ**	
parA (parA-A)	CFVF	Forward primer	CCCAGTTATCCCAAGCGATCT	McMillen et al. (2006)
	CFVR	Reverse primer	CG**TT G**GGATT**G**TAAATTTTAGCTTG**T**T *	
	CFVP1	Probe	**FAM**-CATGTTATTTAATACCGCAA-**MGB-NFQ**	
parA (parA-B)	parA-B_F	Forward primer	CGGCGATGATACGCTTTTAGT	This study
	parA-B_R	Reverse primer	GAGCTATCTGCTCTAATGTCCGTAAAT	
	parA-B_P	Probe	**VIC**-CGATCCACAAAGAAGTAT-**MGB-NFQ**	
fic1	fic1_F	Forward primer	CGATGTCATTGTTGCGCAGT	This study
	fic1_R	Reverse primer	AGCAACTAGCGAGCGTGAAT	
fic2	fic2_F	Forward primer	TGACCTTTTGGGCTGTTTGG	This study
	fic2_R	Reverse primer	GAGCTTGGCGATATGCTGGA	
virB9	nC1165g2F	Forward primer	TGACAAAGATGAGCGGATAG	Moolhuijzen et al. (2009)
	nC1165g4R	Reverse primer	TACCTGTTCGCCGTTTTC	
virB11	nC1165g4F	Forward primer	AGGACACAAATGGTAACTGG	Moolhuijzen et al. (2009)
	nC1165g4R	Reverse primer	GATTGTATAGCGGACTTTGC	
nahE	nahE-F	Forward primer	TGTTATGGTGATCAAAATAGCTGTTG	van der Graaf-van Bloois et al. (2013)
	nahE-R	Reverse primer	GAGCTGTTTTTATGGCTACTCTTTTTTA	
	nahE-P	Probe	**VIC**-TGTATATGCACTTTTAGCAACTT-**MGB-NFQ**	

Annex II

Agreement between parA-A and parA-B assays.

parA-A	parA-B		Total
	Positive	**Negative**	
Positive	11 (7.8%)	29 (20.6%)	40 (28.4%)
Negative	0 (0 %)	101 (71.6%)	101 (71.6 %)
Total	11 (7.8%)	130 (92.2%)	141

Agreement between ISC-A and ISC-B assays.

ISC-A	ISC-B		Total
	Positive	**Negative**	
Positive	24 (17.0%)	59 (41.8 %)	83 (58.9%)
Negative	0 (0%)	58 (41.1%)	58 (41.1%)
Total	24 (17.0%)	117 (83.0%)	141

Annex III

Primers designed in this study for detection of putative virulence genes and T4SS-encoding genes

Target	Primers	Annealing temperature	Product size (bp)
cadF	Fw: ATGAGCCGTTTAGCGTGGTT	60 ºC	234
	Rv: GAGTTGATGATTGCGCTTTGGA		
ciaB	Fw: TTCACGCTGCGATAGGGTTT	56 ºC	337
	Rv: GAGCGCTACTGCGTGAGTAT		
invA	Fw: GCGTTTGACCATCGTATGGAG		243
	Rv: CTCCAACTTATCCGCTTGAGTG		
pldA	Fw: CGCATAGCATTTGGCAGCAA		300
	Rv: ACTCTTTTGCTATCTGCCACCA		
virB2	Fw: TCTTTTGTTCGCTGCTGGTG	58 ºC	153
	Rv: CTTACTGTTTGACCGCCCCA		
virB3-virB4	Fw: ACTTATGGCGGCAGAGGATG		482
	Rv: CGCCACTTTGACCAAGAACG		
virB5	FW: GCATTCCAGTTGTAGATGGTGC		358
	Rv: GAGCATTGATCTTTTCCGCC		
virB6	Fw: TTCCAACAGCCATACCGCAT	60 ºC	426
	Rv: TTTGGGCTGGACTTATGGGC		
virB7	Fw: CTTGTTGGTTGCACAAGCGT		256
	Rv: GCGCTATCTCCTGATTGCCA		
virB8	Fw: AACCAGCGTAAATGAAGCCG	58 ºC	385
	Rv: TGTCGTAAGCGAACTAGGTTGA		
virB10	Fw: TGATTTCGCTCCACACGACA		741
	Rv: TTGCCTACGAGCAACCAGAG		
virD4	Fw: AATGGCAAAATTCCAAGTCG	56 ºC	286
	Rv: CCTTGCCTGATTGTTCGATT		