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REVIEW ARTICLE

Challenges in the Diagnosis and Management of *Campylobacter fetus* subsp. *venerealis* in Bovine Genital Campylobacteriosis: Resistance and Control Strategies

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ABSTRACT

Campylobacter fetus subspecies *venerealis* (Cfv) causes bovine genital campylobacteriosis (BGC) disease, which is significant in the cattle industry worldwide. Globally, herds infected with Cfv often have reduced pregnancy rates, early embryonic loss, infertility and abortion. Infected herds yield 36% lower profits compared to uninfected herds. Therefore, an accurate diagnosis is required to control BGC globally. Different tests/techniques like biochemical tests, immunofluorescence tests, enzyme-linked immunosorbent assay, and Polymerase Chain Reaction (PCR) techniques have been used to identify and diagnose Cfv. However, diagnostic performance varies across methods, and subspecies-level confirmation remains inconsistent in field samples. Subspecies-level detection remains difficult because Cfv and Cff are highly similar genetically, and commonly used markers perform inconsistently across strains and field samples. Subspecies-level detection remains difficult because Cfv and *C. fetus subsp. fetus* (Cff) are highly similar genetically, and commonly used markers perform inconsistently across strains and field samples. Therefore, more research on advancing real-time PCR in this contemporary setting with higher specificity is needed to understand the genetic diversity of Cfv. This is a narrative review based on peer-reviewed literature identified through structured searching of relevant databases using keywords related to BGC/Cfv, diagnosis, and control strategies. This review provides a critical synthesis of the major reasons for inconsistent diagnostic performance in field settings and highlights practical priorities for improving subspecies-level confirmation and surveillance. Furthermore, this review discusses current diagnostic techniques for Cfv and briefly summarizes reported antimicrobial susceptibility findings relevant to treatment decisions and antimicrobial stewardship.

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INTRODUCTION

Campylobacter is a zoonotic pathogen (Sahin *et al.*, 2017). It was first reported in 1886 from the stools of children. This bacterium, later on in 1913, was also identified in cows, aborted bovine fetuses, swine suffering from diarrhea, and calves having intestinal problems (García-Sánchez *et al.*, 2018). *Campylobacter* infects different animals, including poultry, cattle, pigs, sheep, reptiles, birds (Hlashwayo *et al.*, 2021), dogs, and cats (Karama *et al.*, 2019). Due to significant diversity, many species of these animals serve as natural *Campylobacter* species reservoirs (Paintsil *et al.*, 2022) and act as substantial risks to human health due to the secretion of bacteria through the waste of livestock (Gahamanyi *et al.*, 2020).

Campylobacter has immense potential for new species; therefore, 34 species belonging to this genus have been discovered. *Campylobacter fetus* has two subspecies that have implications for both human and animal health, and these are *C. fetus subsp. fetus* (Cff) and *C. fetus subsp. venerealis* (Cfv) (Wagenaar *et al.*, 2014;

Campylobacteriosis, 2018). Moreover, Cfv consists of a phenotypic variant known as Biovar intermedius (Caroline da Silva Silveira *et al.*, 2018). Cfv is the principal causative agent of a reproductive disease in cattle called bovine genital campylobacteriosis (BGC). It is a sexually transmitted disease characterized by death at a very early embryonic stage, abortion, and mortality (Sahin *et al.*, 2017). In the 1940s, infertility in cattle was first linked to BGC, and by the 1960s, it was acknowledged to be a global problem responsible for infertility (Caroline da Silva Silveira *et al.*, 2018). BGC causes reproductive failure in cattle, resulting in irregular estrus and infertility. Heifers are more prone to this infection resulting from compromised immunity (Jimenez *et al.*, 2011). Herds infected with Cfv infection occasionally have compromised breeding efficiency, death during the embryonic stage, abortions, low conception rate, and prolonged calving interval, as shown in Fig. 1. Abortions are more frequently observed between the 4th and 6th months of gestation. (Konan *et al.*, 2007). BGC results in substantial economic losses due to abortion, reduced calf output, increased insemination costs, and culling of infected animals. In one study, herd profitability was estimated to be 36% lower in Cfv-infected herds than in uninfected herds (McMillen *et al.*, 2006). Many countries require that embryos, semen, cattle, and breeding bulls intended for trade are certified free of Cfv infection. Therefore, reliable diagnostic testing is essential to support control programs and international movement of animals and germplasm.

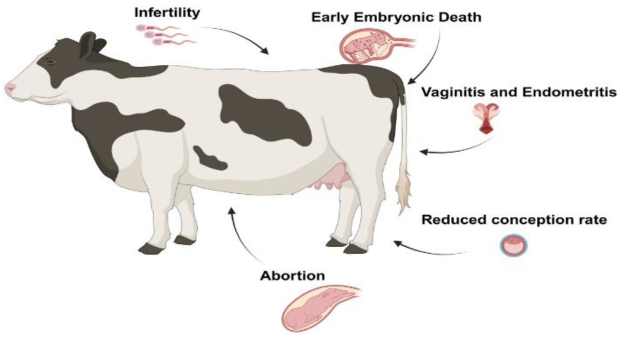

Fig. 1: Symptoms of Bovine Genital Campylobacteriosis. CFV leads to infertility, reduced conception rate, early embryonic death, abortion, vaginitis and endometritis in cattle.

BGC is listed by the World Organization for Animal Health (WOAH), and outbreaks can have major economic consequences and affect international trade of semen and breeding animals (Silva *et al.*, 2020.,Pena-Fernández *et al.*, 2021a). Thus, the success of BGC eradication programs and the international trade of semen and bulls relies on accurate diagnostic methods (EFSA AHAW Panel *et al.*, 2017; Campylobacteriosis, 2018). Precise identification of Cfv is crucial for diagnosis, and WOAH recommends bacterial culture followed by phenotypic characterization as the primary method (Campylobacteriosis, 2018). However, Cfv is a fastidious bacterium and is often overlooked because of the growth of other microorganisms in the sample. This limitation has led to the development of molecular diagnostic methods that target nucleotide sequences specific to Cfv (van der Graaf-van Bloois *et al.*, 2013). Despite distinct epidemiological profiles, Cfv and Cff share more than 90% genomic similarity, complicating the identification of sensitive and specific molecular markers for Cfv. High genetic and phenotypic similarities between these subspecies make it difficult to comprehend their ecological and pathogenic differences (Sahin *et al.*, 2017). The mechanisms involved in the pathogenicity of Cfv are not yet completely known. Both subspecies of *C. fetus* possess genes encoding several well-studied virulence factors associated with other *Campylobacter species*. Furthermore, a genomic island containing a type IV secretion system (T4SS) and FIC-domain proteins, linked to the formation of filaments triggered by cyclic adenosine monophosphate (cAMP), has been found only in Cfv (Ali *et al.*, 2012).

Despite various available tests, the definitive identification of Cfv remains challenging**.** Traditional culture is slow, labor-intensive, and often insensitive for Cfv, as overgrowth by commensals can mask Cfv colonies (Silva *et al.*, 2020). Classical biochemical assays (1% glycine tolerance and H_2S production), used to differentiate Cfv (negative) from Cff (typically positive) are not fully reliable. Certain Cfv strains can acquire glycine tolerance, and intermediate biovars can display partial positivity, leading to misidentification (Silva *et al.*, 2020; Pena-Fernández *et al.*, 2024). Molecular methods (PCR and real-time PCR) have improved detection sensitivity, but subspecies discrimination remains problematic. Due to high genetic similarity, Cfv-specific targets like the *parA* gene or insertion element *ISCfe1* have been found in other *Campylobacter* spp., causing false positives (Silva *et al.*, 2020). Indeed, no current PCR assay achieves 100% accuracy in distinguishing Cfv from Cff, underscoring the need for more specific molecular markers. These diagnostic limitations carry significant veterinary and economic consequences. Undetected or misdiagnosed infections contribute to herd infertility, early embryonic loss, and abortion, which reduce reproductive performance and incur substantial economic losses (Pena-Fernández *et al.*, 2024). Inconsistent testing protocols across regions further risk misclassification of herd health status and trade disputes (M. A. van Bergen *et al.*, 2005). Although antibiotic therapy is not the primary control for BGC, monitoring of antimicrobial resistance (AMR) in Cfv has become an important adjunct. Recent surveillance indicates that Cfv isolates remain largely susceptible to most antimicrobials, showing only intrinsic resistances to antibiotics such as nalidixic acid and trimethoprim, whereas Cff isolates often carry multiple acquired resistance genes. Notably, plasmid-borne resistance elements (such as a *tet*(*O*) tetracycline resistance gene) have been detected in Cff and could spread to Cfv, highlighting the importance of continued AMR surveillance. (van der Graaf-van Bloois *et al.*, 2023)

Although BGC has been discussed previously, persistent field-level challenges remain in confirming Cfv and differentiating it from Cff using routine laboratory approaches. This review synthesizes available evidence on diagnostic approaches (culture/biotyping, DIF, ELISA and PCR-based assays), summarizes key reasons for inconsistent field performance (fastidious growth, phenotypic overlap and limitations of commonly used molecular targets), and highlights recent genomics-informed advances that can improve subspecies discrimination in BGC control programs. We also summarize current evidence on antimicrobial susceptibility in Cfv relevant to adjunct management practices, including semen processing. A schematic diagram of this review is described in Fig. 2.

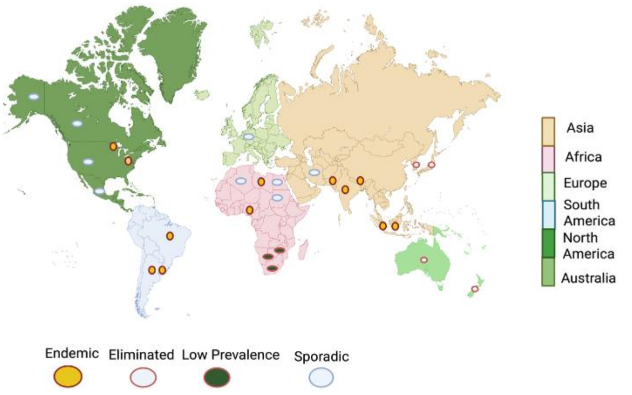

Fig. 2: Worldwide distribution of BGC. Endemic in Brazil, Argentina, Uruguay; South Africa, Nigeria, India, Pakistan, Bangladesh and Indonesia. Sporadic/occasional in the USA, Canada, Mexico; Germany and other Western European countries; and parts of the Middle East/North Africa (Iran, Egypt, Sudan, Algeria) and there is also low prevalence in Zimbabwe, Botswana, Kenya and controlled or near-eliminated in Japan, South Korea, Australia, and New Zealand (and most intensively managed herds in North America and Western Europe).

Literature search and selection: This article is a narrative review. Relevant peer-reviewed literature was identified through structured searches of major databases such as PubMed, Scopus, Web of Science, and Google Scholar using combinations of keywords including "bovine genital campylobacteriosis," "*Campylobacter fetus* subsp. *venerealis*," "Cfv," "diagnosis," "culture," "biotyping," "DIF," "ELISA," "PCR," "real-time PCR/qPCR," "*ISCfe1*," "*parA*," and "control." Studies were prioritized when they addressed bovine genital infection and/or subspecies-level detection of Cfv. Non-bovine or non-genital studies were included only when they directly informed marker specificity, assay performance, or interpretation relevant to Cfv/BGC. Information extracted included sample type, diagnostic target/assay format, and reported outcomes (sensitivity/specificity or comparative performance), along with implications for herd-level control.

Epidemiology, Global Distribution and Economic Impact of BGC: BGC is globally distributed and imposes substantial economic losses through reduced reproductive performance, making the epidemiological context essential for interpreting diagnostic and control priorities. BGC is a disease that must be reported to the WOAH. BGC has been reported in Asia, Europe, America and Oceania (OIE, 2019). However, no specific study has been conducted region-wise. Worldwide distribution of BGC is shown in Fig. 3. In Africa and the Middle East, BGC persists in regions with natural breeding and limited surveillance. In Asia, countries like Japan and India show underreported infection due to low artificial insemination (AI) adoption and minimal routine testing. Europe exhibits varying prevalence, with low rates in Sweden and Latvia due to AI and testing, while Spain reports 12.2% herd-level prevalence, especially in extensive beef systems (Pena-Fernández *et al.*, 2021a). In South America, Brazil, Uruguay, and Argentina have high endemicity, particularly in extensive systems with poor biosecurity (Siqueira *et al.*, 2023). In North America, localized outbreaks are more accurately reported due to better diagnostic infrastructure (Mshelia *et al.*, 2010). Oceania sees endemic BGC in the beef and dairy sectors, but structured control and widespread AI use help limit the disease's impact (Ong *et al.*, 2022).

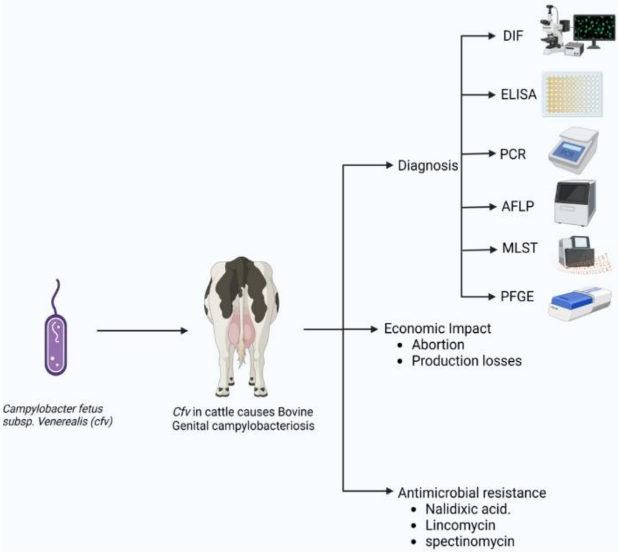

Fig. 3: Schematic overview of the scope of this review on BGC. The diagram summarizes the main themes discussed: (i) diagnostic approaches used for Cfv detection and subspecies differentiation by direct immunofluorescence (DIF), enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). (ii) **Economic impact** associated with reproductive failure (e.g., infertility/abortion) and production losses; and (iii) antimicrobial resistance considerations, highlighting antimicrobials commonly assessed in susceptibility studies (e.g., nalidixic acid, lincomycin and spectinomycin).

Key challenges in diagnosing and managing BGC are the lack of sensitive and specific diagnostic tests and the absence of monitoring programs in numerous countries (Silveira *et al.*, 2018). Since natural breeding is frequently used, developing countries have a higher incidence of this disease (Mshelia *et al.*, 2010).

Differences in reported BGC prevalence likely reflect variation in farming systems and control practices (e.g., higher risk with extensive herds and natural mating versus lower risk with AI using screened semen), as well as differences in surveillance intensity and veterinary infrastructure. Diagnostic capacity also plays a major role. Regions with limited access to validated tests and routine screening may under-detect infection, while use of assays with differing sensitivity/specificity can inflate or underestimate apparent prevalence, making cross-study comparisons difficult (Abdel-Glil *et al.*, 2023).

BGC can have a significant negative financial impact on affected herds because the profitability of herds depends on the number of calves raised by a cow each year. BGC has earned the tag "the quiet profit taker" due to its subclinical presentation, typically not recognized until low calving rates become evident (Blaser *et al.*, 2008). The reduction in gross profit can reach up to 66 and 33%, respectively, in the first and subsequent years following the introduction of BGC (Hum, 2007). In Australia, BGC is estimated to cost the beef industry approximately AUD 43.7 million annually (Juli *et al.*, 2024). The lack of official surveillance hinders accurate economic assessments in Brazil, but the high prevalence suggests significant potential losses. Similarly, in Argentina, BGC is a leading cause of abortion in cattle, further emphasizing its economic burden. Despite the production losses, herds infected with BGC are liable for the costs of managing and diagnosing the illness (Truyers *et al.*, 2014). These epidemiological patterns and economic impacts reinforce the need for sensitive, subspecies-specific diagnostics and coordinated surveillance to support effective control.

Diagnosis of BGC: The C. fetus subspecies displayed various host tissue modifications, but at the gene level, they are difficult to distinguish (Moolhuijzen *et al.,* 2009). According to genetic information, the genomes of the C. fetus subspecies are highly homologous, with an average identity of 92.9% (Kienesberger *et al.,* 2014). Currently, BGC diagnostic tools include a variety of procedures and techniques such as immune enzymatic assays and vaginal mucus agglutination tests. These techniques identify the presence of Cfv (i.e., bacterial culture) or its components (i.e., direct immunohistochemistry or immunofluorescence) for surface protein detection and PCR-based assays for the detection of genes. Smegma may be collected from the prepuce and penile mucosa for the presence of Cfv in bulls because Cfv gets established with very persistent colonization in the preputial crypts (Silveira *et al.,* 2018). For the diagnosis of Cfv in cattle and aborted fetuses, cervicovaginal mucus, fetal tissue, or fetal fluid are adequate samples. Morphologic identification of the bacterial colonies is insufficient for identifying *Campylobacter* at the species and subspecies levels. Cff, which is also clinically significant but unrelated to BGC, can develop in the same selective medium and exhibit comparable colony traits to Cfv. Based on biochemical studies, these subspecies should be recognized by their molecular tests of phenotypic characteristics (BonDurant, 2005). Cff produces H2S and can be grown in 0.1 and 15% sodium selenite glycine, respectively, while Cfv does not show these characteristics. For the identification, there are serious concerns about the validity of these biochemical traits because some strains of Cfv had developed the characteristics of glycine tolerance by a mutation event and transduction. However, some strains of Cfv have gained the glycine tolerance trait, but there is doubt about the validity of these biochemical traits due to unambiguous identification (Marcel AP van Bergen *et al.,* 2005). Reported Cfv positivity/prevalence estimates and the diagnostic methods used across studies are summarized in Table 1. The wide variability among studies likely reflects differences in sampling strategy (bull vs. herd-level sampling), host population and the diagnostic assays applied, rather than true geographic differences alone.

Direct immunofluorescence (DIF): Direct immunofluorescence (DIF) remains a widely utilized technique for diagnosing BGC, particularly on clinical samples such as cervicovaginal mucus, aborted fetuses, preputial smegma, and uterine tissue. This method employs fluorescently labeled antibodies specific to Cfv, allowing for the direct visualization of the bacterium under a fluorescence microscope. WOAH recommends DIF as a reliable diagnostic tool for international bull trade, emphasizing its global applicability (García *et al.*, 2021).

While DIF has proven useful, its sensitivity and specificity can vary significantly across different studies. A study showed a sensitivity of 69.4% and specificity of 94.4% when testing preputial smegma and cervicovaginal mucus (Campero *et al.*, 2017). Conversely, a similar study in Brazil reported a higher sensitivity of 92.59% but a lower specificity of 88.89% (Figueiredo *et al.*, 2002). These discrepancies suggest that factors such as sample type, environmental conditions, and the quality of the antibodies used may contribute to variability in test performance. In a study DIF test was explored in experimentally infected bulls and reported a specificity of 100%, with no cross-reactivity against non-Cfv strains in the preputial microbiota. However, the study's sensitivity was reported at 79, with 83% concordance compared to bacteriological culture, which highlights DIF's limitation in detecting lower bacterial loads (García *et al.*, 2021).

Table 1: Reported study-specific Cfv positivity/prevalence estimates in bovine genital campylobacteriosis (BGC) and the diagnostic methods used across studies. The table summarizes host group/sample origin (e.g., bulls/cows/fetuses), assay type (e.g., DIF/IFAT, PCR and real-time PCR) and the proportion of test-positive animals reported in each study. Because studies differ in sampling strategy, herd population, case definition and test performance, values should be interpreted as study-specific estimates and not as directly comparable prevalence across regions or years.

Year of study	Samples origin	Technique	Animals positive	Reference
2024	Bull	PCR/RT-PCR	Bull=95.3% Total animals=485	(Pena-Fernández *et al.*, 2024)
2023	Bull	PCR	67.67%	(Siqueira *et al.*, 2023)
2022	Bull	IFAT/PCR/RT-PCR	IFAT=13.03%,PCR=22.1% RT-PCR= 12.4%, Total animals=267	(Mederos *et al.*, 2022)
2021	Bull	PCR	7.7% (400/5182)	(Pena-Fernández *et al.*, 2021b)
2020	Bull/Cow	PCR	Cow=42.2%, Bull=1.52% (58/200)	(Ishtifaq *et al.*, 2020)
2018	Cow	PCR	7.7% (21/273)	(de Oliveira *et al.*, 2018)
2018	Bull	PCR	17.5% (35/200)	(Botelho *et al.*, 2018)
2015	Bull	PCR	1.8% (7/383)	(de Oliveira *et al.*, 2015)
2014	Bull/COW/Fetus	PCR	10.9% (89/816)	(Ziech *et al.*, 2014)
2013	Bull	DIF	1.5% (437/29178)	(Molina *et al.*, 2013)
2012	Bull/Cow	DIF	11.1% (44/398)	(Leal *et al.*, 2012)
2009	Bull	DIF/Isolation	35.9%/10.3% (14 and 4/39)	(Rocha *et al.*, 2009)
2005	Bull	DIF	50.8% (61/120)	(Miranda, 2005)
2005	Bull	DIF/Isolation	28.05% and 12% (492/1754)	(Experto, 2005)
2003	Cow	DIF	25.5% (40/157)	(Stynen *et al.*, 2003)
2002	Bull	DIF	51.7% (171/327)	(Pellegrin *et al.*, 2002)

IFAT: Immunofluorescence Antibody Test, PCR: Polymerase Chain Reaction, RT-PCR: Real-time Polymerase Chain Reaction, DIF: Direct Immunofluorescence

In a more recent study, Mederos *et al.* (2022) observed a marked decrease in sensitivity (11.7%) despite high specificity (94.5%), which raises critical questions about the factors influencing these results. The disparity in sensitivity across studies may stem from differences in sample collection methods, the type of test used (e.g., antibody specificity), and inherent differences in Cfv load across cattle populations. Sampling time, the stage of infection, and handling conditions may all play a role in these variabilities (Silveira *et al.*, 2018).

The DIF test offers rapid results with high specificity and minimal observer variability, making it valuable in situations where quick decision-making is required, such as during herd management. However, its limitations include lower sensitivity compared to traditional bacteriological culture methods, leading to potential false negatives, particularly in cases of low bacterial load or early-stage infection. The test also requires specialized equipment (e.g., fluorescence microscopy) and trained personnel to ensure proper execution and interpretation of results. While DIF remains a useful tool, its limitations in sensitivity and dependence on sample quality underscore the need for supplementary diagnostic methods or repeated testing to ensure accurate diagnosis, particularly in cases of low infection prevalence (Abdel-Glil *et al.*, 2023).

Enzyme-linked Immunosorbent assay (ELISA): Enzyme-linked immunosorbent assay (ELISA) is a widely employed diagnostic method for detecting antibodies against Cfv in clinical samples, such as vaginal secretions. The assay principle relies on the use of specific antigens to capture antibodies present in the sample, followed by enzyme-labelled secondary antibodies that produce a colorimetric signal, which is quantitatively measured. ELISA can be designed to detect different classes of immunoglobulins, with IgA and IgG being the most common targets in BGC diagnosis (Azam *et al.*, 2023).

In the context of BGC, an IgA-based ELISA was used to detect antibodies against the Cfv antigen in vaginal secretions. This approach demonstrated a strong local antibody response in the vaginal and uterine mucosa of infected cows, particularly in those recently aborted. The specificity of the IgA-ELISA was reported to be 98.5%, although the sensitivity was not accurately determined, suggesting the need for further validation (Devenish *et al.*, 2005). It is important to note that IgA antibodies are primarily involved in mucosal immunity, making IgA-ELISA particularly relevant for detecting infections in mucosal surfaces like the cervix and vagina (Corthesy *et al.*, 2013).

While IgA-ELISA offers specificity in identifying local immune responses, its sensitivity is generally lower in comparison to IgG-based ELISAs, which are often employed for systemic immune detection. IgG-ELISA, which targets systemic antibodies, can offer high sensitivity but may suffer from cross-reactivity due to the presence of antibodies against other bacterial species in the bull's preputial microbiota. This cross-reactivity can lead to false positives, especially if the bulls have been previously exposed to other *Campylobacter* strains or immunized. Furthermore, the timing of antibody production is critical; IgG responses typically appear later in the infection process, potentially leading to missed diagnoses during early stages or before the onset of an antibody response (Kelley *et al.*, 2011).

In herd screening for BGC, ELISA is a valuable tool, particularly for detecting antibodies in cows exhibiting infertility or abortion symptoms. ELISA's high sensitivity (98%) in detecting *C. fetus* in cervicovaginal samples has been shown to surpass microbiological culture (74%) (Brooks *et al.*, 2004). However, both ELISA and direct immunofluorescence (DIF) serve as preliminary tests, with limitations in distinguishing between Cfv and other *C. fetus* subspecies. As a result, these methods can provide useful initial diagnostic insights but must be complemented by more definitive techniques, such as PCR or culture, to confirm the diagnosis and distinguish between subspecies.

Moreover, the use of ELISA in preputial smegma testing in bulls is complicated by the transient nature of the immune response in carriers. Since bulls may harbor *Cfv* without showing overt clinical signs, antibody levels may fluctuate, leading to intermittent detection (Yarokhno *et al.*, 2015). Additionally, the immune response in vaccinated animals may further complicate the interpretation of results, as immunization with *Campylobacter* vaccines does not affect IgA responses in the vaginal mucus but can result in the absence of antibodies in the preputial smegma (Chapwanya *et al.*, 2016).

Polymerase Chain reaction (PCR): Polymerase Chain Reaction (PCR), multiplex PCR, and real-time PCR (RT-PCR) are powerful molecular tools for the detection of Cfv and are considered more sensitive and specific than conventional methods such as microbiological culture (Marcel AP van Bergen *et al.*, 2005; Guerra *et al.*, 2014). These techniques use specific primers to amplify Cfv-specific DNA sequences, providing a faster and more accurate diagnosis. Despite their advantages, variations in sensitivity and specificity across different studies highlight the complexities of PCR-based diagnostics for BGC.

The multiplex-PCR targets both the *C. fetus* species-specific *cstA* gene and the Cfv subspecies-specific *parA* gene (Hum *et al.*, 1997). However, discrepancies in performance across studies have raised concerns about the robustness of this approach. While *cstA* and *parA* serve as reliable markers for *C. fetus* and Cfv identification, studies have shown limitations, such as primers targeting the *cstA* gene failing to detect *C. fetus* subsp. *testudinum* (Iraola *et al.*, 2016). This suggests that strain variation and the genetic diversity within *C. fetus* could be contributing to the observed discrepancies. Similarly, RT-PCR using the *parA* gene probe, which is believed to be specific to Cfv, has been reported to produce false positives when cross-reacting with *C. hyointestinalis* strains (Spence *et al.*, 2011). The presence of such cross-reactivity points to the challenge of designing primers and probes that are truly species and subspecies-specific, especially in mixed microbial populations where genetic overlap may occur.

A recent study by van der Graaf-van Bloois *et al.* (2013) reported that PCR assays targeting the *parA* gene missed 47% of *Cfv* strains, while those using primers from Hum *et al.* (1997) missed 42%, demonstrating that these commonly used genetic markers do not offer complete sensitivity. These results underscore the importance of selecting appropriate genetic targets and recognizing that no single target gene may provide comprehensive coverage of Cfv strains, particularly in diverse or field-based samples (van der Graaf-van Bloois *et al.*, 2013). The identification of Cfv in different studies using the *parA* gene is listed in Table 2.

The incorporation of the insertion sequence *ISCfe1*, commonly used as a Cfv-associated marker, has provided an additional target for more specific diagnostics. Research by Abril *et al.* (2007) highlighted the usefulness of *ISCfe1* as a diagnostic marker for Cfv species identification, with studies showing that this element is not only present in Cfv but also varies in composition among different strains, reflecting its role in genome transposition. However, despite the promise of *ISCfe1*, the use of this marker alone may not fully address the challenges of strain variation, as certain Cfv strains may possess distinct genetic profiles that are not captured by this element (Abdel-Glil *et al.*, 2020). The identification and differentiation of Cfv and Cff by using *ISCfe1* gene is described in Table 3.

The sensitivity and specificity of PCR methods also appear to be influenced by the sample type. In a study conducted in Uruguay, conventional PCR showed better sensitivity (53.7%) than RT-PCR (36.1%), although RT-PCR exhibited higher specificity (98.4%) for detecting Cfv in non-infected bulls (Mederos *et al.*, 2022). This suggests that PCR may perform better in field samples, where bacterial loads can vary, while RT-PCR is more effective at confirming negative results in non-infected animals. Discrepancies between PCR and RT-PCR performance could also be attributed to differences in assay sensitivity to variations in bacterial DNA quantities, amplification efficiency, and primer specificity. Primers and probes used in different studies for Cfv identification by RT-PCR are described in Table 4.

Moreover, the issue of cross-reactivity remains a concern, especially when using genes such as *parA*, which is also present in Cff and other closely related *Campylobacter* species. The inability to distinguish between Cfv and Cff based on the *parA* gene, as demonstrated by Graaf-van Bloois *et al.* (2016), further complicates the accurate identification of Cfv in mixed or complex samples (Graaf *et al.*, 2013). This limitation calls for the development of additional molecular markers or the integration of multiple markers to achieve higher specificity.

Recent advances have addressed some of these challenges. Recently, researchers identified a unique 6-bp sequence in the *asd* gene that is specific to Cfv, providing a more reliable target for subspecies differentiation (van der Graaf-van Bloois *et al.*, 2024). The use of multiple genetic targets, such as *parA, ISCfe1*, and *asd*, in combination may offer a more reliable diagnostic approach, improving both the sensitivity and specificity of PCR-based assays for BGC.

Table 2: Cfv identification through the *parA* gene. Primer sets, validation panels (strains/clinical samples), and reported performance measures are summarized. Across studies, sensitivity and field performance are inconsistent, indicating that parA-based assays alone may be insufficient for reliable subspecies-level confirmation in diverse field samples

Study	Primer	Strains/Samples	Performance
(Sanhueza *et al.*, 2014)		222 preputial samples/31 herds	28.8 % of samples were positive; No relationship between PCR and pregnancy ratio
(van der Graaf-van Bloois *et al.*, 2013)		83 Cff, 60 Cfv, 12 non-C. fetus strains	100% specificity 53 % sensitivity
(McMillen *et al.*, 2006)	CFVF/CFVR	249 samples from prepuce and 120 samples cervicovaginal mucus	PCR positive 39 / 9 detected by bacterial culture
(Guerra *et al.*, 2014)		From virgin bulls prepuce 300, From 13 infected bulls, 260 repeated samples	85 % specificity; 85 % sensitivity
(van der Graaf-van Bloois *et al.*, 2013)		83 Cff, 60 *Cfv* 12 non-*C. fetus* strains	58 % sensitivity; 83 % specificity
(Chaban *et al.*, 2012)		401 samples from prepuce Total animals 377	Limit of detection 10^3 CFU/mL; Two different *parA* gene were identified
(Abril *et al.*, 2007)	VensF/VensR	26 Cfv, 27 Cff strains	96 % specificity 96 % sensitivity
(Schulze *et al.*, 2006)		81 Cfv,22 Cff strains	100 % match with phenotypic diagnosis
(Willoughby *et al.*, 2005)		44 Cff, 32 Cfv, 16 non-C. fetus strains	100 % specificity 45 % sensitivity
(Wagenaar *et al.*, 2001)		69 *C. fetus* strains	98.6 % match with AFLP results , 88 % match with phenotypic results
(Hum *et al.*, 1997)		59 Cfv and 40 Cff strains	Only 2.02% of the results showed discrepancies when compared to PFGE or conventional and probabilistic phenotyping methods.

F: Forward Primer; R: Reverse primer; Cff: *Campylobacter fetus subspecies fetus*; Cfv: *Campylobacter fetus subspecies venerealis*; AFLP: Amplified fragment-length polymorphism; PFGE: Pulse field gel-electrophoresis.

Table 3: *ISCfe1*-based PCR assays used for detection and differentiation of Cfv. This table summarizes commonly used ISCfe1 primer sets and reported diagnostic performance. Most validation studies report high specificity (and often high sensitivity), supporting ISCfe1 as a useful marker, although performance depends on primer design and the composition of the reference panel

Primers	Performance	Strains	Reference
ISC1- F/ISC1-R	-	142 Cfv	(Siqueira *et al.*, 2023)
CampF7/CampR7	98.7 % sensitivity 99.8 % specificity	223 Cfv, 485 Cff, and 363 non *C. fetus* strains	(McGoldrick *et al.*, 2013)
ISC2-F/ISC2-R	100 % sensitivity 98 % specificity	60 Cfv, 83 Cff, and 12 non *C. fetus* strains	(van der Graaf-van Bloois *et al.*, 2013)
ISC1-F/ ISC1-R	97 % sensitivity 100 % specificity	60 Cfv, 83 Cff, and 12 non *C. fetus* strains	(van der Graaf-van Bloois *et al.*, 2013)
CVEN-L/CVEN-R2	97 % sensitivity 100 % specificity	60 Cfv, 83 Cff, and 12 non *C. fetus* strains	(van der Graaf-van Bloois *et al.*, 2013)
CVEN-L/CVEN-R2	96 % sensitivity 100 % specificity	26 Cfv, 27 Cff strains, and 23 non *C. fetus* strains	(Abril *et al.*, 2007)

Table 4: Probes and primers used in real-time PCR assays for the identification Cfv. This table lists representative primer/probe sets, sequences, and gene targets reported for real-time PCR assays. The diversity of targets reflects ongoing efforts to improve subspecies specificity and highlights the need for standardized validation across laboratories and field sample types.

Reference	Primer/probe	Sequence	Target gene
(McMillen *et al.*, 2006)	CFVF	CCCAGTTATCCCAAGCGATCT	
	CFVR	CGTTGGGATTGTAAATTTTAGCTTGTT	*parA (parA-A)*
	CFVP1	FAM-CATGTTATTTAATACCGCAA-MGB-NFQ	
(Moolhuijzen *et al.*, 2009)	nC1165g2F	TGACAAAGATGAGCGGATAG	
	nC1165g4R	TACCTGTTCGCCGTTTTC	*virB9*
(van der Graaf-van Bloois *et al.*, 2013)	nahE-F	TGTTATGGTGATCAAAATAGCTGTTG	
	nahE-R	GAGCTGTTTTTATGGCTACTCTTTTTTTA	*NahE*
	nahE-P	IC-TGTATATGCACTTTTAGCAACTT-MGBNFQ	
(van der Graaf-van Bloois *et al.*, 2013)	ISC1-F	AGGCGAAGAGAATGTTAAATTTGAA	
	SC1-R	CCATAAAGCCTAGCTGAAAAAACTG	*ISCfe1 (ISC-B)*
	ISC_P	FAM-CCAAAGATGTCTTAGAAATA-MGB-NFQ	
(Silva *et al.*, 2021)	ISC-A_F	AAACCAAACAATAAAGCAATCACTCA	*ISCfe1 (ISC-A)*
	ISC-A_R	ACACCTTGCTTATAATACTCTTGCCATT	
	ISC-A_P	FAM-TTGGCTGTTCTCGTTTAG-MGB-NFQ	
(Iraola *et al.*, 2016)	16SFw	5′GCACCTGTCTCAACTTTC3	*16SRNA*
	16SRv	5′CCTTACCTGGGCTTGAT3′	
	16SPb	5′-VIC-ATCTCTAAGAGATTAGTTG-MGB/NFQ-3′	
(Siqueira *et al.*, 2023)	ISC1- F	5′GGTGGAGAGCGTAGATATAAATTAG-3′	*ISCfe1*
	ISC1-R	5′ CCATAAAGCCTAGCTGAAAAAACTG-3′	

Amplified Fragment Length Polymorphism (AFLP): This genetic technique involves the chromosome's enzymatic digestion, fluorescence-labelled primer amplification, and product separation via polyacrylamide gels to create bands that are usually between 50 and 500 nucleotides long (TM *et al.*, 2000). A study used the technique to differentiate *C. fetus* to the subspecies level. However, one study showed that Cff and Cfv could not be differentiated (On and Harrington *et al.*, 2000). Despite being more effective in distinguishing species and subspecies in taxonomic and epidemiological contexts, the technique's labour-intensive process and reliance on expensive software, equipment, and sterile cultures render it impractical for most clinical laboratories (OIE, 2018).

Multi-Locus Sequence Typing (MLST): Multi-Locus Sequence Typing (MLST) is a genotyping method that uses the nucleotide sequences of multiple housekeeping genes to characterize bacterial species. The principle of MLST is based on the amplification and sequencing of several housekeeping genes that are highly conserved across species but show sufficient genetic diversity to distinguish between different strains. Each unique allele at a particular locus represents a sequence type (ST), allowing for the identification of clonal relationships among bacterial isolates. This method has been widely used in bacterial epidemiology and is valuable for tracking the spread and evolution of pathogens (Marcel AP van Bergen *et al.*, 2005).

MLST has proven useful in identifying *C. fetus* and its subspecies, as it provides a high degree of resolution in differentiating between strains. For example, the method was employed to classify 55 of 57 *C. fetus* isolates, including those associated with ST-7 and ST-12, offering a reliable tool for understanding the genetic diversity within *C. fetus* populations. Despite its success, MLST has some limitations, particularly when distinguishing closely related subspecies of *C. fetus*, such as Cff and Cfv, due to the lack of unique sequence variations in some isolates. Following the detection of an ST-4 Cff strain isolated from a rural laborer, the ability of MLST to accurately diagnose subspecies of *C. fetus* was called into question (Iraola *et al.*, 2015).

Despite these challenges, MLST continues to provide valuable epidemiological insights, especially when analyzing the clonal distribution and geographic spread of Cfv. A recent study by Abdel-Glil *et al.* (2023) utilized MLST to investigate the genomic epidemiology of *Cfv* in Germany, uncovering important details about the clonal nature and spread of this pathogen, which can help inform control measures and surveillance strategies (Abdel-Glil *et al.*, 2023).

However, one significant limitation of MLST is its reliance on a small number of genetic loci. While it is highly effective in discriminating between different strains within a species, the method's resolution may be insufficient when distinguishing subspecies or highly similar strains (Maiden *et al.*, 2013). Additionally, MLST is a relatively labor-intensive process that involves multiple PCR steps, sequencing, and allele calling, which can be time-consuming and prone to errors if not performed carefully (Anwer *et al.*, 2024).

In contrast, whole-genome sequencing (WGS) provides a much higher resolution by analyzing the entire bacterial genome, enabling the detection of even small genetic variations that MLST might miss (Quainoo *et al.*, 2017). WGS also offers the advantage of not being limited by pre-determined loci and can identify variations across the entire genome, making it particularly useful for distinguishing between subspecies, studying genetic diversity, and detecting novel strains. Moreover, WGS can provide a comprehensive analysis of antibiotic resistance profiles, virulence factors, and other relevant genetic traits, which are not captured by MLST (Bonvegna *et al.*, 2022).

While WGS offers superior resolution and a broader scope, it is more expensive and computationally demanding than MLST. MLST, with its lower cost and simpler methodology, remains a useful tool for large-scale epidemiological studies, where the differentiation of strains or subspecies is not as critical (Brlek *et al.*, 2024). The choice between MLST and WGS depends largely on the specific objectives of the study, whether broad epidemiological surveillance or in-depth genomic analysis is required.

Pulse Field Gel Electrophoresis (PFGE): When bacterial chromosomal DNA is cleaved in agarose using restriction enzymes to lessen random breaks, the process is known as pulse field gel electrophoresis (PFGE). According to Wassenaar and Newell (2000), the product is separated by gel electrophoresis to create a genotypic profile. This method has been applied to various *Campylobacter* species, including *C. jejuni*, *C. coli*, and *C. fetus* (On and Harrington, 2001). However, different PFGE profiles for the identical isolates have been found in other labs due to variations in the electrophoresis conditions and restriction enzymes used. The detection of *C. fetus* subspecies and biovar sizes depends mainly on PFGE. This work demonstrated via PFGE that the genome size of Cff is 1.1 MB, compared to 1.3 MB for Cfv and 1.5 MB for its biovar Intermedius. PFGE offers high-resolution genotyping that can differentiate closely related Cfv strains, a powerful tool for epidemiological studies and outbreak investigations. It is standardized and widely accepted in molecular epidemiology, allowing for the identification of clonal clusters and the tracking of transmission events. However, PFGE is labor-intensive, time-consuming, and requires specialized technical expertise, making it impractical for routine diagnostics. Additionally, its limited throughput and the subjective nature of interpreting the patterns can pose challenges in large-scale applications. PFGE remains a valuable tool for in-depth molecular analysis of Cfv despite these limitations (On and Harrington *et al.*, 2001).

Diagnosing *C. fetus* subspecies is essential for determining its burden on the public health system and implementing effective control programs. The genetic evolution of *C. fetus* in mammals is still poorly understood despite molecular tests created for definitive laboratory diagnosis. Because diverse methods produce inconsistent findings, most protocols can't be confidently applied directly to DNA extracted from field samples. Phenotypic assays are incompatible with some strains' genetic features and have low reproducibility. Therefore, further extensive research is required to verify the viability of molecular methods.

Antibiotic Resistance in Cfv: Antimicrobial resistance (AMR) is a global problem (Rafiq *et al.*, 2024). Overuse and misuse of antibiotics in livestock is increasing the AMR in *Campylobacter* species (Rafiq *et al.*, 2025). While studying the antibiotic susceptibility of Cfv in India, it was discovered that Cfv isolates showed sensitivity to cephalothin and metronidazole and resistance to nalidixic acid, fluoroquinolones, and tetracyclines. Those results supported other researchers who noted a higher level of fluoroquinolone, macrolide, penicillin, and tetracycline resistance in *Campylobacter* isolates from different species (Ishtifaq *et al.*, 2020). Another study reported the antibiotic susceptibility of Cfv isolated from bovine specimens between 2000 and 2009. According to this study, various antibiotics, streptomycin (10µg), gentamicin (10µg), spectinomycin (25µg), penicillin (10µg), lincomycin (10µg), ciprofloxacin (5µg), erythromycin (30µg), and tetracycline (30µg), having different sensitivities, were utilized to treat semen. A disk diffusion susceptibility test was conducted to assess the sensitivity of 50 microorganisms. It was proven that gentamicin was more effective against all strains. Some antimicrobial drugs, such as lincomycin and spectinomycin, showed the most significant frequency against seven of the 50 isolates (14%) (Hänel *et al.*, 2011). In a study by Zerbato *et al.* (2024), it was reported that Cfv isolates exhibited resistance to ciprofloxacin (MIC >4 mg/kg) and tetracyclines (MIC >16 mg/kg), while remaining susceptible to macrolides such as erythromycin, clarithromycin, and azithromycin (resistance rates ranging from 2% to 4.8%. Another investigation of 21 Brazilian Cfv isolates showed resistance to enrofloxacin and lincomycin and intermediate sensitivity to polymyxin B, neomycin, and lincomycin. A culture sensitivity test showed that all isolates were sensitive to semen, carrier bull penicillin, and streptomycin (Vargas *et al.*, 2005).

While researching the pathogenicity of Cfv, it was found that they contain tetracycline and streptomycin resistance genes such as *Tet (44)* and *ant (6)-Ib*. Genes such as *Tet (44)*, a 640-amino-acid protein, induce ribosomal protection against tetracycline and minocycline. Aminoglycoside nucleotide transferases include the 286-amino-acid streptomycin resistance determinant ANT(6)-Ib. Gene inactivation and expression showed resistance (Abril *et al.*, 2010). A crucial factor in Campylobacter*'s* resistance to several antimicrobials and hazardous substances is the multidrug efflux system or *CmeABC*. One study functionally characterized the cmeABC homologs in five different *Campylobacter species*, including *C. jejuni, C. coli, C. lari, C. upsaliensis*, and *C. fetus*. The findings showed that all five *Campylobacter* species had *cmeABC,* and all five *Campylobacter* species share a similar genetic structure for this efflux operon. All five *Campylobacter* species became more susceptible to a broader range of antimicrobials after insertional mutagenesis of *cmeB*. The *Campylobacter* species investigated in this study showed genetic and functional conservation of the *CmeABC* efflux system, demonstrating the crucial role that *CmeABC* plays in *Campylobacter* pathobiology. AMR data on Cfv remain limited and region-specific. Few surveillance studies have been published. One German study reported virtually no acquired resistance in 50 isolates (Hänel *et al.*, 2011), whereas a small Brazilian sample showed high fluoroquinolone resistance. Interpretation of susceptibility results is hampered by the absence of Cfv-specific MIC breakpoints, forcing reliance on criteria from other bacteria. Moreover, studies often use small sample sizes, and known resistance genes do not always correlate with phenotype. For instance, some Cfv strains harbor efflux pump genes yet exhibit no detectable drug resistance. Future efforts should prioritize establishing standardized susceptibility testing and Cfv-specific breakpoints, integrating whole-genome sequencing (WGS) to identify resistance determinants and monitor their evolution (van der Graaf-van Bloois *et al.*, 2023), and implementing broad, longitudinal AMR surveillance to inform semen processing protocols and bull treatment strategies. More complete and up-to-date information would aid in developing global antibiotic resistance control strategies and address campylobacteriosis in humans and animals (Hlashwayo *et al.*, 2020). In Fig. 4, the antibiotic resistance mechanism in *Campylobacter* is described.

Diagnostic Limitations: BGC diagnosis faces significant limitations. Traditional culture and phenotypic tests (e.g. H_2S production, glycine tolerance) often misidentify or fail to distinguish *Campylobacter fetus* subspecies (Pena-Fernández *et al.*, 2024). Available molecular assays have shown suboptimal sensitivity and specificity for differentiating Cfv from Cff (van der Graaf-van Bloois *et al.*, 2024), and tests like immunofluorescence cannot discriminate subspecies. Field diagnostics also suffer from low sensitivity. For example, a latent-class study found conventional PCR detected only 4% of infected bulls (Mederos *et al.*, 2022). To address these issues, research is focusing on novel molecular markers and subspecies-specific PCR assays (Pena-Fernández *et al.*, 2024), rigorous validation of diagnostics under field conditions, and genomic epidemiology approaches (e.g. whole-genome sequencing) to improve Cfv detection and trace its spread.

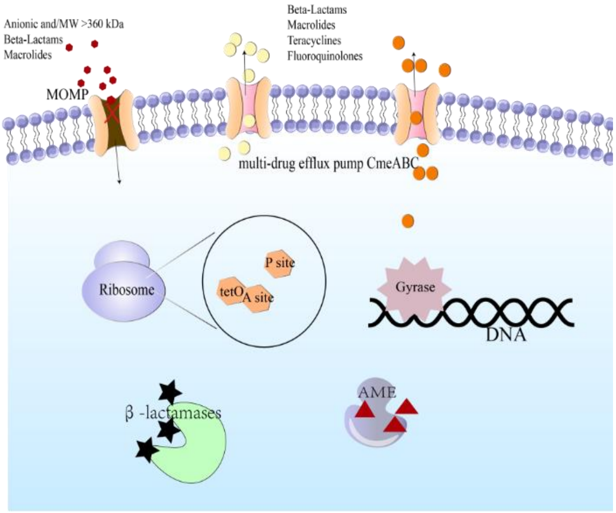

Fig. 4: Antibiotic resistance mechanisms in Campylobacter. *OXA-61* β-lactamases contribute to β-lactam resistance, while aminoglycoside resistance is mediated by modifying enzymes, particularly aminoglycoside phosphotransferases. The *CmeABC* efflux pump enhances resistance to tetracyclines, macrolides, and fluoroquinolones. Tetracycline resistance occurs when *tet(O)* protein prevents binding to the ribosome's A site, and macrolide resistance is caused by point mutations in the *23SrRNA*. The outer membrane protein restricts entry of large or negatively charged antibiotics like ceftriaxone. Fluoroquinolone resistance is mainly due to a mutation in DNA gyrase (Thr-86-Ile).

Future Prospects: Looking ahead, a stronger focus on both advanced diagnostics and proactive surveillance will be essential to overcome current gaps in controlling BGC. Many existing diagnostic tools have notable shortcomings. For example, the high genomic similarity between Cfv and Cff (over 90%) has made it difficult to develop subspecies-specific tests, and some PCR assays have consequently failed to detect a large proportion of Cfv strains in practice. To address this, more robust and specific diagnostic methods are a top priority. This includes developing real-time PCR assays targeting unique genetic markers of Cfv and introducing rapid pen-side tests (e.g., LAMP-based isothermal amplification) for on-site detection and differentiation of Cfv. Equally important is strengthening surveillance efforts. Coordinated monitoring programs and routine herd screening would enable earlier outbreak detection and better tracking of infection spread is crucial, since many countries currently lack formal BGC surveillance systems. Such programs should also include longitudinal monitoring of antimicrobial resistance in Cfv, as continuous surveillance is vital to catch emerging drug resistance before it becomes widespread. Another priority is to evaluate vaccine-based interventions. According to WOAH, some commercial and autogenous vaccines are available; their real-world efficacy remains, so dedicated studies are needed to determine whether vaccination can effectively complement biosecurity and treatment measures. Moreover, the *asd* gene holds significant promise as a future diagnostic marker for Cfv, offering a high degree of specificity and potential for improved detection compared to existing markers. Further studies are needed to assess its performance in diverse epidemiological contexts and to develop optimized diagnostic assays based on this gene. The incorporation of the asd gene into PCR-based diagnostic tests could represent an important advancement in the accurate and timely detection of Cfv, ultimately contributing to better control and eradication strategies for bovine genital campylobacteriosis. By pursuing these targeted improvements in diagnostics, surveillance, and prevention, the field can move closer to effective control and eventual eradication of BGC.

Conclusions: BGC continues to cause significant reproductive and economic losses globally, yet its diagnosis remains challenging due to the fastidious nature of Cfv and its genetic similarity to related subspecies. While molecular techniques have improved detection sensitivity and specificity, further advancements such as more specific RT-PCR assays, are needed. Antimicrobial resistance in Cfv appears limited, but ongoing surveillance is essential to prevent emerging resistance. Strengthening diagnostic capabilities and implementing coordinated global monitoring are crucial steps toward effectively controlling and eradicating BGC. These epidemiological patterns and economic impacts reinforce the need for sensitive, subspecies-specific diagnostics and coordinated surveillance to support effective control.

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