IMPROVING DIAGNOSTIC TECHNIQUES FOR VENEREAL DISEASES IN BULLS

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Saskatoon, Saskatchewan, Canada

By

Álvaro García Guerra, D.V.M.

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ABSTRACT

Infectious disease continues to cause significant problems on reproductive efficiency in the cattle industry. The purpose of this project is to evaluate new testing strategies for *Tritrichomonas foetus* and *Campylobacter fetus* subsp. *venerealis.*

This thesis describes the result of three studies that evaluated the use of real-time PCR for the identification of *Tritrichomonas foetus* and *Campylobacter fetus* subsp. *venerealis* in carrier bulls. The first study evaluated the specificity of a real-time PCR test for *T. foetus* in individual culture enriched samples, and the sensitivity of the assay for use in pooled samples of up to 25 bulls. Specificity estimates were 98.8% (95% CI 97-99.4) and 100% (95% CI 98.9-100) for culture and real-time PCR, respectively. The sensitivity of the real-time PCR assay for pooled preputial samples was: 96.8% (83.8-99.4) for pool ratios 1/3 and 1/5; 93.5% (79.3-98.2) for pool ratios 1/2, 1/15, 1/20 and 1/25; and 90.3% (75.1-96.6), and were not significantly different. However, 13 of the 217 pools tested were negative and 9 of these negative testing pools contained the same positive sample. The media in this positive sample showed evidence of contamination and could potentially explain the failure to detect *T. foetus*.

The second study evaluated the sensitivity of a real-time PCR for the detection of *T. foetus* in individual and pooled direct preputial samples. Sensitivity of individual samples tested by culture, real-time PCR in direct and culture enriched samples were determined from 121 samples obtained from 9 infected bulls. Sensitivity estimates were: 95.0% (95% CI: 89.6% to 97.7%) for culture, 95.9% (95% CI: 90.7 to 98.2) for real-time PCR in cultured enriched samples, and 90.1% (95% CI: 83.5 to 94.2) for direct preputial samples and did not differ (P=0.12). Sensitivity estimates for direct pooled samples in groups of 5 or 10 were: 83.6% (95% CI: 75.6

to 89.4) and 77.3% (95% CI: 68.6-84.1), respectively and were not significantly different (P=0.08). The use of repeat sampling tested in pools by real-time PCR increased the sensitivity to 100% and 96% for 3 consecutive samples (pools of 5 or 10, respectively). The use of pooled direct preputial samples although sensitive, still requires the use of repeated sampling.

The third study determined the sensitivity and specificity of a recently developed real-time PCR (qPCR) tests for Cfv. A total of 300 virgin bulls were tested by both culture and qPCR. Specificity estimates were 85% (95% CI: 80.5 to 88.6) for qPCR and 100% (95% CI: 98.7 to 100) for culture, and were significantly different (P<0.01). A total of 4 naturally infected bulls and 9 artificially infected bulls were sampled serially to obtain positive samples for a sensitivity analysis. Sensitivity estimates and 95% confidence intervals are as follows: qPCR (85.4%, 95% CI: 80.6-89.2); direct culture on blood agar (82.3%, 95% CI: 77.2-86.5), DFAT (72.1%, 95% CI: 66.2-77.4), direct culture on Skirrow agar (32.7%, 95% CI: 27.2-38.7), TEM and blood agar (30%, 95% CI: 23.4-37.5), and TEM and Skirrow agar (38.1%, 95% CI: 31-45.9). The sensitivity of the different tests evaluated varied significantly with different ambient temperatures (P<0.01). The sensitivity of the qPCR was significantly higher than any other test when temperatures exceeded 5°C. The use of repeated sampling at weekly intervals significantly improved the sensitivity of the qPCR.

The real-time PCR assay for the detection of *T. foetus* in both individual and pooled samples appears to be highly sensitive and specific. Moreover, the possibility of using direct preputial samples provides a cost-effective diagnostic strategy. Real-time PCR in direct preputial samples for BGC diagnosis in bulls has good sensitivity and specificity. However, the use of repeated sampling maybe needed in order to maximize the ability to detect carrier bulls.

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DEDICATION

To my parents Jorge and Juana, for their unconditional love and support. They have taught me that honesty, perseverance and hard work are the key for a successful career.

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"Ad astra per aspera"

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
°F	Degrees Fahrenheit
AFLP	Amplified Fragment Length Polymorphism
Cff	*Campylobacter fetus* subsp. *fetus*
CFU	Colony Forming Units
Cfv	*Campylobacter fetus* subsp. *venerealis*
CI	Confidence interval
Ct	Cycle threshold
DFAT	Direct fluorescent antibody test
DNA	Deoxyribonucleic acid
h	Hours
kbp	Kilobase pairs
ml	Millilitre
MLST	Multi Locus Sequence Typing
n	Number
OR	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDS	Prairie Diagnostic Services
PFGE	Pulse Field Gel Electrophoresis
qPCR	Quantitative real-time polymerase chain reaction
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
s	Standard deviation
SSCP	Single Strand Conformation Polymorphism
µl	Microlitre

1. INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Reproduction is one of the most important factors affecting beef and dairy cattle production [1-4]. More specifically, failure to conceive and early pregnancy loss have been identified as the primary cause for reduced calf crop in beef cattle, and the major cause of reproductive losses in dairy cattle [1,5]. Infectious causes of infertility, embryonic and fetal death are a significant cause of reduced reproductive performance in cattle [6].

Infectious causes of embryonic and fetal mortality in cattle include an extensive list of bacterial, fungal, viral and protozoan agents [7]. Several infectious agents in which venereal transmission occurs have been described in cattle such as: *Leptospira* spp., *Campylobacter fetus* subsp. *venerealis*, *Histophilus somni*, *Ureaplasma diversum*, *Mycoplasma* spp., *Bovine herpesvirus I*, *Bovine viral diarrhea virus* (BVDV), and *Tritrichomonas foetus* [6,7]. However, there are only two common reproductive diseases in which the venereal route is almost exclusively the only means of transmission, and the causing agents are obligate parasites of the reproductive tract of cattle, and these are Bovine trichomonosis (BT) and Bovine Genital Campylobacteriosis (BGC).

Bovine trichomonosis is caused by *Tritrichomonas foetus* and was first described by Kunstler in 1888 (as reviewed by [8]), while BGC is caused by *Campylobacter fetus* subsp. *venerealis* originally described in cattle as *Vibrio fetus* by Smith in 1918 (as reviewed by [9]). Both diseases are characterized in the female by infertility [10,11], early embryonic and fetal loss [12-14], reduced pregnancy rates [15,16] and abortions [17,18]. In contrast, bulls do not show any clinical signs and are considered asymptomatic carriers [19,20]. Venereal diseases are transmitted during

mating, and as a result have been of great concern in areas where cattle are managed extensively under range conditions [19]. Decreased incidence in intensively managed cattle has been attributed to the use of artificial insemination (AI) [21], with strict control in the semen collection and freezing procedure at accredited centers [22,23].

An important distinction must be made regarding the two *Campylobacter fetus* subspecies, *fetus* and *venerealis*. The differentiation between the two subspecies was originally made based on epidemiological characteristics of two distinct diseases. As mentioned above Cfv is the agent identified as responsible for BGC. On the other hand *Campylobacter fetus* subsp. *fetus* (Cff) is responsible for sporadic abortions in cattle and sheep. The organism is a common inhabitant of the intestine in cattle [24] and abortion is thought to be the result of bacteremia and subsequent placentitis, and usually occurs late into gestation [25,26]. Although, this organism is not thought to be transmitted venereally conflicting evidence has been reported in the literature. Isolation of Cff from preputial samples has been reported, and the organism could also be established in the vagina and cervix of heifers [27-29]. In addition, infertility was observed in at least one study and one other indicated this possibility based on history of isolates [28,29]. Although there is at least some evidence for the venereal transmission of Cff, accurate determination of the subspecies has traditionally been based on phenotypic characteristics of the isolates which have poor reproducibility [30]. It is not the intention of the present review to explore the possibility of Cff as a venereal pathogen; however reference will be made solely for the purpose of diagnostic differentiation from Cfv.

Significant economic losses have been attributed to trichomonosis in both beef [31] and dairy [32] cattle. In 1989 based on an estimated calf loss of 5%, the cost to the U. S. beef industry was estimated as 650 million dollars a year [31]. A simulation model developed by Rae et al. [33],

indicated that depending on bull prevalence and with a reduction in the calf crop of 14 to 50%, the return per cow exposed to breeding was decreased by 4 to 10%, respectively. However, economic losses can be even greater if the cost of culling of cows and bulls and the cost of testing and vaccination are included. Conversely, the amount of knowledge in regards to the economic impact of BGC is somewhat limited. In 1960 it was estimated that 4% of the cows in the U. S. failed to conceive due to BGC and economic losses associated were $167 million (as reviewed by [25]). Recent data from an outbreak of BGC in a herd in Saskatchewan indicated that pregnancy rates could be reduced by 16 to 62% when Cfv infected bulls are used [16]. In light of these recent findings it is reasonable to assume that economic losses due to BGC are comparable to those reported for BT.

The present review is not intended to encompass all aspects of the two main venereal diseases in cattle. Several published reviews are present in the literature that covers the most important aspects of the disease and its diagnosis in both male and female [6,34-36]. The objective of the present paper is to provide a summary of the most important aspects of the epidemiology and pathogenesis of these two diseases in the bull and most importantly to outline and analyze the different diagnostic methods available for the detection of BT and BGC in the bull. Finally, the control strategies based on identifying carrier bulls will be briefly explored.

1.2 ETIOLOGIC AGENTS

Tritrichomonas foetus

T. foetus is a motile flagellated protozoan of the order trichomonadida, featuring 3 anterior flagella (11-17µm in length), and one posterior flagellum associated to an undulating membrane. Dimensions are approximately 10-25 x 3-15µm and present a characteristic rolling and jerky

type of movement key to its identification in culture [37,38]. It is an aerotolerant anaerobe and carbohydrates are its main energy source [38]. It is more commonly seen its trophozoite form, spindle shaped, although under adverse conditions adopts a pseudocyst form in which the flagella are internalized [39]. Several of these pseudocyst forms are multinucleated and can generate new organisms by a budding process [40]. Its primary natural host is cattle, although its presence in other species has been reported [41,42]. In addition, significant evidence has been provided that shows that *T. foetus* and *T. suis* are the same organism [43].

Three serotypes have been described for *T. foetus*, named var. *brisbane*, var. *belfast* and var. *manley* by agglutination [44]. However, protein profiles and antigenic profiling show a high degree of similarity between isolates and antigenic cross reactions [45].

Campylobacter fetus subsp. *venerealis*

Campylobacters are Gram negative epsilonproteobacteria highly adapted to mucosal surfaces [46,47]. They are spiral slender curved rods 0.2-0.3 x 1.5-5 μm, and some spirals are as long as 8 μm. Other forms can also be observed such as S-shaped and gull winged. They present a single polar flagellum on one or both ends, and have a rapid corkscrew motility. They are microaerophilic with a respiratory metabolism, and energy is obtained from amino acids and tricarboxylic acid cycle intermediates. The type species is *Campylobacter fetus* [26,46], and is composed of two subspecies *Campylobacter fetus* subsp. *fetus* (Cff, previously known as *Vibrio fetus* subsp. *intestinalis*) and *Campylobacter fetus* subsp. *venerealis* (Cfv, previously known as *Vibrio fetus* subsp. *venerealis*). Both are oxidase and catalase positive, and are slow growing fastidious organisms [9,48]. Differentiation between subspecies has been based on the ability of Cff to grow in the presence of 1% glycine and to produce H_2S in sensitive medium

(supplemented with 0.02% cysteine-HCl), while Cfv is sensitive to glycine and does not produce H_2S [9]. However, intermediate strains have been described based on their ability to produce H_2S and named *Campylobacter fetus* subsp. *venerealis* biovar *intermedius* (Cfvi) [9,49]. More recently, some strains of this biovar have been shown to tolerate 1% glycine [50]. As a result the subspecies differentiation has become increasingly confusing as current literature refers to Cfvi based on its ability to tolerate glycine, while the traditional description has been based on its ability to produce H_2S in sensitive media. The primary host of Cfv is cattle and has also been described in buffalo [51].

By means of molecular based tools new information has been incorporated to the knowledge of this complex species. DNA sequence similarity between subspecies has been shown to be very high, thus preventing subspeciation by this method [52]. Genomic size is quite similar between subspecies although most Cfv strains are approximately 228 kbp larger [50]. Recently, complete genome comparison revealed a core-genome shared between Cfv and Cff, and several genes specific to each subspecies [53]. Although both subspecies are genetically closely related, they differ in tissue and host specificity (Cfv is very host specific). Based on the identification of pathogenicity islands in the Cfv genome, thought to be mobile genetic elements, Cfv is believed to be a bovine clone of Cff [47,53].

A serotyping scheme for *C. fetus* has been described based on the O-specific polysaccharide chain with repeating oligosaccharide units present in the lipopolysaccharide (LPS), known as the O-antigen [54]. Two main heat stable serotypes are named A and B. Cff can present serotype A, B or AB, while Cfv presents the serotype A only [49,55]. Within serotype A the LPS structure is identical and is not possible to differentiate between Cff and Cfv based on this characteristic [55].

1.3 EPIDEMIOLOGY OF BOVINE VENEREAL DISEASES

1.3.1 Geographic distribution and prevalence

Bovine trichomonosis and BGC have been described in Europe [56-58], Africa [59], North America [16,60], South America [61-63], Australia [19,64,65], and Asia [21,66]. Both BT and BGC are listed under the OIE list of notifiable diseases and reports of absence or presence should be filed every six months [67]. In spite of the fact that the use of AI has decreased the incidence of these two diseases, especially in dairy cattle, it continues to be a significant problem in extensively managed cattle worldwide.

The individual prevalence of BT in bulls is shown in Table 1.1. Individual bull prevalence is usually under 5% with the exception of a few studies. A few different distinctions can be made between the studies reporting higher prevalence. The study conducted in north-eastern Australia is almost 40 years old and newer reports may indicate lower prevalence as shown in the case of South Africa in which newer estimates indicate a decrease in prevalence. It is noteworthy in the case of Spain in which the prevalence appeared to increase within the same region between 1998 and 2012, and this has been attributed to a re-emergence of the disease in this region of extensively managed cattle. The use of prevalence estimates should be taken with caution as most of these studies have been based on samples received by diagnostic laboratories or abattoir surveys and the introduction of bias cannot be ruled out. Herd prevalence estimates range from 0.76 to 44.1% [19,58,68-71] and within herd bull prevalence estimates range from 4 to 83% [19,71-74]. The within herd bull prevalence has been used as a measure of the spread of the disease between bulls on an infected herd. However, one of the major limitations in this estimate is usually the lack of reference to the size of the herd, the number and size of breeding groups,

and the bull to cow ratio used. Moreover, the within herd prevalence usually does not exceed 50%, and is thought to be due to the higher proportion of younger bulls in the bull population on any given herd [75].

Individual prevalence estimates of BGC reported in bulls in different geographic areas are shown in Table 1.2. Prevalence estimates shown were obtained by means of different tests, and subspeciation was not performed in all studies, as a result the prevalence may be overestimated by the inclusion of Cff. The presented prevalence estimates have been obtained over a prolonged period of time. Prevalence estimates in Canada have been obtained mainly based on bulls in AI centers and the latest study identified only Cff. As a result, current prevalence estimates in commercial bulls are needed in order to properly assess the distribution of this disease. Herd prevalence has been reported from 9 to 89% [64,69,76-79]. Although herd prevalence reports have been relatively high, care must be taken as often estimates reported are based on herds with a history of poor reproductive performance. Within herd bull prevalence in Australia was estimated to be 15%; however it ranged from 3.7% to 48% [78].

The presence of dual infections with BT and BGC has been reported and prevalence estimates range from 0.7 to 18% [78,80]. Moreover the herd prevalence of dual infection in Australia was found to be over 50%, which indicates the importance of a correct diagnosis [78].

1.3.2 Transmission

Bovine trichomonosis and BGC are transmitted mainly during coitus [81,82]. In addition, transmission has been shown to occur by the use of contaminated semen collection devices [83,84], and artificial insemination with contaminated semen [85,86]. It would appear that strict

biosecurity practices in the collection and processing of semen make the last two routes less likely under current procedures [22].

Mounting between males in bulls housed together occurs although its frequency tends to decrease with time [87]. Spread of BGC infection between bulls has been suggested based on high rates of infection within AI studs [83], and has since become a common belief. In this study this was suggested as a possibility due to the fact that some of the infected bulls were housed in groups in large pens. However, no mention of the number of bulls or distribution of infection between individually and grouped housed bulls was made. Moreover, the authors suggest the semen collection procedure to be the most likely cause of spread of the disease, and this is consistent with other reports [88].

On the other hand, transmission of BT between bulls has been more rarely mentioned. Parker et al. [89] reported the isolation of *T. foetus* from one virgin yearling bull housed with infected bulls. The bull was seen mounting other bulls, but organisms could only be isolated from the bull's prepuce for a period of 26 days. Others have reported housing infected and non-infected bulls together for long periods of time without detecting any new infection [6]. Although the possibility of transmission by mounting between bulls may be a possibility, there is not enough evidence to support it. Furthermore, the sensitivity of these organisms to environmental conditions makes this an unlikely possibility, and its importance in the spread of the disease would appear to be minor [6]. As a result, virgin bulls are considered to be free of infection [37].

Passive transmission, with the bull acting as a mechanical vector, has been shown to occur between vaccinated bulls and Cfv infected heifers, and could potentially occur between bulls resistant to infection and susceptible heifers. However, the effect of this passive transmission on

herd fertility is not clear. One study conducted with Cfv indicated that the transmission rate is low and only occurred after a bull was hand mated to an infected heifer and a naïve heifer within 30 minutes [90]. Conversely, one other study found that 28% of heifers were found to be infected by passive transmission, although the bull to female ratio was 1:8 [91]. Whether this form of transmission is a significant concern remains unclear, however evidence collected to date suggests that transmission rates would be low and thus its role under range conditions may be minor.

1.3.3 Risk factors

Higher prevalence of *T. foetus* in older bulls compared to younger bulls has been reported in several studies [71,75,92,93]. The prevalence of infected bulls has been shown to increase from 1.8% in 2 year old bulls to as high as 43% in bulls > 4 years old [75,92]. Bulls > 3 years old are 3.5 to 17 times more likely to be *T. foetus* carriers than bulls ≤ 3 years [58,74]. However, it is important to point out that bulls as young as 1-2 years can be carriers and this should be taken into account when establishing control programs [94,95]. Clark et al. [95] were successful in establishing infection in 92% of bulls ≥ 3 years and only in 16% of bull 1 to 2 years.

The relationship between age and risk of infection with BGC is less clear. The prevalence in AI studs was reported to be 1.7% and 46.7% for bulls < 6 years and those ≥ 6 years of age [83]. However, a clear distinction between *C. fetus* subspecies was not made in this study. More recently, the purchase of new bulls was associated with a 35% decrease in the risk of BGC. It is not clear if this association is due to purchase of young virgin bulls or is a reflection of a better management practices in those herds [61]. Bulls as young as 20 months of age have been successfully infected with Cfv [96]. In this study 22 bulls were repeatedly exposed starting at 18

to 47 months of age and 45% became carriers before reaching 4 years of age. Success in establishing a carrier state was achieved in 60%, 83% and 83% of bulls, when challenge was started at either 20, 34 or 47 months, respectively. Similarly, the rate of infection after instillation of pure cultures was 75% in bulls of 41-49 months and 100% in those 66-74 months [97].

Although young bulls can be successfully infected, the success rate in establishing infection is higher in older bulls. Moreover, the two studies mentioned above used artificial exposure and it is not known whether the size of the inoculum reflects accurately what occurs under natural conditions. As a result, further research is needed to establish whether age plays an important role in the establishment of the carrier state as was shown for BT.

Commingled grazing has been commonly associated with an increase risk of disease occurrence and if appropriate preventative measures are not taken this can lead to reduced pregnancy rates [98]. Commingled grazing is a common practice in western Canada and it is performed on both public and private land. Moreover, the first report of BT in western Canada originated in a community pasture [99]. The use of commingled grazing has been associated with *T. foetus* infection (OR=2.3), and it was demonstrated that it was the number of herds that shared the grazing land and not the mere use of public land that was associated with an increased risk of BT [100]. Sharing livestock between producers has been responsible for transmission of BT between herds in two cases reported in Ontario [101] and a similar association was reported in a large study in Argentina (OR=5.4) [63]. It is plausible that the same conditions would hold true for BGC, however little information has been reported in the literature. One study conducted in Argentina observed that trespassing of bulls between neighboring herds increase the odds of detecting Cfv by 2.0 times [61]. Based on this observation it would appear that commingling and sharing of breeding animals could also be a risk factor in the occurrence of BGC.

Breed has been reported as being associated with BT infection in some studies, with *Bos taurus* bulls 6 times more likely to be infected [74,93]. Similarly, a higher prevalence of BT in *Bos taurus* than in *Bos indicus* or their crosses was found in California. In addition, none of the pure *Bos indicus* bulls in this study was identified as infected. However, these results must be interpreted with caution since the sample distribution of breed types was unequal, thus preventing adequate comparisons [71]. In contrast, a study of a large cattle operation in Florida showed no difference in prevalence between *Bos taurus* and *Bos indicus* crosses [75]. The reasons for differences in the potential susceptibility of different breeds remain unclear; however it has been proposed that *Bos taurus* breeds may be more prone to be carriers due to the higher frequency of mating as compared to *Bos indicus* breeds.

Lower incidence in smaller and more intensively managed herds was the proposed explanation for the difference in prevalence between two regions in Australia [19], and similar results have been reported in the US [71,93]. In addition, a greater bull to cow ratio was associated with an increased risk of BT in herds in Florida (OR=12.8) [93]. The higher herd prevalence of BT in bulls in larger operations could be explained at least in part by the rotation of bulls between breeding groups, and greater number of bulls per breeding unit. The rotation of bulls increases the number of sexual contacts and was identified as an unconditional risk factor in one study in Argentina (OR=2.4) [63]. Increased number of bulls per breeding unit also results in a greater number of sexual contacts and thus increases the individual bull prevalence [93]. Based on these reports it appears that management practices rather than the actual herd size would be responsible for the difference in prevalence between small and large herds. The effect of herd size and risk of infection with BGC has not been reported in the literature. However, similarities between both diseases would suggest that the same risk factors would hold true for BGC.

1.4 PATHOGENESIS IN THE BULL

1.4.1 Anatomy of the penis and prepuce

The penis of the bulls is of the fibroelastic type and is enclosed by the tunic albuginea [102]. The free part of the penis is 11.3 ± 0.3 cm and lies within the preputial cavity or "sheath" [103,104]. The total length of the penis in the bull is 105 ± 6 cm in *Bos taurus* breeds and is significantly longer in *Bos indicus* breeds [104]. The preputial cavity is formed between the lamina interna of the prepuce and the free part of the penis, and is on average 39.2 cm in length, although breed variations have been described where polled breeds have a longer preputial cavity [103,104]. The mucous membrane lining the prepuce is continuous with the skin of the free part of the penis, and is covered with stratified squamous epithelium. The lamina interna forms longitudinal and in some places transverse folds and the attachment to the free end of the penis forms the fornix of the prepuce [103,105]. The epithelial infoldings have been shown to be present in both the penis and lamina interna of the prepuce [106]. During fetal and early postnatal life the internal lining of the prepuce and the penis are attached, and detachment is usually complete by 12 months of age or after the first breeding [102,107]. The orifice of the prepuce is pendulous, covered in coarse hair and located caudal to the umbilicus [102,108]. The prepuce presents cranial and caudal preputial muscles, although the caudal muscles could be absent in some polled breeds [108,109]. The cranial muscle constricts and elevates the preputial orifice while the caudal muscle retracts the lamina interna by its attachment at the fornix of the prepuce [108].

1.4.2 Distribution of *T. foetus* and *Campylobacter fetus* subsp. *venerealis*

The infection of the bull with either Cfv or *T. foetus* does not produce clinical signs or recognizable pathogenicity [88,110]. The organisms are distributed along the mucosal surface of

both penis and prepuce, and can be occasionally found in the cranial portion of the urethra [20,95,110]. The number of Cfv and *T. foetus* in infected bulls tend to be greater near the fornix of the prepuce, and dorsal part of the penis, and decreased towards the preputial orifice. However, great variability between bulls has been reported [20,110,111]. Histological examination reveals organisms are located superficially and do not penetrate the epithelium, although *T. foetus* has been observed within layers of the epithelium [20,112]. *Tritrichomonas foetus* and Cfv are commonly seen within the crypts of the penile epithelium and to a lesser extent in those of the prepuce [20,112]. The presence within crypts is thought to be due to a more favorable environment due to lower oxygen tension, pH and temperature that would favor the development of Cfv [20]. It has been suggested that the establishment of a carrier state is directly dependant on the presence of crypts [20,111].

The presence of a greater number and depth of epithelial crypts as the bull mature has been suggested in the literature [20], thus explaining the greater incidence of long term carriers in older bulls found in some studies [83,95]. However, a recent study, in a small number of bulls, showed that the area of the preputial and penile epithelia, the area of infoldings, or the total number of infoldings were not significantly different between 2 year old bulls and those $\geq$ 5 years [113]. Another hypothesis to explain the greater prevalence of venereal disease in older bulls has been related to increased opportunities to acquire infection as the number of sexual contacts increases with age. Nevertheless, the establishment of infection appears to be highly variable, particularly for Cfv, and supports the idea that individual characteristics of the animal appear to play an important role [95,96]. Moreover, the progeny of certain sires appears to be more susceptible to BGC than others suggesting a genetic predisposition [96]. However, the basis for the predisposition of certain animals to become long term carriers remains unknown.

Observations of *T. foetus* made on studies in mice, suggests that although different isolates are able to establish genital infections, there are variations in the duration of the infection at least in females [114]. The combination of varying degrees of pathogenicity of some strains and the different degrees of susceptibility of certain bulls can lead to temporary carriers. Cfv temporary carriers have been reported for periods of up to 29 days [96]. Similarly, *T. foetus* has been shown to cause a temporary carrier status in 1-2 year old bulls for 1 to 3 months [95]. Age seems to be the common factor for the reports of temporary carrier status, although the mechanisms behind this resistance remain unknown.

1.4.3 Level of infection

The level of infection of the preputial cavity with either pathogen appears to be variable between bulls and samples within bulls [20,115]. The amount of *T. foetus* per ml of smegma can range from 0 to over 400,000, thus consecutive examinations of preputial samples from the same bull may yield different results [115,116]. Definite peaks in the concentration of organisms have been reported to occur at 4 to 10 days intervals for *T. foetus* [115]. Similarly, the number of Cfv organisms isolated from preputial samples range from 0 to over 200,000 CFU/ml [20,35].

Fluctuation in number of organisms in the preputial cavity has been responsible for the need for repeated sampling as a means to identify carrier bulls accurately. The cause of fluctuations remain unclear, however suggestions have been made at least for *T. foetus*. Potential factors affecting the population of *T. foetus* or its recovery are: recent mating, contamination (due to masturbation or intermittent prolapse of the prepuce), variations in pH and bacterial flora, development of resistance, and limitations of diagnostic techniques [116].

1.4.4 Effect on bull fertility

The presence of *T. foetus* and Cfv in semen is believed to be due to contamination with preputial secretions, as it has not been isolated from the epididymis or accessory glands [110,112]. As a result neither of these organisms has been associated with semen quality. However, recent studies conducted *in vitro* have shown that at least *T. foetus* has some detrimental effects on sperm. *T. foetus* was found to adhere to sperm causing agglutination, phagocytosis and motility reduction after co-incubation [117,118]. Moreover, it was shown that *T. foetus* expresses a cysteine protease that is secreted to the environment and is in part responsible for the cytotoxic effect upon sperm [118]. Conversely, the effect of Cfv on sperm cells has not been determined. In view of these facts it is possible that at least part of the infertility of females mated to infected bulls could be due to an effect on sperm. However, it is not known if these studies conducted *in vitro* reflect what occurs *in vivo* as the sperm to *T. foetus* ratio used is probably higher than what occurs under natural conditions.

1.5 DIAGNOSIS

1.5.1 Sample collection

Sampling methods are aimed to obtain preputial material or smegma. One of the first methods described and most widely adopted was Bartlett's aspiration method [116]. This technique consists of a long glass pipette fitted to a rubber bulb. The device is placed into the prepuce and scraping of the surface of the penis and prepuce is performed by moving the pipette back and forth while suction is applied. The volume of preputial fluid obtained ranges from 0.5 to 2 ml [119]. This method was lately modified to be used with plastic pipettes attached to a syringe as described by Parker et al [120]. The scraping of the preputial cavity and penis 10 times yields

excellent samples and no advantage has been reported when using a greater number of scrapings [121].

Preputial washings have also been described and consist in the introduction of large volumes of warm solutions (200 ml) into the prepuce cavity, the preputial orifice is then closed and the prepuce massaged. The fluid is recovered in a beaker and centrifugation is necessary to concentrate the organisms [122]. This method has been later modified by using smaller volumes (30 ml) and combining it with aspiration [123]. Despite minor differences, both procedures are usually referred to as preputial washings. One of the major disadvantages of the original method is that it is laborious, and although the latter modification tended to simplify the collection procedure a centrifugation step is still required.

A metal brush, formed by a long handle and 6 cm cylinder with 13 grooves, has been used to obtain preputial samples by scraping [120,124]. The major disadvantage of this method is that the metal brush is reusable and requires sterilization between samplings usually accomplished by immersion in boiling water [120]. However, recently a plastic disposable version of the metal brush has been used and appears to be useful and practical [125,126]. Other less common methods used are cotton swabs [122], and rinsing of the artificial vagina after semen collection [84].

Comparisons of the efficacy between sampling techniques have been done extensively, although most of them in regards of *T. foetus* diagnosis. The use of the aspiration method or preputial washings has resulted in no differences in sensitivity when culture or PCR was used for *T. foetus* [123,127,128]. One study found the washings to result in higher sensitivity using direct examination, however large volumes of saline were used and the procedure was deemed to be to

inappropriate for application in the field [122]. On the other hand, aspiration has resulted in better recovery of Cfv than preputial washings and less contamination of the culture medium [129].

Tedesco et al. [130], compared the metal brush vs. the aspiration method for *T. foetus* diagnosis, showing that the former yielded more positive results when direct evaluation was used, although no difference was observed when samples were evaluated after culture. These results are supported by another study where the pipette method and metal brush resulted in a sensitivity of 91.6% and 93.3% respectively after culture [120]. In addition, the use of the plastic brush or the aspiration method resulted in excellent agreement when tested by either culture or PCR ($k \geq 0.88$) [58].

Conversely, the use of the brush was able to recover a greater number of Cfv organisms than the aspiration when tested by real-time PCR [125]. Similarly, the metal brush was found to be superior to aspiration for Cfv detection when culture was used, and the degree of contamination of culture plates was lower with the metal brush [129]. However, the suction in the aspiration method was accomplished by a mouthpiece instead of a syringe and methods were performed in alternate weeks thus potential differences in the population of organisms could occur. Given the laborious characteristics of using the metal brush or the washing methods, the pipette method seems to be a faster and simpler method [119,128].

Overall, the most practical method and most widely adopted method in North America is the aspiration method. This method allows for collection of adequate samples by a single operator. Although the metal brush technique appears to provide at least some benefit for the recovery of Cfv, the fact that the brush needs to be sterilized after each use has not made it very popular.

However, recently the same device has become available in a disposable plastic form, making it an excellent alternative to the aspiration method.

1.5.2 Transport of samples

Under field conditions preputial samples are often subjected to prolonged transport times and unfavorable conditions. Both Cfv and *T. foetus* are temperature sensitive and conditions in North America range from high temperatures in the summer to extremely low temperatures during the winter [131,132]. Thus, the lack of proper conditions during transport may result in false negative results [133,134]. In addition, preputial samples are an extremely complex sample due to the normal microflora plus possible contaminants from feces or other sources. Although the ecology of the preputial flora is not well understood, control of contaminants is an essential step in the diagnosis of venereal diseases, especially when viable organisms are needed.

Transport of samples for *Tritrichomonas foetus*

Separation of *T. foetus* from contaminants is an important factor for the success of culture methods. The addition of agar combined with keeping the media undisturbed will allow the motile *T. foetus* to migrate to the bottom of the tube while contaminants will remain on the surface [94]. However, keeping inoculated media undisturbed during transport is extremely difficult.

A wide range of transport media has been reported and their efficacy to maintain viable organisms varies from 24 to 96 hours. Some types of media and their efficacy are: modified Stuart's medium for up to 48 hours [130,135]; Kupferberg media or broth up to 84 h [136], buffered saline with fetal serum up to 24 to 48 h, lactated Ringer's up to 48 h and cow's milk 96

h [94,137]; Thioglycolate with calf serum and the InPouch™ TF® up to 96 h [131]. Transport at higher temperatures (22-37ºC) requires animal products (milk or serum) and antibiotics [137]. Furthermore, freezing should be avoided during transport as viability declines rapidly and no organisms are seen after 3 h [131]. Transport of samples in non-nutrient media has been reported to decrease sensitivity by 14% compared serum or milk supplemented media [92].

Since the introduction of the InPouch™ TF® culture kit, it has become the preferred method for transport of samples as it serves also as the culture medium, thus avoiding the need for transfer of samples upon arrival to the laboratory. The temperature at which the InPouch™ TF® should be kept during transport has been previously studied. Bryan et al. [131] indicated that transport at 22 to 37°C for up to 4 days yielded 100% recovery rates when microscopic detection was used. In contrast, transport and refrigeration at 4°C appeared to not affect the sensitivity if it did not exceed 72 h. However variability between different *T. foetus* isolates was observed [131,138]. In addition, detection was also possible by means of real-time PCR [138]. Samples collected in PBS for testing by PCR can be held at 4°C for up to 30 h without significant decrease in sensitivity [127,139]. Longer storage times at either 4°C or room temperature for samples collected in PBS for PCR have been shown to be acceptable [126,140], although a decline in sensitivity after storage for 5 days has been reported [126,127].

Temperatures higher than 42 °C for less than 24 h have been shown to be detrimental for the survival of *T. foetus*. However, the use of real-time PCR still allows for the identification of carrier animals since it does not require organism viability [132,138]. Temperatures of between 20 and 37°C appear to be the optimal range to maintain the viability of *T. foetus*, and although refrigeration at 4°C can be used it should be kept to a minimum [131,138]. If PCR is to be used,

samples can be kept at 4°C or room temperature. However, further research is needed to evaluate storage conditions for these types of tests.

Transport of samples *Campylobacter fetus* subsp. *venerealis*

The use of transport media for the recovery of Cfv is essential when transport times exceed 6 to 8 h and culture is being used as the diagnostic method [135]. The poor survival of the organism, due to its fastidious nature, has been identified as one of the major problems in the diagnosis of BGC [48,134,141]. Transport media should maintain the viability of Cfv, enhance its multiplication and prevent overgrowth by contaminants [135]. A survey indicated that 73% of diagnostic laboratories used an enrichment media although the composition was extremely variable [142]. Several transport enrichment media have been described in the literature however only two of them have been widely accepted and have shown superior performance [134,143].

The modified Weybridge transport enrichment media (mWTEM) contains charcoal, lysed blood, a combination of antibiotics and antifungal agents plus FBP supplement (a mixture of ferrous sulphate, sodium metabisulphite and sodium pyruvate) [144]. The use of FBP has been deemed essential as it allows Cfv to tolerate the aerobic conditions [145]. This media has been shown *in vitro* to allow detection when as few as 10 organisms were inoculated. The antibiotic combination controls some strains of *Proteus* and *Pseudomonas*. However, complete control of *Pseudomonas* overgrowth remains an issue and use of filtration techniques has been found useful [146,147].

The mWTEM is inoculated with the sample and incubated at 37°C for 3 days before plating on the culture media, however the delay between inoculation and incubation has to be minimized in order to avoid the decrease in viability of the organism [144]. The use of mWTEM yielded a

similar sensitivity as direct culture under ideal conditions [146], and performed better when extended transport was required [66]. However, more recent studies showed that direct culture was significantly more sensitive than culture after incubation in mWTEM for 24 h [147].

A simpler version of this media without the FBP, known as Weybridge, was shown to maintain the viability of Cfv for 96 h at room temperature or refrigerated [143]. However, a recent study indicated that the use of Weybridge media with 4 h transport, and avoiding the incubation at 37°C allowed for significantly better isolation rates than other transport medium and longer transport times. In addition, the use of the antibiotic combination present in the Weybridge media was significantly better in controlling for contaminants than other media [134].

Another type of transport media, known as the Australian TEM, was developed by Clark et al. [148]. This media is based on serum plus a combination of antibiotics and antifungal agents. In addition, the air in the tube containing the medium has to be replaced to provide a microaerophilic environment. The Australian TEM should be transported at 18 to 37°C for no longer than 2 days and subsequently incubated at 37°C for 4 days. In addition, two steps of filtration have been recommended before the TEM suspension is plated on culture media [148]. However, a certain degree of controversy exits between reports in regards of the time the TEM is able to maintain Cfv viability. One study reported to maintain Cfv viability for 24 h only at room temperature and not when refrigerated [143], while another study indicated good recovery after 2 to 4 days at room temperature, depending on the inoculum size [149]. It was suggested that this variability was due to differences in the modified atmosphere within different tubes, and two tubes should be inoculated.

Comparison of the Australian TEM with the mWTEM indicated that the latter was significantly more efficient, with isolation rates of 25% and 75% respectively, and better control of contamination [150]. Moreover, the viability of Cfv strains could be maintained for a longer period in the mWTEM [143]. Although studies comparing both media are scarce, evidence indicates that the mWTEM can maintain the viability of Cfv for a longer period of time and under a wider range of temperatures. Furthermore, given the need to replace the atmosphere in the tube to provide a microaerophilic environment and the laborious procedure for its preparation, use of the Australian TEM has been discouraged by some authors [143,150].

The superiority of mWTEM compared to the Australian TEM could at least partially be explained by the reduced concentration of polymixin B, known to be inhibitory for some Cfv strains [150]. In this regard, Hardwood et al. [151], developed the Thomann (TTE) media by replacing some of the ingredients of the mWTEM and decreasing the antibiotic concentrations by 4 or 5 times. *In vitro* studies with the TTE medium show survival times of up to 8 days of transport at temperatures from 8 to 37°C. Although this medium appears promising, further studies in field conditions are required.

The majority of the transport media developed have been aimed at maintaining the viability of Cfv for subsequent culture. The development of other diagnostic tests has not necessarily resulted in new media being developed but rather continues to use the ones available. The use of transport media for PCR testing should be evaluated carefully. At least some evidence exists that ingredients such as blood can act as inhibitors for the PCR thus affecting its performance [151]. As a result, PCR assays developed to be used directly on preputial samples have suggested the use of buffered saline as a transport media, as viability of the organism is not needed [125,152].

1.5.3 Culture based methods

Culture methods for *Tritrichomonas foetus*

Identification of *T. foetus* is done based on the observation of characteristic morphology and jerky movement by bright field microscopy (100X) [94]. Sensitivity of direct examination from preputial secretions has been reported to be 68-90% [115,116]. However, direct examination of preputial smegma is effective and practical for diagnosing trichomonosis only when few bulls are to be examined and laboratory facilities are nearby [94].

Diagnostic methods based on culture are significantly more effective than direct examination of preputial samples [130]. Culture of *T. foetus*, regardless of the medium used, is usually carried out for up to 10 days at 37°C, performing daily microscopic examinations [119,153-156]. Culture sensitivity has been estimated to be 73-97% for bulls [36,119,123,154]. Several media have been described that are suitable for *T. foetus* growth such as: Schneider egg shell [157]; modified Plastridge [155]; Claussen's; Diamond's [153]; and InPouch™ TF® [158] and Trichomonas CM161 [159].

The InPouch™ TF® test became available in 1990 [158], and is now one of the most widely used diagnostic methods [160]. This test is a flexible plastic pouch formed by two chambers containing 4 ml of a proprietary medium. The sample is inoculated into the upper chamber, and the liquid forced into the lower chamber. The pouch is then incubated in a vertical position concentrating the organisms in the bottom. Microscopic evaluation is performed directly through the pouch walls using the re-usable clip viewer. This method has several advantages compared to other culture media such as: smaller inoculum is required to yield a positive result; less time consuming; early detection; used both as a transport and culture media; less susceptible to

breaking and leakage; can be stored at room temperature for one year, and prevents contamination by avoiding the need to open the pouch for examination [154,158,160].

Sensitivity estimates for some of the common culture media used are shown in Table 1.3. Diamond's media and the InPouch^{TM} TF are the two most commonly used media. Although some studies have shown no difference in sensitivity when Diamond's media was used compared to the InPouch^{TM} TF® [123,158,161], one study showed an improved sensitivity when samples were cultured on the InPouch^{TM} TF® than when a transport medium (thioglycolate) plus Diamond's medium was used (OR=6.9) [154]. Moreover, several studies have shown that samples inoculated in the InPouch^{TM} TF® are detected within 36 to 72 hours after incubation inoculation due to greater initial growth rate than in Diamond's medium [58,74,158,160]. Samples inoculated in the InPouch^{TM} TF® allowed earlier detection than in Diamond's medium, even though fewer organisms were inoculated in the InPouch^{TM} TF, and organisms survived longer in the InPouch ^{TM} TF [160]. In addition, contaminant overgrowth is better controlled by the InPouch^{TM} TF, than by Diamond's medium [58].

The specificity of culture techniques has been assumed to be 100% [36,123]. However, the finding of motile trichomonads similar to *T. foetus* indicates that the current "gold standard" may not be 100% specific [37,156]. Recent estimates of specificity with different culture media indicate a specificity of 91 to 98% [139,156,162]. The presence of non-*T. foetus* trichomonads has been reported to be responsible for up to 17% of false positive cultures in breeding bulls [163]. Although this report was based on samples sent for confirmation of the identity of organisms seen during routine examinations, it still indicates that the occurrence of false positive cultures should be considered.

Several other trichomonads, thought to be gastrointestinal inhabitants, have been detected in preputial samples from both mature and virgin bulls, e.g., *Tetratrichomonas* spp., *Pseudotrichomonas* spp. and *Pentatrichomonas hominis* [164]. The presence of these organisms in the preputial cavity of virgin bulls suggests that they are transmitted by mounting and sodomizing, a common behavior in young bulls, however the reason for their presence in mature bulls remains unknown [37,163]. Morphology of these organisms is similar to *T. foetus*, although a more rounded shape, usually 4 or 5 anterior flagella, plus a slower growth rate and decreased survival in subculture are distinct features of these organisms [37,156,164]. Unfortunately these characteristics are not easily seen by routine microscopy. Therefore, more complex and time consuming techniques such as stained smears [165] or electron microscopy [156] should be performed. Challenge studies with *P. hominis* and *Tetratrichomonas* spp. indicated that these organisms could not persistently colonize the prepuce or the vagina and were non-pathogenic [166,167].

Culture methods for *Campylobacter fetus* subsp. *venerealis*

Isolation by culture methods is the most common technique used for the diagnosis of BGC [142]. Given that Cfv is microaerophilic it requires an atmosphere composed of 85% nitrogen, 10% carbon dioxide and 5% oxygen [88]. Two different selection methods have been used in order to allow growth of Cfv while preventing overgrowth by saprophytic bacteria and other contaminants. These two methods are the use of filtration of the sample or the use of selective culture media containing antibiotics.

Selective media used for the isolation of Cfv have a nutrient agar base with inclusion of blood at 5 to 10% [88,168]. The antibiotic combination used has been the most variable component,

although usually differences between media rely on the exchange of one drug or the modification of its concentration. The two most common media are campylobacter selective agar (CSA) and Skirrow's agar [150].

CSA contains novobiocin, bacitracin, polymixin B, and cycloheximide [169]. The sensitivity of CSA ranges from 64 to 94% when samples are plated within few hours of collection [135,150,169-171]. Skirrow's agar has been extensively used for the isolation of thermophilic campylobacters and contains vancomycin, trimethoprim and a higher concentration of polymixin B, than CSA agar [172]. Sensitivity of Skirrow's agar ranges from 32 to 68% [134,147,150]. Although there appears to be a great difference in the performance of both media, only one study has compared them directly [150]. In this study, CSA was found to be more sensitive, but only 19 samples were evaluated. Given that some strains of Cfv have been found to be susceptible to polymixin B, the use of CSA, which has a lower concentration of this antibiotic, may explain the improved isolation rates [150].

Filtration of the samples before culture with a 0.65 μm pore size filter has also been recommended for the isolation of Cfv [173]. Traditionally this method was performed by forcing the sample through the filter using positive pressure [168]. The use of this method has resulted in isolation rates similar to those obtained with selective agar in some studies and slightly lower in others [168,169,174]. The greatest concern when using this method has been the reduction of colony counts by up to 90% [169]. The use of positive pressure may cause obstruction of the filter by tissue or other particulates in the samples. An improved method using passive filtration, allowing for the motile Cfv to migrate through the filter onto the surface of the culture plate, has been described and used extensively for the isolation of campylobacters from other types of samples [175-178]. More recently, this approach has been used in preputial samples and resulted

in a sensitivity of 87%, performing significantly better than Skirrow's agar when direct culture was used [147]. This method provides a simple and cost effective technique for the isolation of Cfv.

One of the major drawbacks of using culture methods lies in the common overgrowth by contaminants. Contaminants commonly found are *Pseudomonas aeruginosa*, *Proteus* spp. and fungi [134,147,168]. Although the selective media previously described contain antibiotics to control for these organisms, *Pseudomonas* continues to be a problem as it appears that at least certain isolates are resistant and complete control is not achieved [134,168]. The use of higher concentrations of agar in the media has been shown to improve the control of spreading organisms [135]. The use of passive filtration has been shown to control overgrowth of both organisms effectively [147].

Contamination with fungi reduces the surface available for recovery of Cfv, and the use of CSA appears to control fungal contaminants by the addition of cycloheximide. Conversely, Skirrow's agar commonly available from commercial sources does not contain any antifungal agents and as a result it has been recommended that either cycloheximide or amphotericin B be added to the formula [173]. As with bacterial contaminants, the use of filtration techniques can control fungal overgrowth [147].

The use of culture methods requires that upon isolation of colonies consistent with Cfv, phenotypic identification procedures be performed for identification. *Campylobacter fetus* are generally catalase positive, do not produce H_2S in conventional media, are sensitive to cephalothin, negative for hippurate hydrolysis and do not grow at 42°C or in the presence of 3.5% NaCl [179]. In order to differentiate Cfv and Cff, tolerance to 1% glycine and H_2S

production in cysteine media have been recommended [9]. However, the World Organization for Animal Health indicates that glycine tolerance is the gold standard method for subspeciation [173].

Phenotypic characterization of Cfv is influenced by the inoculum size and the basal media used; resulting in poor reproducibility [30,180]. As a result, intermediate strains of Cfv, tolerant to 1% glycine, have been described [50,181]. Some authors suggest that this could be due to the lack of test standardization [182]. Moreover, the use of molecular techniques such as PCR and AFLP has raised questions about whether tolerance to glycine is an appropriate method for subspeciation of *C. fetus* [183].

Culture methods are time consuming and their sensitivity decreases when samples are not processed within a few hours of collection. The use of both selective agar and filtration methods has been suggested by some authors in order to increase sensitivity, however this increases cost and time [168].

1.5.4 Immuno-based diagnostics

Tritrichomonas foetus antigen detection tests

The production of antibodies in response to *T. foetus* infection has been reported [184] and an ELISA test has been reported for detection of antibodies in preputial samples [112], although it has not been used for diagnostic purposes.

Corbeil et al. [163] developed an immunofluorescent assay (IFA) using the monoclonal antibody (MAb) TF1.15 in a two step method along with culture to detect *T. foetus* and avoid false positive results by non-*T. foetus* trichomonads in preputial samples. Since IFA had a complete

agreement with 5.8S rRNA PCR, this monoclonal antibody has the potential for use in development of immunodiagnostic assays that could be used in labs with no access to PCR [163]. However, there is a lack of information on the sensitivity and specificity of the IFA assay.

Campylobacter fetus subsp. *venerealis* antigen detection tests

The challenges faced with culture methods, particularly with prolonged transport times, led to development of diagnostic tests based on the recognition of Cfv antigens thus removing the need to maintain the viability of Cfv.

The direct fluorescent antibody test (DFAT) detects Cfv by means of a polyclonal antibody labeled with fluorescein isothiocyanate (FITC) [185]. Preputial samples are subjected to centrifugation, fixed to slides and subsequently incubated with the conjugated antibody [186]. Slides are visualized with a fluorescent microscope and Cfv is identified based on fluorescence and morphological characteristics. One of the advantages of this test is that the sample is collected in a buffered formaldehyde solution and can be maintained refrigerated in this media for several days [61]. In addition, results can be obtained within hours of receiving the sample. Although the DFAT is less time consuming than culture methods, examination of a large number of samples is laborious and expertise is needed in order to correctly identify the organism.

The DFAT has a detection limit of 10^2 cells/ml of sample when centrifugation is used [186]. The use of DFAT has been shown to have comparable or greater sensitivity when compared to culture methods [185,187,188]. The sensitivity of this test has been reported to range from 80 to 94% [135,169,186]. On the other hand, the specificity of the DFAT has been estimated at 89%, and one of the major concerns has been the cross reaction with Cff serotype A [186,187]. Although subspeciation is not possible with this test, the advantages related to transport

conditions and turnaround time for results have made this test very popular in South America and Australia [61,169].

A MAb capture ELISA was recently developed for the detection of *C. fetus* in preputial samples. This assay uses 4 monoclonal IgG antibodies against epitopes in the LPS. Two of these MAb react with the O-antigen of *C. fetus* serotype A, one with Serotype B and the remaining one (MAb M1825) identifies a core epitope of the LPS common to both serotypes [189]. A preliminary assessment of this assay indicated a detection limit 10^5-10^7 CFU/ml depending on the strains. The sensitivity was significantly higher than culture methods and estimated at 98 to 100% [27,189]. In order to be applied to large number of samples, MAb M1825 is used and those testing positive are serotyped with all four MAb. The specificity was estimated to be 99.5%, however the assay is unable to differentiate between Cfv and Cff as they share the serotype A. Although this test appears to be suitable as a screening method for large numbers of animals, samples must still be collected in to TEM [189]. Whether simpler transport media could be used is unknown and requires further research.

1.5.5 Molecular diagnostic techniques

Tritrichomonas foetus molecular based tests

Conventional diagnostic techniques are based on identification of *T. foetus* by morphological characteristics and thus are subjected to specificity problems [190]. The *T. foetus* ribosomal RNA (rRNA) operon is repeated 12 times in tandem [191]. Discrimination of trichomonads relies on size variations of the internal transcribed spacer (ITS) regions within the rRNA operon, as well as sequence differences. *T. foetus* shares less than 70% sequence identity with commonly detected *P. hominis* and *Tetratrichomonas* [192].

Comparative sequence analysis of the 5.8S rRNA gene and flanking ITS regions using primers TFR1 and TFR2 showed *Tritrichomonas* species produced a 372 bp amplicon, while products from other trichomonads were slightly smaller or larger [192,193]. These results were confirmed by a later study [192] where three distinct clusters were determined, formed by *T. foetus* isolates, *P. hominis* and the last one thought to be related to *Tetratrichomonas* sp.. The results obtained by Felleisen et al [193] lead to a later study by the same group where a PCR assay was developed targeting the 5.8S rRNA gene and ITS regions of *T. foetus*. In this assay, primers TFR3 and TFR4 yield a 347 bp product from *T. foetus*. A DNA enzyme immunoassay (DEIA) was used as a detection system, with probe TFR8-Bio targeting a portion of the 5.8S rRNA gene.

The assay developed by Felleisen et al. showed excellent specificity, but the detection limit was lower than cultured samples due to inhibition when direct samples were used. By using in vitro propagated culture as a sample the combined sensitivity of the test was improved [190]. However, others have found that direct samples could be used successfully when using commercial DNA extraction kits, resin and agar, or even without DNA isolation [58,140,194]. In addition, it has been suggested that some samples needed to be diluted 1:10 after extraction, likely to dilute the effect of inhibitors, without affecting availability of the target [195].

The PCR assay with primers TFR3 and TFR4 became one of the most widely used tests for the detection of *T. foetus*. These primers have been applied to a variety of samples and objectives such as: formalin fixed tissues and cervico-vaginal mucus (CVM) [196], semen samples [197], and to differentiate *T. foetus* from other trichomonads after culture in a two-step method [89,156,163]. The detection limit of this assay is 50-100 organisms/ml [127,195]. Excellent agreement between culture and PCR using primers TFR3 and TFR4 has been reported [58,68,72,198]. This PCR is believed to have comparable or even improved sensitivity over

culture, and sensitivity estimates range from 66 to 98% [68,127,139]. The main disadvantage of this technique is that it requires the processing of the sample after amplification so as to be visualized in agarose gels, meaning the testing of large number of samples at once is time consuming.

The intensive study of the rDNA unit of *T. foetus* led to the design of a 5' Taq nuclease assay using a fluorescent probe with a 3' minor groove binder targeting the ITS-1 region [126]. The detection limit of this real-time PCR is a single cell equivalent per reaction and is significantly better than conventional PCR [126,199]. Extraction of DNA from preputial samples for this assay has been performed successfully in column based kits, automated magnetic bead extraction systems and the heat lysis method [199]. The heat lysis method yielded the best detection limit, although is more time consuming than the automated systems [126,199].

It is of particular interest that enrichment culture prior to real-time PCR showed a lower analytical sensitivity than when direct samples were used [126]. Although this is thought to be related to a potential increase in inhibitors when culture is performed, the assay does not appear to be affected by contaminants such as bacteria, semen or blood when using crude heat lysis extracts [126]. This assay allows for reliable quantification of the number of DNA copies. However, no conclusions on the number of organisms in the actual prepuce can be drawn until a standardized method of sampling is adopted.

The use of the Taq nuclease assay has been widely accepted and shows a 96-97% agreement with culture in the InPouch [200,201]. The analytical sensitivity of this real-time PCR is significantly better than conventional culture methods [126]. Furthermore, it is believed to be more sensitive than culture and microscopic examination. However, estimates of clinical

sensitivity are not reported in the literature [200]. Finally this type of technique avoids the processing of the sample post amplification, thus reducing the possibility of contamination in the laboratory and eliminates the potential of false negative results when low amounts of sample are loaded in the gel for visualization [196].

The specificity of conventional PCR has been estimated to be 98 to 100% [139,195]. However, the introduction of the real-time PCR has brought about questions regarding its specificity. These have mainly originated from a field study in which culture, conventional PCR and real-time PCR were compared [198]. In this study, the gold standard was a positive result in both culture and conventional PCR, and as a result animals positive only with the real-time PCR were classified as false positives. Whether these are true false positives or reflect the greater sensitivity of the real-time PCR test is not known and further research is needed. However, the assay has been tested with several organisms commonly found in preputial samples and none result in amplification [167]. In addition, even samples with high threshold cycle values ($C_{t),}$ as a result of lower concentration of the PCR target in the sample, have been confirmed to be *T. foetus* by amplicon sequencing [202].

An interesting feature of PCR based methods is the high sensitivity this type of technique provides. As a result, research has been done to test the possibility of pooling samples. A study by Kennedy et al. [203] tested pools of 5 individual samples using primers TFR3 and TFR4 in conventional PCR. Results from this study are quite promising showing sensitivity and specificity relative to individual PCR of 100%. Moreover, pools of five samples have been tested after 48 h incubation resulting in a sensitivity of 100% and a specificity of 97.2% [204]. However, further research should be performed on the feasibility of larger pool size and whether pre-enrichment in culture is needed.

Other PCR methods targeting the 5.8S rRNA or the 18S rRNA genes have been developed but information on their performance on clinical samples is limited [205-207]. In addition to PCR, other molecular diagnostic techniques have been used, such as RFLP, RAPD and SSCP [133,208,209]. However, these techniques are usually more time consuming than PCR and have been utilized for discrepant results when developing new tests [210] or for characterizing isolates in epidemiological studies [133]. The latest technique adopted for *T. foetus* diagnosis is LAMP (loop mediated isothermal amplification) a technique that does not require expensive equipment. This assay showed a detection limit similar to that of conventional PCR; however, further research on clinical samples is needed. This assay could provide a valuable tool in smaller laboratories without access to PCR [211].

Campylobacter fetus subsp. *venerealis* molecular based tests

The development of molecular diagnostic techniques for the detection of Cfv has been based on the need for faster and more accurate methods. Given the fastidious requirements of Cfv, culture diagnosis is difficult under field conditions. The development of molecular techniques can potentially overcome this problem as there is no need to maintain the organism in a viable state. In addition, the development of molecular based techniques has been aimed at allowing subspeciation and as a result shed some light on the differences between Cff and Cfv.

Several species-specific PCR methods have been developed for *C. fetus,* especially when looking at fecal samples in order to differentiate from other campylobacters or from other pathogens. PCR assays targeting the 23S rDNA unit and cpn60 have been developed and applied mainly to fecal samples [212,213]. Use of the 16S rDNA unit as a target has also been studied; and

although useful at the species level the high similarity between subspecies has precluded its use for Cfv detection [214-216].

Hum et al. [217] described a multiplex PCR test for the detection and subspeciation of *C. fetus* that has been used worldwide. This test uses two pairs of primers: MG3F/MG4R, which targets the carbon starvation gene present in both Cff and Cfv, and VenSF/VenSR, which targets the parA gene present in Cfv [217]. The agreement between this PCR test and phenotypic characterization of *C. fetus* isolates has been reviewed elsewhere [152]. Overall agreement with the glycine tolerance test has ranged between 70 and 100% in over 600 isolates [62,182,183,217-221]. Moreover the agreement with alternative typing methods such as AFLP, MLST, PFGE and other PCR tests ranged from 71 to 100% [183,218,219,221,222]. Although the primers designed by Hum seem to have good overall agreement with phenotypic methods, some significant discrepancies have been reported. One of these discrepancies was on 17 isolates classified as Cfv based on phenotypic characteristics, obtained from heifers or aborted fetuses in the UK [220], and the other case was on 40 Cfv biovar *intermedius* isolates from South Africa [181]. It is not clear whether differences in isolates from these two geographic locations could account for the failure of the Hum PCR to classify them as Cfv or whether differences in the conditions of the phenotypic tests could lead to misidentification as previously suggested [30,222].

The multiplex PCR described by Hum has been applied extensively. However, the use of this test has been usually limited to the identification of isolates after culture [147]. Only one study has reported the use of this assay in preputial samples collected in mWTEM. The assay showed a sensitivity of 85.7% and a specificity of 99% as compared to culture. Prolonged storage in mWTEM media beyond 24 h was detrimental for the detection of Cfv, and this was attributed to contamination with *Pseudomonas* which produced DNase enzymes [181]. On the other hand,

contaminants such as blood, urine and semen did not affect the performance of the assay. However, the presence of more than 2% of feces caused a 10 fold decrease in analytical sensitivity [181]. Given the need for processing after PCR for the detection of amplicons, the use of this conventional PCR would be time consuming when a large number of samples are required to be processed.

Two real-time PCR tests for the detection of Cfv have been reported both targeting the *parA* gene [125,152]. The first one is a 5' Taq nuclease assay using a fluorescent probe [125], while the second one uses the Cfv specific primers designed by Hum, validated with several hundred isolates, adapted to a SYBR Green qPCR platform [152]. Both assays used a heat lysis DNA extraction method in direct preputial samples with a detection limit of a single cell per reaction. The detection limit is significantly better than that of the conventional Hum multiplex PCR and culture. Moreover, in field trials the real-time PCR appeared to be more sensitive than culture [125].

Although the performance of these tests appears to be useful for the diagnosis of Cfv in bulls, no estimates on clinical sensitivity and specificity have been reported for either test. Both assays target the same gene, but primer annealing sites are different. The report of a novel Cfv sequence type found recently would result in mismatches in one of the primer landing sites with the Taq nuclease assay, but not with the SYBR Green assay [152]. An interesting feature of these tests is the possibility of quantification of Cfv in samples, providing valuable information on the pathogenesis of the disease. However, the lack of standardized methods of sample collection that would provide a measurable volume of samples precludes the applicability of this information to a better understanding of Cfv pathogenesis [152].

Of greater concern has been the report of misidentification of a *C. hyointestinalis* isolate as Cfv, found in bovine feces, by both sets of primers used in the real-time PCR assays [223]. The *parA* gene is believed to be included in a genomic island unique to Cfv [47,53]. This island has been identified as a pathogenicity island containing virulence factors specific for Cfv; however, mobility genes have also been identified [47]. The presence of these mobility genes indicates the possibility of horizontal gene transfer. Whether this phenomenon is responsible for the misidentification reported previously remains unknown and warrants further research.

Another target for the detection of Cfv is an insertion element named ISCfe1 [221]. This element is present one or more times in the genome of Cfv. A set of primers named CVEN-L and CVEN-R2 have been developed using ISCfe1 as a target, and have shown 100% agreement with phenotypic characterization based on glycine tolerance, and 98% agreement with the Hum primers. Other primers have been designed based on the ISCfe1 element and used to develop a LAMP assay that had 100% agreement with phenotypic profiling [224]. Furthermore, a Taqman real-time PCR has been recently developed targeting the ISCfe1 element and insertion sites for use on samples collected in a commercial culture kit used routinely for *T. foetus* diagnosis [225]. However, the use of assays targeting the ISCfe1 directly in preputial samples has not been adopted with the exception of the Taqman test, and sensitivity and specificity estimates are not available.

The complete genome of the Cfv type strain (ATCC 19438) has been recently sequenced [226]. As a result, full genome comparisons between Cff and Cfv have been made possible. The first report of the comparison of both organisms has been published and the two type strains show a core-genome shared by both subspecies. In addition, three pathogenicity islands were particularly studied due to differences between Cff and Cfv [53]. One of these genomic islands

was previously described and contains the *parA* gene used as a target for both conventional and real-time PCR assays [47,152]. This island also contains several genes involved in a type IV secretion (T4SS) system involved in transmission of proteins to host cells [47]. Some of the genes (e.g. VirB11) involved in T4SS have been selected as potential targets for Cfv detection and applied in a PCR test for Cfv and Cfv biovar *intermedius* detection in samples from aborted fetuses [227]. Further research is needed to evaluate the use of this assay in preputial samples.

1.6 CONTROL STRATEGIES

Several control strategies for venereal diseases are available including identification and culling of infected animals, treatment and preventative or therapeutic vaccination. Vaccination and treatment strategies will not be discussed here, but rather this discussion will focus on strategies based on the identification of carrier bulls.

The identification of carrier bulls and subsequent culling has been used extensively [228]. However, several factors can affect the ability to accurately detect infected bulls such as appropriate sample collection and handling, transport time and conditions, test used and its sensitivity, and variations in the level of infection. Despite significant research done in optimizing sample collection, transport and developing sensitive diagnostic tests, the use of a single sample has been unsuccessful in identifying all carrier bulls in a given herd. Periods as long as 4 or 8 months where infection cannot be detected in *T. foetus* infected bulls have been reported in some bulls [116]. Although these bulls are referred to as atypical bulls, other studies showed that only 50 to 68% of the bulls are consistently positive in every sample collected [115,198]. This has also been demonstrated in Cfv infected bulls although precise estimates were not reported [88].

Repeated sampling has been deemed necessary in order to circumvent this issue. Recommendations on the number of consecutive tests needed to certify that a bull is free of infection have ranged from two to up to six [22,61,162,171]. The use of up to four consecutive samples test by culture for *T. foetus* increased the sensitivity from 73 to 99% [75]. Repeated sampling and testing by conventional PCR has also resulted in increased sensitivity [68,139]. Information regarding repeated sampling for Cfv detection is scarce in the literature. One study indicated that the sensitivity of culture of semen samples increased from 39% to 95% when three consecutive samples were used [88].

Despite general recommendations provided in the literature, the resulting strategy should be carefully assessed based on each situation. The sensitivity and specificity of the test to be used and the pre-test probability of infection should dictate whether to perform a single or multiple tests [120]. These factors determine the predictive value of the test; as a result the estimated pre-test probability or prevalence will dictate the strategy to take. For example, in a closed herd where virgin bulls are consistently used, the pre-test probability is low and as a result the negative predictive value will tend to be higher and a single test may be sufficient to establish that a certain bull is free of infection. Conversely, when the prevalence is high multiple tests may be necessary.

Another strategy used has been the performance of two tests in parallel. This approach has been suggested to be used for the detection of *T. foetus* by combining culture and conventional PCR [139]. This approach resulted in sensitivity comparable to that of two cultures or two PCR tests in samples from consecutive weeks. The use of multiple tests in parallel has been proposed for Cfv detection as well by using DFAT and culture or by using two culture methods such as selective media and filtration [146,187]. The addition of PCR methods to this strategy has not

been reported, however, it is possible to expect a similar increase in sensitivity as that seen for *T. foetus*.

Another study used conventional PCR and culture for *T. foetus* detection but interpreted in series when used in 3 consecutive samples. The use of this approach maximized the positive predictive value and allowed for the identification of 87, 95 and 100% of the infected bulls in 1, 2 and 3 consecutive samples [198]. Recently, the establishment of mandatory testing for bulls entering a state resulted in the requirement of either 3 culture negative samples or a single negative PCR test to demonstrate freedom from infection. The results from the study by Ondrak et al. [198], does not support this decision when using culture and conventional PCR, at least for regulatory purposes. On the other hand, their recommendation was based on a study conducted on an infected herd with relatively high bull prevalence.

During the past 10 years several states within the United States have incorporated mandatory testing of bulls for *T. foetus*. Although the regulations vary by state, most of them require testing for bulls being imported from other states and several have also included bulls being sold for breeding purposes within the state [68,229]. The use of mandatory testing has yielded promising results as reported in New Mexico where the prevalence of *T. foetus* in bulls has decreased from 6.3% to 2.8% in two years by using real-time PCR [230]. For Cfv, no current estimates of prevalence have been reported for North America, thus making it difficult to assess the current status of the disease. The lack of estimates is probably a reflection of the small use of traditional diagnostic methods available due to their poor performance when laboratories are in distant locations. It is possible that the implementation of newer tests such as real-time PCR will allow for large epidemiological studies to be conducted and prevalence estimates obtained.

Although *T. foetus* prevalence is declining in those states with mandatory testing, testing bulls alone will probably not be enough to eradicate the disease. The reasons for this were recently described and include the lack of consistency in sample collection, the lack of a test with 100% sensitivity, and the time and conditions during transport to the diagnostic laboratory [231]. In addition, carrier cows have been described, although there incidence is low [232]. As a result other measures that could aid in the control of this disease should be considered and added to current control programs.

1.7 RESEARCH OBJECTIVES

1. To determine the specificity of a real-time PCR test for the detection of *T. foetus* in individual preputial samples collected in a commercial culture kit.
2. To determine the sensitivity of a real-time PCR test for *T. foetus* in cultured enriched samples pooled in groups of up to 25.
3. To determine the sensitivity of a real-time PCR test in individual preputial samples with or without culture enrichment for the detection of *T. foetus*.
4. To determine the sensitivity of a real-time PCR for the detection of *T. foetus* in direct preputial samples pooled in groups 5 and 10 samples.
5. To determine the sensitivity and specificity of a real-time PCR for the detection of *Campylobacter fetus* subsp. *venerealis* in direct preputial samples.

Table 1.1. Bovine trichomonosis individual prevalence estimates in bulls of different geographic areas.

Country	Region	N	Test	Prevalence	Source†	Study
Argentina	La Pampa	5313	culture	1.3	Screening	Fort et al., 2004 [233]
Australia	WA	504	culture	1.2	Abattoir	Turnbull et al., 1977 [234]
Australia	NT/Qld	265	culture	30.2	Abattoir	Ladds et al., 1973 [235]
Botswana		210	PCR	3.3	Laboratories	Madoroba et al., 2011 [59]
Canada	SK/MB	569	culture	8.4	Laboratories	Copeland et al., 1994 [236]
Colombia		146	culture	13.7	Screening	Griffiths et al., 1984 [69]
Costa Rica		378	culture	5.3	Screening	Perez et al., 1992 [74]
Namibia		1201	PCR	3.7	Laboratories	Madoroba et al., 2011 [59]
S. Africa	Transkei	87	culture	26.4	Screening	Pefanis et al., 1988 [80]
S. Africa		1999	PCR	4.5	Laboratories	Madoroba et al., 2011 [59]
S. Africa		193	culture/PCR	5.2	Laboratories	Mukhufhi et al., 2003 [127]
Spain	NW Spain	103	culture/PCR	32	Screening	Mendoza-Ibarra et al., 2012 [58]
Spain	NW Spain	70	culture	2.9	Screening	Martin-Gomez et al., 1998 [237]
USA	14 states	2909	culture	0.17	Abattoir	Grotelueschen et al., 1994 [238]
USA	FL	1975	culture	6.0	Survey	Rae et al., 2004 [93]
USA	TX	31202	qPCR	3.7	Mandatory	Szonyi et al., 2012 [60]
USA	NV	2389	culture	4.7	Screening	Kvasnicka et al., 1989 [70]
USA	AL	347	culture/PCR	0.27	Laboratories	Rodning et al., 2008 [239]
USA	CA	729	culture	4.1	Screening	BonDurant et al., 1990 [71]
USA	WY	8222	culture/PCR	0.21	Mandatory	Yao et al., 2011 [68]
USA	LA	126	culture	5.5	Screening	Richardson et al., 1986 [240]

†Mandatory (refers to estimates obtained from government enforced testing of breeding bulls); Abattoir (refers to samples collected from bulls sent for slaughter); Laboratories (refers to estimates obtained from diagnostic laboratories from samples received in a defined period of time); Screening (based on random or convenient testing of a population of bulls from different herds); Survey (based on a stratified random sampling procedure of herds in a defined geographical area).

Table 1.2. Bovine genital campylobacteriosis individual prevalence estimates in bulls of different geographic areas.

Country	N	Test	Subspeciation	Prevalence	Source†	Study
Argentina	6763	DFAT	No	0.9	Screening	Fort et al., 2004 [233]
Australia	504	culture	Cfv	4	Abattoir	Turnbull et al. 1977 [234]
Belgium	329	culture/DFAT	NR	12.5	Screening	Bouters et al., 1973 [56]
Brazil	327	DFAT	No	52.3	Screening	Pellegrin et al., 2002 [79]
Canada	67	culture/DFAT	NR	29.8	AI center	Ruckerbauer et al., 1974 [187]
Canada	105	culture/DFAT	NR	16	AI center	Garcia et al., 1983 [141]
Canada	200	culture/DFAT	NR	0.5	Abattoir	Finlay et al., 1985 [241]
Canada	529	Elisa/culture	Cff	3.4	AI center	Devenish et al., 2005 [27]
Colombia	146	culture	Cfv	15	Screening	Griffiths et al., 1984 [69]
Namibia	1201	PCR 16SrRNA	No	2.08	Laboratories	Madoroba et al., 2011 [59]
Nigeria	585	culture	Cfv	2.1	Screening	Bawa et al., 1991 [76]
S. Africa	87	culture	Cfvi	17.2	Screening	Pefanis et al., 1988 [80]
S. Africa	1912	PCR 16SrRNA	No	1.78	Laboratories	Madoroba et al., 2011 [59]
Tanzania	58	culture	Cfv	5.1	Screening	Swai et al., 2005 [242]
Zambia	41	PCR 16SrRNA	No	2.44	Laboratories	Madoroba et al., 2011 [59]

† Abattoir (refers to samples collected from bulls sent for slaughter); Laboratories (refers to estimates obtained from diagnostic laboratories from samples received in a defined period of time); Screening (based on random or convenient testing of a population of bulls from different herds); AI center (based on sampling for control purposes in artificial insemination centers).

Table 1.3. Sensitivity estimates of different culture media used for the detection of *Tritrichomonas foetus* in preputial samples. PBS (Phosphate buffered saline), TFTM (Thioglycolate transport media).

Culture media	Bulls	N° samples from carrier bulls	Transport media	Source	Sensitivity (%)	Study
Clausen's	14	167	PBS	Infected bulls	73.5[B]	Schonmann et al., 1994 [123]
Diamond's	195	225	Lactated Ringer	Field samples	81.6	Skirrow et al., 1985 [92]
	150	126	Diamond's	Field samples	96.8	Appell et al., 1993 [161]
	14	555	PBS	Infected bulls	93.2	Schonmann et al., 1994 [123]
	21	168	TFTM	Infected bulls	76.8[B]	Parker et al., 2003 [154]
	86	39	Diamond's	Infected bulls	97.4	Thomas et al., 1990 [158]
	10	44	PBS	Infected bulls	81.8	Ho et al., 1994 [243]
InPouch^{TM} TF	1383	NR	InPouch	Field samples	73	Rae et al., 1999 [75]
	86	54	InPouch	Field samples	70.4	Peter et al., 1995 [244]
	150	126	InPouch	Field samples	98.4	Appell et al., 1993 [161]
	14	167	PBS	Infected bulls	88[A]	Schonmann et al., 1994 [123]
	21	168	InPouch	Infected bulls	95.8[A]	Parker et al., 2003 [154]
	86	39	InPouch	Infected bulls	94.9	Thomas et al., 1990 [158]
	79	180	InPouch	Infected bulls	67.8	Cobo et al 2007 [139]
Mod. Plastridge	2832	NR	Mod. Plastridge	Field samples	72	Perez et al., 2006 [162]
	3	98	Peptone	Infected bulls	98.9	Tedesco et al., 1979 [130]
	13	143	Bact. broth	Infected bulls	97	Clark et al., 1971 [119]
Trichomonas CM161	3	30	PBS	Infected bulls	96	Irons et al., 2002 [128]
	832	115	PBS	Field samples	100	Ribeiro et al.,1990 [159]
	5	29	PBS	Infected bulls	83	Mukhufhi et al., 2003 [127]

[A,B] Different superscripts between estimates obtained in the same study indicate significant differences ($P<0.05$).

2. USE OF POOLED PREPUTIAL SAMPLES ENRICHED BY CULTURE FOR THE DETECTION OF *TRITRICHOMONAS FOETUS* BY REAL TIME POLYMERASE CHAIN REACTION

Alvaro García Guerra, DVM; Janet E. Hill, BSc, PhD; John Campbell, DVM, DVSc; Cheryl L. Waldner, DVM, PhD; Steven H. Hendrick, DVM, DVSc

From the Department of Large Animal Clinical Sciences (Garcia Guerra, Campbell, Waldner, Hendrick) ; and the Department of Veterinary Microbiology (Hill), Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5B4. Address correspondence to Dr. Hendrick (steve.hendrick@usask.ca).

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2.1 ABSTRACT:

Objective—To determine the sensitivity of a real-time PCR for the detection of *Tritrichomonas foetus* in pooled preputial samples enriched by culture in groups of up to 25 bulls. And to determine the specificity of the real-time PCR in individual cultured enriched samples.

Design—Cross-sectional study.

Animals—188 bulls and 150 steers.

Procedure— Preputial scrapes were collected from 150 steers and 187 virgin bulls using the aspiration method. Samples were placed into a commercial culture kit, transported to the lab within 4 h, and incubated at 37°C (98.6°F) for 7 days. In addition, weekly samples were collected from a naturally infected bull. Cultures were examined microscopically on alternate days. All samples were tested individually by real-time PCR. Pools of preputial samples were made by including fixed aliquots of known positive and negative samples in ratios of 1/2, 1/3, 1/5, 1/10, 1/15, 1/20, and 1/25. Thirty one pools were made for each ratio. Specificity of individual samples collected into a commercial culture kit was calculated for both culture and real-time PCR. Sensitivity of pooled samples at different ratios was determined by the proportion of pools testing positive, and differences between pool ratios were determined by generalized estimating equations.

Results—Specificity estimates in individual culture enriched samples were 98.8% and 100% for culture and real-time PCR respectively, and were not significantly different. Sensitivity of real-time PCR for different pool ratios, created using positive samples from one bull, was not significantly different, with an overall sensitivity 94%.

Conclusions and Clinical Relevance—The evaluated real-time PCR assay for trichomonosis presents a high specificity and has good sensitivity in pools of up to 25 samples.

2.2 INTRODUCTION

Reproductive performance is one of the most important factors affecting the economic success of cow-calf operations [1,2,245]. Early pregnancy loss and failure to conceive have been identified as the primary cause for poor reproductive performance [1]. Bovine trichomonosis is a venereal disease of cattle caused by *Tritrichomonas foetus,* commonly identified in herds with prolonged calving seasons, reduced pregnancy [15,198] and calving rates [228]. As expected this disease has a significant impact in the cow-calf enterprise profitability with an estimated loss of 5 to 35% return per cow exposed to breeding [33].

Control of trichomonosis has been aimed at identifying and subsequently eliminating infected "carrier" bulls from the herd [119]. Identification of carrier bulls is commonly accomplished by collection of preputial scrape samples, culture and microscopic identification. Although culture remains the "gold standard", sensitivity of culture methods is quite variable (ranging from 67.8 to 98.9%) [92,119,130,139,154,161], depending on factors such as sample collection, transport conditions and culture method used. Most recently, PCR methods have been introduced to replace [126] or complement culture based methods [156].

Polymerase chain reaction methods have a lower detection limit than culture in both conventional and real time platforms [126]. This has led to the introduction of testing strategies such as the use of pooled samples. Previous studies have estimated the sensitivity of pooled preputial samples in groups of 5 bulls (only one positive sample included in each pool) to be 100% using both conventional and real-time PCR [203,204]. However, there is a need for additional information on the utility of larger pool sizes to optimize the application of this testing strategy. Other questions about the specificity of real-time PCR have also been reported in the

literature [198]. Given the economic consequences of culling a healthy bull, further study regarding the potential for false positive results with the real-time PCR assay is needed. The objectives of the present study were to: a) determine the specificity of a real-time PCR assay for the detection *Tritrichomonas foetus* in preputial samples collected in a commercial culture kit, and b) to determine the sensitivity of individual preputial samples collected in the culture kit, incubated and pooled in groups of up to 25 bulls tested by means of a real-time PCR assay.

2.3 MATERIALS AND METHODS

Experiment 1 - Specificity of real time PCR for T. foetus in individual preputial samples enriched by culture

Animal procedures were performed in accordance with the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee. Preputial samples were collected in June 2010 from 150 steers, housed at the University of Saskatchewan Beef Research & Teaching Unit, and from 187 virgin bulls in March and April 2011 housed at the Agri-Environment Service Branch bull stations (Maple Creek and Spring Creek). The breed and approximate age was recorded for each animal. Preputial samples were collected by means of the aspiration method [120,139]. Two preputial samples were collected from virgin bulls, however only one was used for the purpose of the present study. Whether the first or the second sample was collected into the culture media kit, and used for the present study, was alternated with each new bull entering the chute and recorded.

Briefly, an individually wrapped 25" plastic pipette[a] attached to a 20 ml syringe was inserted into the prepuce and the plastic sheath was pulled back. The scrape was performed by moving the pipette back and forth 10 times while applying 15 ml of suction. At completion, the pipette was

withdrawn and rinsed into the upper chamber of a self contained *T. foetus* culture pouch[c]. Pouches were closed as per manufacturer instructions and transported to the lab in a warm cooler (approximately 25°C [77°F]) within 4 to 7 hours of collection.

Samples were subjected to two tests: real-time PCR and culture with microscopic examination. Pouches were placed upright in an aerobic incubator at 37°C ([98.6°F], day 0) and cultured for 7 days. All pouches were examined using a bright field microscope (100X) by scanning along the seam of the pouch starting 1 cm from the bottom on one side, continuing down and along the bottom and up the other side to approx 1 cm from the bottom of the pouch [198]. Examinations were performed on days 1, 3, 5 and 7 of incubation and the results recorded.

On day 3, a 500 µl aliquot was taken from all samples and submitted for real-time PCR testing at a commercial diagnostic laboratory[e]. DNA extraction was performed from a 200 µl aliquot using a commercial DNA extraction kit[b] and an automated extraction system[d]. The assay used is a 5' Taq nuclease real-time PCR with minor groove binder modified probe and was performed as previously described [126]. Samples were considered positive when a C_t value of < 40 was obtained. Further identification of samples with visible *T. foetus*-like organisms but with a negative real-time PCR result was performed using a conventional PCR assay as previously described [207], with minor modifications. Briefly, reactions were carried in a 50 µl volume, the temperature profile used was 3 minutes incubation at 94°C (201.2°F) followed by 40 cycles of 94°C (201.2°F) for 30 s, annealing at 58°C (136.4°F) for 20 s, and extension at 72°C (161.6°C) for 30 s. A final extension step of 10 minutes at 72°C (161.6°F) was added. Samples were visualized in a 2.5% agarose gel.

Experiment 2 - Sensitivity of a real time PCR assay for T. foetus in pooled preputial samples enriched by culture

Samples collected in Experiment 1 from steers were used as known negative samples. After culture of these samples, the contents of the pouch were transferred to a 4 ml cryovial and stored at -80°C (-112°F). Known positive samples were collected from a bull that was confirmed as naturally infected based on both multiple positive cultures and PCR results. This bull was housed at the Western College of Veterinary Medicine Animal Care Unit. Two consecutive preputial samples were collected from the infected bull once a week and processed for culture and microscopy by the method described above. Pouches from the infected bull were withdrawn from the incubator at different times (day 0 to 5) and subjected to real-time PCR testing as described above.

Pools were made by including fixed aliquots of individual test-verified (culture and real-time PCR) positive and negative samples in ratios of 1/2, 1/3, 1/5, 1/10, 1/15, 1/20, and 1/25 to a total volume of 500 µl. The concentration of organisms in samples collected from the infected bull were determined by real-time PCR [126] and distributed to insure that all ratios contained equal numbers of pools with positive samples ranging from high (10^7 organisms/ml) to low concentration (10^3 organisms/ml) samples. Thirty one pools were made for each ratio and were tested by real-time PCR as described above.

Data analysis

Crude specificity estimates for real-time PCR and culture were determined by calculating the proportion of non-breeding animals, virgin bulls and steers, which tested negative for each assay. Confidence intervals were calculated based on the Wilson score method [246]. The animals

tested in experiment 1 had never been exposed to breeding and were assumed to be true negatives and not infected with *T. foetus*. Exact logistic regression[i] was used to evaluate potential differences in the specificity of the two tests. The animal category (steer or bull) was added as a covariate in the model. First order interactions with test type were evaluated and retained if statistically significant. Order of scrape was added to the model and retained if significant. The difference in prevalence of non-*T. foetus* trichomonads between bulls and steers was evaluated using Fisher's exact test[f].

Crude sensitivity estimates for each pool ratio were determined by calculating the proportion of the total samples testing positive. Confidence intervals were calculated as described above. Generalized Estimating Equations[g] were used to compare the sensitivity estimates between different pool ratios, after accounting for repeated use of the same sample in multiple pools, and to assess whether sensitivity varied by replicate, order of scrape, number of days the positive sample was incubated, and concentration of organisms in the positive sample. Variables other than pool ratio were retained if they were either significant ($P<0.05$) or acted as important confounders. Risk factors were considered to be confounders if removing or adding the factor from the model changed the regression coefficient for another risk factor of interest by more than 10% [247]. Analysis of potential differences in cycle threshold values among pool ratios was performed using a linear mixed model[h], after accounting for repeated use of the same sample in multiple pools. Plots of standardized residuals compared with predicted values were used to assess the models for extreme outliers. The level of significance used was 5%. One commercial statistics software package was used for all analyses[j].

2.4 RESULTS

Experiment 1

The 187 virgin bulls included 58% yearlings and 24% 2-year olds; the remaining bulls were ≤ 2 years, although the exact age was unknown. Breed composition was 69% Angus, 29% Charolais, and 2% Simmental and Limousin. The samples tested originated from the 1st scrape in 49% of the bulls and from the 2nd scrape in the other 51%. The steer cohort was composed of cross-breed animals between 14-15 mo of age, and all of the samples originated from a 1st scrape.

Contamination of cultured samples, observed as distension of the pouches due to gas accumulation, was reported in 14% (48/337) of samples. All samples were negative based on real-time PCR for *T. foetus*. Four pouches were positive ("false positive") by microscopic examination. False-positive (non-*T. foetus* trichomonads) results were reported on day 5 for 3 steers and on day 7 for 1 bull. Rounded organisms with an undulating membrane and 4 or more anterior flagella were observed using microscopic examination at 400X magnification with phase contrast.

Conventional PCR to identify the non-*T. foetus* trichomonad organisms in false-positive samples was successful only in 2 of the 4 samples. One of the samples had a 142 base pair product consistent with *Pentatrichomonas hominis* and the second sample yielded a product of between 200 and 300 base pairs not previously reported (data not shown). The observed prevalence of non-*T. foetus* trichomonads was 1.2% (4/337), and was not significantly different between bulls (0.5%, 1/187) and steers (2.0%, 3/150) (P=0.22).

There was no significant difference (P=0.94) between the specificity of culture (Specificity = 98.8%, 95% CI: 97.0 to 99.5) and the specificity of the real-time PCR (Specificity = 100%, 95% CI: 98.9 to 100). In addition, there was no difference in the specificity of the tests between steers and bulls (P=0.25).

Experiment 2

There were no significant differences among the sensitivity estimates for the eight pool ratios examined in this study (P=0.70); 1/10 (Sensitivity=90.3%, 95% CI: 75.1 to 96.6); 1/2, 1/15, 1/20 and 1/25 (Sensitivity=93.5%, 95% CI: 79.3 to 98.2); 1/3 and 1/5 (Sensitivity=96.8%, 95% CI: 83.8 to 99.4) (Figure 2.1).

Overall sensitivity was 94% (95% CI: 90.0 to 96.5). However, the sensitivity was significantly associated with the concentration of organisms in the positive sample included in a given pool (P<0.01). For each log increase in the concentration of organisms, the odds of a positive test result increased 3.7 times (95%CI: 2.3 to 6.1). The number of days the positive samples had been incubated was also associated with the sensitivity (P<0.01). For each additional day the sample was incubated, the odds of a positive result decreased by 9.8 times (95%CI: 3.9 to 24.7). The average C_t values were not significantly different among pool ratios (P=0.052) (Table 2.1).

More than 69% (9/13) of all samples that tested negative, regardless of pool ratio, contained the same sample as a positive contributor. Visual assessment of that pouch revealed significant evidence of contamination including change in color of the media to green and development of a large amount of gas. In addition, pools containing this sample as a positive contributor had significantly higher C_t values than pools containing other positive contributors (39.0, 95% CI:35.7-42.3 vs. 31.4, 95%CI:30.8-31.9, P<0.01).

2.5 DISCUSSION

The results of the present study indicate that the use of real-time PCR for the detection of *T. foetus* in bulls is highly specific (100%) and comparable to the results obtained for culture in the pouch (98.8%). In addition, the use of real-time PCR in pooled samples in groups of up to 25 bulls appears to be highly sensitive.

Culture methods used for the detection of *T. foetus* carrier bulls have historically been assumed to be 100% specific [36]. However, recent studies indicated the presence of trichomonads morphologically similar to *T. foetus* (referred as non-*T. foetus*) in preputial samples [37,156]. The origin of these non-pathogenic organisms is believed to be fecal, and the presence in bulls is likely the result of sodomizing and riding activity especially in young bulls [166,192]. The prevalence of non-*T. foetus* trichomonads in preputial samples from virgin bulls was reported to be 8.4% [156]. These findings are higher than those reported in the present study. The difference in the present study could be due to the culture media, individually wrapped pipettes reducing the chances of fecal contamination, or geographic variability in organism prevalence.

To the author's knowledge, this is the first report in which non-*T. foetus* trichomonads were recovered from steers. There was no significant difference in the occurrence of false positive culture results between steers and virgin bulls. The use of steers could provide a safe and readily accessible model for the study of trichomonosis, in particular for specificity determination. However further research is needed to validate this model by means of anatomical and histological examination of the prepuce and penis in both bulls and steers. Future work should investigate whether infection can be induced in steers and if a carrier state can be achieved.

Several methods have been proposed to circumvent the problem of false-positive samples obtained by culture, such as staining [165] and PCR [156,195]. Although the introduction of real-time PCR has made significant improvement in the sensitivity, concerns have arisen suggesting that false-positive results may be more common than with conventional PCR. The incorporation of PCR methods with high analytical sensitivity has raised questions about the possibility of detecting non-viable organisms or extremely low numbers of viable organisms [248]. However, the use of a 5' Taq nuclease real-time PCR with minor groove binder modified probe has been reported to achieve higher specificity than conventional PCR assays [126].

A previous study compared the use of culture, conventional PCR and real-time PCR in an infected herd in Nebraska [198]. The authors suggested that false-positive results were more common with real-time PCR, considering both conventional PCR and culture as the gold standard. This result was not supported by our current findings. In the previous study, the bulls used to assess specificity originated from an infected herd raising the question of whether those were truly false-positive test results or not. In the present study, the population used to calculate specificity had no previous exposure to breeding and was assumed to be negative. There is further evidence supporting the specificity of the real-time PCR from other studies. Samples with high Ct values, which are commonly re-evaluated in commercial laboratories as suspected *T. foetus* positives, are indeed positive as confirmed by product sequencing [202].

Results for the use of pooled samples obtained in the present study are similar to those reported previously for groups of 5 [203,204] and moreover, no difference in sensitivity was detected between different pool ratios in groups of up to 25 samples. The present study also included positive samples with a range of different concentrations of organisms to represent the potential impact of bulls with various levels of infection. Longer incubation time of the samples, up to 5

days, resulted in a reduced chance of identifying a positive sample within a pool. However, care must be taken as the source of positive samples in the present study was a single infected bull, and potential differences in strains were not evaluated.

Most of the pools that tested negative contained the same positive sample regardless of the pool ratio. The culture media for this positive pouch turned green in color, produced a large amount of gas and had the lowest reported concentration of organisms (5.5×10^3 organisms/ml) of any the samples used in this study. The intense contamination observed in this sample could suggest the presence of PCR inhibitors that might have affected the performance of the test. Several substances commonly found in preputial fluid have been identified as potential PCR inhibitors including blood (hemoglobin), urine (urea), and feces. Although the mechanism underlying the inhibition is poorly understood, the result is usually evidenced by reduced analytical sensitivity [249]. Considering that individual characteristics of each sample included in a pool could alter the result of the test, depending on the concentration of inhibitors, further research is needed to investigate the use of pooled samples without prior enrichment by culture. It would be safe to advise that samples showing significant evidence of contamination should not be included in pooled testing but rather be tested individually.

The use of real-time PCR assays provides the possibility of testing a large number of samples in a timely and cost-effective manner, with reduced possibility of artifactual results relative to testing protocols that require more sample manipulation. The use of real-time PCR for the detection of trichomonosis carrier bulls in preputial samples is highly specific and the use of pooled samples appears to be relatively sensitive in pools of up to 25 bulls, provided samples are collected and incubated individually before pooling.

[a]Continental Plastics, Cambridge, ON, Canada.

[b]DNeasy Blood & Tissue Biosprint 96 One-for-all Vet kit, Qiagen, Mississauga, ON, Canada.

[c]InPouch™ TF, Biomed Diagnostics, San José, CA.

[d]MagMAX™ Express-96 Standard Magnetic Particle Processor, Applied Biosystems, Life Technologies Inc., Burlington, ON, Canada.

[e]Prairie Diagnostic Services, Saskatoon, SK, Canada.

[f]PROC FREQ.

[g]PROC GENMOD

[h]PROC MIXED

[i]PROC LOGISTIC

[j]SAS Software Version 9.2, SAS institute, Cary, NC.

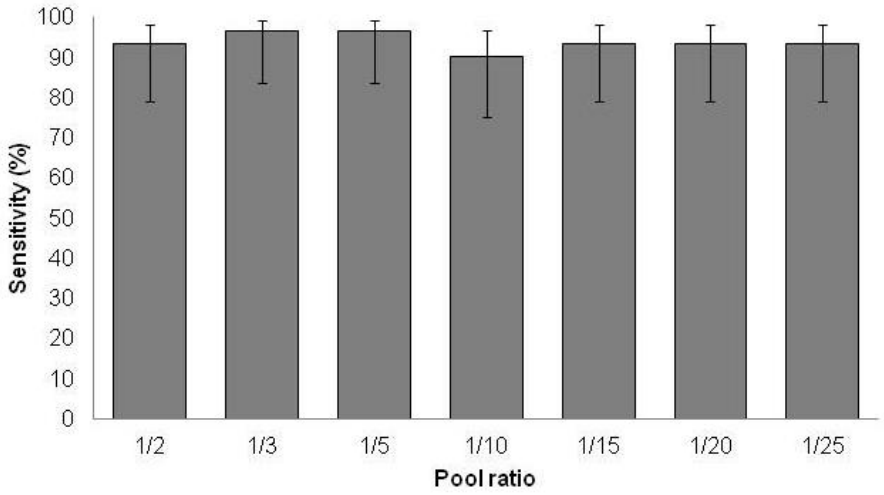

Figure 2.1. Crude sensitivity estimates and 95% confidence intervals of pooled preputial samples at different ratios collected in the culture pouch and tested by real-time PCR (N=31 pools per ratio).

Table 2.1. Mean and 95% confidence intervals of threshold cycle (C_t) estimates of pooled preputial samples at different ratios collected in the culture pouch and tested by real-time PCR.

Pool ratio	N	Mean C_t	95% Confidence Intervals
1/2	29	30.8	29.3-32.3
1/3	30	30.1	28.7-31.6
1/5	30	30.8	29.3-32.3
1/10	28	31.3	29.8-32.9
1/15	29	32.4	30.9-33.9
1/20	29	32.6	31.1-34.1
1/25	29	33.1	31.6-34.6

3. SENSITIVITY OF A REAL TIME POLYMERASE CHAIN REACTION FOR *TRITRICHOMONAS FOETUS* IN DIRECT INDIVIDUAL AND POOLED PREPUTIAL SAMPLES

A. García Guerra[1], J. E. Hill[2], C. L. Waldner[1], J. Campbell[1], S. Hendrick[1*]

[1]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5B4

[2]Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5B4

[*] Corresponding Author: 52 Campus Drive, WCVM, Saskatoon, Saskatchewan, Canada S7N 5B4 FAX: (001) 306 966 7159; e-mail: steve.hendrick@usask.ca

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3.1 ABSTRACT

The objective of this study was to evaluate the sensitivity of a commercially available real-time PCR test for the detection of *Tritrichomonas foetus* in individual and pooled direct preputial samples. Two preputial samples were collected from each of 1016 bulls and placed alternatively into an InPouch^{TM} TF, or into 2 ml of phosphate buffered saline (PBS). Upon arrival to the lab pouches were cultured and evaluated as per manufacturer instructions. In addition, once a week two samples were collected and processed from 9 *T. foetus* infected bulls to complete a total of 121 samples. Samples collected in PBS, referred to as direct preputial samples were submitted for individual real-time PCR testing to a commercial diagnostic lab. Direct preputial samples were blocked by order of sampling and randomly divided for pooling into groups of 5 and 10 samples ensuring that every pool had one sample from a known infected bull. Sensitivity of individual samples tested by culture, real-time PCR in direct and culture enriched samples, were determined from 121 samples obtained from 9 infected bulls. Sensitivity estimates were: 95.0% (95% CI: 89.6% to 97.7%) for culture, 95.9% (95% CI: 90.7 to 98.2) for real-time PCR in cultured enriched samples, and 90.1% (95% CI: 83.5 to 94.2) for direct preputial samples and did not differ (P=0.12). Sensitivity estimates for direct pooled samples were: 83.6% (95% CI: 75.6 to 89.4) and 77.3% (95% CI: 68.6-84.1) for pools of 5 or 10 samples, respectively (n=110 per group). There was no significant difference in the sensitivity between pool ratios (P=0.08). The use of 3 sequential samples, collected in PBS, at weekly intervals and tested in pools by real-time PCR increased the sensitivity to 100% and 96% for pools of 5 or 10, respectively. In conclusion, results obtained indicate that the use of direct preputial samples collected in PBS and tested by real-time PCR individually have a comparable sensitivity to culture and real-time PCR in enriched samples. The use of pooled direct preputial samples appears to be relatively sensitive.

However, this strategy still requires repeat sampling in order to increase sensitivity. Real-time PCR testing of preputial samples collected directly into PBS with the option of pooling would decrease the cost associated with screening bulls, and increase the feasibility of large epidemiological studies and active surveillance.

Keywords: *Tritrichomonas foetus*, PCR, pooled samples.

3.2 INTRODUCTION

Bovine trichomonosis is a venereal disease of cattle caused by the flagellated protozoan parasite *Tritrichomonas foetus*. The disease has been reported worldwide [58,59,63,71,74,99,250,251], especially wherever extensive management and natural breeding is used [19,124]. Significant economic losses have been attributed to this disease in both the beef and dairy industry [31,32]. Some of the control strategies currently available for cattle producers include: testing of all breeding bulls and culling of positive animals, use of exclusively virgin bulls, introduction of AI programs, culling of open cows, and maintenance of a closed herd [231]. Mandatory testing for both interstate and intrastate movement of bulls has been incorporated in several regions within the United States in an attempt to limit the spread of the disease and move towards eradication [68,229].

Historically, testing has been based on culture and microscopic examination with a minimum of three consecutive negative samples recommended to classify a bull as negative [120]. However, some bull studs require up to 6 consecutive negative results depending on the age of the bull at the time of testing to certify the bull as free of the parasite [22]. Factors contributing to testing costs include sample collection, the culture media used, and subsequent personnel time for incubation and sequential microscopy. Repeated individual animal testing is a substantial expense for the producer.

Over the last decade PCR methods have been widely adopted. However, most protocols still require samples to be collected and incubated in culture media [139,156,190]. The combination of laboratory expenses for PCR and culture media have in many cases increased the cost per test to producers. The cost of testing is a substantial deterrent, often limiting its use in beef herds

unless there is evidence of reproductive failure. Recently, changes have been introduced to trichomonosis regulations in some states allowing for a single PCR test to be equivalent to three consecutive culture tests, attempting to simplify and reduce the cost of testing [60,252]. However, a recent study showed that a single PCR test was not sensitive enough to eliminate the use of repeat sampling [198]. Testing of pooled preputial samples by PCR has been proposed recently and shown to have high sensitivity [203]. Pooling could be a valuable option for limiting the per test laboratory costs in large herds.

The introduction of a Taq nuclease real-time PCR assay targeting the ITS1 region has had a positive impact in trichomonosis testing due to its high analytical sensitivity, specificity and high throughput capabilities [126]. The use of this assay for pooled samples in groups of five has been reported to be highly sensitive [204]. However, the reported method required that the scraping from each bull was collected into a commercial culture kit so that pooling only decreased the laboratory portion of the testing cost.

A recent study by our research group determined that real-time PCR testing of preputial samples in groups of up to 25 bulls was highly sensitive. However, contamination of individual samples by the presence of PCR inhibitors could decrease the performance of the test. It has been shown that real-time PCR has a lower detection limit (1 organism/reaction) when direct preputial samples, collected in PBS, are tested as compared to culture enriched samples [126]. The use of direct preputial samples would decrease the cost of testing and potentially improve the sensitivity of the test. The objectives of the present study were to: a) determine the sensitivity of a real-time PCR for the detection *T. foetus* in individual direct preputial samples and samples collected in a commercial culture kit, and b) to determine the sensitivity of pooled direct preputial samples in groups of 5 or 10 bulls by means of a real-time PCR assay.

3.3 MATERIALS AND METHODS

Experiment 1 - Sensitivity of culture and real time PCR for T. foetus in individual preputial samples with or without enrichment by culture

All animal procedures were performed in accordance with the Canadian Council on Animal Care and approved by the University of Saskatchewan Animal Care Protocol Review Committee. Preputial samples were collected at weekly intervals from 9 beef bulls infected with *T. foetus* housed at the University of Saskatchewan Animal Care Unit. A bull was defined as infected for the purpose of this study if he tested positive at least once by both culture and PCR as described below. Five bulls were naturally infected and originated either from two neighboring herds in Alberta (n=4) or from a herd in Saskatchewan (n=1). The remaining 4 bulls were artificially infected with either the Saskatchewan isolate (n=3) or the Alberta isolate (n=1). Artificial infection was performed as described previously with minor modifications [95,120]. Briefly, field isolates of *T. foetus* were cultured in modified Diamond's medium at 37°C for 3 days. The culture was centrifuged on day 3 at 650 X g for 5 minutes. Half the supernatant was discarded and the pellet was resuspended in the remaining supernatant. A 1:10 dilution was made in PBS (20 mM phosphate, 150 mM NaCl) to establish the concentration of organisms by means of a hemacytometer (Improved Neubauer, Hausser Scientific, Horsham, PA, USA). The inoculum was diluted with PBS to reach a concentration of approximately 1 x 10^6 organisms per ml. Inocula were transported in a styrofoam container at 37°C within 20 minutes to the site where inoculation was performed. A 25" (63.5 cm) plastic pipette was used to deposit 2 ml of the inoculum at the fornix of the prepuce and the preputial opening was held closed for 30 seconds.

Samples were collected from all bulls at weekly intervals. Ambient temperature and time from sampling to arrival at the lab were recorded for each sample. Preputial samples were collected by means of the aspiration method [120,139]. Briefly, an individually wrapped 25" plastic pipette (Continental Plastics, Cambridge, ON, Canada) attached to a 20 ml syringe was inserted into the prepuce and the plastic sheath was pulled back. The pipette was moved back and forth 10 times while applying 15 ml of suction. Two consecutive preputial samples were collected from each bull at every sampling. One sample was rinsed into the upper chamber of an InPouch^{TM} TF (Biomed Diagnostics, San Jose, California, USA) and one into 2 ml of PBS (direct preputial samples), and the collection order was alternated every week and recorded. Samples in the InPouch^{TM} TF were transported to the laboratory in a warm styrofoam container (25.8°C ± 2.4°C), while samples collected in PBS were placed in a styrofoam box with ice packs. At arrival, direct samples were placed in a -80°C freezer until tested by real-time PCR. Pouches were placed in an aerobic incubator at 37°C (day 0) and cultured for 7 days. All pouches were examined using a bright field microscope (100×) as previously described [198]. Examinations were performed on days 1, 3, 5 and 7 of incubation. After the last examination, pouches were stored at -80°C until real-time PCR was performed. Direct preputial samples and pouches were subjected to real-time polymerase chain reaction (real-time PCR) at a commercial diagnostic lab (Prairie Diagnostic Services, Saskatoon, SK). DNA was extracted from a 200 µl aliquot of medium using a commercial kit (DNeasy Blood & Tissue BioSprint 96 One-For-All Vet kit, Qiagen, Mississauga, ON, Canada). The real-time PCR assay was performed as described by McMillen et al. 2006 [126], using a Stratagene Mx3005P QPCR (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Samples were considered positive if the threshold cycle (C_t) value was < 40.

Experiment 2 - Sensitivity of a real-time PCR assay for detection of T. foetus in pooled direct preputial samples

Direct preputial samples collected in Experiment 1 from infected bulls were used as known positive samples. In addition, 1016 bulls from 23 locations in Saskatchewan were sampled. Two preputial samples were collected as described above from each bull and placed alternatively into an InPouch^{TM} TF (Biomed Diagnostics, San Jose, California, USA) or into a 4 ml cryovial containing 2 ml of PBS (direct preputial samples). Pouches were closed as per manufacturer instructions and placed in a warm cooler (24.6 ± 1.4 °C), while cryovials were placed in a styrofoam box with ice packs. All samples were transported to the lab within 0.5 to 14 h (7.5 ± 3 h, mean ± sd). Upon arrival at the laboratory, pouches and direct preputial samples were processed as described in Experiment 1. An aliquot of 200 µl of individual direct preputial samples was submitted for real-time PCR testing.

Upon obtaining individual results from all field samples, those negative by both culture and real-time PCR, were blocked by order of sampling and randomly divided into groups to construct pools of 5 and 10 samples that had one and only one sample from a known carrier bull (samples from Experiment 1). Pools were made by combining aliquots (200 µl) of prepuce scraping samples collected directly into PBS to a total volume of 1 or 2 ml resulting in pools of 5 and 10 bulls, respectively. One hundred and ten pools were made for each ratio, and submitted to the commercial diagnostic lab. DNA extraction and real-time PCR procedures were performed as described for Experiment 1.

Data analysis

Crude sensitivity estimates for Experiment 1 were calculated using samples collected from known infected bulls and were determined for each method as the proportion of samples that tested positive. Confidence intervals were calculated by the Wilson score method [246]. Samples included in the estimation of sensitivity for each bull were considered starting with the first sampling that tested positive by culture ("gold standard") until the last culture positive sample obtained or the completion of the study. All samples collected during that period from each bull were considered to originate from a carrier bull regardless of tests results to account for expected fluctuations in organism numbers [115]. Potential differences in sensitivity between test types were evaluated using generalized linear mixed models with a binomial distribution and logit link function. The calculations were performed using the GLIMMIX procedure (SAS 9.2, Cary, NC, USA). Within-bull and sampling within bull clustering were accounted for with random intercepts. The proportion of total variance explained by differences between bulls ($\rho=\sigma^2_b /(\sigma^2_b + \sigma^2_s +\pi^2/3)$) and sampling within bull ($\rho=(\sigma^2_b + \sigma^2_s)/(\sigma^2_b + \sigma^2_s +\pi^2/3)$) was also estimated [247]. Breed, age, type of infection (natural or artificial), ambient temperature, order of sample (1^{st} or 2^{nd} preputial sample collected on the same day) and transport time (> 1 h or ≤ 1 h), were evaluated as covariates in the model.

Crude sensitivity estimates and 95% confidence intervals for each pool ratio in Experiment 2 were determined as described for Experiment 1, but using the proportion of total pools for each ratio (5 or 10) that tested positive. Generalized Estimating Equations (GEE) were used to evaluate potential differences in the sensitivity between the pool ratios, after accounting for the expected correlation among aliquots from the same infected bull sample used as the positive for both pools of 5 and 10 samples. The GEE model also examined whether sensitivity of the

pooling protocol varied with the order of sample collection or natural compared to artificial infection. The calculations were performed using the GENMOD procedure (SAS 9.2, Cary, NC, USA).

Threshold cycle (C_t) values are negatively correlated with the amount of target DNA sequence present in the samples. Analysis of potential differences in C_t values between tests in Experiment 1, and between pool sizes in Experiment 2 was performed using a linear mixed model by means of the MIXED procedure (SAS 9.2, Cary, NC, USA) after accounting for repeated use of the same positive sample.

The final question addressed involved assessing the importance of the current recommendation that three sequential tests will improve the sensitivity of detection and are needed to provide adequate negative predictive value in situations with moderate or high pretest probability of infection [120]. The effect of repeated sampling on the sensitivity of real-time PCR in pooled samples was analyzed by identifying groups of 3 sequential pools for each positive bull based on the date of collection of the positive sample included in the pool. The resulting sets of three samples from successive weekly intervals that had been tested in pools of 5 or 10 were obtained for each bull, and cumulative sensitivity was calculated for 1, 2 or 3 consecutive samplings. Differences in cumulative sensitivity between pools of 5 or 10 samples and between 1, 2 or 3 consecutive samplings were evaluated by generalized linear mixed models with a binomial distribution and logit link function using the GLIMMIX procedure (SAS 9.2, Cary, NC, USA). Within bull clustering was accounted for with a random intercept.

The models were evaluated for outliers using plots of residuals compared with predicted values. Covariates included in the models were retained if they were either significant ($P<0.05$) or acted

as important confounders. Risk factors were considered to be confounders if removing or adding the factor from the model changed the regression coefficient for another risk factor of interest by more than 10% [247]. The level of significance used was 5%, and variables were considered statistically different when $P \leq 0.05$.

3.4 RESULTS

Experiment 1

There was no significant difference in the sensitivity of conventional culture using the InPouch^{TM} TF, real-time PCR performed on culture enriched samples, and real-time PCR in direct preputial samples (P=0.12) (Table 3.1). A total of 102 (84%) samples were positive by all three tests, and no samples were negative by all three tests. Fifteen (12.4%) samples were positive for two of the tests and negative for the remaining tests (11 positive for culture and real-time PCR from the culture-enriched sample, 3 positive by real-time PCR from the culture-enriched and direct samples, and 1 positive for culture and direct real-time PCR). A total of 4 samples were positive for only one test (3 positive for direct real-time PCR and 1 for culture). There were no other measured factors associated with the sensitivity of the tests including: type of infection (natural vs. artificial) (P=0.87), breed (P=0.40), order of sampling (P=0.55), sample transport time (≤1 h vs >1 h) (P=0.92), age (P=0.93), and ambient temperature (P=0.69).

Fifty six percent (5/9) of bulls tested positive by culture at every sampling, 78% (7/9) of bulls tested positive at every sampling by real-time PCR in culture enriched samples, every time, and 56% (5/9) of bulls tested positive every time for direct real-time PCR. Differences among bulls were responsible for 31% of the unexplained variation, and sampling within bull was responsible for 42% of the unexplained variation.

Culture samples testing positive were detected after 24 h of incubation in 93% (112/121) of the samples, and none of them were detected after 5 days of incubation.

The mean (± SEM) Ct value for the real-time PCR in direct preputial samples (31.98 ± 0.46) was significantly higher ($P<0.01$) than the mean value for the real-time PCR in culture enriched samples (26.39 ± 0.45).

Experiment 2

The commercial bulls sampled represented 13 cattle breeds as follows: Angus 56% (569/1016), Charolais 28.9% (294/1016), Limousin 6.6% (67/1016), Simmental 4.3% (44/1016), Beef Booster 1.7% (17/1016), Hereford 1.1% (11/1016) and other 1.4% (14/1016) (Galloway, Gelbvieh, Holstein and Black Welsh). Of the total number of bulls sampled, 18.1% (184/1016) were yearlings, 19.5% (198/1016) 2 years old, 48% (488/1016) 3 to 5 years old, 13.3% (135/1016) > 5 year and 1.1% (11/1016) were unknown.

Only three samples (0.29%) were positive by culture in the InPouch^{TM} TF culture kit; however, they were negative by direct real-time PCR. One sample (0.1%) was positive by direct real-time PCR, but negative for culture. These four samples were not included in the subsequent pooling. Gas development within the culture pouch was detected in 18.4% of the samples.

There was no significant difference ($P=0.08$) in the sensitivity of pools of 5 (83.6%, 95% CI: 75.6 to 89.4) compared to pools of 10 (77.3%, 95% CI: 68.6 to 84.1) direct preputial samples analyzed by real-time PCR. The sensitivity of pooling was not associated with the order of sampling ($P=0.89$) or type of infection (natural *vs.* artificial) ($P=0.47$). Similarly, there was no

difference in cycle threshold values between pools of 5 samples (mean,34.1± SEM,0.3) and pools of 10 samples (34.3±0.3) (P=0.66).

There was no significant difference in the cumulative sensitivity between pools of different sizes at 1, 2 or 3 consecutive samplings (P=0.99). Regardless of pool size there was a significant increase in cumulative sensitivity between 1 and 2 consecutive samplings (P<0.01). However, no significant difference was detected between 2 and 3 consecutive samplings (P=0.99) (Fig. 3.1).

3.5 DISCUSSION

The sensitivity of culture, and real-time PCR performed on both direct and culture-enriched preputial samples was comparable. Culture sensitivity was consistent with previous reports under similar conditions [154]. However, care must be taken when interpreting culture results as transport of samples was conducted under ideal conditions and transport time was in all cases ≤ 2 h. This was further supported by the detection of 97.4% of the positive samples within 24 h of incubation at 37°C. This high rate of identification early in the incubation period is slightly higher than previous results [58,74,160], and could be explained by reduced transport time at the appropriate temperature. Providing temperatures between 22°C and 37°C during transport allows for optimal development of *T. foetus* [131]. However, samples collected in areas where cattle are managed extensively are often subjected to prolonged transport times and a wide range of temperatures depending on the time of the year and geographic location [132,133,138]. Recent reports have demonstrated reversible pseudocyst forms of *T. foetus*, characterized by internalized flagella, adopted in the face of adverse conditions such as limited nutrient availability, temperature changes and presence of certain drugs. This form has been reported to be adopted in >55% of organisms in fresh samples [39,40]. It is reasonable to expect that samples exposed to

prolonged transport times and extreme temperatures would tend to develop pseudocysts form. As a result, microscopic detection of these forms would be impaired by the lack of the characteristic motility, one of the main criteria for the identification of *T. foetus*. The use of real-time PCR in both direct and culture methods would potentially allow for the detection of these forms in samples that have been exposed to inadequate transport conditions.

Sample collection is commonly carried out in the field under unfavorable conditions [133]. However, there is a lack of evidence regarding the effect of ambient temperatures at the time of sample collection. Based on data from the present study in which samples were collected under a wide range of ambient temperatures (-25°C to 25°C), there was no reduction in culture or real-time PCR sensitivity when collection was performed at low temperatures when the samples were immediately stored at 25°C and transported in less than 2 h. Temperatures above 37°C are extremely rare in western Canada and were not evaluated, as a result, research is needed to evaluate the effect of high temperatures during sample collection on test sensitivity.

None of the individual samples were negative on all three tests. However, there were several samples from known infected bulls that were only positive for one or two of the three tests during the study period. This observation could be explained by differences in the concentration of organisms in a sample from any particular time due to either variations in the level of infection in the bull or an inadequate sampling procedure. The number of organisms in the preputial cavity has been shown to be quite variable not only between bulls but also between samples from individual bulls [115]. In the present study, half of the bulls were consistently positive in all samples when tested by culture or direct real-time PCR, with almost 80% of the bulls being positive at every sampling by real-time PCR from culture-enriched samples.

These findings are consistent with previous studies in which direct microscopic examination [115] or culture and conventional PCR [198] were used. The reason for fluctuations in the level of parasites in the preputial cavity remains unknown, and peak numbers have been observed every 4-10 days [115]. Consequently, infected bulls rarely have more than two consecutive negative test results. However, occasionally atypical bulls have been observed, that present prolonged periods in which they test negative [116]. In the present study, none of the bulls had more than two consecutive negative results when tested by culture or real-time PCR from enriched samples. However, one artificially infected bull had four consecutive negative results by direct real-time PCR. This period was coincident with the first four samples after infection and could potentially indicate lower levels of organisms until the infection became well established.

Conventional PCR methods for *T. foetus* detection in direct preputial samples have a reported sensitivity range of 86 to 98% [127,140]. In addition, the use of real-time PCR in this type of sample has been shown to improve the detection limit from 50 to a single organism per reaction compared to real-time PCR in culture enriched samples [126]. Sensitivity estimates for real-time PCR in direct preputial samples obtained in the present study are similar to those reported above. The use of direct preputial samples had comparable sensitivity to that of real-time PCR in culture-enriched samples. The direct method would reduce the cost of the test as no special media would be required, simplify the temperature requirements for transport of the samples, and reduce the overall turnaround time due to the fact that incubation of the samples would no longer be needed.

The failure to observe an improvement in the sensitivity of real-time PCR in direct preputial samples as compared to samples enriched by culture could be due to optimal conditions during

transport and culture of samples collected in the InPouch^{TM} TF. These conditions allowed for appropriate growth and multiplication of *T. foetus* as demonstrated by the significantly lower C_t values obtained for samples tested after culture enrichment. However, samples transported under adverse conditions could potentially have reduced multiplication of *T. foetus*, eliminating any potential advantage of the culture-enriched samples. For example, the pouch could be frozen during transport without an optimal incubation period. As a result further research is needed to provide optimal regional sampling strategies for practitioners and diagnostic laboratories.

The use of pooled samples enriched in culture and tested by PCR has been shown to be highly sensitive in groups of 5 bulls with a single positive bull included in the pool [203,204]. In addition, a recent study showed that the sensitivity of a real-time PCR assay did not decrease when pool sizes were increased to 25 bulls [253]. However, the composition and condition of individual samples included in any given pool could potentially decrease the sensitivity of the test due to the presence of inhibitors. The use of direct preputial samples was expected to result in comparable or improved sensitivity due to removal of potential inhibitors generated during the culture enrichment.

The sensitivity of pools of 5 or 10 direct preputial samples tested by real-time PCR was lower in the present study compared to previous studies [204]. However, there was an important difference in the protocol when compared to previous studies. Positive samples in the pools were from known carrier bulls, but the samples were not necessarily PCR or culture positive when tested individually. The difference in how samples from infected bulls were defined in this study could explain at least in part the lower sensitivity reported here. The present design better reflects the potential performance of this protocol in a field situation, by including samples of infected bulls regardless of the individual sample test results. As described previously, samples collected

from infected bulls will not always be consistent due to variations in the level of infection or variations in sample collection technique.

Historically multiple consecutive tests have been required to confirm that a bull is free from infection. This strategy was developed to optimize the probability of detecting positive animals. Current recommendations indicate between 3 and 6 consecutive samples at weekly intervals [22,94]. Given the sensitivity estimates obtained for pooled direct preputial samples tested by real-time PCR in the present study, the effect of repeat sampling was investigated. Results indicated an increase in sensitivity when > 2 consecutive samples at weekly intervals were collected and tested in pools of 5 or 10. The relative increase in sensitivity with each additional sampling is consistent with previous reports using individual samples tested by culture [75] and PCR [198]. However, Parker et al. [120] noted that pre-test probability of infection was the most important factor when interpreting individual test results. Repeat testing will increase the overall sensitivity, the probability of detecting an elusive carrier, and increase the final negative predictive value. Ultimately, the strategy to be used in each situation should be evaluated based on the pre-test probability of infection, the acceptable level of uncertainty in a negative result, and considering the cost of multiple sample collection and testing.

3.6 CONCLUSIONS

The present study indicates that the use of real-time PCR in individual direct preputial samples has comparable sensitivity to that of conventional culture and real-time PCR performed on culture-enriched samples. The use of direct preputial samples precludes the need for expensive culture media thus reducing the cost of testing. In addition, the use of pooled direct preputial samples in groups of 5 and tested by real-time PCR should be a suitable strategy for monitoring

herds and performing large-scale epidemiological studies in a cost effective manner. The present pooling strategy could be also used to determine prevalence estimates in geographic areas to provide useful information towards the establishment of control and eradication programs. However, repeated sampling at weekly intervals is still required to maximize the sensitivity of the described testing strategy.

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Table 3.1. Proportion of samples from known *T. foetus* carrier bulls testing positive by means of culture and real-time PCR from both culture enriched samples or direct preputial samples (95% Confidence Intervals).

Type	ID	Breed	Age (years)	Samples collected	Culture	Pouch real-time PCR	Direct real-time PCR
Artificial	004	Black Angus	7	22	95.5% (78.2-99.2)	90.9% (72.2-97.5)	59.1% (38.7-76.7)
	10	Red Angus	4	16	93.8% (71.8-98.9)	100% (80.6-100)	93.8% (71.8-98.9)
	173	Black Angus	7	9	100% (70.1-100)	100% (70.1-100)	100% (70.1-100)
	218	Black Angus	6	10	100% (72.3-100)	100% (72.3-100)	100% (72.3-100)
Natural	18T	Charolais	5	8	75.0% (40.9-92.9)	100% (67.6-100)	100% (67.6-100)
	11T	Charolais	5	9	100% (70.1-100)	100% (70.1-100)	88.9% (56.5-98.0)
	45R	Red Angus	7	30	100% (88.7-100)	100% (88.7-100)	100% (88.7-100)
	71U	Limousin	4	9	100% (70.1-100)	100% (70.1-100)	88.9% (56.5-98.0)
	MLZ	Charolais	6	8	75.0% (40.9-92.9)	62.5% (30.6-86.3)	100% (67.6-100)
			Total	**121**	**95.04% (89.6-97.7)**	**95.87% (90.7-98.2)**	**90.08% (83.5-94.2)**

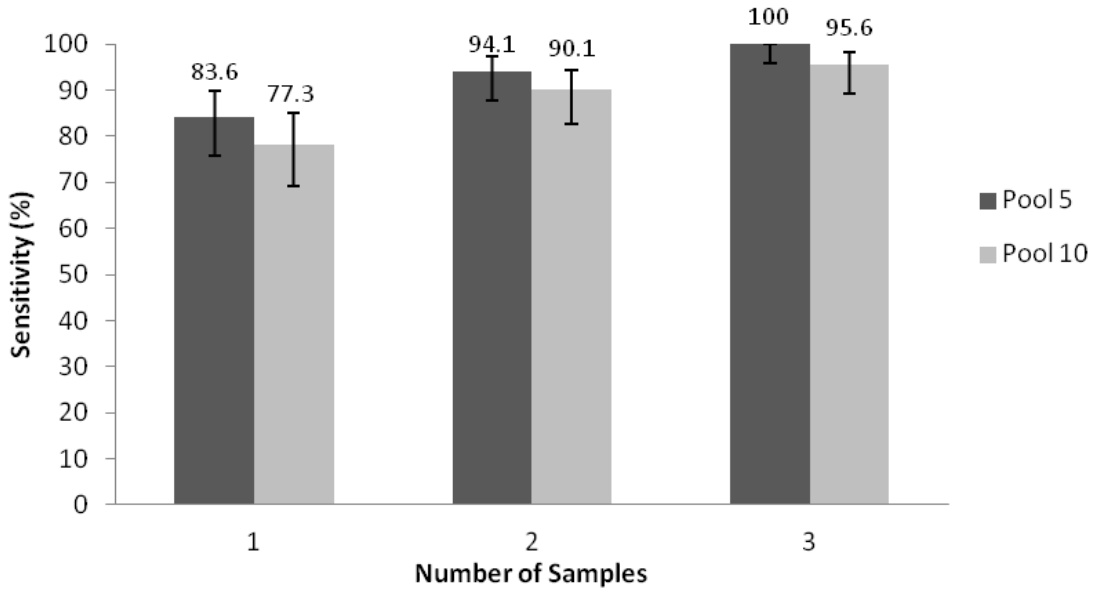

Figure 3.1. Cumulative sensitivity estimates and 95% confidence intervals of pooled direct preputial samples in groups of 5 or 10 and tested by real-time PCR, collected at weekly intervals.

4. CLINICAL SENSITIVTY AND SPECIFICITY OF A REAL TIME PCR ASSAY FOR BOVINE GENITAL CAMPYLOBACTERIOSIS IN DIRECT PREPUTIAL SAMPLES

A. García Guerra[1*], B. Chaban[2], J. E. Hill[2], C. L. Waldner[1], S. Hendrick[1]

[1]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5B4

[2]Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5B4

[*] Corresponding Author: 52 Campus Drive, WCVM, Saskatoon, Saskatchewan, Canada S7N 5B4 FAX: (001) 306 966 7159; e-mail: a.garciaguerra@usask.ca

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4.1 ABSTRACT

The diagnostic methods commonly used for the detection of *Campylobacter fetus* subsp. *venerealis* (Cfv) in bulls require maintaining viable organisms. Field conditions and distance to diagnostic laboratories have limited the success of these methods due to the fastidious requirements of Cfv. The objective of the present study was to determine the clinical sensitivity and specificity of a recently developed real-time PCR (qPCR) assay for direct preputial samples collected in phosphate buffered saline (PBS) and transported on ice. Preputial samples were collected from 300 virgin bulls and from 13 Cfv infected bulls. Specificity of the qPCR was calculated from samples collected from virgin bulls and compared to culture with the use of a transport enrichment media (TEM). Sensitivity of the qPCR was obtained from multiple samples collected at weekly intervals from infected bulls. In addition, the qPCR was compared to the direct fluorescent antibody test (DFAT), direct culture and culture after incubation in TEM. Specificity estimates were 85% (95% CI: 80.5 to 88.6) for qPCR and 100% (95% CI: 98.7 to 100) for culture, and were significantly different ($P<0.01$). The average sensitivity of the qPCR was 85.4% (95% CI: 80.6-89.2), direct culture in blood agar 82.3% (95% CI: 77.2-86.5), DFAT 72.1% (95% CI: 66.2-77.4), direct culture in Skirrow agar 32.7% (95% CI: 27.2-38.7), TEM and blood agar 30% (95% CI: 23.4-37.5), and TEM and Skirrow agar 38.1% (95% CI: 31-45.9). The difference between tests varied with ambient temperature ($P<0.01$). The sensitivity of the qPCR increased with increasing ambient temperatures, and was greater than all other tests ($P<0.05$) when ambient temperature exceeded 5°C. The use of repeated sampling significantly increased the sensitivity of the qPCR when 2 consecutive samples at weekly intervals were used ($P<0.01$). In conclusion, the use of qPCR as a screening test on direct preputial samples appears to be highly sensitive. Although the improvement of the qPCR over direct culture is dependent on

temperature, care must be taken as transport times that allow direct culture are unlikely to be seen under field conditions. However, the use of repeated sampling is still required in order to maximize sensitivity. As a result, the qPCR test would provide a fast and reliable method for the screening of Cfv in bulls.

4.2 INTRODUCTION

Bovine genital campylobacteriosis (BGC) is a venereal disease caused by *Campylobacter fetus* subspecies *venerealis* (Cfv), and characterized by temporary infertility, reduced pregnancy rates and abortions [10,16,77,254]. This disease occurs worldwide, wherever natural service is used for breeding [77,254,255]. In addition, the occurrence of BGC is a major limitation to the international trade of semen and embryos [27]. Detection of Cfv is usually focused on bulls as they are asymptomatic carriers and act as one of the main components of the epidemiology of BGC [20]. As a result, most diagnostic tests have been aimed at identifying Cfv in preputial samples.

Current diagnostic techniques available for the detection of Cfv in preputial samples include culture [134], DFAT (direct fluorescent antibody test) [186], ELISA [27] and PCR [152,217]. Bacterial culture and subsequent phenotypic identification remains the "gold standard" for *Campylobacter fetus* subspecies *venerealis* detection [173]. However, isolation of *C. fetus* is challenging in most field situations due to its fastidious nature and sensitivity to environmental conditions [180]. As a result, the accuracy of culture depends on the inoculum size, the presence of competing microflora in the sample, environmental conditions during transport, and laboratory techniques [135,141,256,257]. Cattle management conditions often result in samples being collected in distant locations where samples must undergo transport times greater than 24 h.

Due to these circumstances, special transport media have been developed to improve culture sensitivity by providing an appropriate environment for Cfv and selectively inhibit competing microflora. Two of the most common transport enrichment media (TEM) used are Clark's TEM

[257] and modified Weybridge TEM [144]. However, sensitivity of culture using TEM is quite variable ranging from 25% to 90% [146,147,150]. Moreover, the use of TEM requires special media with a limited shelf life and special conditions during transport, some of which are cumbersome to obtain in most field situations.

Following arrival at the diagnostic laboratory, the samples need to be processed and placed onto a culture medium with a modified atmosphere. Several selective agar media are available for the isolation of Cfv such as Campylobacter selective agar (CSA) [169] or Skirrow selective agar (SK) [172]. These selective agar contain Polymixin B among other antibiotics and susceptibility of some Cfv strains to this antibiotic has been reported [150], and overgrowth by contaminants is also common [134,147]. An alternative method has been the use of filtration and non-selective agar media [175,176].

Upon isolation, subsequent identification needs to be performed to confirm the presence of Cfv. Currently, the internationally accepted test to differentiate *C. fetus* subspecies is the tolerance to 1% glycine [173]. The use of this phenotypic characteristic has resulted in significant controversy as intermediate strains of Cfv have been described [50], and the result of the test is strongly influenced by the inoculum size and basal medium used [179,180]. In addition, tolerance to glycine has been reported to be a characteristic susceptible to acquisition by phages or by spontaneous mutation [258]. As a result, the use of tolerance to glycine has been discouraged by many authors as a means for subspecies determination [183,218].

The introduction of molecular based diagnostic techniques provided the necessary tools to develop a fast and reliable test that would be useful in field investigations. Although several PCR tests have been developed for the detection of *C. fetus,* there are only a few of them that are able

to identify Cfv specifically [125,217,221,222,224]. Primers VenSF and VenSR, developed by Hum et al. [217] as part of a multiplex PCR test, amplify a 142 bp region of the *parA* gene within a genomic island present only in Cfv [47]. These primers have been the most extensively used and validated with hundreds of isolates from different countries (as reviewed by [152]). However, these primers have been mainly used for the identification of isolates and their use in field samples has been limited. A recent study adapted primers VenSF/VenSR to the SYBR green qPCR platform and optimized the sample processing for direct preputial sample testing [152]. In addition, the analytical sensitivity of this assay was determined to be 10^3 CFU (colony forming units) per ml of sample, which translates to a single cell per reaction. This test provides the possibility of a sensitive, simple and cost-effective procedure that eliminates the need for special transport media and would be suitable for screening large numbers of bulls [152].

Currently, little is known about the diagnostic sensitivity and specificity of the qPCR test in clinical samples and how it compares to other available tests. Thus, the objective of the present study was to determine: a) the clinical sensitivity and specificity of a qPCR assay for the detection of *Campylobacter fetus* subsp. *venerealis* in direct preputial samples; b) the performance of the test compared to culture with or without transport enrichment media and the direct fluorescence antibody test; c) the effect of ambient temperature at the time of sample collection on the sensitivity of different diagnostic tests.

4.3 MATERIALS AND METHODS

Animals

Preputial samples were collected from 300 virgin beef bulls housed at the Agri-Environment Service Branch Maple Creek and Spring Creek bull stations in Saskatchewan, Canada. In

addition, samples were collected at regular intervals from 13 bulls infected with *Campylobacter fetus* subsp. *venerealis* (Cfv), housed at the University of Saskatchewan Animal Care Unit. Four of the bulls were purchased from two infected herds in Saskatchewan, and were determined to be Cfv carriers by PCR [152], culture and phenotypic evaluation [182]. Isolates recovered from the naturally infected bulls were used to artificially infect 9 additional bulls as described by Bier et al. [97] with minor modifications. Briefly, Cfv cultures were grown for 3 days and harvested into phosphate buffered saline (PBS, 20 mM phosphate, 150 mM NaCl) using a sterile swab. The culture was dilute with PBS to an OD_{600} of 0.4 and 2 ml of this inoculum was deposited in the fornix of the prepuce using a sterile plastic pipette. The inoculum was also plated on 5% sheep blood agar plates (BBL Columbia SB agar, BD diagnostics, Mississauga, Ontario, Canada) to determine the colony forming units (CFU) administered. Initial challenge was performed as a series of two exposures approximately 1 week apart. In cases where initial challenge was not successful, a second series of two exposures was performed. All animal procedures were performed in accordance with the Canadian Council on Animal care and approved by the University of Saskatchewan Protocol Review Committee.

Data collection

Data on breed, age, order of the samples collected, and time between collection and sample processing at the laboratory was recorded for every bull sampled. In addition, ambient temperature at the time of sample collection was obtained from the National Climate Data and Information Archive, generated by the Weather Office of Environment Canada [259]. Data were obtained from the closest weather station relative to where the bulls were sampled. During the winter months the temperature value used was adjusted for the effect of wind as provided in the wind chill index [259]. For the virgin bulls, temperature data were obtained for every hour spent

during sample collection on a given day and averaged. For the Cfv infected bulls, temperature data was recorded for each sample collection day.

Sample collection and transport

Preputial samples were collected by means of the aspiration method [120,139]. Briefly, an individually wrapped 25" plastic pipette (Continental Plastics, Cambridge, ON, Canada) attached to a 20 ml syringe was inserted into the prepuce and the plastic sheath was pulled back. The scrape was performed by moving the pipette back and forward 10 times while applying 15 ml of suction. The preputial material was then rinsed into the appropriate media. Two samples were collected from all virgin bulls; however only one was used for the purpose of this study. Samples collected from virgin bulls were rinsed into 2 ml of PBS. Immediately, a 300 µl aliquot was transferred into 10 ml of modified Weybridge transport enrichment media (TEM) [134,144]. Direct preputial samples collected into PBS were placed in a styrofoam box containing ice packs, and samples in TEM were transported in an insulated container with warm water bags at approximately 25°C. Samples were transported to the laboratory for further processing within 24 h.

Two preputial samples were collected at weekly intervals from 13 Cfv infected bulls by the method described above. One sample was rinsed into 5 ml of a 1% buffered formaldehyde solution for direct antibody fluorescent test (DFAT), and placed in a refrigerator at 4°C within 2 h until further processing. The remaining sample was rinsed into 2 ml of PBS and an aliquot placed immediately into TEM, as described above. The order in which samples for either media were collected was alternated for each bull weekly. Both samples were placed in a styrofoam container with warm water bags at 27.6 ± 2.1 °C. All samples collected from infected bulls were

transported to the lab within 2 h, and those in TEM were left at room temperature (24.6 ± 1.1 °C) for 24 h [134].

Culture procedures

Samples collected in TEM from virgin bulls were plated onto Campylobacter Agar Skirrow (Oxoid, Nepean, Ontario, Canada) (N=191) or campylobacter selective agar (CSA, n=109) [135,260]. Campylobacter selective agar was prepared as described by Clark et al., 1972 [135] and two plates were made with 1.5% and 4% agar. A 300 µl aliquot was placed on the edge of the plate and spread using a disposable loop.

Direct preputial samples collected from Cfv infected bulls were plated onto Campylobacter Agar Skirrow, as described above, and 5% sheep blood agar (BA) plates (BBL Columbia SB agar, BD diagnostics, Mississauga, Ontario, Canada) overlaid with a 0.65 µm mixed cellulose ester membrane filter (Millipore, Billerica, Massachusetts, USA) [152]. A 300 µl aliquot was layered on the filter and plates were incubated aerobically at 37°C for 30 min to allow motile cells to migrate through the membrane, after which filters were removed and plates placed in an incubator. Samples placed on TEM for 24 h were then cultured in both Skirrow's and BA as described for direct preputial samples.

All plates were placed at 37°C for 72 h in microaerophilic conditions using a GasPak™ EZ CampyPouch™ System (BD diagnostics, Mississauga, Ontario, Canada). Culture plates were then removed and Cfv colonies were identified as smooth, 1-2 mm in diameter, convex, white-grayish translucent colonies [46] and microscopically examined by Gram stain. Gram-negative cells with *Campylobacter*-like morphology, straight or curved thin rods, were confirmed as Cfv by a conventional multiplex PCR as previously described [217]. Comparison of culture methods

using a subset of the samples presented here, has been reported elsewhere [147], and the use of the data here is for the purpose of comparison with the developed qPCR.

Direct fluorescent antibody test (DFAT)

All samples were processed within 10 days of collection. The test was performed using a fluorescein isothiocyanate (FITC) conjugated polyclonal antibody against *Campylobacter fetus* subsp. *venerealis* (CampyAzul-conjugado, Laboratorio Azul Diagnostico S. A., Azul, Buenos Aires, Argentina). The technique was performed as described previously with minor modifications [173,185]. Briefly, samples were centrifuged at 600 g for 10 minutes, and the supernatant was subjected to a second centrifugation at 2700 g for 30 minutes. The pellet was resuspended in 500 µl of PBS and 20 µl of the suspension was applied to a 12-well slide in duplicate. Samples were air-dried and fixed in ethanol for 15 minutes. The labeled antibody was applied to each well and incubated at 37°C in a dark humid chamber for 30 minutes. The slides were then washed three times in PBS for 10 minutes each. For each batch of samples processed a suspension of Cfv and *C. jejuni* were included as positive and negative controls, respectively. Samples with at least one fluorescent organism with morphology consistent with *Campylobacter* in either one of the duplicates was considered a positive sample.

Quantitative real time PCR (qPCR)

Extraction of DNA from direct preputial samples was performed by the heat lysis method as previously described [125,152]. Real time PCR reactions were carried out as previously described using primers VenSF and VenSR [152]. Data obtained was analyzed using computer software (iQ5 Optical System, Bio-Rad, Mississauga, ON, Canada). End point analysis was performed by calculating the average relative fluorescent units (RFU) for the last 5 cycles.

Negative controls (no template and extraction negative) were used to define unknown samples. Samples were considered positive if there average RFU value was greater than the negative control average plus a tolerance level. The optimal tolerance level was determined by using a receiver operating characteristic (ROC) curve as described below. Samples were considered positive if at least one of the duplicates had an average RFU value greater than the tolerance level. In addition, all positive samples were evaluated to determine if the correct melt peak signal was generated (78.5°C ± 0.5°C) [152].

Data analysis

In order to determine the optimal cutpoint for the qPCR assay a non-parametric receiver operating characteristic curve was developed [247]. The values used as cut point were defined as the tolerance above the average negative controls and expressed as percentage of the range of relative fluorescent units (RFU) for a given qPCR run. The percentage of the range (PR) above the negative controls for each sample was calculated by the following formula; {PR= (RFU $\overline{X_S}$ - RFU $\overline{X_N}$) / RFU_{range}}. Where RFU $\overline{X_S}$ is the average RFU for the duplicates of the sample, RFU $\overline{X_N}$ is the average RFU for the duplicates of the no template and extraction negative controls, and RFU_{range} is the highest RFU value of the run minus the RFU $\overline{X_N}$. The PR value was determined for every sample from both known Cfv infected bulls and virgin bulls. In those samples where only one of the duplicate had a C_t (Cycle threshold) value, only the RFU value of that duplicate was used in the calculation of the PR.

The receiver operating characteristic (ROC) curve was obtained using the LOGISTIC procedure, (SAS 9.2, Cary, NC, USA). In addition, a plot of sensitivity and specificity against the different

cut points was produced. For each cutpoint value the sensitivity and specificity was calculated using the FREQ procedure, and plots were produced using the GPLOT procedure (SAS 9.2). The optimal cut point was determined as the value that would maximize both sensitivity and specificity.

Crude specificity estimates for qPCR and culture were determined by calculating the proportion of virgin bulls that tested negative for each assay. Confidence intervals were calculated based on the Wilson score method [246]. The animals tested for specificity determination had never been exposed to breeding and were assumed to be true negatives and not infected with Cfv. Potential differences in the specificity between tests were evaluated by exact logistic regression using the LOGISTIC procedure (SAS 9.2). The order of the sample collected and the breed of the bulls were evaluated as covariates in the model.

Sensitivity estimates for each test were determined by calculating the proportion of samples collected from Cfv infected bulls testing positive. Confidence intervals were determined by the method described above. Eligible samples from Cfv infected bulls were defined as those obtained within the first sampling that tested positive by culture ("gold standard") until the last culture positive sample obtained or the completion of the study. Samples collected during the period defined were considered to originate from a known Cfv infected bull and were included regardless of individual test results. Clearance of infection was determined when 4 consecutive negative culture samples were obtained [96]. Potential differences in sensitivity between tests were evaluated using generalized linear mixed models with a binomial distribution and logit link function. The calculations were performed using the GLIMMIX procedure (SAS 9.2). Within-bull and sampling within bull clustering were accounted for with random intercepts. The proportion of total variance explained by differences between bulls ($\rho=\sigma^2_b /(\sigma^2_b + \sigma^2_s +\pi^2/3)$)

and sampling within bull ($\rho=(\sigma^2_b + \sigma^2_s)/(\sigma^2_b + \sigma^2_s +\pi^2/3)$) was also estimated [247]. The ambient temperature at the time of sample collection was included as a fixed effect in order to evaluate its influence in test sensitivity and first order interaction was evaluated. In addition, a series of potential risk factors were evaluated including: age, breed, order of sample collection, source of infection (natural or artificial) and transport time (≤1 h or >1 h).

The potential improvement in sensitivity with repeated sampling and testing by qPCR was analyzed by identifying groups of 3 sequential samples for each Cfv infected bull based on the date of sample collection. The resulting sets of three samples from successive weekly samples were used to calculate the cumulative sensitivity for 1, 2 or 3 consecutive samples. Potential differences in cumulative sensitivity between the numbers of consecutive samplings were evaluated by generalized linear mixed models with a binomial distribution and logit link function using the GLIMMIX procedure (SAS 9.2). Within bull clustering was accounted for with a random intercept.

Variables evaluated as potential risk factors in each model were retained if they were either significant ($P<0.05$) or acted as important confounders. Risk factors were considered to be confounders if removing or adding the factor from the model changed the regression coefficient for another risk factor of interest by more than 10% [247]. The level of significance used was 5%, and variables were considered statistically different when $P \leq 0.05$.

4.4 RESULTS

Determination of the cut point value for the qPCR

The resulting receiver operating characteristic curve is shown in Figure 4.1. The line depicted at a 45 degree angle represents a test with a 50% discriminating ability. The displacement of the curve towards the upper left corner indicates an improved discriminating ability. The cut point at which sensitivity and specificity are maximized was determined at 2.5% as shown in Figure 4.2, and corresponds to a sensitivity and specificity of 85%. The minimum cut point value that resulted in a sensitivity > 90% was 0.5%. However, the specificity at this value was 70.8%. Conversely, the minimum cut point value that determined specificity > 95% was 17%, but the sensitivity at this cut point decreased to 61.9%.

Specificity

The group of virgin bulls for the determination of specificity represented 5 breeds of beef cattle as follows: Angus 57% (172/300), Charolais 38% (114/300), Gelbvieh 2.3% (7/300), Simmental 1.7% (5/300), and Limousin 0.7% (2/300). Out of 300 samples collected from virgin bulls, 36% (109/300) were from 1 year olds, 33% (99/300) were 2 year olds, and 31% (92/300) were ≤ 2 years old, but the exact age was undetermined. Samples collected originated from a 1^{st} or 2^{nd} scrape in 69% (207/200) and 31% (93/300) of the bulls, respectively. The average ambient temperature for the days samples were collected from the virgin bulls was 5.3 ± 0.9 °C. Crude specificity estimates were 85% (95% CI: 80.5 to 88.6) for qPCR and 100% (95% CI: 98.7 to 100) for culture, and were significantly different (P<0.01). There was no significant effect of order of sampling (P=0.45) and thus sampling order was removed from the model.

Characteristics of Cfv infected bulls

The characteristics of Cfv infected bulls are described in Table 4.1. The mean age of the bulls was 5.2 years (range 3 to 7). Eight of the artificially challenged bulls were successfully infected with one series of two exposures. The remaining bull required a second series of two exposures. The mean number of colony forming units (CFU) inoculated into the prepuce was: $1.01x10^9$ CFU for the 1st exposure, $4.08x10^8$ CFU for the 2nd exposure, $6.4x10^8$ CFU for the 3rd exposure, and $3.3x10^9$ CFU for the 4th exposure. The mean interval between first exposure and the first culture positive sample obtained from the artificially infected bulls was 14.5 days (range 7 to 36 days).

Sensitivity

Crude sensitivity estimates for the different tests evaluated were: qPCR (85.4%, 95% CI: 80.6-89.2); direct culture on blood agar (82.3%, 95% CI: 77.2-86.5), DFAT (72.1%, 95% CI: 66.2-77.4), direct culture on Skirrow agar (32.7%, 95% CI: 27.2-38.7), TEM and blood agar (30%, 95% CI: 23.4-37.5), and TEM and Skirrow agar (38.1%, 95% CI: 31-45.9).

The mean ambient temperature for sample collection days from Cfv infected bulls was 5.1°C (range 27.5°C to -34°C). The effect of ambient temperature on sensitivity varied by test type ($P<0.0001$) (Figure 4.3) (Table 4.2). Overall, the qPCR assay and direct culture on blood agar plus filter had superior sensitivity. However, when temperatures increase above 5°C the sensitivity of the qPCR was significantly higher than culture. Conversely, at progressively colder temperatures, direct culture on blood agar had higher sensitivity. The sensitivity of the qPCR increased as ambient temperature increased; whereas, the performance of direct culture on blood agar remained relatively constant.

The sensitivity of DFAT was superior to culture using TEM and direct culture on Skirrow selective agar; however, it was lower than qPCR and direct culture on blood agar. The increase in sensitivity of the DFAT with increasing temperature is similar to that described for qPCR. Both direct culture and the use of TEM in Skirrow selective agar showed a decrease in sensitivity as temperature increases. Conversely, the use of TEM and blood agar had higher sensitivity at increasing temperatures.

There were no other measured factors associated with the sensitivity of the tests including: order of sampling (P=0.92), type of infection (natural vs. artificial) (P=0.35), breed (P=0.75), age of the bull (P=0.34), or sample transport time (≤1 h vs >1 h) (P=0.25). Differences among bulls were responsible for 12.8% of the unexplained variation, and differences among samples within bull were responsible for 35.2% of the unexplained variation.

Performance of the qPCR and repeated testing

Only 2 of the 13 bulls (15.4%) were positive by qPCR in every sample collected. The percentage of positive samples and the mean quantity of organisms per ml of sample for each bull is shown in Table 4.3. There was wide variation in the percentage of positive samples obtained by qPCR for each bull, ranging from 46 to 100%. The mean quantity of organisms per ml of sample as determined by qPCR was 7.0×10^7 (range 0 to 2.5×10^8).

The use of repeated testing at weekly intervals significantly improved the sensitivity of the qPCR test (P<0.0001). However, the increase was significant for up 2 samplings, and no further improvement was seen when 3 samples were collected (P=0.051) (Figure 4.4).

4.5 DISCUSSION

The cut point chosen for the described qPCR assay was a tolerance level of 2.5%, which optimized both sensitivity and specificity. Further comparisons with culture and DFAT in the present study have been performed using this cut point and interpretation will change with different cut points [261]. The optimal cut point for an assay can be adjusted depending on the intended goal of the testing. The choice of cut point should be based on the pre-test probability in the population of interest as well as the consequences of false positives and false negatives [261]. For example, screening the bull battery in a commercial cow-calf operation in which Cfv is suspected could require a cut point that maximizes sensitivity as the pre-test probability is high and the cost of false negative results can be very high. On the other hand, a test used for a pure bred bull from a semen collection centre should initially optimize specificity to avoid unnecessary culling of a valuable animal. Ultimately, the objective of the testing strategy and the pre-test probability of the infection should dictate the selected cut point.

The sensitivity of 85% for the qPCR assay used in this study was comparable to direct culture under optimal conditions, and similar to the sensitivity of a published study based on a multiplex PCR using the same primers [262]. However, in that study, the multiplex PCR was performed on samples inoculated into TEM and estimates were obtained by comparison to culture. The only real time PCR assays described for Cfv detection directly from preputial samples are the SYBR Green based assay used in this study and a 5' Taq nuclease assay described by McMillen et al. [125]. Although the analytical sensitivity of the two assays is comparable, there is currently no information available on clinical sensitivity and specificity for the McMillen assay. One potential limitation of the Taq nuclease assay is that the primers used by McMillen targeting the *parA*

gene have been shown to have mismatches with certain Cfv sequence types and have also been shown to cross react with a strain of *C. hyointestinalis* [152,223].

The internationally recognized gold standard for BGC diagnosis in the bull is isolation of Cfv by culture of preputial secretions [173]. The use of direct culture has been relatively successful when samples are processed within 2 to 4 h [147,169]. However, sensitivity reports for this method are quite variable and range from 32% to 94% when using selective media [97,135,146,147,169,171]. An alternative method using passive filtration and non-selective media has been successful for the isolation of other campylobacter from feces [175,176]. A recent report by our research group, using samples collected for the present study, compared the use of this filtration system and Skirrow selective agar. Sensitivity of direct culture using the filtration technique was significantly higher than using Skirrow agar [147]. However, transport times in that study were less than 2 h and samples were kept at a relatively constant temperature. Such conditions are hard to obtain in most field situations. Diagnostic laboratories are often located at great distances from where the samples are collected, thus requiring shipment of the samples through courier services and transport times greater than 24 h.

Considering the limitations of direct culture in field situations, several strategies have been developed to allow for the detection of Cfv in field samples. The use of transport enrichment media (TEM) provides an appropriate environment for Cfv. Although it improves the recovery rate during prolonged transport times, its sensitivity in the present study was significantly lower than direct culture, DFAT and qPCR. Moreover, the use of TEM requires special media and transport conditions, and is time consuming. Although its composition is directed towards supporting the growth of Cfv and inhibiting contaminants, overgrowth by species like *Pseudomonas aeruginosa* remains an issue [147].

A monoclonal antibody capture ELISA has been developed as a screening test for the detection of *C. fetus*, and has been reported to have a sensitivity and specificity of 100% and 99.5%, respectively [27]. However, this test does not allow for the differentiation between Cfv and *Campylobacter fetus* subsp. *fetus* (Cff) and still requires samples to be collected into TEM and maintained under strict transport conditions. Another alternative has been the use of the DFAT; this test does not require samples to be collected in laborious media and has a relatively high sensitivity. Previous reports have indicated a sensitivity > 90% for this test [169,186], while the results of the present study indicate a lower estimate. A potential explanation might be based on the degree of experience of the operator as positive samples are not only detected by positive fluorescence, but also based on morphological characteristics. However, the main disadvantage of this technique is its cross-reaction with certain Cff strains of serotype A [186]. The qPCR test described here presents the advantage that no special media is required, is relatively fast and has an improved sensitivity compared to most other tests available.

Specificity estimates obtained for the qPCR are also relatively high. However, the proportion of virgin bulls that tested positive by this method was higher than expected (15%). For the purpose of this study, virgin bulls were assumed to be negative for BGC due to the lack of exposure to breeding as reported by the owners. It is possible that some of these bulls had been exposed to breeding without notice by the owner. Further research should be conducted on bulls kept in isolation from the time of birth to properly asses this possibility. Bull to bull transmission has also been suggested in the literature as a potential means of transmission based on the frequent mounting behavior observed, particularly in younger bulls [263]. However, to the best of our knowledge, there has been no scientific data to support this assumption, and the high susceptibility of the organism to environmental conditions makes this unlikely [6].

A recent report indicated a positive reaction of a *Campylobacter hyointestinalis* strain in New Zealand with primers VenSF/VenSR used in this study [223]. The target of these primers, the *parA* gene located in a genomic island [47], could have been acquired by this strain of *C. hyointestinalis* as the genomic island is believed to be a mobile element [223]. Although this has been the only reported misclassification with these primers, there is a need for further research. In addition, a Cff strain recently isolated from veal calves contains at least part of the genomic island believed to be exclusive to Cfv, and that a truncated portion of the *parA* gene appears to be included [264]. Whether the assay described herein would result in the misidentification of the new Cff strain as Cfv is not known and further research is needed to evaluate this possibility. Both Cff and *C. hyointestinalis* are commonly isolated from feces of cattle [24,265], and could potentially contaminate the prepuce in young bulls, where mounting and sodomizing is a common behavior [266], thus explaining the positive reactors to the qPCR found in the present study. Despite the potential limitations, the qPCR reported here is the only real time PCR assay described for which clinical sensitivity and specificity have been reported.

The relationship between ambient temperature at the time of sample collection and sensitivity of the tests described provides novel information that could aid in the design of testing strategies that would maximize the opportunity to detect carrier bulls. To the best of our knowledge, this is the first report that identifies this type of relationship. The improvement in sensitivity observed for the qPCR and DFAT when temperatures increase could be explained by differences in the population of Cfv organisms within the preputial cavity. Variations in the number of organisms recovered between examinations and between bulls have been described [20]. It is possible that ambient temperatures could affect the level of infection in bulls by changes in the temperature within the preputial cavity, resulting in higher numbers of Cfv in warmer temperatures. As a

result the likelihood of identifying carrier bulls would increase. However, accurate information regarding quantity of organisms in the preputial cavity are lacking due to the fact that sampling methods currently used do not generate consistent sample volumes. Thus, further research is needed to properly evaluate the number of organisms present and whether fluctuations are associated with ambient temperatures.

The relationship between culture and ambient temperature presents some interesting differences. When Skirrow media was used directly or with TEM, sensitivity decreased as ambient temperatures increased. The occurrence of fungal overgrowth in selective agar for Cfv has been previously described, and can result in significant reductions in recovery rates [134,147]. The overgrowth by fungus could potentially explain why sensitivity decreases at progressively warmer temperatures, as the warmer temperatures provide better fungal growth conditions. Seasonal variations in the occurrence of fungal contamination have been previously suggested [169].

The influence of temperature when using the passive filtration system in non-selective agar differed depending on whether culture was performed directly or after transport in TEM. The relationship with passive filtration in non-selective agar when using TEM is similar to that described for qPCR and DFAT and might be a reflection of differing numbers of organisms in the prepuce. However, when culture was done directly on non-selective agar, there was almost no influence of temperature. This could be due to the short transport time and appropriate control of contaminants, maximizing the opportunity for isolation. Although several organisms have been commonly isolated from preputial samples such as *C. sputorum* bv. *bubulus* [9], *Proteus spp.* [134,171], and *Pseudomona aeruginosa* [134,147] little is known about the microbial

population of the preputial cavity and how the ecology could influence the establishment and persistence of Cfv.

The low proportion of bulls testing positive at every sampling, plus the variability in the number of organisms within samples from the same bull, indicate that a single test is likely not enough to ensure identification of all carrier bulls. Because variations in the level of infection have been reported [20], reliance on a single test is discouraged [187]. The examination of 3 to 6 consecutive samples collected at weekly intervals by culture or DFAT has been the most common recommendation [22,171]. The cumulative sensitivity of the qPCR in 2 consecutive preputial samples showed a significant improvement as compared to the sensitivity of a single sample. Although there was no significant improvement when 3 examinations were performed as compared to 2, the use of 3 samples would allow identification of almost 100% of infected bulls. The use of repeated sampling has been a common practice in the diagnosis of *Tritrichomonas foetus* [120] and both practitioners and producers are already familiar with this strategy. Moreover, the use of direct preputial samples collected in PBS would allow for the evaluation of the two main venereal diseases from the same sample using the qPCR described here and a previously reported real-time PCR for *T. foetus* [253].

4.6 CONCLUSIONS

Current diagnosis of BGC in extensive cattle operations is cumbersome due to prolonged transport times and adverse conditions during such transport. The present study describes the performance of a high throughput qPCR assay from direct preputial samples, with a relatively high sensitivity and specificity. Moreover, the influence of ambient temperature at the time of sample collection would suggest that samples should be collected in preferably above freezing

temperatures. Finally, although the sensitivity of the qPCR described is relatively high, repeated sampling might still be required to accurately identify carrier and non-carrier bulls.

Acknowledgments

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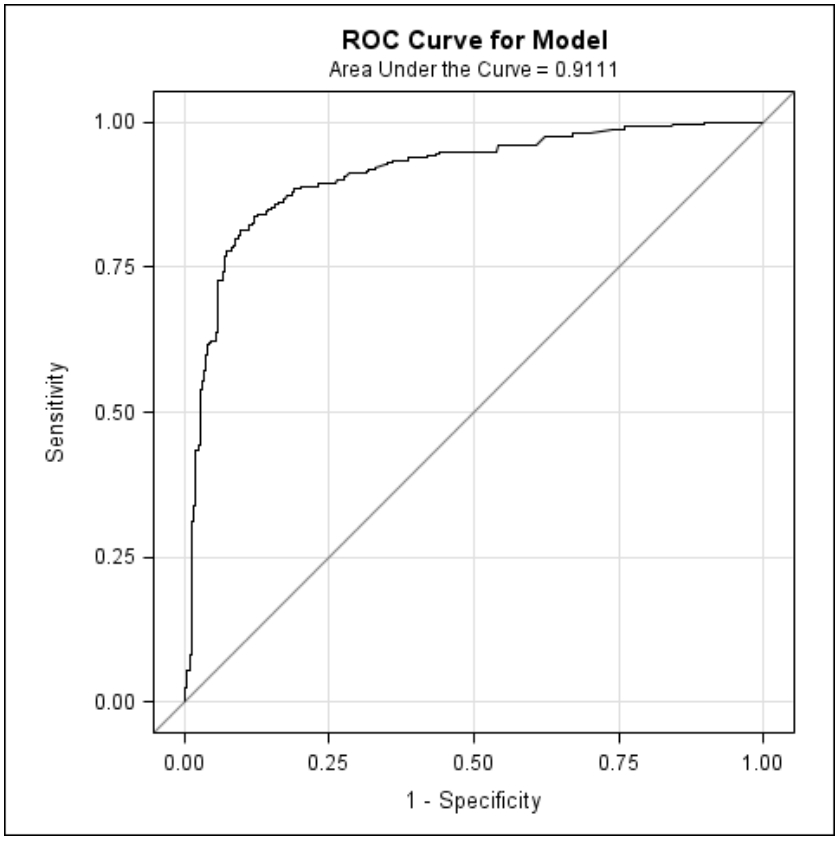

Figure 4.1. Receiver operating characteristic (ROC) curve for different tolerance levels used as cut points for the qPCR assay (N=560).

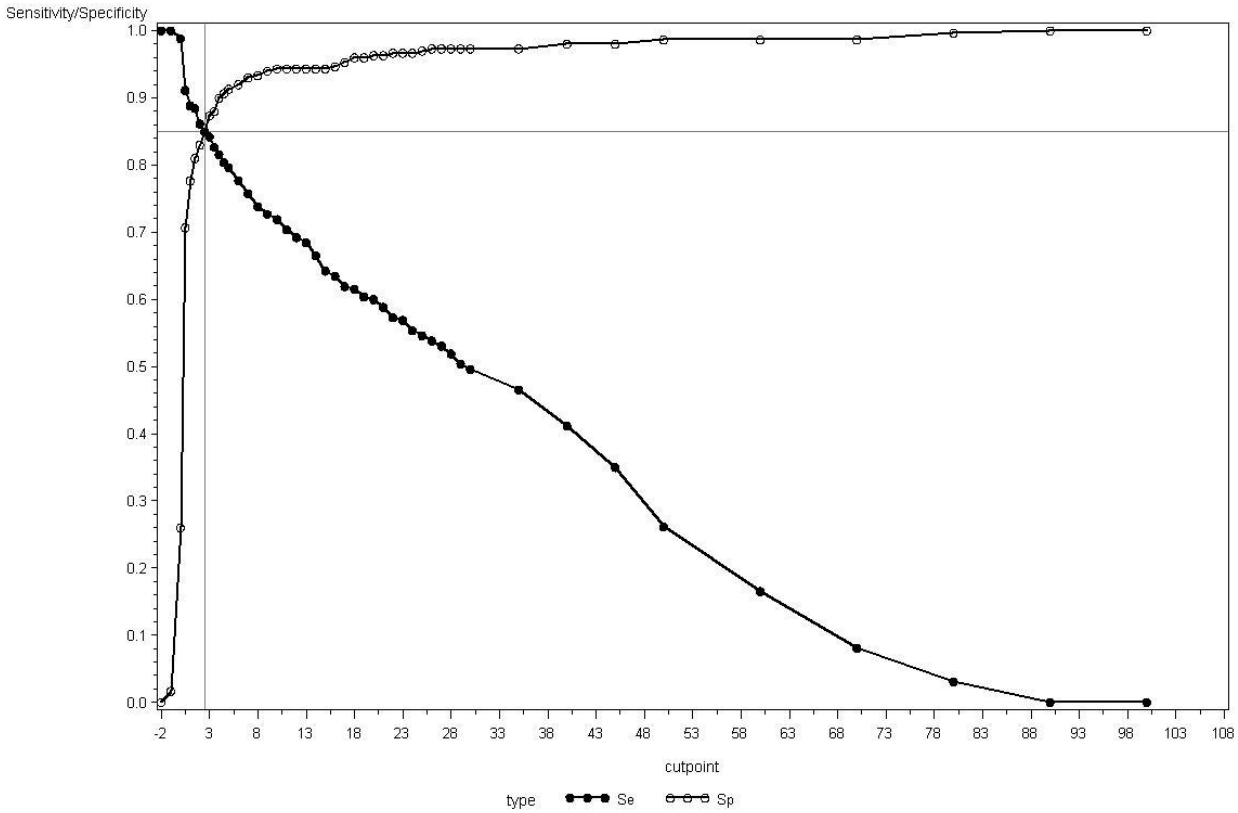

Figure 4.2. Sensitivity (Se) and Specificity (Sp) of the qPCR assay for different cut points. The vertical line indicates the cut point value at which Se=Sp, and the Se/Sp value is indicated by the horizontal line.

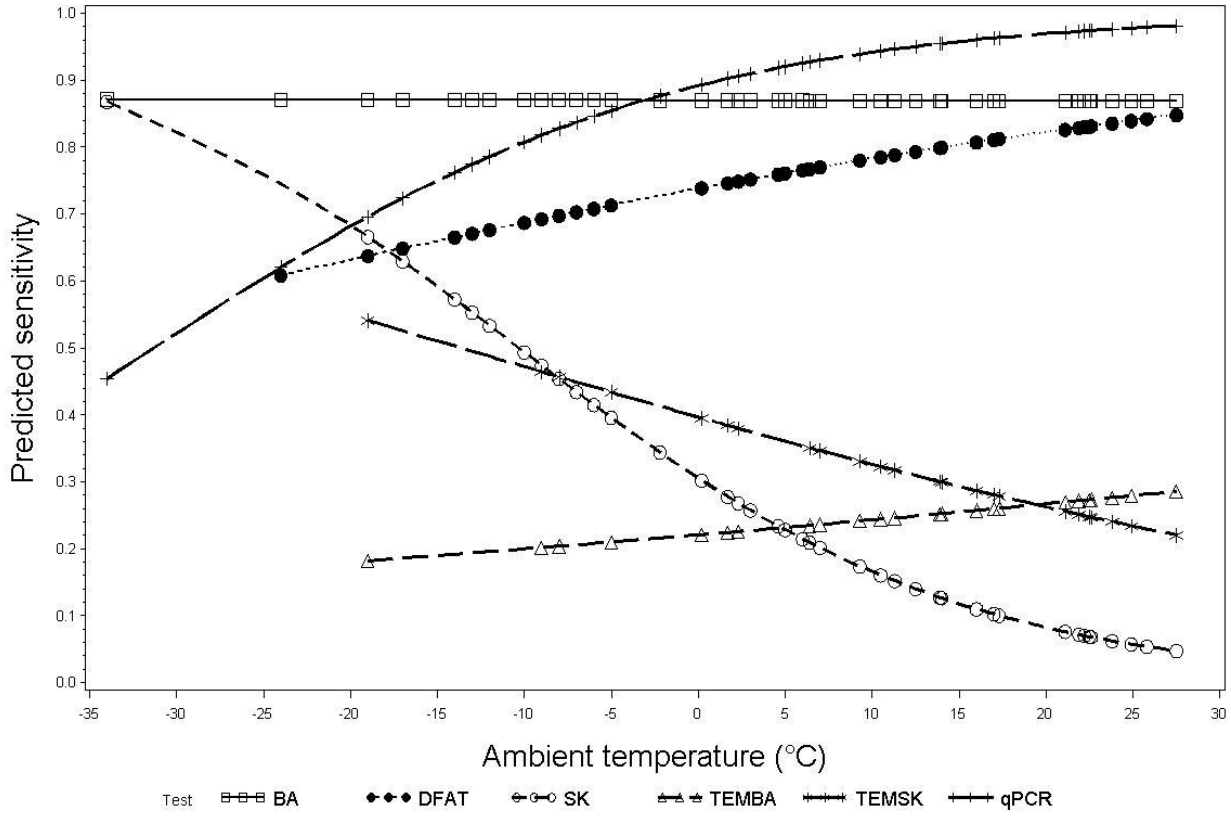

Figure 4.3. Predicted sensitivity estimates for 6 different diagnostic tests in preputial sample at different ambient temperatures. Data was obtained from the generalized linear mixed model. BA (direct culture with blood agar plus filter, N=260), DFAT (direct fluorescent antibody test, N=244), SK (direct culture in Skirrow selective agar, N=251), TEMBA (transport enrichment media and subsequent culture in blood agar plus filter, N=160), TEMSK (transport enrichment media and subsequent culture in Skirrow selective agar, N=160), qPCR (quantitative real-time PCR, N=260).

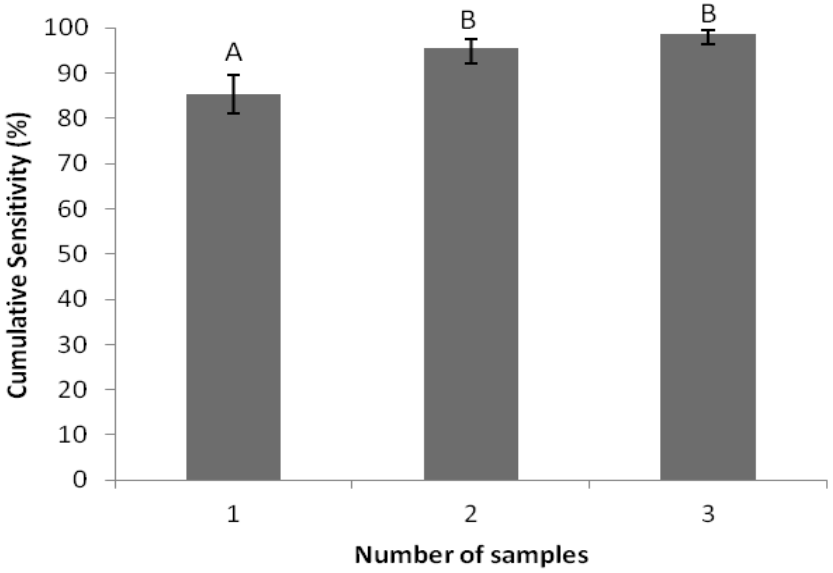

Figure 4.4. Cumulative sensitivity estimates and 95% confidence intervals of 1 (n=260), 2 (n=247) or 3 (n=234) direct preputial samples collected at weekly intervals and tested by qPCR (quantitative real-time PCR). ^{A,B} indicate significant differences between number of samples ($P<0.05$).

Table 4.1. Individual characteristics of bulls infected with *Campylobacter fetus* subsp. *venerealis* used to estimate the sensitivity of 3 diagnostic methods.

Type	ID	Breed	Age (yrs)	Samples collected	Duration of infection*	N° of exposures	Interval between exposures
Artificial	Jim3	Angus	5	4	21†	2	7
	25	Gelbvieh	7	13	83	2	8
	75	Angus	7	13	83	2	8
	94	Limousin	4	11	70	2	8
	173	Angus	7	5	28†	2	7
	953	Angus	4	13	83	2	8
	5201	Angus	7	41	287	2	7
	5206	Angus	3	6	46†	4	6 (17)‡
	5215	Limousin	3	28	188	2	28
Natural	19uB	Angus	4	20	307†		
	19uY	Angus	4	43	342		
	66Y	Angus	6	21	140		
	TD	Angus	6	42	335		

*Determined as the period of time in days between the first and the last culture positive sample obtained or the end of the study. Animals that cleared the infection are marked with†.

‡ The number in brackets indicates the interval between the two series of two exposures.

Table 4.2. Adjusted sensitivity estimates and 95% confidence intervals for culture, DFAT (direct fluorescent antibody test) and qPCR (quantitative real-time PCR) under different ambient temperatures during sample collection.

			Ambient temperature (°C)				
	Test	**N**	**-5**	**0**	**5**	**10**	**15**
Direct Culture	**Blood agar (Filter)**	260	87.0 [A] (77.5-92.9)	87.0 [A] (78.1-92.6)	87.0 [A] (78.3-92.5)	87.0 [B] (77.9-92.6)	86.9 [B] (77.0-92.9)
	Skirrow	251	39.6 [C] (26.1-54.8)	30.5 [D] (19.6-44.3)	22.8 [D] (13.9-35.0)	16.6 [E] (9.4-27.4)	11.8 [D] (6.1-21.4)
TEM + Culture	**Blood agar (Filter)**	160	21.0 [D] (10.6-37.3)	22.1 [DE] (12.2-36.6)	23.2 [D] (13.5-36.7)	24.3 [DE] (14.4-37.9)	25.5 [C] (14.7-40.4)
	Skirrow	160	43.4 [C] (26.7-61.7)	39.6 [CD] (25.2-56.1)	36.0 [C] (23.1-51.4)	32.6 [D] (20.4-47.6)	29.3 [C] (17.4-45.0)
	DFAT	244	71.3 [B] (57.1-82.3)	73.8 [B] (60.8-83.6)	76.1 [B] (63.7-85.2)	78.3 [C] (65.9-87.1)	80.3 [B] (67.5-88.9)
	qPCR	260	85.5 [A] (75.5-91.8)	89.2 [A] (81.3-94.0)	92.0 [A] (85.4-95.8)	94.2 [A] (88.4-97.2)	95.8 [A] (90.7-98.1)

[A,B] Different superscripts within column are significantly different (P<0.05).

Table 4.3. Mean organisms per ml of preputial sample and proportion of samples testing positive by qPCR for each Cfv carrier bull.

Type	ID	Mean (organisms/ml)	Range (organisms/ml)	Positive samples (%)
Artificial	173	$8.95x10^5$	0 - $1.88x10^6$	4/5 (80)
	25	$1.18x10^7$	0 - $6.65x10^7$	8/13 (61.5)
	5201	$4.75x10^6$	0 - $2.98x10^7$	36/41 (87.8)
	5215	$3.29x10^6$	0 - $1.77x10^7$	24/28 (85.7)
	953	$1.14x10^6$	0 - $1.03x10^7$	6/13 (46.2)
	94	$3.26x10^5$	0 - $1.17x10^6$	7/11 (63.6)
	75	$6.94x10^6$	0 - $4.40x10^7$	12/13 (92.3)
	5206	$2.81x10^7$	0 - $1.10x10^8$	5/6 (83.3)
	Jim3	$1.22x10^6$	0 - $2.46x10^6$	3/4 (75)
Natural	TD	$4.86x10^7$	0 - $5.50x10^8$	36/42 (85.7)
	19uY	$3.49x10^8$	$2.08x10^5$ - $2.5x10^9$	43/43 (100)
	19uB	$3.09x10^7$	0 - $1.95x10^8$	18/20 (90)
	66Y	$7.78x10^6$	$4.55x10^4$ - $2.79x10^7$	21/21 (100)

5. GENERAL DISCUSSION AND FUTURE RESEARCH

5.1 DISCUSSION

This thesis describes the evaluation of diagnostic methods for the detection of the two main venereal diseases in bulls. The sensitivity of a real-time PCR assay for the detection of *T. foetus* in both individual and pooled preputial samples with or without prior enrichment by culture was evaluated. In addition, the specificity of such assay was determined in individual samples enriched by culture. Furthermore, the sensitivity and specificity of a recently developed real-time PCR for the detection of *Campylobacter fetus* subsp. *venerealis* was determined and compared to other diagnostic methods currently available.

Diagnostic methods for *T. foetus* have evolved slowly from culture and microscopic detection to the use of PCR based methods. Particularly, the introduction of real-time PCR has had a significant impact on the strategies used in the detection of carrier bulls. Previous studies have described the development of this assay, and to a certain extent the performance with field samples, however, there is a lack of precise estimates of sensitivity and specificity [126,198]. Moreover, the use of pooled samples has been introduced recently and need for further information on size of pools and media in which the samples should be collected is needed [203].

Conversely, diagnostic tests for Cfv in preputial samples continue to rely on isolation and phenotypic identification as recommended by the World Organization for Animal Health [173]. Polymerase chain reaction based tests have been used mainly for the identification of isolates obtained by culture, and have not been applied directly to preputial samples [152]. Recently, real-time PCR tests have been developed for the detection of Cfv in preputial samples [125,152],

and information on their performance in clinical samples is needed in order to be applied adequately for diagnostic purposes.

The purpose of this chapter is to summarize the main findings of this thesis, obtained from three studies. In addition, the strengths and limitations of each of these studies will be presented and potential improvements explored. Finally, further areas of research related to diagnostic strategies for the main venereal diseases in cattle will be discussed as a starting point for improvement in their control.

In the first study (Chapter 2) the specificity of a real-time PCR test for *T. foetus* in culture enriched preputial samples was determined. Given the improved analytical sensitivity of this assay concerns have been raised regarding its specificity. A recent study indicated that false positive results were a common finding when real-time PCR was used. However, the gold standard in this study was a combination of culture and conventional PCR [198]. In this study, they assumed that negative testing bulls from infected herds are indeed negative which poses the question whether the bulls positive by real-time PCR were false positives or not. The results presented in this thesis indicate that the real-time PCR evaluated was highly specific (100%). The use of a population of known negative status, virgin bull and steers, allows for accurate determination of the test specificity. The implementation of PCR methods and their high analytical sensitivity has brought questions on the significance of positive results. These tests allow for the detection of non-viable organisms and very low numbers, however the biological meaning in the development of infection remains unknown [248].

Culture methods for the identification of *T. foetus* remain the gold standard. The improvement of culture methods has been significant and the technique has become more user friendly since the

introduction of the InPouch culture kit [158]. However, the use of this method does present some disadvantages. In the first place, concerns about specificity due to the presence of non-*T. foetus* trichomonads undistinguishable from each other under routine microscopic examinations have been put forward [156]. The percentage of false positives caused by these organisms has been estimated to be as high as 8%. The results presented in this thesis indicate a substantially lower prevalence of non-*T. foetus* trichomonads (1.2%). The difference could be due to differences in prevalence between geographic locations as the cited work was conducted in Argentina, or to differences in sample collection method. In the study by Campero et al. [156], no plastic sheath was used and contamination could be responsible for the difference in prevalence. The specificity of the culture method using the InPouch culture kit was comparable to that obtained with the real-time PCR and as a result is a valid diagnostic tool.

Although the presence of non-*T. foetus* trichomonads has been found in different sample collection dates from the same bull it appears that this is the result of contamination with feces, as colonization of the prepuce with *Tetratrichomonas* has not been achieved after inoculation of pure cultures [156,166]. The development of improved sample collection methods that would maximize the number of *T. foetus* organisms recovered minimizing environmental contamination would be of great benefit to diagnostic accuracy. Although several sampling methods have been developed and compared, little has been done towards reducing contamination. Double guarded pipettes may provide enough protection to avoid collection of manure and dirt commonly present in the preputial orifice.

This is the first report in which non-*T. foetus* trichomonads were recovered from steers. It appears that the prevalence of these organisms in steers is not different than in virgin bulls. These findings provide valuable information to potentially use steers as a model. In the first

place steers could be routinely used as known negative animals for the determination of specificity of newly developed tests. Whether steers can be successfully infected with *T. foetus* remains unknown, but further research is needed to evaluate this possibility. The use of steers as a model would provide a safer and readily available source of animals for research.

Sensitivity of culture tests for *T. foetus* reported in the literature vary greatly from as low as 67% to 100% [139,159]. Although different media have been used, high sensitivity has been achieved using the InPouch culture kit when optimal transport conditions are provided [154]. Similarly, the results obtained in the second study (Chapter 3) indicate that culture techniques can have high sensitivity (95%) when transport times are reduced and temperature is controlled. Unfortunately these conditions are difficult to obtain when collecting samples in commercial herds, and diagnostic laboratories are located at great distances from the sampling location. As a result, care must be taken when interpreting the results presented, and a decrease in sensitivity could be expected especially during extreme weather conditions [131,132].

Contaminating organisms may affect the ability to detect *T. foetus* in culture. The InPouch kit contains a proprietary medium and its composition is not publicly available. Previous studies showed that this culture kit provides better control of bacterial and fungal contamination than other media [58]. Although the effect of contaminants on the sensitivity of the culture test has not been addressed in the present study, the large proportion of culture pouches with evidence of contamination (18%) is noteworthy. The development of heavy contamination may impair the ability of *T. foetus* to develop and thus become detectable. As a result, care must be taken when examining contaminated samples and the possibility of re-testing these animals explored. Improvement in sample collection methods to reduce contamination would minimize this problem.

Overall the use of culture and microscopic examination for *T. foetus* diagnosis in preputial samples appears to remain a useful method. However, the evaluation of cultured samples is time consuming as examination is performed usually over 5 to 7 days, especially when a large number of samples need to be processed. In addition, this results in an important delay for the communication of results. Furthermore, the cost associated with the culture medium plus the labor needed to perform the examinations increases the cost of testing.

Since the development of PCR based methods for *T. foetus* diagnosis these techniques have been applied mainly to culture enriched samples [156,195]. The development of the 5' Taq nuclease real-time PCR assay indicated that testing of direct preputial samples allowed for improved analytical sensitivity when compared to culture enriched samples [126]. The potential explanation for this was based on the fact that culture enrichment may not only allow for multiplication of the organism but also for greater concentrations of potential PCR inhibitors. However, the potential substances responsible for inhibition have not been identified and as a result the variation in their concentration during culture remains unknown. In the study presented in Chapter 3 no difference in sensitivity was found between real-time PCR performed on direct and culture enriched preputial samples.

The lack of difference in sensitivity of the real-time PCR test between direct and cultured enriched preputial samples could be due to the short transport time provided, allowing for optimal conditions for rapid multiplication of the organisms. As mentioned before, transport under field conditions involves prolonged times and extreme temperatures. Thus it is possible that multiplication of the organism in the culture media would be impaired and the sensitivity of the real-time PCR test in culture enriched samples be reduced. As a result, further research is needed to compare the performance of real-time PCR in both types of samples when prolonged

transport times and adverse conditions are provided. Despite the lack of difference in sensitivity, the major advantage of using direct preputial samples would be the cost reduction for testing. Given that no special media is required and that PBS can be easily prepared at low cost, this approach would provide a cost effective strategy.

The use of pooled preputial samples has been previously described and shown to have high sensitivity when pools of 5 samples are tested by either conventional or real-time PCR [203,204]. Results presented in Chapter 2 indicate that pools of up to 25 samples can be made without compromising sensitivity when samples are collected individually and cultured before testing by real-time PCR. Although this study included positive samples of a wide range of *T. foetus* concentrations they all originated from a single infected animal. The use of a single animal as a source of positive samples presents certain limitations as different strains may colonize and persist in different levels, and individual variation between animals will result in different levels of infection [115]. Consequently, increasing the number of bulls providing positive samples and the use of different isolates would be required to further validate this strategy.

One of the major findings of the study presented in Chapter 2 was the most of the pools of culture enriched samples that tested negative by real-time PCR regardless of pool size contained the same contributor as a positive sample. This sample showed evidence of overgrowth by contaminants as the media had turned green in color and a large amount of gas was present. It is possible that given the high degree of contamination present this would result in the presence of potential PCR inhibitors thus preventing the detection of the organism by reducing the analytical sensitivity [249]. A previous study has shown that the presence of > 2% of feces in the sample will result in a 10 fold decrease in analytical sensitivity of a PCR test for Cfv performed in

preputial samples [181]. In summary, it would be important to avoid pooling samples that show evidence of contamination so as to maximize the opportunity to identify carrier bulls.

Based on the results presented in Chapter 2, a means of avoiding overgrowth by contaminants and their potential inhibition is to use direct preputial samples collected in a non-nutrient media. As mentioned previously the use of direct samples have resulted in a higher analytical sensitivity [126]. The study presented in Chapter 3 intended to address this strategy by evaluating the sensitivity of real-time PCR in pools of 5 or 10 direct preputial samples. The sensitivity obtained was lower than previous reports using culture enriched samples [204] and those presented in Chapter 2. A potential contributing factor for this difference could be the use of samples from a carrier bull but irrespective of their individual test status at a particular sampling. Although this approach more accurately reflected sampling in a herd, it might result in lower sensitivity as not all bulls are consistently positive. Despite this difference, the sensitivity of real-time PCR on direct pooled preputial samples is relatively high and did not decrease when the pool size was increased from 5 to 10 samples.

The use of real-time PCR in pooled samples provides the possibility of testing a large number of samples in a timely and cost-effective manner. Moreover, the use of direct preputial samples would, as mentioned before, further reduce the cost as no special media is required. In the proposed strategy, samples are to be collected individually and pooled later in the laboratory. Although pooling of samples chute-side would further reduce costs, the need for re-sampling if a pool is positive and the inability to evaluate appropriately the degree of contamination of a sample before pooling would probably be more expensive and affect the sensitivity of the test.

The concentration of *T. foetus* in preputial fluid is quite variable between and within bulls. The median number of organisms per ml of preputial fluid for individual bulls ranges from 80 to 44,000 [115]. In that same study several bulls had samples in which *T. foetus* could not be detected, although later samplings revealed that the bull remained a carrier. Approximately 50 to 70% of the bulls yield consistently positive samples and these results have been obtained by either direct microscopic examination or culture and conventional PCR [115,198]. In the study described in Chapter 3, similar estimates were obtained depending on the test used. The occurrence of these variations has led to the use of repeated testing in order to maximize the probability of identifying carrier bulls.

Repeated testing has been commonly applied to individual testing and, as shown in the results presented in Chapter 3, it appears to be a valuable strategy when using pooled samples. The use of 2 consecutive samples significantly increased the cumulative sensitivity when direct preputial samples were pooled. Based on these results, two consecutive direct preputial samples should be collected when using pools of 5 or 10 in order to maximize sensitivity. In Chapter 2 this assessment was not done due to the limited number of pools tested for each ratio and because all positive samples originated from a single carrier bull. The relative increase in the sensitivity of repeated sampling when culture enriched samples are pooled and tested by real-time PCR remains unknown and further research is needed to evaluate this strategy.

Identification of Cfv carrier bulls has been a major limitation in the control of BGC, particularly in extensively managed cattle. Culture techniques present several disadvantages when samples are not processed in the laboratory within a few hours of collection [147]. Currently in North America there is a lack of alternative diagnostic methods with the exception of an ELISA test for antigen detection [27]. However this assay is not able to distinguish between Cfv and Cff, and as

a result has not been widely adopted. Recently a real-time PCR was developed for use in direct preputial samples and shown to have high analytical sensitivity [152]. The results presented in Chapter 4 describe the clinical sensitivity and specificity of this test. The assay presented an overall sensitivity and specificity of 85%. Sensitivity was better than culture after incubation in transport enrichment media (TEM) and DFAT. As a result this test provides a useful alternative for samples collected at distant locations of the diagnostic laboratory.

One of the main findings of the study presented in Chapter 4 was the relationship between ambient temperature and the sensitivity of the different tests. The influence of ambient temperature could potentially have a great impact on testing strategies. As presented in Chapter 4 increasing temperatures tend to increase the sensitivity of both the real-time PCR and DFAT. However, care must be taken as the range of temperatures evaluated is representative of climate in Western Canada. The impact of higher temperatures, as those present in southern areas of North America, have not been evaluated. Whether the ambient temperature influences the level of organisms present in the preputial cavity remains unknown, due to the lack of a sample collection method providing consistent measurable volumes of preputial fluid.

The correct identification of *C. fetus* subspecies is one of the greatest challenges for BGC diagnosis. Traditional phenotypic testing has proved to be unreliable due to the potential of strains acquiring the characteristic of interest by mutation [258]. The identification of both subspecies has been of great interest particularly for the international trade of semen and embryos. Molecular diagnostic techniques have been used in an attempt to resolve this issue, however the problem remains as agreement between techniques is variable [219]. Given the great similarity between both subspecies of *C. fetus*, the most common targets used for PCR tests are located in what are believed to be mobile elements acquired by Cfv during its evolution.

Recently some of these targets, like the one used in the study presented in Chapter 4, have been found in Cff strains and *C. hyointestinalis* [223,264]. This could potentially explain the lower specificity found for the real-time PCR test used in Chapter 4. Although this has been reported in very few cases it is of great concern as acquisition of the PCR target by other *Campylobacter* species will render the test unfit for correct diagnosis. The recent completion of the genome sequencing of Cfv has allowed for a more comprehensive comparison between Cff and Cfv [53], and the sequencing of more strains in the future will provide useful information on the selection of the most appropriate targets for diagnostic assays.

The effect of repeated sampling on the sensitivity of a real-time PCR test for Cfv detection was also evaluated. Similar to the findings described for *T. foetus* the testing of two consecutive samples increases the sensitivity of the test. The proportion of bulls testing positive in every single examination is extremely low. This is likely due to variations in the level of infection within the preputial cavity as reported previously [20,88]. As a result, the use of repeated testing has been recommended when designing diagnostic strategies for carrier bulls. As mentioned previously the pre-test probability of infection along with the sensitivity and specificity of the test chosen should dictate the number of samples from each animal that need to be examined.

The use of direct preputial samples has the potential to allow for the testing of both *T. foetus* and Cfv from the same sample. The results presented in Chapter 3 and 4 support the use of this type of sample for the detection of both pathogens by use of real-time PCR methods. Traditionally, the diagnosis of both pathogens needed different media and required either the collection of two samples or the splitting of the sample after collection which usually did not occur until several hours after. The use of a single sample would reduce the costs associated with sample collection

and use of special media, and the use of sensitive tests with the report of results in a timely manner.

5.2 FUTURE RESEARCH

The collection of adequate samples is the first step needed for the diagnosis of venereal diseases in bulls. Several methods have been developed in order to maximize the number of organisms recovered. However, control of potential contaminants has been shown to be a key point for the successful identification of both *T. foetus* and Cfv. Samples are usually collected in a chute system and contamination with feces and dirt is a common issue. The preputial orifice is often covered in manure or mud and renders the collection of a clean sample cumbersome. The use of plastic pipettes individually wrapped in plastic sheaths appears to provide a certain degree of protection, however, when withdrawn from the prepuce the tip is exposed to the environment. The use of double guarded pipettes could potentially provide the necessary cover to avoid contamination. The decrease in contaminants would not only increase the success of culture methods but also reduce potential inhibitors for PCR based tests.

Results presented in this thesis have indicated that direct preputial samples collected in a non-nutrient media can be used successfully for the detection of both *T. foetus* and Cfv by real-time PCR. The use of this medium provides a significant reduction in the cost of testing; however there is a lack of information on the best transport conditions needed to maximize the identification of carrier bulls. Some evidence has indicated that samples collected in PBS can be maintained at room temperature for several days before testing by real-time PCR, although the number of organisms detected tend to decrease with time [125,126]. In western Canada samples do not reach the diagnostic laboratory until 24 h after collection, and usually longer times are

required. The evaluation of different temperatures and transport duration could provide accurate recommendations for practitioners that would aid in maximizing the ability to identify carrier bulls.

The use of pooled preputial samples has proven to be highly sensitive for the identification of *T. foetus* carrier bulls. The results presented on the real-time PCR test for Cfv are encouraging and the use of pooled samples for Cfv diagnosis should be explored. Pool sizes of 5 and 10 bulls would be useful for most herd sizes in western Canada. Moreover, the development of pooling strategies may allow for the detection of both *T. foetus* and Cfv in pooled samples thus simplifying the collection of samples and reducing the costs associated with need of multiple samples.

The use of real-time PCR assays using Taqman probes creates the possibility of multiplexing, allowing for testing for both diseases in a single reaction. As described previously the real-time PCR test used for *T. foetus* is already established in a Taqman platform [126], on the other hand the real-time PCR used for Cfv is not [152]. However, a Taqman probe for the Cfv target could potentially be developed and used in combination with the primers VenSF/VenSR evaluated in this thesis. The use of Taqman probes with different fluorescent markers would allow for the detection of both organisms in a single reaction, thus reducing cost significantly and reducing the time to reporting of results. As a result this strategy would encourage producers and practitioners to screen for both diseases actively, providing useful information on the prevalence of both *T. foetus* and Cfv.

5.3 GENERAL CONCLUSIONS

The real-time PCR assay for the detection of *T. foetus* carrier bulls in individually collected and cultured enriched samples is highly specific. Sensitivity of this assay in individual direct or culture enriched samples is high and comparable to culture under optimal conditions. The use of the real-time PCR in culture enriched pooled samples appears to be relatively sensitive in groups of up to 25 bulls. Furthermore, the use of pooled direct samples in groups of 5 or 10 is relatively sensitive. However, repeated sampling is still needed in order to maximize sensitivity. The use of direct samples and pooling of samples provides the possibility of testing a large number of samples in a timely and cost-effective manner, and precludes the need for special media.

The real-time PCR described for BGC diagnosis in bulls appears to have good sensitivity and specificity. However, the use of repeated sampling maybe needed in order to maximize the ability to detect carrier bulls. The influence of ambient temperature at the time of sample collection suggests that samples should be collected above freezing temperatures. Finally, this assay provides a valuable tool suitable for use for BGC detection, particularly when extended transport times are expected.

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