Rediscovering the silent enemy in cattle reproduction health: from the latest findings about sexually transmitted trichomoniasis and campylobacteriosis to the current control and future therapeutics

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The objective of this review is to discuss sexually transmitted diseases (STDs) caused by Tritrichomonas foetus (T. foetus) and Campylobacter fetus (C. fetus) subsp. venerealis focusing on prevalence, pathogenesis in cows and bulls and diagnosis. Later, we examine the research on prophylactic systemic immunization of bulls and cows with antigens of T. foetus and C. fetus subsp. venerealis and the efficacy in protecting, preventing or sometimes even clearing pre-existing infections in the genital tract. An analysis of the latest advances in bovine trichomoniasis and campylobacteriosis may establish common knowledge to pursue united efforts for further control of STDs.

Keywords: Cattle, sexually transmitted diseases, trichomoniasis, campylobacteriosis, vaccines

What are bovine sexually transmitted diseases?

Bovine STDs comprise trichomoniasis caused by flagellated obligate extracellular protozoon T. foetus and campylobacteriosis (i.e., bovine vibriosis) caused by gram-negative bacterium C. fetus. Two species of C. fetus are relevant in cattle health: C. fetus subsp. fetus and C. fetus subsp. venerealis. C. fetus subsp. venerealis, including the biotype intermedius inhabits exclusively the genital tract of cattle and is the causative agent of campylobacteriosis. However, C. fetus subsp. fetus may also provoke sporadic genital infection and abortion as it inhabits the intestine but it can colonize the genital tract via ascending genital infections or venereal route. Coitus is the primary route of transmission of T. foetus and C. fetus subsp. venerealis, although T. foetus and C. fetus subsp. venerealis can survive in raw and processed bull semen and be also spread via artificial insemination (AI). Both STDs cause early pregnancy loss, infertility and late abortion. The STDs are responsible for important economic losses due to reproductive losses, including poor conception rates (from slightly subnormal to 50 percent or lower), reduced calf crops, increased days-to-conception, extended calving seasons, cost of replacement bulls, loss of genetic potential due to culling, and subsequently lighter weaning weights. A study of the impact of bovine trichomoniasis predicted a reduction of 14 to 50% in annual calf crop, a prolonged breeding season, a reduction of 5 to 12% in weight gain in the suckling / growing period, a reduction of 4 to 10% in pounds of marketable calf crop at weaning, a reduction of 4 to 10% in monetary return per calf born, and a substantial reduction of 5 to 35% in the return per cow confined with a fertile bull.

Are bovine STDs still important and prevalent?

Bovine STDs are distributed worldwide with high disease incidence in developing countries, where natural breeding of cattle is widely practiced. However, STDs are also still endemic in developed countries, where natural breeding is commonly practiced in beef herds. Latest studies reported bulls infected with T. foetus in herds from countries around the world, including USA, Argentina, Spain, Australia, and Transkei. Likewise, bulls infected with C. fetus subsp. venerealis have been reported during the last decades in USA, Australia, Great Britain, Colombia, Tanzania, Nigeria, Canada, Argentina and Transkei. Recent sporadic outbreaks of bovine abortions associated with C. fetus have been reported in developing areas of Africa, Asia, and South America and even in developed countries of Europe, Oceania and North America. Most importantly, infections by T. foetus and C. fetus subsp. venerealis are clearly underestimated in many regions, including in North America, because there is no global governmental monitoring programs for STDs, lack of reports in most countries and/or testing is limited for some animal categories or situations. For instance, trichomoniasis testing in several US states is required only for bulls 18 months of age and older sold through a public sale yard (there are no federal requirements in place), ignoring private treaty sales and the presence of the disease in
cows or herds that do not trade livestock. Lastly, STDs are intrinsically associated with cattle production systems deficient in management and veterinary assistance which makes it more difficult to identify and control and; to make matters worse, they usually lack the proper laboratory facilities for the routine diagnosis. Thus, areas with extensive cattle raising and natural breeding, commonly found in Latin America, Africa, Asia, western North America and some countries of Europe, still have a high prevalence of trichomoniasis and campylobacteriosis of unknown magnitude, spread and economic importance.

What is known about bovine STDs?

Bulls—the silent and permanent carrier

_Trichomonas foetus_. _T. foetus_ persistently and asymptptomatically colonize the epithelium of the prepuce, penis, and rarely the urethral orifice of bulls. One study showed that _T. foetus_ was cultured from the preputial and penile epithelial surfaces of 24/24 bulls infected with _T. foetus_ (15 naturally and 9 experimentally), as shown by culture of smegma samples, but from the urethral orifice of only 4/24 infected bulls.²⁶ Likewise, in other study with 24 bulls naturally infected with _T. foetus_ and carriers at slaughter or within four months before, as shown by smegma culture, _T. foetus_ was detected by immunohistochemistry on the epithelial surface of preputial (5/24 bulls) and penile (14/24 bulls) crypts, but never in the penile and prostatic urethra, seminal vesicles, prostate or epididymis.²⁷ However, it appears that _T. foetus_ is restricted to mucosal surfaces and incapable of invading tissue. No whole trichomonads were found in genital tissue sections by immunofluorescence with anti _T. foetus_ immunoglobulins or by histological staining in 24 _T. foetus_-infected bulls.²⁶ Similarly, trichomonad antigens but not whole trichomonads have been detected by immunohistochemistry a few cell layers deep and occasionally below the basement membrane in the preputial and penile epithelial crypts of 24 _T. foetus_-infected bulls.²⁷ The superficial location of _T. foetus_ suggests that antigen presenting cells (APCs) could capture _T. foetus_ antigens from genital surfaces by using their dendritic projections or as a result of contacting with genital epithelial and subepithelial cells, which have shown to take up _T. foetus_ antigens in bulls²⁷ and interact with stromal APCs, at least in female rats.²⁸

Experimental and natural genital infections with _T. foetus_ in bulls caused no clinical signs or gross pathological changes.²⁶ The microscopic response in the prepuce and penis of bulls infected with _T. foetus_, characterized by infiltration of intraepithelial lymphocytes and subepithelial lymphocytes and plasma cells,²⁶²⁷ did not show any more inflammation than that observed in normal bulls.²⁹ Genital infection with _T. foetus_ is persistent since _T. foetus_ was consistently isolated by culture of smegma samples up to the time of slaughter at 4-18 months post-infection in 9 experimentally infected bulls (2-7 years old) and at 3-12 months from initial diagnosis in 15 naturally infected bulls (3-7 years old).²⁶ In addition, most bulls (8/9; 3-6 years old) challenged with different concentrations of _T. foetus_ (10²-10⁶) became persistently infected until collection was terminated after 5 to 14 months.³⁰ In this same study, all bulls (5/5; 2-7 years old) challenged with 2.5 x10⁶ _T. foetus_ were also infected until slaughter 2 months later.³⁰ However, young bulls seem to be more resistant to _T. foetus_ infection or eliminate it faster since, in one study, 16/18 young bulls (1-2 years old) were refractory to infection and the remaining 2 bulls were temporary carriers for less than 4 months.³⁰ Normal modifications in the preputial and penile epithelium of aged bulls (>5 years old), including more mucosal folds and deeper crypts, may provide a reduced oxygen tension niche that increases susceptibility to infections with the anaerobic _T. foetus_.³⁰

Infection of bulls with _T. foetus_ is considered to have limited or no effect on male fertility because _T. foetus_ does not inhabit the male urethra²⁶²⁷ and its presence in semen is improbable.²⁶ Although bovine sperm cells (1 x10⁸) were damaged or killed by exposure in vitro (30 min-6 h at 37°C) to _T. foetus_ (1 x10⁶; other concentrations of parasites or sperm were not reported),³¹ this cytotoxic effect of _T. foetus_ would be greatly reduced (i.e., diluted) in natural infections in which trichomonads are rarely found in semen²⁶ and bulls usually ejaculate 6 x10⁹ sperms per coitus. Genital antibody responses to _T. foetus_ infection in bulls, even when measurable, were incapable of eliminating genital infection since surface antigen specific IgG1, IgA and IgM antibodies in smegma of bulls naturally infected with _T. foetus_, measured by ELISA at one time point, coexisted with _T. foetus_ positive smegma cultures.²⁷ Thus,
T. foetus appears not to be killed or induces pathogen specific inflammation in bulls since it persistently inhabits the lower genital tract for long periods of time with no clinical signs.

Campylobacter fetus subsp. venerealis. As described for T. foetus, C. fetus subsp. venerealis also persistently and asymptotically colonizes the epithelium of the prepuce and penis of bulls. In one study, C. fetus subsp. venerealis was identified by immunofluorescence on impression smears and epithelial scrapings in the prepuce and penis of 6/6 bulls (5.5-12 years old), 5 of them as carriers as shown by culture of smegma samples, but only 1/6 bulls were colonized in the distal urethra. In addition, immunofluorescent studies on tissue sections showed that C. fetus subsp. venerealis colonized the preputial and penile epithelial surface of the lumen and the crypts but never the deep epithelium or subepithelium.32

Infection with C. fetus subsp. venerealis in bulls was not associated with any clinical signs, altered semen quality,33,34 or gross genital abnormalities.35 Microscopically, two studies, each with 6 C. fetus subsp. venerealis-infected bulls, agreed that the subepithelium of the prepuce and penis were infiltrated by lymphocytes and plasma cells, but this inflammatory response was non pathognomonic for C. fetus subsp. venerealis.32,35 In agreement, the number of total non-antigen specific plasma cells in the subepithelium of the prepuce were similar between 4 bulls infected with C. fetus subsp. venerealis and 79 control bulls.39

Genital infection with C. fetus subsp. venerealis among mature bulls (>4 years old) appeared to be persistent, as determined by consecutive C. fetus subsp. venerealis positive smegma cultures. After intrareputual challenge with C. fetus subsp. venerealis (4.8 x 10^8), 4/4 old (66-74 months old) and 3/4 young (41-49 months old) bulls were infected until slaughter, at 9-10 and 16-18 weeks after challenge, respectively, while the remaining young bull was infected for only 4 weeks. In agreement, 4/6 bulls (5 years old) intrapreputially challenged with C. fetus subsp. venerealis (3 times with 2.5-4.5 x 10^9 organisms) were infected for at least 4 months in an experiment terminated at 4 months.6 It is uncertain whether the genital infection with C. fetus subsp. venerealis is influenced by the age of bulls. In one study, the incidence of C. fetus subsp. venerealis in semen was higher in old (> 6 years old) (65/139) than in young (< 6 years old) (4/233) bulls (with a variable number of cultures per bull) but, in another, the preputial and penile mucosa was microscopically similar between old and young bulls, both similarly infected with C. fetus subsp. venerealis.35

Antibody response to C. fetus subsp. venerealis in bulls, even when measurable, did not clear the infection. Genital agglutinating antibodies specific to C. fetus subsp. venerealis coexisted with C. fetus subsp. venerealis positive cultures in smegma of two naturally infected bulls. On the other hand, in other studies, specific C. fetus subsp. venerealis agglutinins were absent in smegma of 6 naturally infected bulls, and the levels of specific agglutinins were similar between 8 experimentally infected bulls (7 of them carriers as shown by smegma culture) and 2 uninfected bulls. Likewise, no systemic immune response was detected by tube agglutination tests (O somatic-cell wall and superficial K heat-labile antigens) in 4 bulls infected with C. fetus subsp. venerealis for at least 4 months after challenge. These studies demonstrate that C. fetus subsp. venerealis could inhabit the lower genital tract of bulls for at least a few months and in the absence of an effective specific antibody response.

Cows-pregnancy loss due to STDs

Trichomonas foetus. Infection with T. foetus in bovine females, in contrast to bulls, provoked genital inflammation including vaginitis, cervicitis and endometritis and, in pregnant cows, fetal death in the first trimester of gestation or later. In one experimental study, this genital inflammation and pregnancy loss occurred after 7 weeks of infection. Moreover, infection with T. foetus in female cattle does induce a measurable antigen specific antibody response in the vagina. Intra-vaginal inoculation of non-pregnant heifers with T. foetus (7 x 10^6) induced whole T. foetus cell specific IgG1 and IgA antibodies in vaginal secretions at 7-9 weeks and IgA persisted longer for 24 weeks after infection or until the time of genital clearance. Lower intra-vaginal doses of T. foetus (1 x 10^6) similarly induced vaginal IgG1 and IgA antibodies specific to TF1.17 antigens at 5-10 weeks and to whole T. foetus cell antigens at 5-8 weeks. Also differing from bulls, the genital infection in heifers was limited to 13-28 weeks (vaginal
challenge of $7 \times 10^6$ *T. foetus*) \cite{45} and many of the infected heifers cleared infection by 6-10 weeks in studies terminated at 8-12 weeks after challenge (vaginal challenge of $1 \times 10^6$ *T. foetus*). \cite{38,40-43,47} The above studies suggested that the immunity acquired by infection with *T. foetus* in bovine females usually results in a brief genital infection compared to bulls; however, it cannot prevent reproductive failures.

Such non-protective natural genital antibody response to *T. foetus* might be due to parasite factors capable of affecting local immunity by masking antigens or digesting proteins involved in innate and acquired immunity. Virulent factors of *T. foetus* include secreted extracellular cysteine proteinases \cite{48} which, *in vitro*, digest fibrinogen, fibronectin, albumin, lactoferrin, \cite{49} the third component of the complement (C3) \cite{50} and IgG1 and IgG2 antibodies. \cite{49} Since these cysteine proteinases preferentially cleave IgG2*α* allotypes rather than IgG2*β*, \cite{51} long-term *T. foetus* infection with low levels of local IgG2 antibodies may be due to a host genetic predominance of the susceptible IgG2*α* allotype. Otherwise, *T. foetus* non-specifically binds bovine IgG2 and to a lesser extent IgG1 isotypes \cite{52} and this mechanism may possibly shield the parasite from antibody recognition of masked antigens in the effector stage of the immune response.

*Campylobacter fetus* subsp. *venerealis*. Natural infection with *C. fetus* subsp. *venerealis* in cows is associated with reproductive failure, including irregular estrus, transient infertility and, in pregnant cows, embryonic-fetal death. \cite{25,34,53-56} Moreover, experimental intrauterine and cervico-vaginal infection with *C. fetus* subsp. *venerealis* in female cattle provoked different grades of genital inflammation, including vaginitis, cervicitis, endometritis, and salpingitis. \cite{57} Genital infection with *C. fetus* subsp. *venerealis* in female cattle, differing from bulls, does induce a detectable antibody response in the lower genital tract (i.e., vagina). In one study, using indirect fluorescence antibody assays, intra-vaginal inoculation of non-pregnant heifers with *C. fetus* subsp. *venerealis* ($1 \times 10^6$) induced antigen specific transient IgM followed by persistent IgA and IgG antibody responses in cervico-vaginal secretions and a IgG1 response in uterine secretions. \cite{58} Likewise, higher intra-vaginal doses of *C. fetus* subsp. *venerealis* (2-4 $\times 10^6$ organisms) induced specific agglutinating and immobilizing antibodies in cervico-vaginal secretions of heifers. \cite{59} In addition, a vaginal specific IgA antibody response was detected by ELISA after abortion in 7 heifers naturally infected with *C. fetus* subsp. *venerealis*. \cite{55} Genital infection with *C. fetus* subsp. *venerealis* in female cattle, contrasting with bulls, was limited to weeks or months in the uterus and oviducts where there was a predominance of IgG antibodies \cite{53,57,58} but persisted longer, for 6-18 months or even 24 months, in the vagina where there was a predominance of IgA antibodies. \cite{53,58,59} These carrier cows could maintain the infection in vaginal mucus for longer periods, perhaps from one breeding season to another. However, some cows (up to 5 per cent), may be resistant to infection after repeated exposure to carrier bulls or the deposition of large numbers of viable organisms into the reproductive tract. \cite{60}

**How to diagnose of bovine STDs?**

Culture and polymerase chain reaction for detecting STDs in genital secretions

Vaginal and preputial secretions are usually sampled by introducing an insemination/infusion pipette inside the vaginal fornix or preputial cavity and moving it back and forth in short strokes while aspirating the secretions. \cite{61} Phosphate buffered saline solution (PBS) may be added to wash the cavity and recover higher amounts of samples, although it may dilute the sample. Alternatively, preputial secretions can be taken by scraping the cavity using a metal brush with no significant differences in culture sensitivity (Se) compared with the pipette. \cite{62} Diagnosis of trichomoniasis in genital secretions, and campylobacteriosis although much less commonly utilized, are mostly based on culture and/or polymerase chain reaction (PCR), as described below.

**Culture of Tritrichomonas foetus.** The most routinely employed diagnostic test for bovine trichomoniasis is culturing preputial smegma or vaginal secretions into selected media, such as Diamond’s. \cite{63} Plastridge. \cite{64} In Pouch TF® (Biomed Diagnostics, San Jose, CA), \cite{65} or liver infusion broth. \cite{66} Samples are incubated at 37°C (98.6°F) and examined on days 1, 3, 5 and 7 after sampling by placing a
drop on a glass slide and observed at 40-100x magnification using light microscopy. Samples are considered positive when living trichomonads with size, shape and a wave-like, rapid and irregular jerky movement of the protozoan body compatible with *T. foetus* were observed. Although diagnostic Se of In Pouch TF® and Diamond's for detecting *T. foetus* in bulls differ among studies (88%-98.4%; 81.6%-93.2%), differences between the culture media sensitivities were not significant.

Polymerase chain reaction of Tritrichomonas foetus. Diagnosis of *T. foetus* in smegma of bulls by PCR is theoretically as sensitive and specific as cultures because it relies on the amplification of DNA from the organism and not on the successful culture of live organisms. Polymerase chain reaction methods targeting the 5.8S rRNA gene and the flanking internal transcribed spacer (ITS1 and ITS2) regions of *T. foetus* has found wide acceptance and has been applied to detected *T. foetus* from cultured isolates, cervico-vaginal mucus, and tissues from female genitalia; and, in vitro, it differentiated *T. foetus* from other morphologically similar trichomonads. This PCR includes a set of primers, TFR1 and TFR2 complementary to conserved sequences of the 3'-end of the 18S subunit rRNA gene and the 5'-end of the 28S subunit rRNA gene, that define the Trichomonadidae family by yielding a product of 372-bp. Other set of primers, TFR3 complementary to the 5' end of the 28S rRNA gene and TFR4 to the 18S rRNA gene and ITS1, specifically target *T. foetus* yielding a product of 347 bp. The in vitro biological Se of TFR3/4 was approximately 90% and the specificity (Sp) of 98%, detecting as little as 0.03 pg of purified *T. foetus* DNA or approximately 2 organisms/mL of sheath-wash samples under laboratory conditions. Other PCR-based tests include a loop mediated isothermal amplification targeting the 5.8S rDNA subunit designed for the specific identification of *T. foetus* in smegma that was as sensitive and specific as PCR amplification with TFR3 and TFR4 primers. A *T. foetus*-specific 5' Taq nuclease assay using a 3' minor groove binder-DNA probe (TaqMan MGB) targeting conserved regions of the ITS-1 is also available for detecting *T. foetus* in genital secretions. This probe-based real time PCR assay was more sensitive than InPouch TF® culture and the conventional TFR3-TFR4 PCR assay detecting *T. foetus* in spiked smegma or mucus specimens or in pooled protozoal cultures of preputial scraping samples. Thus, although variability in methodologies used by the laboratories affects the Se and Sp of qPCR, this test seems to be rapid, quantitative, reliable and accurate in detecting *T. foetus* and, in addition it has been recently implemented by California Department of Food and Agriculture (CDFA) for diagnosis of bovine trichomoniasis. As a general rule, genital secretions sent to the laboratory for diagnosing STDs should be transported at room temperature for culture and at 4°C for PCR.

A new concern in the diagnosis of bovine trichomoniasis-the false trichomonads

Culture isolation of trichomonads from preputial secretions of bulls, others than *T. foetus*, infers a risk of false Sp in the diagnosis of *T. foetus* by culture. Trichomonads morphologically similarly under low microscopic observation to *T. foetus* were isolated from cultured preputial secretions of virgin bulls from widely dispersed geographical areas, including the western of USA, Canada, and Argentina. Since the virgin bulls had not been used for breeding, these non-sexually transmitted trichomonads were generalized as “non-*T. foetus* trichomonads”. Transmission and scanning electron microscopy identified *Tetratrichomonas* spp. in non-*T. foetus* trichomonads isolates. Fortunately, amplified sequences of the 5.8S rRNA gene and ITSs and restriction fragment length polymorphism (RFLP) tests in lieu of DNA sequencing differentiated *T. foetus* from *Pentatrichomonas hominis* (*P. hominis*) and *Tetratrichomonas* spp. recovered from the bovine preputial cavity.

Confronted with this new problem, the persistence and pathogenicity of tetratrichomonads and *P. hominis* has been investigated in bulls and cows. These studies showed tetratrichomonads are only detected intermittently in the female genital tract and produced no histological lesions. Likewise, *Tetratrichomonas* spp. did not survive in experimentally infected bulls or were only detected for a short period in naturally infected bulls. Trichomonads including *Tetratrichomonas* spp are commensal species in the bovine intestinal tract. Consequently, the intermittently finding of *Tetratrichomonas* spp. in genital secretions but frequent isolation in feces suggests a fecal-genital route of contamination. This fecal-genital route of contamination is supported by the fact that tetratrichomonads may appear in
the lower genital tract in bulls due to homosexual behavior. Thus, “non-\textit{T. foetus} trichomonads” including \textit{Tetra trichomonas} spp. from the intestinal tract could sporadically contaminate the genital tract as a consequence of defecation and physical contact among cows and bulls, inhabiting temporarily the genital tract and confusing the diagnosis of trichomoniasis.

\textbf{Combination of culture and PCR for a faster and reliable diagnosis of bovine trichomoniasis} 

Diagnosis of \textit{T. foetus} by culture only is a limited approach because Se varied from 84% to 96% under experimental conditions\textsuperscript{63,86-90} or even lower under field suboptimal conditions\textsuperscript{7} and it lacks Sp since \textit{Tetra trichomonas} spp. can be isolated from cultured preputial smegma\textsuperscript{67,80,81,87}. As a result, the US AI industry prescribes for bovine trichomoniasis a rigorous protocol of six weekly \textit{T. foetus} negative cultures for bulls older than 365 days of age.\textsuperscript{91} This diagnostic routine of six weekly cultures for diagnosing \textit{T. foetus} in bulls has proven to be highly effective in controlling disease, with a Se of 86.7% and Sp of 97.5%.\textsuperscript{61} However, the use of culture and PCR, individually on the same sample split in two, one for culture and one for PCR, for three consecutive weeks (Se 87.5%, Sp 95.6%) appeared to be similar to this standard of six weekly cultures.\textsuperscript{61} The same study proved that a single culture or PCR seems to be equally sensitive detecting \textit{T. foetus} (Se of 67.8% and 65.9%, respectively) with Sp greater than 90%.\textsuperscript{61} The similar performance of culture and PCR agrees with in vitro studies where PCR made from 5-day cultures of male genital secretions agreed 92.9% with culture.\textsuperscript{73} Combination of culture and PCR for \textit{T. foetus} diagnostics offers high Se and improved Sp and may require less time and perhaps less cost in the surveillance of AI bulls and beef bulls prior to the breeding season.

\textit{Culture of \textit{C. fetus} subsp. \textit{venerealis}.} For isolating \textit{C. fetus} subsp. \textit{venerealis}, samples obtained from preputial or vaginal secretions, or fetal stomach contents, lungs, liver and placenta, for abortion investigations, are transported to the laboratory in transport enrichment medium (TEM) at room temperature. In the laboratory, samples are cultured in selective culture media, such as 5% sheep blood agar, Skirrow’s agar\textsuperscript{92} or Clark’s selective agar in microaerophilic conditions containing 5–7% oxygen, 5–15% carbon dioxide and 65–90% nitrogen and incubated at 37 ±2°C for 48h. Passive filtration of fresh preputial scrapings onto blood agar yielded higher recovery rates of \textit{C. fetus} subsp. \textit{venerealis} than direct plating.\textsuperscript{93} Subspecies of \textit{Campylobacter} are differentiated biochemically by tolerance to 1% glycine and production of hydrogen sulfide through the utilization of cysteine in which presumptive \textit{C. fetus} subsp. \textit{fetus} colonies but not \textit{C. fetus} subsp. \textit{venerealis}, will growth with 1% glycine or with NaCl and cysteine.\textsuperscript{94} However, culture of \textit{C. fetus} subsp. \textit{venerealis} has severe practical limitations and is not widely used as diagnostic test because it has low Se and is laborious and time-consuming. Also, some strains of \textit{C. fetus} subsp. \textit{venerealis} are sensitive to polymyxin B (common in both TEM and selective media), \textit{C. fetus venerealis} biovar “intermedius” can tolerate higher concentrations of glycine, and commensal bacteria easily overgrow into these culture media. In addition, transport of samples containing \textit{C. fetus} subsp. \textit{venerealis} is critical as excessively cool or hot temperatures or transit times longer than 24 hours will make recovery of \textit{C. fetus} subsp. \textit{venerealis} very unlikely.\textsuperscript{95}

\textit{PCR for \textit{C. fetus} subsp. \textit{venerealis}.} Diagnosis of campylobacteriosis by PCR-based test should not only identify \textit{C. fetus} subsp. \textit{venerealis} but also differentiate it from \textit{C. fetus} subsp. \textit{fetus}. Misidentification of \textit{C. fetus} subsp. \textit{venerealis} as \textit{C. fetus} subsp. \textit{fetus} results into the spread of \textit{C. fetus} subsp. \textit{venerealis} into cattle populations and \textit{C. fetus} subsp. \textit{fetus} as \textit{C. fetus} subsp. \textit{venerealis} results in economic losses.\textsuperscript{96} \textit{C. fetus} subsp. \textit{venerealis} has high sequence identity (92%) with \textit{C. fetus} subsp. \textit{fetus} but possess unique elements that include a pathogenicity island (30-kb element) for encoding genes phylogenetically related to the VirB–VirD4 operon for bacterial type IV secretion system and mobility genes such as phage integrase and insertion sequence (IS) transposase.\textsuperscript{97-100} These genomic particularities of \textit{C. fetus} subsp. \textit{venerealis} have been useful for designing several PCR primer sequences for identification and differentiation of \textit{C. fetus} subsp. \textit{venerealis}.\textsuperscript{92,96,101} One of the most reliable PCR and quantitative PCR test includes one primer set (MG3F/MG4R) that amplifies a 750-960 base pair fragment of the \textit{C. fetus} carbon starvation protein gene, found in both \textit{C. fetus} subspecies, and other primer set (VenSF/VenSR) that amplifies a 142 bp fragment of the parA gene, exclusive of \textit{C. fetus} subsp. \textit{venerealis}.\textsuperscript{102} A SYBR Green qPCR based on VenSF/VenSR primer set was likewise optimized for
detecting *C. fetus* subsp. *venerealis* directly in preputial samples. A multiplex PCR assay using a set of primer to amplify a *C. fetus*-specific 764-bp sequence and other set of primers (nC1165g4F/nC1165g4R) to amplify a 233-bp sequence that is only present in *C. fetus* subsp. *venerealis* also detected and differentiated between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* in samples of abomasal liquid of aborted bovine fetuses without any pre-enrichment step. Likewise, two real time SYBR Green PCR assays for the detection and discrimination of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* showed high Se and Sp for *C. fetus* (CampF4/R4; 100% and 99.6%, respectively) and *C. fetus venerealis* (CampF7/R7; 98.7% and 99.8%, respectively) on 1071 bacterial isolates. However, molecular analysis by amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) may be still recommended to identify *C. fetus* subspecies isolates as other real-time PCR assay targeting gene *nahE* showed 100% Se and Sp for *C. fetus* species but the subspecies *venerealis* specific real-time PCR (ISCfe1) failed due to sequence variation of the target insertion sequence. Thus, diagnosis of campylobacteriosis still needs further investigation to be routinely used for control and eradication because culture is impractical and PCR is not widely standardized. The recent complete sequencing genome of *C. fetus* subsp. *venerealis* will lead to new improved molecular diagnostic tools (e.g. real-time PCR, PCR) for identification of *C. fetus* subsp. *venerealis*.

**Control of STDs by testing and vaccination**

There is no effective legal treatment for *T. foetus* since nitro-imidazole drugs showed some efficacy but they are not allowed in cattle. In addition, antibiotic treatments for *C. fetus* subsp. *venerealis* are impractical and with doubtful efficacy. Thus, control of bovine STDs involves diagnostic testing of animals to identify positive animals and depending on the local laws, culling of infected animals. For instance the control program disposed by the CDFA in partnership with the livestock industry declares that: bulls infected *T. foetus* should be permanently quarantined until they go to slaughter and herdmate bulls should be quarantined until one negative real-time PCR test or three consecutive negative trichomoniasis culture tests. Importantly, both STDs need to be reported to local authorities. The veterinarian approved for trichomoniasis sampling that collects the samples is required to report all positive and negative test results to CDFA within two days of the final laboratory reading date, and negative tests must be reported within 30 days. Campylobacteriosis belongs to list B of the notifiable disease of the International Office of Epizootics (OIE) and animals or animal products must be certified as *C. fetus* subsp. *venerealis*-free for international trade. Hence, the AI industry demands rigorous testing standards and protocols for assuring semen free of pathogens, as bulls are the epidemiologic natural reservoir and a major factor in maintaining and transmitting STDs. For controlling *C. fetus* subsp. *venerealis* and *T. foetus* infections, for example in the USA, the Certified Semen Services, Inc. (a subsidiary of the National Association of Animal Breeders) requires a series of weekly negative cultures of smegma from each bull with an age-dependent regime (1 test for bulls <6 month old, 3 tests for bulls 6 month-1 year old, 6 tests for bulls >1 year old). Furthermore, the AI industry routinely adds antibiotics to the semen in the freezing process although cases of bacterial antibiotic resistance have been reported for *C. fetus* subsp. *venerealis*. Thus, diagnosis of *C. fetus* subsp. *venerealis* and *T. foetus*, isolation of infected animals and notification to authorities are foundational steps to achieve success programs of control and eradication of STDs.

Systemic vaccination with *T. foetus* and *C. fetus* subsp. *venerealis* in cattle has been associated with prevention and cure of genital infection by inducing measurable systemic IgG antibodies, which may translocate to genital secretions. We continue to review important studies investigating vaccines against STDs in cows and bulls.

**Vaccines against Tritrichomonas foetus.** Several studies have been shown that systemic immunization in bulls with antigens of *T. foetus* prevents or clears genital infections, based on culture of smegma samples. In one study, whole *T. foetus* cell antigens in a mineral oil adjuvant were systemically given 3 times at monthly intervals to age susceptible bulls (>4 years old). Then, bulls were challenged with *T. foetus* by mating with infected cows or by intrapreputial inoculation at 1 and 6 months after the third vaccine dose and considered free of infection after 5 consecutive weekly negative
smegma cultures and persistently infected after 9 consecutive $T. \textit{foetus}$ positive smegma cultures. The results showed that whole $T. \textit{foetus}$ cell antigens prevented or shortened infection in 37/48 vaccinated bulls whereas merely 18/38 control unvaccinated challenged bulls remained free of infection or infected for a short time.\textsuperscript{106} The same study showed that therapeutic immunization with this whole $T. \textit{foetus}$ cell antigen cleared infection in 11/16 vaccinated bulls while only 1/8 untreated bulls eliminated infection.\textsuperscript{106} This whole $T. \textit{foetus}$ cell vaccine was most effective in bulls less that 5.5 years of age\textsuperscript{106} because young bulls may be more difficult to infect since their preputial crypts less deep would not favor microaerophic/anaerobic microbes. Studies using other types of $T. \textit{foetus}$ antigens showed also some efficacy. In one study, membrane preparation (500 µg/dose) or purified membrane glycoprotein (160 µg/dose) antigens of $T. \textit{foetus}$, both in a mineral oil adjuvant, were systemically given 3 times at monthly intervals to bulls with pre-existing genital $T. \textit{foetus}$ infection.\textsuperscript{107} The results showed that 3/4 bulls vaccinated with membrane and 3/4 bulls vaccinated with membrane glycoprotein eliminated infection at week 2 after the second vaccine dose while the 2 remaining vaccinated bulls and 8/8 infected unvaccinated control bulls were infected for 2 months after the last dose.\textsuperscript{107} The 3 bulls vaccinated with $T. \textit{foetus}$ membrane antigen that cleared infection and an additional 3 non-infected, non-vaccinated control bulls were subsequently challenged with $T. \textit{foetus}$ and none of the vaccinated bulls became infected while 2/3 control bulls acquired infection.\textsuperscript{107} In a more recent study, systemic immunization of bulls with subcutaneous inoculation of 2 mL of a commercial vaccine containing whole-cell killed $T. \textit{foetus}$ in oil adjuvant prevented trichomonad colonization of the preputial and penile mucosa of 4/4 bulls vaccinated and challenged with $T. \textit{foetus}$.\textsuperscript{108} The vaccinated-challenged bulls had systemic and preputial $T. \textit{foetus}$ LPG/protein $T. \textit{foetus}$ antigen specific IgG1 and IgG2 and slight preputial IgE and IgA antibody responses that determined the resistance to trichomonad genital colonization.\textsuperscript{108} These high and persistent levels of serum IgG antibodies in vaccinated bulls together with additional genital IgE antibody responses after the systemic vaccine doses imply that vaccine-induced systemic antibodies were significant contributors to the luminal IgG antibody response. The peak of preputial IgE antibody in vaccinated bulls before challenge, at the same time as the IgG1 and IgG2 antibodies, may aid translocation of serum IgG into smegma. Immunoglobulin E antibodies would cross-link $T. \textit{foetus}$ antigens on mast cell receptors to activate and release mediators increasing endothelial and epithelial permeability. This would facilitate systemic IgG antibody translocation from the bloodstream and across the genital epithelium into secretions as was proposed in infected heifers.\textsuperscript{40} A significant genital specific IgA antibody response was detected before challenge in vaccinated bulls that could contribute to the resistance to trichomonad colonization.\textsuperscript{108} These vaccinated-challenged bulls also had increased epithelial antigen presenting cells (MHC II$^{+}$ and CD205$^{+}$), CD3$^{+}$ and CD8$^{+}$ T lymphocytes, and subepithelial B cells, IgG1 and IgA containing cells in the prepuce that may induce local responses as a part of cell-mediated immune response that prevent genital colonization.\textsuperscript{108}

Systemic vaccination of non-pregnant heifers with an immunoaffinity purified lipophosphoglycan (LPG)/protein complex (TF1.17) antigen of $T. \textit{foetus}$ induces specific IgG1 and IgA antibodies in vaginal and uterine secretions that coincide with clearance of experimental genital infections, usually before 7 weeks.\textsuperscript{38,41,42,47} Genital clearance before 7 weeks in female cattle likely prevents reproductive failures since inflammation and pregnancy loss did not occur until after 7 weeks of infection in an earlier study.\textsuperscript{39} Likewise, systemic vaccination with whole cell\textsuperscript{109-115} or membrane antigens\textsuperscript{114} in cows mated with $T. \textit{foetus}$ infected bulls and experimentally infected shortened genital infection and improved calving rates compared with unvaccinated cows. In female cattle, booster vaccination with $T. \textit{foetus}$ antigens in the nasal\textsuperscript{41} or vaginal mucosa\textsuperscript{42} similarly elicited genital immunity. In these studies, virgin heifers were systemically vaccinated twice with immune purified TF1.17 antigen (100 µg), boosted with formalized whole $T. \textit{foetus}$ cells (10$^9$) given intranasally (6 heifers)\textsuperscript{41} or intravaginally (9 heifers),\textsuperscript{42} and then intravaginally challenged with $T. \textit{foetus}$ (10$^9$) 2-3 weeks later (all vaccine doses in Quil A and given 3 weeks apart). The results showed that systemic priming with vaginal or nasal boosting similarly induced a shortened infection and antigen specific vaginal IgA (3-10 weeks after challenge) and uterine IgA and IgG1 antibodies (10 weeks after challenge).\textsuperscript{41,42} The same vaccination/challenge scheme with the booster given systemically in 6\textsuperscript{41} and 10\textsuperscript{42} heifers also induced shortened infection but mainly vaginal and uterine
IgG1 antibodies. Other specific *T. foetus* antigens, such as Tf190, also resulted in a lower number of infected heifers after experimental inoculation and elicited systemic IgG1 and IgG2 when injected subcutaneously and genital IgA when intra-nasally applied.\textsuperscript{116} Thus, vaccination against trichomoniasis offers some advantages but better antigens and further research evaluating them in controlled and field conditions may be needed as a recent meta-analysis of the efficacy of whole-cell killed *T. foetus* vaccines in beef cattle showed that the impact of vaccination is still limited/low on infection and abortion risk in heifers and on infections in bulls.\textsuperscript{117}

**Vaccines against Campylobacter fetus subsp. venerealis.** Systemic vaccination of bulls with *C. fetus* subsp. *venerealis* antigens has also resulted in genital protection.\textsuperscript{36,118,119} In one study, whole *C. fetus* subsp. *venerealis* cell antigens (~40 mg dry matter weight) in mineral oil adjuvant were systemically given to bulls (14-18 months old) in twice a 2 month intervals and then annually.\textsuperscript{118} The bulls were intraperiutally challenged with *C. fetus* subsp. *venerealis* every 6 months for a total of 5 times and considered free of infection after 4 consecutive weekly negative smegma cultures.\textsuperscript{119} The results showed that 16/16 vaccinated bulls were free of infection but, of 17 control unvaccinated challenged bulls, 13 were infected for 3 or more months and only 2 were free of infection and 2 infected for less than 2 weeks.\textsuperscript{119} The ages at which bulls became infected ranged from 2-6 years.\textsuperscript{119} Likewise, in another study, a dual vaccine containing *C. fetus* subsp. *intermedius* and *C. fetus* subsp. *venerealis* antigens (20 mg of each) was given to bulls (20-34 months old) using the same vaccination/challenge scheme.\textsuperscript{120} The results showed that 5/5 bulls challenged with *C. fetus* subsp. *intermedius* and 5/5 bulls challenged with *C. fetus* subsp. *venerealis* remained free of infection while 9/10 challenged unvaccinated control bulls were infected for at least 5 weeks after challenge.\textsuperscript{120} The ages at which bulls became infected ranged from 3.5-5.5 years.\textsuperscript{120} Therapeutic immunization against *C. fetus* subsp. *venerealis* infection in bulls has also demonstrated some effectiveness. In one study, a commercial whole *C. fetus* subsp. *venerealis* cell antigen vaccine (~40 mg dry matter weight) was systemically given in twice a month intervals to bulls already infected (5 years old).\textsuperscript{36} Infection status was determined by smegma culture for 8 weeks after primary vaccination and by culture of genital secretions of a virgin heifer after natural service by the tested bull. The results showed that 6/6 infected vaccinated bulls cleared infection while 4/4 infected and unvaccinated bulls remained infected for 4 months, at which time they were vaccinated and 2 of them cleared the infection.\textsuperscript{36} In another study in which infection status was evaluated by smegma culture and immunofluorescence tests, a vaccine containing whole *C. fetus* subsp. *intestinalis* cell antigens in incomplete Freund’s adjuvant (concentration unreported) was systemically given once to 288 bulls serving in a *C. fetus* subsp. *venerealis*-infected area and none of the bulls acquired infection.\textsuperscript{118} Moreover, this same vaccine given twice cleared infection by 42 days after the second dose in 41/41 bulls already infected with *C. fetus* subsp. *venerealis* and exposed to infected females and, prevented re-infections in 8/8 bulls that previously cleared infection by vaccination and were intraperiutally challenged 2-8 times with *C. fetus* subsp. *venerealis* (unreported doses).\textsuperscript{118} However, in this study, results from control (unvaccinated) bulls were not reported, antigenic similarity between the infecting *C. fetus* subsp. *venerealis* and the immunogen *C. fetus* subsp. *intestinalis* was undetermined, and mating in an endemic area may not have been a sufficient challenge. Regarding immunity induced by *C. fetus* subsp. *venerealis* antigens, systemic vaccination with whole *C. fetus* subsp. *venerealis* antigen, given twice at a monthly interval, induced higher titers of serum agglutinins to heat-labile K antigens in 10 vaccinated bulls (agglutination range 500-1280, with peak at 6 weeks after the first vaccine dose) than in 2 unvaccinated bulls (agglutination range 60-80).\textsuperscript{36} Likewise, systemic vaccination with whole *C. fetus* subsp. *venerealis* cell antigen, given twice at a 2 month interval and annually, induced higher titers of serum agglutinins in 12/17 vaccinated bulls (agglutination range 40-40,960, with peak at 3 weeks after the second vaccine dose) than in 17 unvaccinated control bulls (agglutination range usually less than 20 and the highest only 160).\textsuperscript{119}

Systemic immunization of cows with bacterins of *C. fetus* subsp. *venerealis* has been associated with prevention and even cure of infection. Systemic vaccination with *C. fetus* subsp. *venerealis* and biotype *intermedius* (20 mg dry weight of each one) in mineral oil adjuvant vaccine protected cows against genital experimental infection with either organism.\textsuperscript{121} Heifers vaccinated with bacterin...
containing K antigen were resistant to experimental infection with *C. fetus* subsp. *fetus*. \(^{122}\) Moreover, systemic immunization with killed *C. fetus* cells in incomplete Freund’s adjuvant also cured infection in 6/8 cows previously infected with *C. fetus* subsp. *venerealis*. \(^{123}\) This protective and curative effect of systemic vaccination has been largely associated with the induced antibody response as described in bulls. Systemic vaccines with *C. fetus* bacterins in oil adjuvant stimulated high level of systemic IgG1 and IgG2, and genital IgG1 and IgG2. \(^{39,124}\) However, some vaccine failures have been reported too. Two commercial vaccines containing *C. fetus* subsp. *venerealis* applied subcutaneously in female cattle naturally challenged by serving with infected bull during 60 days did not protect them against infection of *C. fetus* subsp. *venerealis*. \(^{125}\) In spite of the vaccination, vaccinated and control groups showed a high percentage of infected heifers and both groups showed a poor reproductive performance. Likewise therapeutic vaccination failed in a 4 year old bull infected with *C. fetus* subsp. *venerealis*, since he remained infected after systemic immunization with 2 different commercial whole cell vaccines, given twice and 3 times, respectively. \(^{126}\) However, after this bull was cured by antibiotic treatment, he remained free of infection to an intrapreputial challenge. \(^{126}\)

Failures in vaccines against campylobacteriosis may rely on two factors: antigenic differences between regional and standard strains and/or insufficient content of dry weight *C. fetus* cells. Variation on the surface antigens of *C. fetus* subsp. *venerealis* may impede recognition by the immune system and the consequent limited immune response to infection. Isolates of *C. fetus* subsp. *venerealis* from smegma of 3/4 relatively young (41-49 months old) and 3/4 older (66-74 months old) infected bulls modified their superficial antigens, in that rabbit antiserum specific to whole heat labile surface *C. fetus* subsp. *venerealis* antigens showed decreased agglutination titers to individual antigens of subsequent isolates compared with the infecting strain. \(^{35}\) Superficial antigenic variation was also evidenced by agglutination tests in *C. fetus* subsp. *venerealis* isolates from cervico-vaginal mucus of 2 heifers over several months of infection. \(^{127}\) In this study, the isolates, sampled throughout infection, reacted with rabbit antiserum of various specificities and the specificity of the cervico-vaginal agglutinating antibodies of the heifers varied during infection. \(^{127}\) As alternative mechanisms of evasion, *C. fetus* subsp. *venerealis* may bind bovine IgA specific antibodies, thus escaping the complement and phagocytosis-mediating properties of IgG \(^{128}\) and bacterial surface glycoproteins, in the absence of specific antibodies, inhibit ingestion by macrophages. \(^{129}\) Hence, microbial antigenic variation and capacity for blocking inflammatory-immune effectors (e.g., complement, IgG, macrophages) may promote persistence of *C. fetus* subsp. *venerealis* and failure of vaccines with international strains only. In addition, many commercial vaccines include *C. fetus* subsp. *venerealis* only and lack *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* biotype *intermedius* although they were also associated with reproductive problems such as infertility, lowered pregnancy rates, and abortion in cattle. \(^{2,3,56,94,130}\) Dry weight cells may also determine the effectiveness of the vaccine as experimental vaccines gave a good protection against genital infection only when they contained at least 40 mg of dry weight per dose in oil adjuvant. \(^{121}\) However, the main limitation about vaccination against campylobacteriosis in cows and bulls is the lack of more updated reports and the testing of the current as well as new vaccines.

There is little information about the effectiveness of vaccines containing both *C. fetus* subsp. *venerealis* and *T. foetus* antigens. One study evaluated systemic vaccination with whole cells of *C. fetus* subsp. *venerealis* and *T. foetus* in cows mated for 90 days with bulls infected with *C. fetus* subsp. *venerealis* and *T. foetus* (from day 0 to day +90) plus an additional vaginal instillation of both pathogens at Day +39. \(^{115}\) Vaccines were administrated subcutaneously at days -30 and +11 and into the vaginal submucosa at day -9 of the mating period. Vaccinated animals showed elevated systemic and vaginal IgG antibody response followed by shorter infections with both pathogens and improved pregnancy rate. \(^{115}\) This vaccination scheme combining systemic subcutaneous and mucosal vaginal doses near and within breeding period could induce lasting immune response that will cover the critical risk period in a 2-3 month service program, from the last part of breeding season until one month after it, when most of pregnancy losses occur. \(^{115}\)
Why IgG antibodies induced by systemic immunization may be the key for successful vaccines?

A protective role of serum IgG antibodies specific to T. foetus and C. fetus subsp. venerealis has been reported from in vitro and in vivo studies. Bovine whole T. foetus cell antigen specific serum inhibited trichomonad adherence to bovine vaginal epithelial cells and immobilized and agglutinated T. foetus. In this same study, the relevance of IgG1 was evidenced since whole T. foetus cell antigen specific serum IgG1-enriched fractions, but not IgG2, inhibited adherence of T. foetus to bovine vaginal epithelial cells. In addition, bovine immune serum or its IgG2 fraction in combination with complement enhanced the neutrophil-mediated destruction of T. foetus, while bovine IgG antibody, but not IgA, stimulated the polymorphonuclear-mediated destruction of C. fetus subsp. venerealis. Furthermore, bovine whole T. foetus cell antigen specific serum enhanced bovine complement-mediated destruction of T. foetus. This enhanced complement-mediated T. foetus killing in presence of antibodies is likely via the classic pathway since bovine serum IgG1 and IgG2 immunoglobulins similarly fixed bovine complement via the classical pathway and bovine serum IgG2 (IgG2b more than IgG2a) induced complement mediated-lysis of anti guinea pig red blood cells via the same pathway. However, it should be considered that complement alone (i.e., in the absence of antibodies) might kill T. foetus by the alternative pathway. Besides, low quantity of antibodies may trigger complement-mediated T. foetus by the classic pathway and additionally, enhance complement-mediated T. foetus by the alternative pathway.

A protective role of IgG and IgA antibodies was also revealed in heifers vaccinated with TF1.17 antigen, in which antigen specific systemic IgG1 and vaginal and uterine IgG1 and IgA accelerated clearance of T. foetus infection before the time when lesions and reproductive failure occurs. This vaccine-induced serum IgG1 antibody against 50-70 kDa and 150-200 kDa shed surface LPG T. foetus antigens is similar to previously reported reactivity of antibodies to TF1.17 and TF190 LPG/protein adhesions. Antibodies specific to surface LPG/protein antigens should protect against different strains since TF1.17 and TF190 antigens are conserved among different isolates of T. foetus. Thus, IgG antibody induced by vaccination by preventing adherence, activating complement and opsonizing pathogens for phagocytosis may define the outcome of STDs in the genital tract of bulls and cows.

Conclusion

Bovine STDs, trichomoniasis and campylobacteriosis, are still endemic mostly in beef cattle, causing silent economical detriment due to reproductive losses. Diagnosis of STDs is complicated and combinations of culture and PCRs consecutive times may be necessary for an adequate identification of diseased animals. Moreover, diagnosis of campylobacteriosis is especially difficult and currently not routinely used. Thus, it is very likely that STD prevalence and impact is underestimated, even more in areas with limited professional and economical resources. With no available treatment, identification and culling of infected animals is the only solution for the problem. Vaccines against STDs have been developed and few of them are on the market. However, the deficiencies in the diagnosis and still limited number of trials evaluating those vaccines impede to measure the real benefits of vaccinations. Sexually transmitted diseases have been known for decades but they continue to be the main reproductive diseases in cattle health. Collective actions among farmers, governments, industry and academia are needed for establishing and controlling regulations for further investigation in diagnosis, treatment and prophylaxis of STDs.

References


