Theriogenology 85 (2016) 781–791

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Review

A review of sexually transmitted bovine trichomoniasis and campylobacteriosis affecting cattle reproductive health

Aubrey N. Michi [a], Pedro H. Favetto [b], John Kastelic [a], Eduardo R. Cobo [a,*]

[a] Department of Production Animal Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada
[b] Private Veterinarian (Dairy Veterinary Services), Escalon, California, USA

ARTICLE INFO

Article history:
Received 29 June 2015
Accepted 28 October 2015

Keywords:
Sexually transmitted disease
Tritrichomonas foetus
Campylobacter fetus venerealis
Cattle
Vaccine

ABSTRACT

The objective is to discuss sexually transmitted diseases caused by *Tritrichomonas foetus* (*T foetus*) and *Campylobacter fetus* (*C fetus*) subsp. *venerealis*, with a focus on prevalence, pathogenesis, and diagnosis in cows and bulls. Diagnosis and control are problematic because these diseases cause severe reproductive losses in cows, but in bulls are clinically asymptomatic, which allows the disease to flourish, especially in the absence of legislated control programs. We review research regarding prophylactic systemic immunization of bulls and cows with antigens of *T foetus* and *C fetus venerealis* and their efficacy in preventing or clearing preexisting infections in the genital tract. Current diagnostic methods of *C fetus venerealis* and *T foetus* (microbial culture and PCR) should be improved. Review of the latest advances in bovine trichomoniasis and campylobacteriosis should promote knowledge and provide an impetus to pursue further efforts to control bovine sexually transmitted diseases.

1. Introduction

The two bovine sexually transmitted diseases (STDs) discussed in this review are *Tritrichomonas foetus* (*T foetus*) and *Campylobacter fetus* (*C fetus*). *Tritrichomonas foetus* is an extracellular flagellated protozoan parasite that inhabits the prepuce of bulls and induces trichomoniasis. *Campylobacter fetus* is a gram-negative, motile, spiral or S-shaped bacterium (i.e., bovine vibriosis), which induces campylobacteriosis. There are two species of *C fetus* relevant in cattle health: *C fetus* subsp. *fetus* and *C fetus* subsp. *venerealis* [1,2]. *Campylobacter fetus venerealis*, including the biotype *intermedius*, exclusively inhabits the genital tract of cattle and causes campylobacteriosis [1–3]. Generally, *C fetus fetus* inhabits the intestine but may migrate to the genital tract via an ascending genital infection or venereal route. The primary mode of transmission of *T foetus* and *C fetus venerealis* is coitus; however, they can survive in raw and processed bull semen, which makes them transmissible via artificial insemination (AI) [4,5]. In bulls, *T foetus* and *C fetus venerealis* are usually clinically asymptomatic. However, adverse effects of *T foetus* and *C fetus venerealis* in cows are characterized by genital infection, which can cause abortion [5–7]. These STDs decrease productivity of cattle by inducing reproductive losses, reduced conception rates (from slightly subnormal to $< 50\%$), reduced calf crops, increased days to conception, extended calving seasons, increased costs of replacement bulls, loss of genetic potential due to culling, and lighter weaning weights. In this regard, bovine trichomoniasis was reported to cause a prolonged breeding season, 5% to 12% reduction in weight gain during the sucking/growing period, 4% to 10% reduction in weaning weights, 4% to 10% reduction in monetary returns per calf born, 14% to 50% reduction in annual calf crop, and 5% to 35% reduction in financial returns per cow, compared to those exposed to a fertile bull [8].

1.1. Prevalence

Bovine STDs have an uneven distribution worldwide, with a high disease incidence in developing countries where cattle are bred predominantly by natural service. In

* Corresponding author. Tel.: +1 (587) 897 4499; fax: +1 (403) 210 6693.
E-mail address: ecobo@ucalgary.ca (E.R. Cobo).

0093-691X/$ – see front matter
http://dx.doi.org/10.1016/j.theriogenology.2015.10.037

contrast, in developed countries, these STDs are more likely to be endemic in beef herds that rely on natural service [9]. Recent studies have reported bulls infected with *T foetus* in herds from the following countries: United States of America [10–12], Argentina [13], Spain [14–16], Australia [17], and the Republic of Transkei [18]. Additionally, in the past decades, bulls infected with *C fetus venerealis* have been reported in the USA [19], Australia [17], Great Britain [20], Colombia [21], Tanzania [22], Nigeria [23], Canada [9,24], Argentina [25,26] and the Republic of Transkei [18]. There have been sporadic outbreaks of bovine abortions induced by *C fetus venerealis* in developing nations in Africa, Asia, and South America, as well as in developed countries in Europe, Oceania, and North America [9].

Currently, there are no global governmental monitoring programs to track the incidence and prevalence of bovine STDs. This lack of monitoring and reporting, in combination with inconsistent testing practices, has caused the worldwide cattle industry to underestimate the adverse effects of these STDs. For instance, several US states only require trichomoniasis testing in bulls aged greater than 18 months being sold through a public sale yard. However, these are only state regulations, not federal regulations. In general, there are no regulations requiring testing for bulls sold by private treaty or those resident in privately owned herds. In many cases, the cattle industry has inadequate veterinary oversight and a lack of laboratory facilities for routine testing. Collectively, all of these factors increase the prevalence and transmission of bovine STDs. This review addresses the current status of knowledge for bovine STDs, useful for research and clinical theriogenologists, and encourages new investigation by highlighting gaps and knowledge deficiencies.

2. *Tritrichomonas foetus* in bulls

Tritrichomonas foetus persistently and asymptomatically colonizes the epithelium of the prepuce, penis, and occasionally the urethral orifice of bulls. In one study, *T foetus* was cultured in smegma collected from the preputial and penile epithelial surfaces in 24 bulls infected with *T foetus* (15 naturally and 9 experimentally), whereas only four of those 24 bulls had positive cultures collected from the urethral orifice [27]. In another study, 24 bulls (2–6 years old) were naturally infected with *T foetus* and remained carriers until at least 4 months before slaughter [28]. On the basis of smegma culture, *T foetus* was detected (immunohistochemistry) on preputial epithelial surfaces (five of 24 bulls) and penile crypts (14 of 24 bulls) but not in the penile or prostatic urethra, seminal vesicles, prostate, or epididymis [28]. Furthermore, immunohistochemistry detected trichomonad antigens a few cell layers below the basement membrane in the preputial and penile epithelial crypts of 24 *T foetus*–infected bulls [28]. The superficial location of *T foetus* suggests that antigen-presenting cells could capture *T foetus* antigens from genital surfaces, either by using their dendritic projections or by direct contact with genital epithelial and subepithelial cells. Therefore, it appears that *T foetus* is restricted to mucosal surfaces and is incapable of invading tissues. However, epithelial cells could react with *T foetus* antigens in bulls and interact with stromal antigen-presenting cells, as reported in uterine stromal cells in rats [29].

Whether induced experimentally or naturally, *T foetus* genital infections in bulls are clinically asymptomatic and cause no gross pathologic changes [27]. The histologic response in the prepuce and penis of bulls infected with *T foetus* is limited to infiltration of intraepithelial lymphocytes, subepithelial lymphocytes, and plasma cells [27,28]. It is noteworthy that bulls with *T foetus* did not have more inflammation than that present in unaffected bulls [30]. Persistence of *T foetus* was confirmed with consistent isolation (culture of smegma samples) up to the time of slaughter at 4 to 18 months after infection in nine experimentally infected bulls (2–7 years old) and at 3 to 12 months after initial diagnosis in 15 naturally infected bulls (3–7 years old) [27]. Most bulls (eight of nine, 3–6 years old) challenged with various numbers of *T foetus* (10^2–10^6 organisms) became chronically infected until collection was terminated after 5 to 14 months [31]. In this same study, all five bulls (2–7 years old) challenged with 2.5×10^6 *T foetus* were also infected until slaughter (2 months later) [31]. Young bulls are either more resistant to *T foetus* infection, or they are able to eliminate it more efficiently. In this study, (16 of 18) young (1–2 years) bulls were refractory to infection, whereas the remaining two bulls were only temporary carriers (<4 months) [31]. Age-related changes in the preputial and penile epithelia of aged bulls (>5 years), including more mucosal folds and deeper crypts, may provide a reduced oxygen tension niche that increases susceptibility to infections with the anaerobic *T foetus* [31]. 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 urethra [27,28] and its presence in semen is rare [27]. Notwithstanding, when bovine sperm (1×10^6 cells) were exposed to *T foetus* (1×10^6 organisms) *in vitro* (30 minutes to 6 hours at 37 °C), they were damaged or killed [32]. However, in natural infections, this cytotoxic effect of *T fetus* would be greatly reduced because trichomonads are rarely detected in semen [27], and mature bulls often ejaculate as many as 6×10^9 sperm per coitus. Genital antibody responses to *T foetus* infection in bulls, even when detected, were incapable of eliminating genital infection. Surface antigen–specific IgG1, IgA, and IgM antibodies in the smegma of bulls naturally infected with *T foetus*, measured by ELISA at one time point, occurred concurrent with *T foetus*–positive smegma cultures [28]. Thus, *T foetus* appears to be resistant to the organism, or it does not induce pathogen-specific inflammation in bulls because it primarily inhabits the lower genital tract for prolonged intervals without causing clinical symptoms.

3. *C fetus venerealis* in bulls

Campylobacter fetus venerealis persistently and asymptomatically colonizes the epithelium of the prepuce and penis of bulls. In one study, *C fetus venerealis* was identified by immunofluorescence on impression smears and epithelial scrapings of the prepuce and penis in six of six bulls (5.5–12 years old); although five were carriers (smegma was culture positive), only one of those bulls had

distal urethra colonization [33]. On the basis of immunofluorescent studies conducted on tissue sections, *C fetus venerealis* colonized preputial and penile epithelial surfaces of the lumen and within crypts but neither in the deep epithelium nor in the subepithelium [33].

Infection with *C fetus venerealis* in bulls was not associated with any clinical symptoms, altered semen quality [6,34], or gross genital abnormalities [35]. In two studies, the subepitheliums of the prepuce and penis of six bulls characterized by *C fetus venerealis* were constantly infiltrated by lymphocytes and plasma cells, although this inflammatory response was not pathognomonic for *C fetus venerealis* [33,35]. Numbers of total non–antigen-specific plasma cells in the subepithelium of the prepuce were similar between four bulls infected with *C fetus venerealis* and 79 control bulls [30].

Genital infection with *C fetus venerealis* among mature (aged >4 years) bulls appears to be persistent based on consecutive *C fetus venerealis*–positive smegma cultures. After intrapreputial challenge with *C fetus venerealis* (4.8×10^9), four of four old (66–74 months) and three of four middle-aged (41–49 months) bulls were infected until slaughter, at nine to 10 and 16 to 18 weeks after challenge, respectively, whereas the remaining young bulls were infected for only 4 weeks [35]. Similarly, four of six bulls (aged 5 years) intrapreputially challenged with *C fetus venerealis* (three times with $2.5–4.5 \times 10^8$ organisms) were infected for at least 4 months (termination of the experiment) [36]. It is uncertain whether the genital infection with *C fetus venerealis* is influenced by bull age. In one study, the incidence of *C fetus venerealis* in semen was higher in old bulls (aged >6 years; 65 of 139) than in younger bulls (aged <6 years; four of 233) based on a variable number of cultures per bull [37]. In another study, preputial and penile mucosa were microscopically similar between old and young bulls that were infected with *C fetus venerealis* [35].

Antibody response to *C fetus venerealis* in bulls, even when detectable, did not clear the infection. Genital agglutinating antibodies specific to *C fetus venerealis* coexisted with *C fetus venerealis*–positive cultures in smegma of two naturally infected bulls [20]. However, in other studies, specific *C fetus venerealis* agglutinins were absent in smegma of six naturally infected bulls [33], and concentrations of specific agglutinins were similar between eight experimentally infected bulls (seven were carriers based on smegma culture) and two uninfected bulls [35]. Likewise, no systemic immune response was detected by tube agglutination tests (O somatic cell wall and superficial K heat-labile antigens) in four bulls infected with *C fetus venerealis* for at least 4 months after challenge [36]. Therefore, *C fetus venerealis* can inhabit the lower genital tract of bulls for at least a few months and thrive in the absence of an effective specific antibody response.

4. *T foetus* in cows

In contrast to bulls, infection with *T foetus* in cows provokes genital inflammation, including cervicitis and endometritis [38–43]. In pregnant cows, fetal death occurs in the first trimester [39] or later [7]. In one experimental study, genital inflammation and pregnancy loss occurred after 7 weeks of infection [39]. Moreover, cows infected with *T foetus* induced a detectable antigen-specific antibody response in the vagina. Intravaginal inoculation of nonpregnant heifers with *T foetus* (7×10^6 organisms) induced *T foetus* cell-specific IgG1 and IgA antibodies in vaginal secretions at 7 to 9 weeks [44], with IgA persisting for 24 weeks after infection or until genital clearance [45]. Similarly, lower intravaginal doses of *T foetus* (1×10^6 organisms) induced production of vaginal IgG1 and IgA antibodies specific to TF1.17 antigens at 5 to 10 weeks [38,41,42] and whole *T foetus* cell antigens at 5 to 8 weeks [43,46]. Also, in contrast to bulls, genital infections in heifers were limited to 13 to 28 weeks (after intravaginal challenge with 7×10^6 *T foetus*) [44], and many infected heifers had cleared the infection by 6 to 10 weeks in studies terminated 8 to 12 weeks after intravaginal challenge with 1×10^6 *T fetus* [38,40–43,46]. On the basis of this research, immunity acquired by infection with *T foetus* in cows usually results in a brief genital infection, although it does not consistently prevent reproductive failures.

This nonprotective 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/or acquired immunity. Virulent factors of *T foetus* include secreted extracellular cysteine proteinases [47], which *in vitro*, digest fibrinogen, fibronectin, albumin, and lactoferrin [48], the third component of complement (C3) [49], as well as IgG1 and IgG2 antibodies [48]. Because these cysteine proteinases preferentially cleave $IgG2^a$ allotypes rather than $IgG2^b$ [50], long-term *T foetus* infections provoking only low concentrations of local IgG2 antibodies may be due to host genetic predominance of the susceptible $IgG2^a$ allotype. Otherwise, *T foetus* nonspecifically binds bovine IgG2 and to a lesser extent IgG1 isotypes [51]. This mechanism may shield the parasite from antibody recognition of masked antigens in the effector stage of the immune response.

5. *C fetus venerealis* in cows

Natural infection with *C fetus venerealis* in cows is associated with reproductive failure, irregular estrus, transient infertility, and in pregnant cows, embryonic or fetal death [26,34,52–55]. Moreover, experimental intrauterine and cervicovaginal infection with *C fetus venerealis* in cows provoked varying grades of genital inflammation, including vaginitis, cervicitis, endometritis, and salpingitis [56]. In contrast to bulls, genital infection with *C fetus venerealis* in cows induces a detectable antibody response in the lower genital tract (i.e., vagina). In one study, using indirect fluorescence antibody assays, intravaginal inoculation of nonpregnant heifers with *C fetus venerealis* (1×10^4 organisms) induced antigen-specific transient IgM, followed by persistent IgA and IgG antibody responses in cervicovaginal secretions and an IgG1 response in uterine secretions [57]. Likewise, higher intravaginal doses of *C fetus venerealis* ($2–4 \times 10^6$ organisms) in heifers induced specific agglutinating and immobilizing antibodies in cervicovaginal secretions [58]. Furthermore, a vaginal-specific IgA antibody response was detected by ELISA after abortion

in seven heifers naturally infected with *C fetus venerealis* [54]. Genital infection with *C fetus venerealis* in female cattle was limited to weeks or months in the uterus and uterine tubes where there is a predominance of IgG antibodies [52,56,57] but persisted longer, i.e., 6 to 18 months (up to 24 months), in the vagina, where there was a predominance of IgA antibodies [52,57,58]. These carrier cows could maintain the infection in vaginal mucus for longer intervals, perhaps from one breeding season to another. However, up to 5% of cows may be resistant to infection after repeated exposure to carrier bulls or deposition of large numbers of viable organisms into the reproductive tract [59].

6. Diagnosis of bovine STDs

Trichomoniasis and campylobacteriosis are usually diagnosed using cell culture and/or polymerase chain reaction (PCR). The most efficient method of sampling vaginal and preputial secretions is insertion of an insemination/infusion pipette inside the vaginal fornix or preputial cavity and performing short strokes while concurrently aspirating secretions [60]. The body cavity can be washed with PBS to recover more organisms, although this can dilute the sample. Alternatively, preputial secretions can be collected by scraping with a metal brush, with no significant differences in culture sensitivity (Se) compared to using a pipette [61].

6.1. Culture of T foetus

The most common diagnostic test for bovine trichomoniasis is culturing preputial smegma or vaginal secretions in selected media, e.g., Diamond's [62], Plastridge [63], InPouch TF (Biomed Diagnostics, San Jose, CA, USA) [64], or liver infusion broth [65]. Samples are incubated at 37 °C (98.6 °F) and examined 1, 3, 5, and 7 days after sampling by placing a drop on a glass slide and examining it at magnifications × 40 to × 100 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* are observed [66]. Sensitivity (Se) of InPouch TF and Diamond's for detecting *T foetus* in bulls ranged from 88 to 98% [67] and 81 to 93% [62], respectively, with no significant difference between these two methods [68,69].

6.2. Cell culture and PCR for diagnosis of bovine trichomoniasis

Diagnosis of bovine *T foetus* by culture alone is limited by Se, which ranged from 84% to 96% under experimental conditions [62,70–72] but was often lower under field conditions [73]. Cell culture lacks specificity (Sp) because *Tetratrichomonas* spp. can be isolated from cultured preputial smegma [66,74–76]. The United States AI industry enforces a rigorous protocol of six once-a-week *T foetus*–negative cultures for bulls aged over 365 days [77]; this diagnostic regimen, with an Se of 86.7% and Sp of 97.5% [60], is highly effective in controlling the disease. However, a combination of culture and PCR performed on each sample for three consecutive weeks (Se: 87.5%, Sp: 95.6%) appeared to be very similar to six weekly cultures [60]. Furthermore, in that study, a single culture or PCR test was equally successful at detecting *T foetus* (Se of 67.8% and 65.9%, respectively) with an Sp greater than 90% [60]. Similar performance of culture and PCR confirms *in vitro* studies where PCR conducted on 5-day cultures of male genital secretions had 92.9% agreement with the culture [73]. Diagnostic testing employing both culture and PCR for *T foetus* yields a higher Se and improved Sp; therefore, this may be the most cost-effective and practical approach to assess bulls before the breeding season.

6.3. New concerns in diagnosis of bovine trichomoniasis

Isolation of *T foetus* via culture from preputial secretions of bulls can detect other species of trichomonads, resulting in a false-positive diagnosis. Morphologically similar trichomonads, observed under low microscopic magnification, were isolated from cultured preputial secretions of virgin bulls from widely dispersed geographical areas, including the Western USA [78], Canada [75], and Argentina [76]. These virgin bulls had not been used for breeding; therefore, these nonsexually transmitted trichomonads were categorized as "non-*T foetus* trichomonads." Transmission and scanning electron microscopy identified *Tetratrichomonas* spp. in non-*T foetus* trichomonads isolates [76]. Fortunately, amplified sequences of the 5.8S ribosomal RNA gene and flanking internal transcribed spacer regions and restriction fragment length polymorphism tests, *in lieu* of DNA sequencing, differentiated *T foetus* from *Pentatrichomonas hominis* (*P. hominis*) and *Tetratrichomonas* spp. recovered from the bovine preputial cavity [78,79].

Confronted with this new challenge, persistence and pathogenicity of tetratrichomonads and *P hominis* were investigated in bulls and cows. In these studies, tetratrichomonads were only detected intermittently in the female genital tract, and they produced no histologic lesions [43,80]. Likewise, *Tetratrichomonas* spp. did not survive in experimentally infected bulls [80] and were only detected briefly in naturally infected bulls [75]. Trichomonads including *Tetratrichomonas* spp. are commensal species in the bovine intestinal tract [81–83]. Consequently, the intermittent presence of *Tetratrichomonas* spp. in genital secretions, albeit frequently isolated from feces, suggests a fecal-genital route of infection, consistent with detection of tetratrichomonads in the lower genital tract in bulls following homosexual behavior [75,76,78,79]. Thus, "non-*T foetus* trichomonads", including *Tetratrichomonas* spp. from the intestinal tract, could sporadically contaminate the genital tract as a consequence of defecation and physical contact among cows and bulls, cause transient infections of genital tract, and consequently, false-positive trichomoniasis diagnoses [74,76,80].

6.4. Culture of C fetus venerealis

Isolating *C fetus venerealis* requires samples from preputial or vaginal secretions, fetal stomach contents, lungs, liver and placentomes (abortion investigations), to be transported to the laboratory in transport enrichment

medium at room temperature. In the laboratory, samples are cultured in selective culture media such as 5% sheep blood agar, Skirrow's agar, [84], 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 48 hours. Passive filtration of fresh preputial scrapings onto blood agar yielded higher recovery rates of *C fetus venerealis* than those obtained with direct plating [85]. Subspecies of *Campylobacter* are differentiated biochemically, on the basis of tolerance to 1% glycine and production of hydrogen sulfide through utilization of cysteine. In that regard, presumptive *C fetus fetus* colonies, but not *C fetus venerealis*, will grow with 1% glycine or with NaCl and cysteine [86].

Culturing *C fetus venerealis* is challenging as it is labor intensive, time-consuming, and has a low Se. In addition, some strains of *C fetus venerealis* are sensitive to polymyxin B (commonly present in transport enrichment medium and selective media). Furthermore, *C fetus venerealis* biovar "*intermedius*" can tolerate higher concentrations of glycine, and some commensal bacteria easily thrive in these culture media. Finally, sensitivity of *C fetus venerealis* to excessive temperature fluctuations provides a challenge when the interval from sample collection to its arrival at the laboratory exceeds 24 hours [87].

6.5. PCR for C fetus venerealis

Diagnosing campylobacteriosis by PCR should not only identify *C fetus venerealis* but also facilitate differentiation between *C fetus fetus* and other trichomonads. Misidentification of *C fetus venerealis* as *C fetus fetus* results in the spread of *C fetus venerealis* in cattle populations and subsequent economic losses [88]. Although *C fetus venerealis* has a high sequence identity (92%) with *C fetus fetus,* there are unique identifiers. Differentiating characteristics 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 transposase [89–92]. These genomic particularities of *C fetus venerealis* have been useful for designing several PCR primer sequences for identification and differentiation of *C fetus venerealis* [84,88,93]. One of the most reliable PCR and quantitative PCR tests includes one primer set (MG3F/MG4R) that amplifies a 750 to 960 base pair fragment of the *C fetus* carbon starvation protein gene, present in both *C fetus* subspecies, and another primer set (VenSF/VenSR) that amplifies a 142-bp fragment of the parA gene, exclusive to *C fetus venerealis* [94]. In one study, an SYBR Green quantitative PCR, based on a VenSF/VenSR primer set, was also satisfactory for direct detection of *C fetus venerealis* in preputial samples [84]. A multiplex PCR assay using a set of primers to amplify a *C fetus*–specific 764-bp sequence and another set of primers (nC1165g4F/nC1165g4R) to amplify a 233-bp sequence uniquely present in *C fetus venerealis* also detected and differentiated between *C fetus fetus* and *C fetus venerealis* in samples of abomasal contents of aborted bovine fetuses, in the absence of a pre-enrichment step [86]. Likewise, two real-time SYBR Green PCR assays for detection and discrimination of *C fetus fetus* and *C fetus venerealis* had 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%) on 1071 bacterial isolates [93]. However, molecular analysis by amplified fragment length polymorphism and multilocus sequence typing are still recommended to identify *C fetus* subspecies isolates, as other real-time PCR assay targeting gene nahE had 100% Se and Sp for *C fetus* spp., although the subspecies venerealis specific real-time PCR (ISCfe1) failed due to sequence variation of the target insertion sequence [88]. Thus, diagnosis of campylobacteriosis still needs further investigation to be routinely used for control and eradication, as culture is impractical and PCR is not widely standardized. Hopefully, recent publication of the complete genome of *C fetus venerealis* [95] will facilitate development of improved molecular diagnostic tools (e.g., real-time PCR, PCR) for identification of this organism.

7. Vaccination and management

Currently, there is no effective standardized or legal treatment for *T foetus*–infected cattle. Although there has been some success with nitroimidazole drugs, none are currently licensed for use in cattle. Antibiotic treatments for *C fetus venerealis* are impractical and have limited efficacy. In the absence of effective treatment options, bovine STDs are largely controlled by diagnostic testing, reporting, and culling of infected animals. For instance, the control program implemented by the California Department of Food and Agriculture regulating the livestock industry mandates that bulls infected with *T foetus* should be permanently quarantined until slaughter, and herdmate bulls should be quarantined pending one negative real-time PCR test or three consecutive negative trichomoniasis culture tests. Reporting the incidence of *T foetus* and *C fetus venerealis* is compulsory and must be declared to local authorities. Veterinarians approved to conduct trichomoniasis sampling are required to report all positive test results to the CDFA within 2 days after the reading date. All negative test results must be reported within 30 days.

Campylobacteriosis is categorized as a List B disease by the International Office of Epizootics; this denotes a transmissible disease of socioeconomic and/or public health importance within countries and is significant for international trade of animals and animal products [96]. Therefore, the AI industry demands rigorous testing to ensure pathogen-free semen as bulls are the epidemiologic carrier and major factor in the transmission of STDs. In the USA, 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 age-dependent testing standards (one, three, or six tests for bulls <6 months, 6 months–1 year, and >1 year, respectively). Furthermore, the AI industry routinely adds antibiotics to semen extenders before cryopreservation, although antimicrobial resistance occurs in some strains of *C fetus venerealis* [97]. Overall, successful diagnosis and mitigation of *C fetus venerealis* and *T foetus* are dependent on isolation of infected animals and consistent reporting activity.

Systemic vaccinations against *T foetus* and *C fetus venerealis* in cattle have been associated with prevention and

cure of genital infections by inducing detectable systemic IgG antibodies, which may translocate to genital secretions. Therefore, discussion of important studies investigating vaccines against STDs in cows and bulls is included below.

7.1. Vaccines against T foetus

There have been several studies in which systemic immunization in bulls with antigens of *T foetus* [98,99] prevented or cleared genital infections, on the basis of negative smegma sample cultures. In one study, whole *T foetus* cell antigens in a mineral oil adjuvant were systemically administered three times (monthly intervals) to age-susceptible bulls (>4 years) [98]. Afterward, 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. Bulls were considered free of infection after five consecutive weekly negative smegma cultures; conversely, they were considered persistently infected after nine consecutive *T foetus*–positive smegma cultures [98]. Whole *T foetus* cell antigens prevented or shortened infections in 37 of 48 vaccinated bulls, whereas 18 of 38 control unvaccinated-challenged bulls either remained free of infection or only had a short-duration infection [98]. Furthermore, therapeutic immunization with a whole *T foetus* cell antigen cleared infections in 11 of 16 vaccinated bulls, whereas only one of eight untreated bulls eliminated the infection [98]. This whole *T foetus* cell vaccine was most effective in bulls aged lesser than 5.5 years [98] because younger bulls have shallower preputial crypts, which are less likely to sustain these microaerophilic/anaerobic microbes.

7.1.1. Efficacy of other types of T foetus antigens

Other types of *T foetus* antigens had some efficacy. In one study, a membrane preparation (500 μg/dose) or purified membrane glycoprotein (160 μg/dose) antigens of *T foetus*, both in a mineral oil adjuvant, were systemically given three times at monthly intervals to bulls with pre-existing genital *T foetus* infections [99]. In that study, three of four bulls vaccinated with membrane antigens and three of four bulls vaccinated with membrane glycoprotein eliminated their infection 2 weeks after the second vaccine dose, whereas the two remaining vaccinated bulls and eight of eight infected unvaccinated control bulls were infected for 2 months after the last dose [99]. The three bulls vaccinated with *T foetus* membrane antigen that cleared the infection and an additional three noninfected, nonvaccinated control bulls were subsequently challenged with *T foetus*. None of the vaccinated bulls became infected, whereas two of three control bulls did [99]. In a more recent study, systemic immunization of bulls with subcutaneous inoculation of 2 mL of a commercial vaccine containing whole-cell killed *T foetus* in oil adjuvant prevented trichomonad colonization of the preputial and penile mucosa in four of four bulls vaccinated and subsequently challenged with *T foetus* [100]. The vaccinated-challenged bulls had systemic and preputial *T foetus* lipophosphoglycan (LPG)/protein *T foetus* antigen–specific IgG1 and IgG2 and slight preputial IgE and IgA antibody responses that conferred resistance to trichomonad genital colonization [100]. On the basis of these high and persistent concentrations of serum IgG antibodies in vaccinated bulls, in conjunction with additional genital IgG antibody responses after systemic vaccinations, it was inferred that vaccine-induced systemic antibodies were important contributors to the luminal IgG antibody response. The peak of preputial IgE antibody in vaccinated bulls before challenge, concurrent with the presence of IgG1 and IgG2 antibodies, may promote translocation of serum IgG into smegma. Immunoglobulin E antibodies would cross-link *T foetus* antigens on mast cell receptors to activate and release mediators thereby increasing endothelial and epithelial permeability and facilitating systemic IgG antibody translocation from the bloodstream to the genital epithelium into secretions, as proposed in infected heifers [40]. However, a significant genital-specific IgA antibody response was detected before challenge in vaccinated bulls that could promote resistance to trichomonad colonization [100]. 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 prevents genital colonization [100].

Systemic vaccination of nonpregnant heifers with an immunoaffinity-purified LPG/protein complex (TF1.17) antigen of *T foetus* induces specific IgG1 and IgA antibodies in vaginal and uterine secretions that coincide with clearance of experimental genital infections, usually within lesser than 7 weeks [38,41,42,46]. Genital clearance before 7 weeks in female cattle likely prevents reproductive failures because inflammation and pregnancy loss did not occur until at least 7 weeks after infection [39]. Likewise, systemic vaccination with whole-cell [101–107] or membrane antigens [106] in cows mated with *T foetus*–infected bulls (natural or experimentally infected) had a shortened interval of genital infection and better calving rates compared to unvaccinated cows. In female cattle, booster vaccination with *T foetus* antigens in nasal [41] or vaginal mucosae [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 foetus* cells (10^8) given intranasally (six heifers) [41] or intravaginally (nine heifers) [42] and 2 to 3 weeks later, intravaginally challenged with 10^6 *T foetus* (all vaccines were in Quil A and given 3 weeks apart). In that study, systemic priming with vaginal or nasal boosting similarly shortened duration of infection and increased antigen-specific vaginal IgA (3–10 weeks after challenge) and uterine IgA and IgG1 antibodies (10 weeks after challenge) [41,42]. The same vaccination/challenge scheme with the booster given systemically to six [41] or 10 [42] heifers also induced shortened infection but that was mainly attributed to vaginal and uterine IgG1 antibodies. Other specific *T foetus* antigens, e.g., Tf190, also resulted in fewer infected heifers after experimental inoculation and elicited systemic IgG1 and IgG2 when injected subcutaneously and genital IgA when given intranasally [108]. On the basis of these studies, trichomoniasis vaccination offers a few advantages. However, further research evaluating better antigens may be required, as recent meta-analysis of

the efficacy of whole-cell killed *T foetus* vaccines in beef cattle concluded that vaccination of heifers and bulls still has limited success in mitigating infections and reducing abortion risk [109].

7.2. Vaccines against C fetus venerealis

Systemic vaccination of bulls with *C fetus venerealis* antigens has also conferred genital protection [36,110,111]. In one study, whole *C fetus venerealis* cell antigens (~40-mg dry matter weight) in mineral oil adjuvant were systemically administered to bulls (14–18 months) twice with a 2-month interval and then annually [111]. These bulls were intrapreputially challenged with *C fetus venerealis* every 6 months (total of five times) and considered free of infection after four consecutive weekly negative smegma cultures [111]. In that study, all 16 vaccinated bulls were free of infection, whereas for 17 control unvaccinated-challenged bulls, 13 were infected for at least 3 months, two were infected for lesser than 2 weeks, and only the remaining two were consistently free of infection [111]. The ages at which bulls became infected ranged from 2 to 6 years [111]. Likewise, in another study, a dual vaccine containing *C fetus intermedius* and *C fetus venerealis* antigens (20 mg of each) was given to bulls (20–34 months) using the same vaccination/challenge protocol [112]. In that study, all five bulls challenged with *C fetus intermedius* and all five bulls challenged with *C fetus venerealis* remained free of infection, whereas nine of 10 challenged-unvaccinated control bulls were infected for at least 5 weeks after challenge [112]. The ages at which bulls became infected ranged from 3.5 to 5.5 years [112].

Therapeutic immunization against *C fetus venerealis* infection in bulls also has some effectiveness. In one study, a commercial whole *C fetus venerealis* cell antigen vaccine (~40-mg dry matter weight) was systemically given twice a month to infected bulls (aged 5 years) [36]. Infection status was determined by smegma culture 8 weeks after primary vaccination and by culture of genital secretions of a virgin heifer after mating by the tested bull. In that study, all six infected vaccinated bulls cleared their infection, whereas all four infected and unvaccinated bulls remained infected for 4 months, at which time they were vaccinated and two subsequently cleared the infection [36]. In another study, infection status was evaluated by smegma culture and immunofluorescence tests; a vaccine containing whole *C fetus intestinalis* cell antigens in incomplete Freund's adjuvant (concentration unreported) was systemically given once to 288 bulls serving in a *C fetus venerealis*–infected area; it was noteworthy that none of the bulls subsequently acquired the infection [110]. Moreover, this same vaccine (given twice) cleared infection by 42 days after the second dose in all 41 bulls already infected with *C fetus venerealis* and exposed to infected females and it prevented reinfections in all eight bulls that previously cleared infection by vaccination and were intrapreputially challenged (2–8 times) with *C fetus venerealis* (unreported doses) [110]. Unfortunately, the outcome is difficult to assess, as results from control (unvaccinated) bulls were not reported, antigenic similarity between the infecting *C fetus venerealis* and the immunogen *C fetus intestinalis* was undetermined and mating in an endemic area may not have been a sufficient challenge. Regarding immunity induced by *C fetus venerealis* antigens, systemic vaccination with whole *C fetus venerealis* antigen, given twice (1-month 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 those in two unvaccinated bulls (agglutination range: 60–80) [36]. Likewise, systemic vaccination with whole *C fetus venerealis* cell antigen, given twice (2-month interval and then annually), induced higher titers of serum agglutinins in 12 of 17 vaccinated bulls (agglutination range: 40–40, 960, peaking 3 weeks after the second vaccine dose) than in 17 unvaccinated control bulls (agglutination range: usually lesser than 20, with a maximum of 160) [111].

Systemic immunization of cows with a *C fetus venerealis* bacterin was associated with prevention of infection as well as apparent cures. Systemic vaccination with *C fetus venerealis* and biotype *intermedius* (20-mg dry weight of each) in mineral oil adjuvant vaccine protected cows against genital experimental infection with either organism [113]. Heifers given a bacterin containing K antigen were resistant to experimental infection with *C fetus fetus* [114]. Moreover, systemic immunization with killed *C fetus* cells in incomplete Freund's adjuvant also cured the infection in six of eight cows previously infected with *C fetus venerealis* [115]. These protective and curative effects of systemic vaccination have been largely associated with the induced antibody response, similar to that described in bulls. Systemic vaccines with *C fetus* bacterins in oil adjuvants stimulated high systemic concentrations of IgG1 and IgG2 and genital IgG1 and IgG2 [58,116]. Despite these successes, there have also been reports of vaccine failures. Two commercial vaccines containing *C fetus venerealis* administered subcutaneously in female cattle did not protect them against infection of *C fetus venerealis* when they were naturally challenged by mating with an infected bull for 60 days [117]. In spite of the vaccination, both vaccinated and control groups had a high percentage of infected heifers and both groups had poor reproductive performance. Likewise, therapeutic vaccination failed in a 4-year-old bull infected with *C fetus venerealis* because it remained infected after systemic immunization with two commercial whole-cell vaccines, given twice and three times, respectively [118]. However, after this bull was cured by antibiotic treatment, it remained resistant to infection by intrapreputial challenge [118].

7.2.1. Vaccine failures

Failures in vaccines against campylobacteriosis may be attributed to 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 venerealis* may impede recognition by the immune system and consequently induce a limited immune response to infection. Isolates were created from *C fetus venerealis* from the smegma of three of four relatively young (41–49 months) and three of four older (66–74 months) infected bulls with modified superficial antigens and rabbit antiserum specific to whole heat-labile surface *C fetus*

venerealis antigens which had decreased agglutination titers to individual antigens of subsequent isolates compared to the infecting strain [35]. Superficial antigenic variation was also apparent based on agglutination tests in *C fetus venerealis* isolates from cervicovaginal mucus of two heifers over several months of infection [119]. In this study, the isolates, sampled throughout infection, reacted with rabbit antiserum of various specificities, and the specificity of the cervicovaginal agglutinating antibodies of the heifers varied during infection [119].

As alternative mechanisms of evasion, *C fetus venerealis* may bind bovine IgA-specific antibodies, thus escaping the complement and phagocytosis-mediating properties of IgG [120] and bacterial surface glycoproteins that, in the absence of specific antibodies, inhibit ingestion by macrophages [121]. Hence, microbial antigenic variation and capacity for blocking inflammatory-immune effectors (e.g., complement, IgG, macrophages) may promote persistence of *C fetus venerealis* and failure of vaccines containing only international strains. In addition, many commercial vaccines only include *C fetus venerealis* but lack *C fetus fetus* and *C fetus venerealis* biotype *intermedius*, although the latter were associated with reproductive problems such as infertility, reduced pregnancy rates, and abortion in cattle [2,3,55,86,122]. Dry weight of the cells per dose may determine the effectiveness of a vaccine, as experimental vaccines induced strong protection against genital infection only when they contained at least 40 mg of dry weight per dose in oil adjuvant [113]. Apparently, the primary limitation of vaccines against campylobacteriosis in cows and bulls is the relative lack of ongoing research and testing of current vaccines and development of novel products.

7.2.2. Dual vaccine effectiveness

There is little information regarding the effectiveness of vaccines containing both *C fetus venerealis* and *T foetus* antigens. One study evaluated systemic vaccination with whole cells of *C fetus venerealis* and *T foetus* in cows mated for 90 days with bulls infected with *C fetus venerealis* and *T foetus* (from Days 0 to +90), plus an additional vaginal instillation of both pathogens at Day +39 [107]. Vaccines were administrated subcutaneously on Days −30 and +11 and into the vaginal submucosa on Day −9 of the mating period. Vaccinated cattle had elevated systemic and vaginal IgG antibody responses, shorter durations of infections with each pathogen, and improved pregnancy rate [107]. This vaccination strategy combining systemic subcutaneous and mucosal vaginal doses before and during the breeding period could induce a prolonged immune response, thereby covering the critical risk period in a 2- to 3-month breeding program, namely, commencing in the later part of the breeding season and persisting until 1 month after it ends, when most pregnancy losses occur [107].

7.3. Are IgG antibodies key to successful vaccines?

A protective role of serum IgG antibodies specific to *T foetus* and *C fetus venerealis* has been reported, on the basis of both *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* [123]. Furthermore, in that study, the relevance of IgG1 was apparent, as whole *T foetus* cell antigen-specific serum IgG1-enriched fractions, but not IgG2, inhibited adherence of *T foetus* to bovine vaginal epithelial cells [123]. In addition, bovine immune serum or its IgG2 fraction, in combination with complement, enhanced neutrophil-mediated destruction of *T foetus* [124]. In contrast, bovine IgG, but not IgA, stimulated polymorphonuclear-mediated destruction of *C fetus venerealis* [120]. Furthermore, bovine whole *T foetus* cell antigen-specific serum enhanced bovine complement-mediated destruction of *T foetus* [125,126]. This enhanced complement-mediated *T foetus* killing in the presence of antibodies is likely via the classic pathway because bovine serum IgG1 and IgG2 immunoglobulins similarly fixed bovine complement via the classical pathway [127] and bovine serum IgG2 ($IgG2^b$ more than $IgG2^a$) induced complement-mediated lysis of antiguinea pig erythrocytes via the same pathway [128]. However, complement alone (i.e., in the absence of antibodies) could kill *T foetus* by the alternative pathway [125,126]. Besides, low antibody concentrations may trigger complement-mediated *T foetus* by the classic pathway and additionally enhance complement-mediated *T foetus* by the alterative pathway.

A protective role of IgG and IgA antibodies was also apparent 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 manifestations of lesions or reproductive failure [38,41,42,46]. This vaccine-induced serum IgG1 antibody against 50 to 70 kDa and 150 to 200 kDa shed surface LPG *T foetus* antigens is similar to previously reported reactivity of antibodies to TF1.17 and Tf190 LPG/protein adhesins [129,130]. Antibodies specific to surface LPG/protein antigens should protect against various strains because TF1.17 and Tf190 antigens are conserved among *T foetus* isolates [131,132]. 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.

8. Conclusions

The bovine STDs, trichomoniasis, and campylobacteriosis remain endemic and prevalent mostly in beef cattle causing mild to severe reproductive losses. To effectively diagnose and treat affected cattle, a combination of serial cultures and PCR testing is necessary to establish pathogen identity. In the diagnosis of trichomoniasis or campylobacteriosis, testing methods have not been streamlined to encourage consistent use by the livestock industry. Owing to the lack of efficient testing methods and worldwide knowledge deficiency of bovine STDs, it is likely that STD prevalence and economic impact is highly underestimated, particularly in areas with limited financial and professional resources. Currently, culling infected livestock is the most cost-effective method of mitigating pathogen prevalence. There have been promising vaccine developments, but few are commercially available. Furthermore, deficiencies in diagnostic methods and limited numbers of clinical trials

evaluating vaccine efficacy continue to hamper efforts to understand vaccine benefits. Although bovine STDs have been identified for decades, they continue to be the primary reproductive diseases of cattle. To improve quality of life for livestock while maintaining healthy economic output, collective action between cattle producers, governments, industry, and academia must be established to create regulations for further investigation in diagnosis, treatment, and prophylaxis of bovine STDs.

Acknowledgments

The authors thank the funding support from Alberta Livestock and Meat Agency Ltd. (ALMA) 2015B008R, Canada and the National Scientific and Technical Research Council PICT 2013-0393, Argentina (Spanish: Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET).

Competing Interests

The authors declare that they have no competing interests.

References

[1] Eaglesome MD, Garcia MM. Microbial agents associated with bovine genital tract infections and semen. Part I. *Brucella abortus, Leptospira, Campylobacter fetus* and *Tritrichomonas foetus*. Vet Bul 1992;62:743–75.
[2] Dekeyser J. Bovine genital campylobacteriosis. Boca Raton: CRC Press; 1984.
[3] Iraola G, Perez R, Naya H, Paolicchi F, Harris D, Lawley TD, et al. Complete genome sequence of Campylobacter fetus subsp. venerealis bv. Intermedius, isolated from the prepuce of a bull. Genome Announc 2013;1:1–2.
[4] Eaglesome MD, Sampath MI, Garcia MM. A detection assay for *Campylobacter fetus* in bovine semen by restriction analysis of PCR amplified DNA. Vet Res Commun 1995;19:253–63.
[5] Garcia MM, Eaglesome MD, Rigby C. Campylobacters important in veterinary medicine. Vet Bul 1983;53:793–818.
[6] Skirrow MB. Diseases due to *Campylobacter, Helicobacter* and related bacteria. J Comp Pathol 1994;111:113–49.
[7] Rhyan JC, Stackhouse LL, Quinn WJ. Fetal and placental lesions in bovine abortion due to *Tritrichomonas foetus*. Vet Pathol 1988;25:350–5.
[8] Rae DO. Impact of trichomoniasis on the cow-calf producer's profitability. J Am Vet Med Assoc 1989;194:771–5.
[9] Mshelia GD, Amin JD, Woldehiwet Z, Murray RD, Egwu GO. Epidemiology of bovine venereal campylobacteriosis: geographic distribution and recent advances in molecular diagnostic techniques. Reprod Domest Anim 2010;45:e221–30.
[10] BonDurant RH, Anderson ML, Blanchard P, Hird D, Danaye-Elmi C, Palmer C, et al. Prevalence of trichomoniasis among California beef herds. J Am Vet Med Assoc 1990;196:1590–3.
[11] Rae DO, Crews JE, Greiner EC, Donovan GA. Epidemiology of *Tritrichomonas foetus* in beef bull populations in Florida. Theriogenology 2004;61:605–18.
[12] Szonyi B, Srinath I, Schwartz A, Clavijo A, Ivanek R. Spatio-temporal epidemiology of *Tritrichomonas foetus* infection in Texas bulls based on state-wide diagnostic laboratory data. Vet Parasitol 2012;186:450–5.
[13] Mardones FO, Perez AM, Martinez A, Carpenter TE. Risk factors associated with *Tritrichomonas foetus* infection in beef herds in the Province of Buenos Aires, Argentina. Vet Parasitol 2008;153:231–7.
[14] Martin-Gomez S, Gonzalez-Paniello R, Pereira-Bueno J, Ortega-Mora LM. Prevalence of *Tritrichomonas foetus* infection in beef bulls in northwestern Spain. Vet Parasitol 1998;75:265–8.
[15] Mendoza-Ibarra JA, Ortega-Mora LM, Pedraza-Diaz S, Rojo-Montejo S, Ruiz-Santa-Quiteria JA, Garcia-Pena FJ, et al. Differences in the prevalence of *Tritrichomonas foetus* infection in beef cattle farmed under extensive conditions in northern Spain. Vet J 2013;196:547–9.
[16] Mendoza-Ibarra JA, Pedraza-Diaz S, Garcia-Pena FJ, Rojo-Montejo S, Ruiz-Santa-Quiteria JA, San Miguel-Ibanez E, et al. High prevalence of *Tritrichomonas foetus* infection in Asturiana de la Montana beef cattle kept in extensive conditions in northern Spain. Vet J 2012;193:146–51.
[17] McCool CJ, Townsend MP, Wolfe SG, Simpson MA, Olm TC, Jayawardhana GA, et al. Prevalence of bovine venereal disease in the Victoria River District of the Northern Territory: likely economic effects and practicable control measures. Aust Vet J 1988;65:153–6.
[18] Pefanis SM, Herr S, Venter CG, Kruger LP, Queiroga CC, Amaral L. Trichomoniasis and campylobacteriosis in bulls in the Republic of Transkei. J S Afr Vet Assoc 1988;59:139–40.
[19] Winter AJ, Burda K, Dunn HO. An evaluation of cultural technics for the detection of *Vibrio fetus* in bovine semen. Cornell Vet 1965;55:431–44.
[20] Corbel MJ. Detection of antibodies to *Campylobacter fetus (Vibrio fetus)* in the preputial secretions of bulls with vibriosis. Br Vet J 1974;130:51–3.
[21] Griffiths IB, Gallego MI, De Leon LS. Levels of some reproductive diseases in the dairy cattle of Colombia. Trop Anim Health Prod 1984;16:219–23.
[22] Swai ES, Hulsebosch J, Van der Heijden W. Prevalence of genital campylobacteriosis and trichomonosis in crossbred breeding bulls kept on zero-grazed smallholder dairy farms in the Tanga region of Tanzania. J S Afr Vet Assoc 2005;76:224–7.
[23] Bawa EK, Adekeye JO, Oyedipe EO, Omoh JU. Prevalence of bovine campylobacteriosis in indigenous cattle of three states in Nigeria. Trop Anim Health Prod 1991;23:157–60.
[24] Waldner C, Hendrick S, Chaban B, Guerra AG, Griffin G, Campbell J, et al. Application of a new diagnostic approach to a bovine genital campylobacteriosis outbreak in a Saskatchewan beef herd. Can Vet J 2013;54:373–6.
[25] Molina L, Perea J, Meglia G, Angon E, Garcia A. Spatial and temporal epidemiology of bovine trichomoniasis and bovine genital campylobacteriosis in La Pampa province (Argentina). Prev Vet Med 2013;110:388–94.
[26] Morrell EL, Barbeito CG, Odeon CA, Gimeno EJ, Campero CM. Histopathological, immunohistochemical, lectinhistochemical and molecular findings in spontaneous bovine abortions by *Campylobacter fetus*. Reprod Domest Anim 2011;46:309–15.
[27] Parsonson IM, Clark BL, Dufty J. The pathogenesis of *Tritrichomonas foetus* infection in the bull. Aust Vet J 1974;50:421–3.
[28] Rhyan JC, Wilson KL, Wagner B, Anderson ML, BonDurant RH, Burgess DE, et al. Demonstration of *Tritrichomonas foetus* in the external genitalia and of specific antibodies in preputial secretions of naturally infected bulls. Vet Pathol 1999;36:406–11.
[29] Wira CR, Rossoll RM. Antigen-presenting cells in the female reproductive tract: influence of the estrous cycle on antigen presentation by uterine epithelial and stromal cells. Endocrinology 1995;136:4526–34.
[30] Flower PJ, Ladds PW, Thomas AD, Watson DL. An immunopathologic study of the bovine prepuce. Vet Pathol 1983;20:189–202.
[31] Clark BL, Parsonson IM, Dufty JH. Experimental infection of bulls with *Tritrichomonas foetus*. Aust Vet J 1974;50:189–91.
[32] Benchimol M, de Andrade Rosa I, da Silva Fontes R, Burla Dias AJ. Trichomonas adhere and phagocytose sperm cells: adhesion seems to be a prominent stage during interaction. Parasitol Res 2008;102:597–604.
[33] Samuelson J, Winter A. Bovine vibriosis: the nature of the carrier state in the bull. J Infect Dis 1966;116:581–92.
[34] Clark BL. Review of bovine vibriosis. Aust Vet J 1971;47:103–7.
[35] Bier P, Hall C, Duncan J, Winter A. Experimental infections with *Campylobacter fetus* in bulls of different ages. Vet Microbiol 1977;2:13–27.
[36] Vasquez LA, Ball L, Bennett BW, Rupp GP, Ellis R, Olson JD, et al. Bovine genital campylobacteriosis (vibriosis): vaccination of experimentally infected bulls. Am J Vet Res 1983;44:1553–7.
[37] Wagner WC, Dunn HO, Vanvleck LD. Incidence of vibriosis in an AI stud. Cornell Vet 1965;55:209–20.
[38] Anderson ML, BonDurant RH, Corbeil RR, Corbeil LB. Immune and inflammatory responses to reproductive tract infection with *Tritrichomonas foetus* in immunized and control heifers. J Parasitol 1996;82:594–600.
[39] Parsonson IM, Clark BL, Dufty JH. Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers. J Comp Pathol 1976;86:59–66.

[40] Corbeil LB, Campero CM, Rhyan JC, Anderson ML, Gershwin LJ, Agnew DW, et al. Uterine mast cells and immunoglobulin-E antibody responses during clearance of *Tritrichomonas foetus*. Vet Pathol 2005;42:282–90.
[41] Corbeil LB, Munson L, Campero C, BonDurant RH. Bovine trichomoniasis as a model for development of vaccines against sexually-transmitted disease. Am J Reprod Immunol 2001;45:310–9.
[42] Corbeil LB, Anderson ML, Corbeil RR, Eddow JM, BonDurant RH. Female reproductive tract immunity in bovine trichomoniasis. Am J Reprod Immunol 1998;39:189–98.
[43] Agnew DW, Munson L, Cobo ER, Olesen D, Corbeil LB, BonDurant RH. Comparative histopathology and antibody responses of non-*Tritrichomonas foetus* trichomonad and *Tritrichomonas foetus* genital infections in virgin heifers. Vet Parasitol 2008;151:170–80.
[44] Skirrow SZ, BonDurant RH. Immunoglobulin isotype of specific antibodies in reproductive tract secretions and sera in *Tritrichomonas foetus*-infected heifers. Am J Vet Res 1990;51:645–53.
[45] Ikeda JS, BonDurant RH, Corbeil LB. Bovine vaginal antibody responses to immunoaffinity-purified surface antigen of *Tritrichomonas foetus*. J Clin Microbiol 1995;33:1158–63.
[46] BonDurant RH, Corbeil RR, Corbeil LB. Immunization of virgin cows with surface antigen TF1.17 of *Tritrichomonas foetus*. Infect Immun 1993;61:1385–94.
[47] Thomford JW, Talbot JA, Ikeda JS, Corbeil LB. Characterization of extracellular proteinases of *Tritrichomonas foetus*. J Parasitol 1996;82:112–7.
[48] Talbot JA, Nielsen K, Corbeil LB. Cleavage of proteins of reproductive secretions by extracellular proteinases of *Tritrichomonas foetus*. Can J Microbiol 1991;37:384–90.
[49] Kania SA, Reed SL, Thomford JW, BonDurant RH, Hirata K, Corbeil RR, et al. Degradation of bovine complement C3 by trichomonad extracellular proteinase. Vet Immunol Immunopathol 2001;78:83–96.
[50] Bastida-Corcuera F, Butler JE, Heyermann H, Thomford JW, Corbeil LB. *Tritrichomonas foetus* extracellular cysteine proteinase cleavage of bovine IgG2 allotypes. J Parasitol 2000;86:328–32.
[51] Corbeil LB, Hodgson JL, Widders PR. Immunoglobulin binding by *Tritrichomonas foetus*. J Clin Microbiol 1991;29:2710–4.
[52] Vandeplassche M, Florent AF, Bouters R, Huysmin A, Brone E, Dekeyser P. The pathogenesis, epidemiology, and treatment of *Vibrio fetus* infection in cattle. CR Rech IRSIA 1963;29:1–90.
[53] Peterson J, Newsam I. The histopathology of genital vibriosis in virgin heifers. Br Vet J 1964;120:229–45.
[54] Hum S, Stephens LR, Quinn C. Diagnosis by ELISA of bovine abortion due to *Campylobacter fetus*. Aust Vet J 1991;68:272–5.
[55] Campero CM, Anderson ML, Walker RL, Blanchard PC, Barbano L, Chiu P, et al. Immunohistochemical identification of *Campylobacter fetus* in natural cases of bovine and ovine abortions. J Vet Med B 2005;52:138–41.
[56] Schurig GD, Hall CE, Burda K, Corbeil LB, Duncan JR, Winter AJ. Infection patterns in heifers following cervicovaginal or intrauterine instillation of *Campylobacter (Vibrio) fetus* venerealis. Cornell Vet 1974;64:533–48.
[57] Corbeil LB, Duncan JR, Schurig GG, Hall CE, Winter AJ. Bovine venereal vibriosis: variations in immunoglobulin class of antibodies in genital secretions and serum. Infect Immun 1974;10:1084–90.
[58] Corbeil LB, Schurig GD, Duncan JR, Corbeil RR, Winter AJ. Immunoglobulin classes and biological functions of *Campylobacter (Vibrio) fetus* antibodies in serum and cervicovaginal mucus. Infect Immun 1974;10:422–9.
[59] Dufty JH, Vaughan J. Bovine venereal campylobacteriosis. In: Howard JL, editor. Current Vetereniary Therapy 3 food animal practice. St. Louis, Missouri, USA: W. B. Saunders, Co; 1993. p. 510–3.
[60] Cobo ER, Favetto PH, Lane VM, Friend A, VanHooser K, Mitchell J, et al. Sensitivity and specificity of culture and PCR of smegma samples of bulls experimentally infected with *Tritrichomonas foetus*. Theriogenology 2007;68:853–60.
[61] Parker S, Campbell J, Ribble C, Gajadhar A. Comparison of two sampling tools for diagnosis of *Tritrichomonas foetus* in bulls and clinical interpretation of culture results. J Am Vet Med Assoc 1999;215:231–5.
[62] Parker S, Campbell J, Gajadhar A. Comparison of the diagnostic sensitivity of a commercially available culture kit and a diagnostic culture test using Diamond's media for diagnosing *Tritrichomonas foetus* in bulls. J Vet Diagn Invest 2003;15:460–5.
[63] Martinez AH, Bardon JC, Noseda RP, Cordeviola J. Diagnostico de trichomoniasis en toros. Propuesta de un esquema de diagnostico. Vet Arg 1985;2:966–77.
[64] Borchardt K, Norman B, Thomas M, Harmon W. Evaluation of a new culture method for diagnosing *Tritrichomonas foetus* infection. Vet Med 1992;87:104–12.
[65] Campero C, Catena M, Medina D. Caldo infusion higado para el cultivo de *Tritrichomonas foetus*. Vet Arg 1986;3:80–1.
[66] BonDurant RH, Gajadhar A, Campero CM, Johnson E, Lun Z-R, Nordhausen RW, et al. Preliminary characterization of a *Tritrichomonas foetus*-like protozoan isolated from preputial smegma of virgin bulls. Bovine Pract 1999;33:124–7.
[67] Schonmann MJ, BonDurant RH, Gardner IA, Van Hoosear K, Baltzer W, Kachulis C. Comparison of sampling and culture methods for the diagnosis of *Tritrichomonas foetus* infection in bulls. Vet Rec 1994;134:620–2.
[68] Bryan LA, Campbell JR, Gajadhar AA. Effects of temperature on the survival of *Tritrichomonas foetus* in transport, Diamond's and InPouch TF media. Vet Rec 1999;144:227–32.
[69] Lun Z, Parker S, Gajadhar AA. Comparison of growth rates of *Tritrichomonas foetus* isolates from various geographic regions using three different culture media. Vet Parasitol 2000;89:199–208.
[70] Parker S, Campbell J, Ribble C, Gajadhar A. Sample collection factors affect the sensitivity of the diagnostic test for *Tritrichomonas foetus* in bulls. Can J Vet Res 2003;67:138–41.
[71] Hoevers J, Snowden K, Graham S, Barling K. A comparison of PCR and in vitro culture for detection of *Tritrichomonas foetus* in bovine preputial scrapings. J Eukaryot Microbiol 2003;50(Suppl):699–700.
[72] Kimsey PB, Darien BJ, Kendrick JW, Franti CE. Bovine trichomoniasis: diagnosis and treatment. J Am Vet Med Assoc 1980;177:616–9.
[73] Mukhufhi N, Irons PC, Michel A, Peta F. Evaluation of a PCR test for the diagnosis of *Tritrichomonas foetus* infection in bulls: effects of sample collection method, storage and transport medium on the test. Theriogenology 2003;60:1269–78.
[74] Campero CM, Rodriguez Dubra C, Bolondi A, Cacciato C, Cobo E, Perez S, et al. Two-step (culture and PCR) diagnostic approach for differentiation of non-*Tritrichomonas foetus* trichomonads from genitalia of virgin beef bulls in Argentina. Vet Parasitol 2003;112:167–75.
[75] Parker S, Campbell J, McIntosh K, Gajadhar A. Diagnosis of trichomoniasis in 'virgin' bulls by culture and polymerase chain reaction. Can Vet J 2003;44:732–4.
[76] Cobo ER, Campero CM, Mariante RM, Benchimol M. Ultrastructural study of a tetratrichomonad species isolated from prepucial smegma of virgin bulls. Vet Parasitol 2003;117:195–211.
[77] CSS. Minimum requierements for disease control of semen produced for artificial insemination. Columbia, MO, USA: Certified Semen Services, National Association Animal Breeders (NAAB); 2003.
[78] Hayes DC, Anderson RR, Walker RL. Identification of trichomonadid protozoa from the bovine preputial cavity by polymerase chain reaction and restriction fragment length polymorphism typing. J Vet Diagn Invest 2003;15:390–4.
[79] Walker RL, Hayes DC, Sawyer SJ, Nordhausen RW, Van Hoosear KA, BonDurant RH. Comparison of the 5.8S rRNA gene and internal transcribed spacer regions of trichomonadid protozoa recovered from the bovine preputial cavity. J Vet Diagn Invest 2003;15:14–20.
[80] Cobo ER, Canton G, Morrell E, Cano D, Campero CM. Failure to establish infection with *Tetratrichomonas* sp. in the reproductive tracts of heifers and bulls. Vet Parasitol 2004;120:145–50.
[81] Honigberg BM. Trichomonads of veterinary importance. Parasitic Protozoa. New York: Academic Press; 1978.
[82] Castella J, Munoz E, Ferrer D, Gutierrez J. Isolation of the trichomonads *Tetratrichomonas buttreyi* (Hibler et al. 1960) Honigberg, 1960 in bovine diarrheic feces. Vet Parasitol 1997;70:41–5.
[83] Bagley C, Paskett E. Diagnostic complications from "fecal" trichomonads. Bovine Pract 2003;37:4.
[84] Chaban B, Chu S, Hendrick S, Waldner C, Hill JE. Evaluation of a *Campylobacter fetus* subspecies *venerealis* real-time quantitative polymerase chain reaction for direct analysis of bovine preputial samples. Can J Vet Res 2012;76:166–73.
[85] Chaban B, Guerra AG, Hendrick SH, Waldner CL, Hill JE. Isolation rates of *Campylobacter fetus* subsp *venerealis* from bovine preputial samples via passive filtration on nonselective medium versus selective medium, with and without transport medium. Am J Vet Res 2013;74:1066–9.
[86] Iraola G, Hernandez M, Calleros L, Paolicchi F, Silveyra S, Velilla A, et al. Application of a multiplex PCR assay for *Campylobacter fetus*

detection and subspecies differentiation in uncultured samples of aborted bovine fetuses. J Vet Sci 2012;13:371–6.
[87] Monke HJ, Love BC, Wittum TE, Monke DR, Byrum BA. Effect of transport enrichment medium, transport time, and growth medium on the detection of *Campylobacter fetus* subsp. *venerealis*. J Vet Diagn Invest 2002;14:35–9.
[88] van der Graaf-van Bloois L, van Bergen MA, van der Wal FJ, de Boer AG, Duim B, Schmidt T, et al. Evaluation of molecular assays for identification *Campylobacter fetus* species and subspecies and development of a *Campylobacter fetus* specific real-time PCR assay. J Microbiol Methods 2013;95:93–7.
[89] Gorkiewicz G, Kienesberger S, Schober C, Scheicher SR, Gully C, Zechner R, et al. A genomic island defines subspecies-specific virulence features of the host-adapted pathogen *Campylobacter fetus* subsp. *venerealis*. J Bacteriol 2010;192:502–17.
[90] Moolhuijzen PM, Lew-Tabor AE, Wlodek BM, Aguero FG, Comerci DJ, Ugalde RA, et al. Genomic analysis of *Campylobacter fetus* subspecies: identification of candidate virulence determinants and diagnostic assay targets. BMC Microbiol 2009;9:86.
[91] Abril C, Vilei EM, Brodard I, Burnens A, Frey J, Miserez R. Discovery of insertion element ISCfe1: a new tool for *Campylobacter fetus* subspecies differentiation. Clin Microbiol Infect 2007;13:993–1000.
[92] Kienesberger S, Sprenger H, Wolfgruber S, Halwachs B, Thallinger GG, Perez-Perez GI, et al. Comparative genome analysis of *Campylobacter fetus* subspecies revealed horizontally acquired genetic elements important for virulence and niche specificity. PLoS One 2014;9:e85491.
[93] McGoldrick A, Chanter J, Gale S, Parr J, Toszeghy M, Line K. Real time PCR to detect and differentiate Campylobacter fetus subspecies fetus and *Campylobacter fetus* subspecies *venerealis*. J Microbiol Methods 2013;94:199–204.
[94] Hum S, Quinn K, Brunner J, On SL. Evaluation of a PCR assay for identification and differentiation of *Campylobacter fetus* subspecies. Aust Vet J 1997;75:827–31.
[95] Stynen AP, Lage AP, Moore RJ, Rezende AM, de Resende VD, Ruy Pde C, et al. Complete genome sequence of type strain *Campylobacter fetus* subsp. *venerealis* NCTC 10354T. J Bacteriol 2011;193:5871–2.
[96] World Organisation for Animal Health (OIE). Bovine genital campylobacteriosis. Paris France: World Organisation for Animal Health (OIE); 2010.
[97] Philpott M. The dangers of disease transmission by artificial insemination and embryo transfer. Br Vet J 1993;149:339–69.
[98] Clark BL, Dufty JH, Parsonson IM. Immunisation of bulls against trichomoniasis. Aust Vet J 1983;60:178–9.
[99] Clark BL, Emery DL, Dufty JH. Therapeutic immunisation of bulls with the membranes and glycoproteins of *Tritrichomonas foetus* var. *brisbane*. Aust Vet J 1984;61:65–6.
[100] Cobo ER, Corbeil LB, Gershwin LJ, BonDurant RH. Preputial cellular and antibody responses of bulls vaccinated and/or challenged with *Tritrichomonas foetus*. Vaccine 2009;28:361–70.
[101] Kvasnicka WG, Hanks D, Huang JC, Hall MR, Sandblom D, Chu HJ, et al. Clinical evaluation of the efficacy of inoculating cattle with a vaccine containing *Tritrichomonas foetus*. Am J Vet Res 1992;53:2023–7.
[102] Hudson D, Ball L, Cheney J, Mortimer R, Bowen B, Marsh D, et al. Testing of trichomoniasis vaccine in heifers mated to infected bulls. Theriogenology 1993;39:937–43.
[103] Gault RA, Kvasnicka WG, Hanks D, Hanks M, Hall MR. Specific antibodies in serum and vaginal mucus of heifers inoculated with a vaccine containing *Tritrichomonas foetus*. Am J Vet Res 1995;56:454–9.
[104] Campero C, Medina D, Rossetti O, Marcovecchio F, Cosentino B, Marcone J, et al. Vacunación subcutánea e intravaginal contra tricomoniasis en vaquillonas. Rev Med Vet 1998;79:347–53.
[105] Schnackel J, Wallace B, Kvasnicka W, Hanks D, Hall M. *Tritrichomonas foetus* vaccine immunogenicity trial. Agri Practice 1989;10:11–4.
[106] Cobo ER, Cano D, Rossetti O, Campero CM. Heifers immunized with whole-cell and membrane vaccines against *Tritrichomonas foetus* and naturally challenged with an infected bull. Vet Parasitol 2002;109:169–84.
[107] Cobo ER, Morsella C, Cano D, Cipolla A, Campero CM. Immunization in heifers with dual vaccines containing *Tritrichomonas foetus* and *Campylobacter fetus* antigens using systemic and mucosal routes. Theriogenology 2004;62:1367–82.
[108] Voyich JM, Ansotegui R, Swenson C, Bailey J, Burgess DE. Antibody responses of cattle immunized with the Tf190 adhesin of *Tritrichomonas foetus*. Clin Diagn Lab Immunol 2001;8:1120–5.
[109] Baltzell P, Newton H, O'Connor AM. A critical review and meta-analysis of the efficacy of whole-cell killed *Tritrichomonas foetus* vaccines in beef cattle. J Vet Intern Med 2013;27:760–70.
[110] Bouters R, De Keyser J, Vandeplassche M, Van Aert A, Brone E, Bonte P. *Vibrio fetus* infection in bulls: curative and preventive vaccination. Br Vet J 1973;129:52–7.
[111] Clark BL, Dufty JH, Monsbourgh MJ, Parsonson IM. Immunisation against bovine vibriosis. Vaccination of bulls against infection with *Campylobacter fetus* subsp. *venerealis*. Aust Vet J 1974;50:407–9.
[112] Clark BL, Dufty JH, Monsbourgh MJ, Parsonson IM. A dual vaccine for immunisation of bulls against vibriosis. Aust Vet J 1979;55:43.
[113] Clark BL, Dufty JH, Monsbourgh MJ, Parsonson IM. A dual vaccine for the immunisation of cattle against vibriosis. Aust Vet J 1977;53:465–6.
[114] Border MM, Firehammer BD. Antigens of *Campylobacter fetus* subsp *fetus* eliciting vaccinal immunity in heifers. Am J Vet Res 1980;41:746–50.
[115] Schurig GG, Hall CE, Corbell LB, Duncan JR, Winter AJ. Bovine veneral vibriosis: cure of genital infection in females by systemic immunization. Infect Immun 1975;11:245–51.
[116] Corbeil LB, Schurig GG, Duncan JR, Wilkie BN, Winter AJ. Immunity in the female bovine reproductive tract based on the response to "*Campylobacter fetus*". Adv Exp Med Biol 1981;137:729–43.
[117] Cobo ER, Cipolla A, Morsella C, Cano D, Campero C. Effect of two commercial vaccines to *Campylobacter fetus* subspecies on heifers naturally challenged. J Vet Med B 2003;50:75–80.
[118] Hum S, Brunner J, Gardiner B. Failure of therapeutic vaccination of a bull infected with *Campylobacter fetus*. Aust Vet J 1993;70:386–7.
[119] Corbeil LB, Schurig GG, Bier PJ, Winter AJ. Bovine venereal vibriosis: antigenic variation of the bacterium during infection. Infect Immun 1975;11:240–4.
[120] Corbeil LB, Corbeil RR, Winter AJ. Bovine venereal vibriosis: activity of inflammatory cells in protective immunity. Am J Vet Res 1975;36:403–6.
[121] McCoy EC, Doyle D, Burda K, Corbeil LB, Winter AJ. Superficial antigens of *Campylobacter (Vibrio) fetus*: characterization of antiphagocytic component. Infect Immun 1975;11:517–25.
[122] van der Graaf-van Bloois L, Miller WG, Yee E, Bono JL, Rijnsburger M, Campero C, et al. First closed genome sequence of *Campylobacter fetus* subsp. *venerealis* bv. *intermedius*. Genome Announc 2014;2:1–2.
[123] Corbeil LB, Hodgson JL, Jones DW, Corbeil RR, Widders PR, Stephens LR. Adherence of *Tritrichomonas foetus* to bovine vaginal epithelial cells. Infect Immun 1989;57:2158–65.
[124] Aydintug MK, Widders PR, Leid RW. Bovine polymorphonuclear leukocyte killing of *Tritrichomonas foetus*. Infect Immun 1993;61:2995–3002.
[125] Hodgson JL, Jones DW, Widders PR, Corbeil LB. Characterization of *Tritrichomonas foetus* antigens by use of monoclonal antibodies. Infect Immun 1990;58:3078–83.
[126] Aydintug MK, Leid RW, Widders PR. Antibody enhances killing of *Tritrichomonas foetus* by the alternative bovine complement pathway. Infect Immun 1990;58:944–8.
[127] McGuire TC, Musoke AJ, Kurtti T. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils and skin. Immunology 1979;38:249–56.
[128] Bastida-Corcuera FD, Butler JE, Yahiro S, Corbeil LB. Differential complement activation by bovine IgG2 allotypes. Vet Immunol Immunopathol 1999;71:115–23.
[129] Singh BN, Lucas JJ, Beach DH, Shin ST, Gilbert RO. Adhesion of *Tritrichomonas foetus* to bovine vaginal epithelial cells. Infect Immun 1999;67:3847–54.
[130] Singh BN, BonDurant RH, Campero CM, Corbeil LB. Immunological and biochemical analysis of glycosylated surface antigens and lipophosphoglycan of *Tritrichomonas foetus*. J Parasitol 2001;87:770–7.
[131] Ikeda JS, BonDurant RH, Campero CM, Corbeil LB. Conservation of a protective surface antigen of *Tritrichomonas foetus*. J Clin Microbiol 1993;31:3289–95.
[132] Shaia CI, Voyich J, Gillis SJ, Singh BN, Burgess DE. Purification and expression of the Tf190 adhesin in *Tritrichomonas foetus*. Infect Immun 1998;66:1100–5.