Review

Diagnosis of *Tritrichomonas foetus*-infected bulls, an ultimate approach to eradicate bovine trichomoniasis in US cattle?

Chaoqun Yao

Correspondence
Chaoqun Yao
cyao@uwyo.edu

Department of Veterinary Sciences, Wyoming State Veterinary Laboratory, University of Wyoming, Laramie, WY 82070, USA

Bovine trichomoniasis is a sexually transmitted protozoan disease with a worldwide distribution. It has been endemic in the USA for more than 80 years. Mississippi and all the states west of the Mississippi River, except Iowa and Minnesota, have rules/regulations to reduce the spread of the disease. The core of these regulations consists of testing bulls and prohibiting importation of non-*Tritrichomonas foetus*-free bulls. Factors such as sampling methods and intervals, shipping medium and temperature, and testing techniques are reviewed for their effect on diagnostic accuracy. Finally, a comprehensive approach for controlling and eventually eradicating the disease is presented.

Introduction

Several species of protozoa have been found in parts of the bovine reproductive tract, such as the preputial cavity of bulls. These include *Tritrichomonas foetus*, the causative agent of bovine trichomoniasis, which may be zoonotic and capable of causing opportunistic infections in human beings (see a mini-review by Yao, 2012), and contaminants of the rumen and the intestine such as *Monocercomonas ruminantium*, *Callimastix frontalis* and *Monas obliqua*, as well as free-living organisms in stagnant water such as *Bodo*, *Spiromonas angusta* and *Polytoma uvella* (Taylor *et al.*, 1994). In addition, *Tetratrichomonas* spp., *Pentatrichomonas hominis* (Dufernez *et al.*, 2007; Grahn *et al.*, 2005) and *Pseudotrichomonas* (Dufernez *et al.*, 2007) have been isolated.

T. foetus is sexually transmitted among cattle from bulls to females and vice versa at coitus. A single mating service with an infected bull resulted in 95 % infections among susceptible nulliparous cows (Parsonson *et al.*, 1976). In bulls, a preputial discharge associated with small nodules on the preputial and penile membranes may occur shortly after infection. Nevertheless, chronically infected bulls usually develop no gross lesions and are often clinically asymptomatic, although they carry a small number of the organisms in the preputium with some concentration in the fornix and around the glans penis. They remain asymptomatic carriers of infection for years and, possibly, for life. In contrast, infected heifers and cows exhibit vaginitis, endometritis, early abortion and transient or permanent infertility (Felleisen, 1999). They usually clear the infection within a few months concomitantly with a short-lived partial immunity that may result in delayed conception and pregnancy of some heifers and cows, and

hence a longer breeding season in affected herds. Some females maintain infection through a normal, full-term pregnancy, and for up to 9 weeks into the post-partum period (Skirrow, 1987). Still others remain infected for up to 22 months after initial infection (Alexander, 1953). The latter may play a role in the maintenance of trichomoniasis in a herd by being a source of infection for bulls (Skirrow, 1987), thereby counteracting the culling of infected bulls.

Bovine trichomoniasis is widespread around the world, especially in Asia, Australia, South America and South Africa, where natural service by bulls is used as a major means of breeding. For example, four of 80 aborted fetuses (5 %) in 12 dairy herds in Beijing, China, between 2008 and 2010 were positive, although all four were coinfected with other pathogens such as infectious bovine rhinotracheitis virus (2), bovine viral diarrhoea virus (1), *Brucella abortius* (1) or *Neospora caninum* (1) (Yang *et al.*, 2012). Twenty-seven of 41 herds (65.9 %) were positive, and prevalence ranged from 2.9 to 33.3 % with an average of 11.7 % in bulls in the Victoria River district of Australia between 1985 and 1986 (McCool *et al.*, 1988). Five of 140 cows (3.5 %) were positive in the province of Formosa, Argentina (Mancebo *et al.*, 1995). The incidence of infected bulls in South Africa varied from 0.9 % in the southern Orange Free State to 10.4 % in the north-western Cape province, with an average of 7.1 % (Erasmus *et al.*, 1989). In contrast, the disease has been dramatically decreased or even eradicated from some regions, such as many European countries, where artificial insemination is widely practised. Only two cases have been reported in the UK over two decades (Taylor *et al.*, 1994). A hotspot of *T. foetus* infection in Europe is northern Spain, where natural breeding is still a common practice. In Principado de Asturias and Leon province, 32 % (33/103) and 2.9 % (2/70) of bulls were positive, respectively

(Mendoza-Ibarra *et al.*, 2012). Artificial insemination itself does not stop disease spread. The protozoan survives freezing in the liquid nitrogen required for conservation of semen if it is able to contaminate the semen. Nevertheless, such a scenario rarely happens in developed countries due to extremely strict screening of semen donors in the industry. In the USA, especially in the midwest and west, the disease is endemic. As a result, Mississippi and all states west of the Mississippi River, except Minnesota and Iowa, have already enforced state rules/regulations to curtail the disease (Yao *et al.*, 2011).

T. foetus has recently been comprehensively reviewed, covering aspects from a historical overview to prevention and control (Rae & Crews, 2006). The focuses of the current review are diagnostic tests and related parameters, which are the bases of the US regulations just mentioned above. A better understanding of these may guide us in a more effective strategy in the war against the disease than the current regulations. A core component of these regulations is testing bulls for *T. foetus*, and positive bulls are culled due to lack of effective treatment regimens. Official tests vary from state to state, but generally include culture, PCR detected by gel electrophoresis (gel-PCR) and quantitative real-time PCR (qPCR).

Diagnosing *T. foetus*-infected bulls is complicated, and its specificity and sensitivity may be compromised by many factors between sample collection and testing. These factors include, but are not limited to, how and when samples are collected, what medium is used in sample shipment, how shipment is delayed and what adverse environments such as extreme temperatures are encountered in shipping, how samples are tested, what medium is used in culture, and how DNA is extracted for PCR. Last but not least, contamination by non-pathogenic trichomonads may yield false-positive results in culture, leading to unnecessary culling of bulls. These and other factors such as the age of bulls are critically reviewed here in order to improve diagnostic accuracy. Finally, a comprehensive approach to the control and eventual eradication of the disease is discussed.

Infection in herds

Age of bulls

By inoculating *T. foetus* directly into the posterior of the preputium, Clark and colleagues found that only three of 19 bulls at 1–2 years old were infected, in contrast to 12 of 13 bulls at 3–7 years old (Clark *et al.*, 1974a). The same authors further found that all bulls older than 4 years were infected after three to six natural services, whereas only one of the two 3-year-old bulls was infected after nine services in a field study. They concluded that 3-year-old bulls were not as susceptible as older bulls in natural service (Clark *et al.*, 1974b). The age-specific infection rates in a beef herd were 21.7, 34.1 and 43.4 % for the bulls of 3, 4 and over 4 years old, respectively (Skirrow *et al.*, 1985). The infection rate for trichomoniasis tended to increase with age, with a 30 % infection rate in animals of 10 years or older ($r=0.47$,

$P<0.05$), whereas no age variation was observed for the infection rate of campylobacteriosis (McCool *et al.*, 1988). In a survey of bulls in coastal and western Queensland and the Northern Territory, infection rates in young (9 months to 3 years), mature (3.5 to 7 years) and old bulls (>7 years) were 0, 25 and 37.2 %, respectively (Ladds *et al.*, 1973). The bulls younger than 3 years old had a prevalence rate of 16 % (4/25), whereas in bulls older than 3 years, prevalence was 40 % (27/68) among 103 bulls in 65 herds at Principado de Asturias in northern Spain (Mendoza-Ibarra *et al.*, 2012). In a survey of a large cow–calf operation near Refugio, TX, USA, the odds of bulls 5 years or older being infected were nine times those of younger bulls (Hoevers *et al.*, 2003). The mean age of infected bulls was significantly higher (5.5 ± 1.6 years) than that of uninfected ones (3.9 ± 2.3 years) ($P<0.001$) in a ranch in central Florida with 1383 bulls (Rae *et al.*, 1999). In summary, data from both experimental and natural infections all indicate that young bulls of 3 years old or younger are less susceptible and have a much lower infection rate than bulls older than 3 years.

Conception rates in *T. foetus*-positive herds

Mylrea and colleagues found that the first alone and the first three mating services yielded 37 and 64 % conception rates in positive herds, compared with 76 and 96 %, respectively, in unaffected herds. Furthermore, the average number of services required for each conception was 3.1 and 1.4, respectively (Mylrea, 1962). In a longitudinal study of 4 years, the calf production rate from cows mated with *T. foetus*-infected bulls was 80.4, 71.4, 92.4 and 86 %, respectively, of those mated with a non-infected bull. In addition, there were 40.2, 65.5 and 21.2 days delay in conception for the first 3 years for the former compared with the latter (Clark *et al.*, 1983). In an investigation of a ranch in the Sandhills of western Nebraska with 3000 cows and 121 bulls subdivided into five management groups, infection rates in bulls ranged from 0 to 40 %, and the proportion of nonpregnant cows was 8.3 to 19.2 %. A linear regression ($y=0.264x+8.992$; $r^2=0.97$) existed between the proportion of nonpregnant cows and infection rates in bulls (Ondrak *et al.*, 2010). However, no such association was found by other authors ($r^2=0.093$, $P=0.39$) (Rae *et al.*, 1999). In conclusion, although exceptions exist, there is a positive association between bull infection rates and the proportion of nonpregnant cows and heifers in a herd, i.e. the higher the rate of bull infection, the higher the prevalence of nonpregnant female cattle.

Sampling

Sampling methods

For bulls, samples are usually collected from the preputial cavity. Although many minor modifications have been described in the literature, methods of sample collection can generally be classified into (a) brushing, (b) scraping, (c) washing and (d) swabbing. Swabbing is currently rarely used so it is not further discussed. A brush may be made of

metal or plastic. A metal brush consists of a handle and a solid metal cylinder. The latter is 8 mm in diameter with parallel grooves perpendicular to its long axis. During sample collection a brush is inserted into the prepuce and scraped back and forth 15–20 times. Scrapings accumulated in grooves are washed off and collected (Parker *et al.*, 1999; Tedesco *et al.*, 1979). A plastic version of this cylinder brusher is also available (Mendoza-Ibarra *et al.*, 2012). The most common device for scraping is an artificial insemination pipette connected to a hypodermic syringe. The volume of the syringe varies from 12 to 60 ml. A silicone rubber tube may be used as an adaptor between the pipette and syringe. During sample collection the pipette tip is inserted into the preputial cavity and scraped back and forth 15–20 times while simultaneously applying suction (Appell *et al.*, 1993; Irons *et al.*, 2002; Mendoza-Ibarra *et al.*, 2012). During sample collection by washing, a solution, usually PBS, is instilled into the preputial cavity followed by massaging the preputium vigorously 10–100 times. Afterwards, the fluid is collected (Irons *et al.*, 2002; Schönmann *et al.*, 1994).

Sampling efficiency

Sampling methods have been compared with one other by multiple laboratories (Table 1). In a comparison of washing with scraping, five infected bulls were sampled 29 times with a resting period of 2–4 days between sampling. On each sampling day, washing samples were collected prior to scraping. No superiority in sensitivity of washing to scraping was found when gel-PCR was performed (Mukhufhi *et al.*, 2003). Samples were alternatively collected by washing and scraping from 14 naturally infected bulls and detected by culture. The sensitivity of scraping was not significantly different from that of washing (Schönmann *et al.*, 1994). A similar conclusion was reached from five positive bulls on two large commercial farms (Irons *et al.*, 2002). Samples collected by scraping and brushing showed no difference in sensitivity by culture (Mendoza-Ibarra *et al.*, 2012). Collectively, these data demonstrated that all three methods,

i.e. brushing, scraping and washing, were comparable with each other, with none being significantly superior to the others. Nevertheless, scraping is most frequently used by US veterinarians due to the ready availability of pipettes and syringes, and ease of performance.

An unexpected yet interesting observation is that samples collected from the right side of the preputial cavity of bulls by a right-handed veterinarian by scraping are more likely to be culture-positive by InPouch kit (odds ratio=4.1, $P<0.05$) than the ones collected from the left side (Parker *et al.*, 2003b). This example shows that apparently simple procedures involved in sampling collection can significantly influence test outcome.

Number and interval of samplings

Frequent and intensive sampling at an interval of 2–4 days resulted in a decreased sensitivity for the samples collected at a later stage (Mukhufhi *et al.*, 2003), which appears to emulate the effect of breeding activity on the number of organisms in the preputial cavity.

In the diagnosis of a naturally infected beef herd using smegma scrapings collected weekly for three consecutive weeks and detection by culture, a collective sensitivity of 81.6 % was observed. Specifically, 57.6 % (38/66), 21.2 % (14/66) and 21.2 % (14/66) were culture-positive on all three cultures, two cultures and one culture, respectively (Skirrow *et al.*, 1985). In another study, two bulls were infected and preputial washes were collected weekly between 2 and 6 weeks after infection. Eight of 10 samples (80 %) were positive by culture (Gregory *et al.*, 1990). Samples collected at an interval of 2, 4 or 7 days from 14 naturally infected bulls had a positive culture rate of 84.3, 88.0 and 88.0 % by InPouch kit, or 65, 70.0 and 73.5 % by Claussen's medium, respectively (Schönmann *et al.*, 1994). The sensitivity for four consecutive tests was: one test, 73 %, two tests, 90 %, three tests, 96 %, and four tests, 99 % (Rae *et al.*, 1999). In a field diagnosis of a herd in the San Joaquin Valley of California using scraping

Table 1. Comparison of sampling methods

Abbreviations: Ntot, total number tested; Npos, number positive; %pos, percentage positive.

Test	Brushing			Scraping			Washing			Reference
	Ntot	Npos	%pos	Ntot	Npos	%pos	Ntot	Npos	%pos	
Culture	179	167	93.3	179	164	91.6				Parker *et al.* (1999)
	50	50	100.0	48	47	97.9				Tedesco *et al.* (1979)
				83	65	78.3	84	69	82.1	Schönmann *et al.* (1994)
Direct microscopy	50	45	90.0	48	29	60.4				Tedesco *et al.* (1979)
Gel-PCR				29	24	82.8	29	24	82.8	Mukhufhi *et al.* (2003)

and culture, Kimsey and colleagues concluded that three times at weekly intervals was adequate to identify all infected bulls (Kimsey *et al.*, 1980). Collectively, three consecutive weekly tests performed after a 1-week sexual rest of bulls are warranted to yield an acceptable sensitivity of 95 % or higher. This is true no matter whether culture, gel-PCR or qPCR is used to test samples. A major reason for multiple sampling is that the protozoan is not persistently present in the preputium for reasons yet to be determined.

Sample shipping: influence of medium and temperature

Samples collected by licensed veterinarians at individual ranches are usually submitted to accredited diagnostic laboratories for testing via various carriers, since few veterinarians have in-house facilities and the capacity to perform testing. Sample shipping and delivery usually take a minimum of 1–2 days even under the most favourable circumstances. It is not unusual for samples not to be delivered for 1 week or even longer. During this period of time, two obvious factors may affect testing results. These are the medium in which the samples are stored and the time elapsed between sample collection and testing. At present, the majority of samples in the USA are inoculated into an InPouch kit straight after collection, with the remainder inoculated into Diamond's medium. Since the major components of both the InPouch kit and Diamond's medium are identical, the effect of transfer medium on test outcome should be marginal and hence will not be further discussed. However, it should be pointed out that PBS is apparently not a good transport medium, since no cultivability was observed for samples held at 4 °C for 48 h or at 37 °C for 24 h (Reece *et al.*, 1983).

The time elapsed between sample collection and delivery to the diagnostic laboratory has a significant effect on test outcome, because samples are usually shipped under ambient temperatures that may vary from −20 to 40 °C depending upon geographical region and season. Experiments have been performed to mimic this situation by incubating the protozoa at various temperatures for different lengths of time in the laboratory under controlled conditions.

Exposure to 46.1 °C for 3 h or 54.4 °C for merely 1 h led to no cultivability, although qPCR was still positive for samples held for up to 24 h under the same conditions (Davidson *et al.*, 2011). A reduction of 14 % in cultivability was observed after samples were stored in LR (lactated Ringer's solution)–smegma suspension overnight at 5 °C (Skirrow *et al.*, 1985). Nevertheless, when cells were held in an InPouch kit for up to 72 h at 4, 20 or 37 °C they grew well at 37 °C as well as yielding positive results by qPCR. Once again, when they were held at 42 °C for 24 h, they did not grow, and were not positive by qPCR (Clavijo *et al.*, 2011). Samples held at room temperature for up to 72 h and at 4 °C for up to 7 days were still positive by gel-PCR (Mutto *et al.*, 2006). On the other hand, when samples were exposed to −20 °C either in Diamond's medium or in

an InPouch kit for more than 3 h, among eight samples none was culture-positive in Diamond's medium (Bryan *et al.*, 1999). After being held at 4 °C for 6 or 24 h post-collection, 78.6 and 80 % of washes and 85.7 and 80 % of scrapes tested positive by culture (Irons *et al.*, 2002). Samples collected by scraping and washing were 97.9, 83.3 and 70.8 % and 98, 96 and 90 % positive, respectively, by culture after being held at 4–7 °C for 24, 48 and 72 h (Tedesco *et al.*, 1979). When samples were tested by gel-PCR after being stored at 4 °C for 6 h, 30 h or 5 days, wash samples yielded 90, 69 and 63 %, and scraping samples yielded 83, 62 and 41 %, respectively (Mukhufhi *et al.*, 2003). In conclusion, testing methods should be carefully chosen after taking into consideration costs and delivery conditions. For example, PCR testing is superior to culturing if samples are collected in remote regions and a possible delay is anticipated, such as highway closure due to bad weather conditions.

Diagnostic tests

Many methods, ranging from direct microscopy to molecular techniques, have been devised to diagnose bovine trichomoniasis. At present, three methods, i.e. culture, gel-PCR and qPCR, are considered as the official tests by many US states and are commonly used in various diagnostic laboratories, and they are described below in detail.

Culture

Cultivation of live *T. foetus* is a 'gold standard' in the diagnosis of bovine trichomoniasis in many countries, including the USA. Samples are cultured at 37 °C for up to 120 h and checked daily by microscopy for live protozoa. The characteristic features of the protozoan include three anterior flagella, one free flagellum at the posterior end, and an undulating membrane (Honigberg, 1963) and a rolling motion of live organisms (Skirrow *et al.*, 1985). Dark field is helpful when live organisms are observed (Taylor *et al.*, 1994). An advantage of culture is its easiness. Disadvantages include: (1) it is time consuming, usually taking several days to get a final result; (2) it requires live cells for cultivation; (3) it is impossible to accurately differentiate contamination by related trichomonads, even for experienced personnel, which results in false-positive diagnoses and unnecessary culling; (4) it has low sensitivity.

Accurate calculation of the sensitivity and specificity of culture for the diagnosis of trichomoniasis in bulls is complicated by a lack of knowledge of the true infection status of these animals. Of 29 samples collected from five laboratory-infected bulls with a resting period of 2–4 days, 24 (83 %) were positive (Mukhufhi *et al.*, 2003). In a field sampling of 2832 mature bulls from 124 beef herds in Salado River basin, Buenos Aires, Argentina, the sensitivity and specificity by Bayesian estimation were 72.0 % (59–87 %) and 95.4 % (94–96 %), respectively (Perez *et al.*,

2006). Among 143 samples collected from 13 naturally infected bulls, 139 (97 %) were positive by culture for up to 4 days. Furthermore, three consecutive testings demonstrated infection in all bulls (Clark *et al.*, 1971).

Several culture media have been found suitable for *T. foetus* culture. Currently, the most widely used in the USA are Diamond's medium (Diamond, 1957) and InPouch TF (BioMed Diagnostics). A detailed comparison among different media is summarized in Table 2. An InPouch kit is apparently superior to Diamond's medium or Claussen's medium due to its higher sensitivity (Table 2). It is also widely accepted by veterinarians and diagnostic laboratories due to convenience and ease of use.

Gel-PCR

Primers TFR3 (5′→3′ sequence: CGGGTCTTCCTATA-TGAGACAGAACC) and TFR4 (5′→3′: CCTGCCGTTGG-ATCAGTTTCGTTAA) are often used in gel-PCR (Felleisen *et al.*, 1998; Parker *et al.*, 2001). The primer pairs specifically amplify a 347 bp DNA fragment of ITS1-5.8S rRNA-ITS2 from *Tritrichomonas* spp. including *T. foetus*, *Tritrichomonas suis* and *Tritrichomonas mobilensis* (Felleisen *et al.*, 1998; Parker *et al.*, 2001). The systematics of these three species, and of bovine and feline isolates of *T. foetus*, remain controversial and unresolved even after molecular investigations were comprehensively reviewed recently (Frey & Müller, 2012). *T. foetus* and *T. suis* were previously suggested to be the same organism (Lun *et al.*, 2005; Tachezy *et al.*, 2002). Primers TFR3 and TFR4 do not amplify genomic DNA of other trichomonads such as *Trichomonas vaginalis*, *Trichomonas tenax*, *Trichomonas gallinae*, *Tetratrichomonas gallinarum* and *Pentatrichomonas hominis* (Felleisen *et al.*, 1998), indicating a high degree of specificity of this primer set for *Tritrichomonas* spp. In addition to its specificity, gel-PCR is also very sensitive. It can detect as low as a single organism (Chen & Li, 2001; Felleisen *et al.*, 1998; McMillen & Lew, 2006) (Table 3). Of 29 samples collected from five infected bulls with a resting period of 2–4 days, 24 (83 %) were positive (Mukhufhi *et al.*, 2003). It is worth mentioning that DNA is required for all DNA amplification techniques, including gel-PCR and qPCR. DNA can be isolated using various methods and reagents, ranging from home-made ones to commercial kits. Raw samples may be directly used in the amplification reaction without prior DNA extraction

under certain circumstances. It appears that DNA extraction methods have a marginal effect on test sensitivity (Table 3).

qPCR

Primers and probe sequences are: TFF2 (5′→3′), GCGGCTGGATTAGCTTTCTTT; TFR2, GGCGCGCAAT-GTGCAT; and TRICHP2, 6-FAM-ACAAGTTCGATCTTT-G-MGB-BHQ. The primers enclose a 57 bp DNA fragment in the 5.8S rRNA gene (McMillen & Lew, 2006). The specificity of the test has been shown by the absence of amplification of genomic DNA from a range of related organisms, including *Trichomonas vaginalis*, *Pentatrichomonas hominis* and *Tetratrichomonas gallinarum* (McMillen & Lew, 2006). The test is very sensitive and can detect a single organism with DNA extraction, and the equivalent of one-tenth of a parasite without DNA extraction (McMillen & Lew, 2006) (Table 3).

Comparison among testing methods

During a 6-week period, 30 bulls inoculated with *T. foetus* were checked weekly by both culture and gel-PCR. Twenty-six and 27 bulls were positive at least once, corresponding to 86.7 and 90.0 %, respectively. Two bulls were negative by both culture and gel-PCR for the whole 6 weeks (Cobo *et al.*, 2007). A complete agreement was found between gel-PCR and culture of 10 positive samples out of 193 diagnostic samples submitted to the Onderstepoort Veterinary Institute in South Africa (Mukhufhi *et al.*, 2003). In a comparison of field samples from a Texas ranch by culture in an InPouch kit and gel-PCR, 13 of 14 culture-positive samples out of 155 bulls were gel-PCR-positive. However, the micro-organisms from a single bull did not survive passage (Hoevers *et al.*, 2003). Thirty-three bulls were gel-PCR-positive among 103 bulls in 65 herds, whereas only 29 were positive by culture (Mendoza-Ibarra *et al.*, 2012). During 2009 and 2010 we tested a total of 3090 bovine samples using both culture and gel-PCR. In total, nine samples were PCR-positive. Two PCR-positive samples were culture-negative (Yao *et al.*, 2011). Among 203 preputial wash samples, 67 and 59 were gel-PCR- and culture-positive, respectively, of which 58 were positive by both methods (Mutto *et al.*, 2006). Among 361 preputial samples collected from 121 bulls, the results of culture and gel-PCR were both positive for 58 and both negative for

Table 2. Comparison of different media on paired samples; values shown are sensitivity (%) (positive/total)

NA, Not available.

InPouch kit	Claussen's medium	Diamond's medium	*P* value	Reference
88.0 (73/83)	73.5 (61/83)		<0.005	Schönmann *et al.* (1994)
98.4 (62/63)		96.8 (61/63)	>0.8	Appell *et al.* (1993)
87.9 (29/33)		78.8 (26/33)	NA	Mendoza-Ibarra *et al.* (2012)
95.8 (161/168)		76.8 (129/168)	<0.001	Parker *et al.* (2003a)

Table 3. Analytical limit of PCR for detecting *T. foetus*

PCR	DNA extraction method	Detection limit (no. of organisms)	Reference
Gel-PCR	Modified protease K digestion procedure	1	Felleisen *et al.* (1998)
	Guanidiniumthiocyanate/silica	100	Mukhufhi *et al.* (2003)
	0.05 % Agar and 5 % resin	3	Parker *et al.* (2001)
	0.05 % Agar and 5 % resin	1	Chen & Li (2001)
	DNAzol kit	1	Chen & Li (2001)
	QIAamp	1	McMillen & Lew (2006)
	No DNA extraction	5	Mutto *et al.* (2006)
qPCR	QIAamp	29	Clavijo *et al.* (2011)
	QIAamp	1	McMillen & Lew (2006)
	No DNA extraction	0.1	McMillen & Lew (2006)

295 samples. Three culture-positive samples were gel-PCR-negative; and five gel-PCR-positive samples were culture-negative. Collectively, the extent of agreement between these two assays yielded a *k* value of 0.92. Seventeen qPCR-positive samples were negative by both culture and gel-PCR (Ondrak *et al.*, 2010). In conclusion, the PCR test not only is more sensitive than culture but also can be used to rule out false-positive cultures in bulls, especially virgin bulls (Campero *et al.*, 2003; Corbeil *et al.*, 2008; Parker *et al.*, 2003a).

Current policy

Bovine trichomoniasis has been endemic in the USA for at least 80 years. It was first reported in 1932 (Emmerson, 1932). Since then the disease has been found in more than a dozen states. State regulations have endeavoured to curtail endemic disease and to minimize economic losses brought about by the disease mainly due to reduced calf crop, culling of bulls and veterinary expenses. One central piece of these regulations is to test bulls and allow the importation only of *T. foetus*-free bulls for reproductive purposes. Positive bulls are culled. A key question remains: is this measure sufficient to control endemic disease?

To answer the question one has to bear in mind several important factors associated with bull testing discussed above. Sample collection is not always effective due to the relatively small numbers and intermittent presence of the protozoan in the preputium of bulls. In addition, the frequency and intervals of sampling affect testing efficiency, and no test is 100 % sensitive. An often overlooked and unpredictable factor is the delay in sample delivery to diagnostic laboratories from the time of collection. A further compounding factor is the existence of positive cows/heifers that are not included in the equation at present. We have monitored the annual prevalence of infected bulls in the state of Wyoming for over a decade since the enforcement of the Bovine Trichomoniasis Regulations in 2000. Although a gradual reduction was observed during the period (*r*=0.717, *P*<0.01), a surge of prevalence of 100 % from 0.62 % in 2008 to 1.29 % in 2009 occurred for no obvious reason (Yao *et al.*,

2011). All these factors undoubtedly decrease the effectiveness of bull testing as a major method of controlling bovine trichomoniasis, which makes the goal hard to reach, if not impossible.

By taking into consideration these factors, I have devised a comprehensive approach to controlling and eventually eradicating bovine trichomoniasis from US cattle, which is presented in Fig. 1. In addition to the regulatory component of state rules/regulations, management and animals are included to achieve the goal of controlling the disease. In the context of management, a closed herd and fencing are suggested, which is based on findings that commingling on public lands (Gay *et al.*, 1996) contributes significantly to bovine trichomoniasis in a herd. A closed herd further indicates no introduction of cattle of unknown *T. foetus* status into a herd. Maintaining a good fence probably will not only prevent comingling but also minimize losses due to runaway cattle, yet this effective method is often overlooked by ranchers.

Young bulls should always be used when possible. This is because bulls younger than 3 years old have been shown to be less susceptible and also less likely to become carriers, as demonstrated by data generated in both the field and the laboratory. It should be pointed out that the use of young bulls on infected cows probably will not have much effect on prevalence long-term, and will certainly increase costs. To minimize infection of bulls, open cows in positive herds should be regularly tested or be impregnated by artificial insemination. A more progressive approach is to cull open cows/heifers. This will minimize the bull's risk of infection, although it will come at a price that some ranchers may be not willing to pay. In the case of the 64 % conception rate mentioned above, ranchers would be culling one-third of their herd, an unacceptable rate.

Concluding remarks

Clearly the single measure of testing bulls and prohibiting importation of positive bulls will not be effective enough to control this endemic disease in the USA any time soon. A combination of measures will be needed, which should be
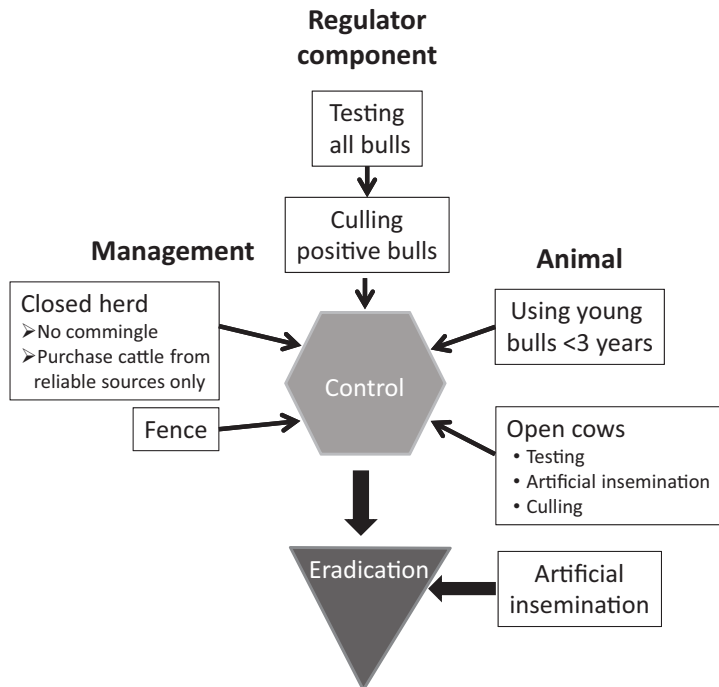
Fig. 1. A comprehensive approach to control and eradicate bovine trichomoniasis.

devised to specifically fit individual cases. In order to achieve eradication, artificial insemination will have to be widely accepted by US ranchers. This is how the disease has been eradicated from many European countries, including the UK. Widespread adoption of artificial insemination throughout the USA is essential to the ultimate eradication of bovine trichomoniasis in this country.

Acknowledgements

References

Alexander, G. I. (1953). An outbreak of bovine trichomoniasis in Queensland and its control. *Aust Vet J* **29**, 61–66.

Appell, L. H., Mickelson, W. D., Thomas, M. W. & Harmon, W. M. (1993). A comparison of techniques used for the diagnosis of *Tritrichomonas foetus* infections in beef bulls. *Agric Practice* **14**, 30–34.

Bryan, L. A., Campbell, J. R. & Gajadhar, A. A. (1999). Effects of temperature on the survival of *Tritrichomonas foetus* in transport, Diamond's and InPouch TF media. *Vet Rec* **144**, 227–232.

Campero, C. M., Rodriguez Dubra, C., Bolondi, A., Cacciato, C., Cobo, E., Perez, S., Odeon, A., Cipolla, A. & BonDurant, R. H. (2003). Two-step (culture and PCR) diagnostic approach for differentiation of non-*T. foetus* trichomonads from genitalia of virgin beef bulls in Argentina. *Vet Parasitol* **112**, 167–175.

Chen, X. G. & Li, J. (2001). Increasing the sensitivity of PCR detection in bovine preputial smegma spiked with *Tritrichomonas foetus* by the addition of agar and resin. *Parasitol Res* **87**, 556–558.

Clark, B. L., White, M. B. & Banfield, J. C. (1971). Diagnosis of *Trichomonas foetus* infection in bulls. *Aust Vet J* **47**, 181–183.

Clark, B. L., Parsonson, I. M. & Dufty, J. H. (1974a). Experimental infection of bulls with *Tritrichomonas foetus. Aust Vet J* **50**, 189–191.

Clark, B. L., Parsonson, I. M. & Dufty, J. H. (1974b). Infection of bulls with *Tritrichomonas foetus* through mating with infected heifers. *Aust Vet J* **50**, 180.

Clark, B. L., Dufty, J. H. & Parsonson, I. M. (1983). The effect of *Tritrichomonas foetus* infection on calving rates in beef cattle. *Aust Vet J* **60**, 71–74.

Clavijo, A., Erol, E., Sneed, L., Sun, F. & Swinford, A. (2011). The influence of temperature and simulated transport conditions of diagnostic samples on real-time polymerase chain reaction for the detection of *Tritrichomonas foetus* DNA. *J Vet Diagn Invest* **23**, 982–985.

Cobo, E. R., Favetto, P. H., Lane, V. M., Friend, A., VanHooser, K., Mitchell, J. & BonDurant, R. H. (2007). Sensitivity and specificity of culture and PCR of smegma samples of bulls experimentally infected with *Tritrichomonas foetus. Theriogenology* **68**, 853–860.

Corbeil, L. B., Campero, C. M., Van Hoosear, K. & Bondurant, R. H. (2008). Detection of trichomonad species in the reproductive tracts of breeding and virgin bulls. *Vet Parasitol* **154**, 226–232.

Davidson, J. M., Ondrak, J. D., Anderson, A. A., Swinford, A. K. & Erol, E. (2011). Evaluation of effects of high incubation temperatures on results of protozoal culture and real-time PCR testing for *Tritrichomonas foetus* inoculated in a commercially available self-contained culture media system. *J Am Vet Med Assoc* **239**, 1589–1593.

Diamond, L. S. (1957). The establishment of various trichomonads of animals and man in axenic cultures. *J Parasitol* **43**, 488–490.

Dufernez, F., Walker, R. L., Noël, C., Caby, S., Mantini, C., Delgado-Viscogliosi, P., Ohkuma, M., Kudo, T., Capron, M. & other authors (2007). Morphological and molecular identification of non-Tritrichomonas foetus trichomonad protozoa from the bovine preputial cavity. J Eukaryot Microbiol 54, 161–168.

Emmerson, M. A. (1932). Trichomoniasis in cattle a preliminary report. J Am Vet Med Assoc 81, 634–640.

Erasmus, J. A., De Wet, J. A. L., Van der Merwe, H. E. & Pienaar, G. C. J. (1989). Bovine trichomoniasis in the north western Cape Province, western Transvaal and the Orange Free State. J S Afr Vet Assoc 60, 51–52.

Felleisen, R. S. (1999). Host-parasite interaction in bovine infection with Tritrichomonas foetus. Microbes Infect 1, 807–816.

Felleisen, R. S., Lambelet, N., Bachmann, P., Nicolet, J., Müller, N. & Gottstein, B. (1998). Detection of Tritrichomonas foetus by PCR and DNA enzyme immunoassay based on rRNA gene unit sequences. J Clin Microbiol 36, 513–519.

Frey, C. F. & Müller, N. (2012). Tritrichomonas – systematics of an enigmatic genus. Mol Cell Probes 26, 132–136.

Gay, J. M., Ebel, E. D. & Kearley, W. P. (1996). Commingled grazing as a risk factor for trichomonosis in beef herds. J Am Vet Med Assoc 209, 643–646.

Grahn, R. A., BonDurant, R. H., van Hoosear, K. A., Walker, R. L. & Lyons, L. A. (2005). An improved molecular assay for Tritrichomonas foetus. Vet Parasitol 127, 33–41.

Gregory, M. W., Ellis, B. & Redwood, D. W. (1990). Comparison of sampling methods for the detection of Tritrichomonas foetus infection in bulls. Vet Rec 127, 16.

Hoevers, J., Snowden, K., Graham, S. & Barling, K. (2003). A comparison of PCR and in vitro culture for detection of Tritrichomonas foetus in bovine preputial scrapings. J Eukaryot Microbiol 50 (Suppl.), 699–700.

Honigberg, B. M. (1963). Evolutionary and systematic relationships in the flagellate order Trichomonadida Kirby. J Protozool 10, 20–63.

Irons, P. C., Henton, M. M. & Bertschinger, H. J. (2002). Collection of preputial material by scraping and aspiration for the diagnosis of Tritrichomonas foetus in bulls. J S Afr Vet Assoc 73, 66–69.

Kimsey, P. B., Darien, B. J., Kendrick, J. W. & Franti, C. E. (1980). Bovine trichomoniasis: diagnosis and treatment. J Am Vet Med Assoc 177, 616–619.

Ladds, P. W., Dennett, D. P. & Glazebrook, J. S. (1973). A survey of the genitalia of bulls in Northern Australia. Aust Vet J 49, 335–340.

Lun, Z. R., Chen, X. G., Zhu, X. Q., Li, X. R. & Xie, M. Q. (2005). Are Tritrichomonas foetus and Tritrichomonas suis synonyms? Trends Parasitol 21, 122–125.

Mancebo, O. A., Russo, A. M., Carabajal, L. L. & Monzon, C. M. (1995). Persistence of Tritrichomonas foetus in naturally infected cows and heifers in Argentina. Vet Parasitol 59, 7–11.

McCool, C. J., Townsend, M. P., Wolfe, S. G., Simpson, M. A., Olm, T. C., Jayawardhana, G. A. & Carney, J. V. (1988). Prevalence of bovine venereal disease in the Victoria River District of the Northern Territory: likely economic effects and practicable control measures. Aust Vet J 65, 153–156.

McMillen, L. & Lew, A. E. (2006). Improved detection of Tritrichomonas foetus in bovine diagnostic specimens using a novel probe-based real time PCR assay. Vet Parasitol 141, 204–215.

Mendoza-Ibarra, J. A., Pedraza-Diaz, S., García-Peña, F. J., Rojo-Montejo, S., Ruiz-Santa-Quiteria, J. A., San Miguel-Ibáñez, E., Navarro-Lozano, V., Ortega-Mora, L. M., Osoro, K. & Collantes-Fernandez, E. (2012). High prevalence of Tritrichomonas foetus infection in Asturiana de la Montaña beef cattle kept in extensive conditions in Northern Spain. Vet J 193, 146–151.

Mukhufhi, N., Irons, P. C., Michel, A. & Peta, F. (2003). 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 60, 1269–1278.

Mutto, A. A., Giambiaggi, S. & Angel, S. O. (2006). PCR detection of Tritrichomonas foetus in preputial bull fluid without prior DNA isolation. Vet Parasitol 136, 357–361.

Mylrea, B. J. (1962). The differential diagnosis of the causes of herd infertility in cattle. Aust Vet J 38, 15–20.

Ondrak, J. D., Keen, J. E., Rupp, G. P., Kennedy, J. A., McVey, D. S. & Baker, W. D. (2010). Repeated testing by use of culture and PCR assay to detect Tritrichomonas foetus carrier bulls in an infected Nebraska herd. J Am Vet Med Assoc 237, 1068–1073.

Parker, S., Campbell, J., Ribble, C. & Gajadhar, A. (1999). Comparison of two sampling tools for diagnosis of Tritrichomonas foetus in bulls and clinical interpretation of culture results. J Am Vet Med Assoc 215, 231–235.

Parker, S., Lun, Z. R. & Gajadhar, A. (2001). Application of a PCR assay to enhance the detection and identification of Tritrichomonas foetus in cultured preputial samples. J Vet Diagn Invest 13, 508–513.

Parker, S., Campbell, J. & Gajadhar, A. (2003a). 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 15, 460–465.

Parker, S., Campbell, J., Ribble, C. & Gajadhar, A. (2003b). Sample collection factors affect the sensitivity of the diagnostic test for Tritrichomonas foetus in bulls. Can J Vet Res 67, 138–141.

Parsonson, I. M., Clark, B. L. & Dufty, J. H. (1976). Early pathogenesis and pathology of Tritrichomonas foetus infection in virgin heifers. J Comp Pathol 86, 59–66.

Perez, A., Cobo, E., Martínez, A., Campero, C. & Späth, E. (2006). Bayesian estimation of Tritrichomonas foetus diagnostic test sensitivity and specificity in range beef bulls. Vet Parasitol 142, 159–162.

Rae, D. O. & Crews, J. E. (2006). Tritrichomonas foetus. Vet Clin North Am Food Anim Pract 22, 595–611.

Rae, D. O., Chenoweth, P. J., Genho, P. C., McIntosh, A. D., Crosby, C. E. & Moore, S. A. (1999). Prevalence of Tritrichomonas fetus in a bull population and effect on production in a large cow-calf enterprise. J Am Vet Med Assoc 214, 1051–1055.

Reece, R. L., Dennett, D. P. & Johnson, R. H. (1983). Some observations on cultural and transport conditions for Tritrichomonas foetus var. brisbane. Aust Vet J 60, 62–63.

Schönmann, M. J., BonDurant, R. H., Gardner, I. A., Van Hoosear, K., Baltzer, W. & Kachulis, C. (1994). Comparison of sampling and culture methods for the diagnosis of Tritrichomonas foetus infection in bulls. Vet Rec 134, 620–622.

Skirrow, S. (1987). Identification of trichomonad-carrier cows. J Am Vet Med Assoc 191, 553–554.

Skirrow, S., BonDurant, R., Farley, J. & Correa, J. (1985). Efficacy of ipronidazole against trichomoniasis in beef bulls. J Am Vet Med Assoc 187, 405–407.

Tachezy, J., Tachezy, R., Hampl, V., Sedinová, M., Vanacová, S., Vrlík, M., Van Ranst, M., Flegr, J. & Kuldaa, J. (2002). Cattle pathogen tritrichomonas foetus (Riedmüller, 1928) and pig commensal Tritrichomonas suis (Gruby & Delafond, 1843) belong to the same species. J Eukaryot Microbiol 49, 154–163.

Taylor, M. A., Marshall, R. N. & Stack, M. (1994). Morphological differentiation of Tritrichomonas foetus from other protozoa of the bovine reproductive tract. Br Vet J 150, 73–80.

Tedesco, L. F., Errico, F. & Del Baglivi, L. P. (1979). Diagnosis of *Tritrichomonas foetus* infection in bulls using two sampling methods and a transport medium. *Aust Vet J* **55**, 322–324.

Yang, N., Cui, X., Qian, W., Yu, S. & Liu, Q. (2012). Survey of nine abortifacient infectious agents in aborted bovine fetuses from dairy farms in Beijing, China, by PCR. *Acta Vet Hung* **60**, 83–92.

Yao, C. (2012). Opportunistic human infections caused by *Tritrichomonas* species: a mini-review. *Clin Microbiol Newsl* **34**, 127–131.

Yao, C., Bardsley, K. D., Litzman, E. A., Hall, M. L. & Davidson, M. R. (2011). *Tritrichomonas foetus* infection in beef bull populations in Wyoming. *J Bacteriol Parasitol* **2**, 117.