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Tritrichomonas foetus Infection in Beef Bull Populations in Wyoming

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Abstract

Tritrichomonas foetus causes bovine trichomoniasis in the reproductive tract of cattle and feline trichomoniasis in the large bowel of the domesticated cat. Bovine trichomoniasis is widespread in the USA especially in the Midwest and West and leads to significant economic losses. Although the disease has been endemic for over three decades in Wyoming, one of the largest beef cattle producing states in the USA little is known about its epidemiology and laboratory diagnosis. We statistically analyzed the data collected from the Wyoming State Veterinary Laboratory and the Wyoming Livestock Board. Individual prevalence in beef bull populations in Wyoming between 1997 and 2010 ranged from 0.21% to 2.69%. A steady decline in prevalence was linearly correlated with year since the enforcement of state laws on the disease began in 2000 (R=0.717, P=0.009). One exception was 2009 when a recurrence occurred. Between 2007 and 2010, average herd prevalence was 2.17%, with 15 of the 23 counties having at least one positive herd. In laboratory diagnosis advanced gel-PCR showed 99.9% agreement with traditional cell culture. This is the first epidemiological study on bovine trichomoniasis in Wyoming and demonstrates that *T. foetus* infection continues to be prevalent in beef cattle in the state where natural service is widely used.

Keywords: *Tritrichomonas foetus;* Bovine trichomoniasis; Wyoming; Protozoan; Cell culture; PCR

Abbreviations: PCR: Polymerase Chain Reaction; qPCR: quantitative PCR; WLSB: Wyoming Livestock Board; WSVL: Wyoming State Veterinary Laboratory

Introduction

Tritrichomonas foetus, the causative protozoan agent of worldwide-distributed trichomoniasis in cattle and the domesticated cat, only has the trophozoite stage in its life cycle. In cattle it is sexually transmitted from bulls to heifers and adult cows at coitus. In susceptible nulliparous cows, 95% become infected after merely a single service of mating with an infected bull [1]. Infected bulls are usually asymptomatic although some develop mild inflammation at the early stage of infection. Nevertheless, they serve as asymptomatic carriers harboring the organisms in the preputial cavity for years, possibly for life. In contrast, infected females upon infection in the vagina, uterus and oviduct exhibit transient or permanent infertility, abortion and pyometra, and often clear infections in one reproductive cycle [2]. However, some cows remain infected throughout apparently normal pregnancies, and for up to 9 weeks into the postpartum period [3]. These cows may play a role in maintaining the disease in herds by serving as reservoirs of uninfected and virgin bulls [3]. Currently no effective regimens are available for treating bovine trichomoniasis although vaccines are available for cows. These vaccines do not prevent cows from becoming infected albeit they elicit very modest protection from abortion [4-8]. At present, testing and culling positive bulls are the main measures of controlling bovine trichomoniasis when natural service is used. It has been shown that control of the disease in a large herd is achieved by using uninfected bulls for service [9]. Alternatively, artificial insemination, if widely adapted, is effective to control and eventually eliminate the disease, as shown in the Great Britain [10]. Curiously, the same organism has been found in domestic cats where it usually infects the large bowel and causes chronic diarrhea [11,12] although it is rarely found in the uterus [13]. Transmission and risk factors in cats are unclear although the fecal-oral route is likely [12,14-16]. There is no reason to believe and no data to support at present that infection of cats and cattle are epidemiologically linked.

Bovine trichomoniasis causes significant economic losses. For example, a 20% lower calf crop in a 100 cow herd may result in an estimate of up to $20,000 in annual losses (http://wlsb.state.wy.us/Animal%20Health /trich% 20brochure%202009.pdf). The disease is often well established in a herd before it is recognized. It is usually signaled by a lower calf crop and a high proportion of open (non-pregnant) cows. The disease has been found in many US states, especially the Midwest and West including Alabama [17], California [18], Colorado [19], Florida [20,21], Idaho [22], Missouri [23], Montana [24], New Mexico [25], Nebraska [26], Nevada [8], Oklahoma [27], as well as Kansas, South Dakota, Utah and Wyoming (Yao C, unpublished data). Due to its widespread distribution and potentially significant economic losses, many states have regulations on bovine trichomoniasis. With Oklahoma and Kansas starting their regulations this year, twenty-one states including all those west of the Mississippi River except Minnesota and Iowa now impose some kinds of regulations on the disease (Figure 1).

Wyoming is one of the largest beef-cattle producing states, per capita, in the US. Annually there were 1.3 to 1.4 million head of cattle in the state between 2006 and 2010 (Wyoming Agricultural Statistics 2010, http://www.nass.usda.gov/Statistics_by_State/Wyoming/Publications/Annual_Statistical_Bulletin/bulletin2010.pdf) with a mere 563,626 residents according to 2010 census (http://2010.census.gov/2010census/data/), which results in an average of 2.3 head of cattle per capita. Bovine trichomoniasis has been known to exist in Wyoming since the 1970s. However, the status of the disease in the state including prevalence and distribution is unknown. In the current study, we investigated prevalence and county distribution as well as sensitivity of tradi-

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Figure 1: US states with bovine trichomoniasis regulations are shown by microscope signs.

tional cell culture versus the more advanced polymerase chain reaction (PCR). From 2007 to 2010, statewide herd prevalence among submitted samples was 2.55%, 3.21%, 2.16% and 0.76%, respectively, with 15 of 23 counties exhibiting a minimum of one positive herd. Although it fluctuated from 0.21% to 2.69% between 1997 and 2010 the statewide prevalence of individual head had been decreasing gradually since the enforcement of state law in 2000. There was a very good agreement between PCR and cell culture in diagnosis of bovine trichomoniasis. This is the first epidemiological study of bovine trichomoniasis in Wyoming beef populations.

Materials and Methods

Data collection

All data were collected from the Wyoming State Veterinary Laboratory (WSVL) and the Wyoming Livestock Board (WLSB). WSVL started testing for *T. foetus* sparsely in the 1970s and has been providing routine testing since the 1990s. Wyoming initiated regulations on bovine trichomoniasis in 2000 and WLSB has always been the executive branch to enforce the regulations ever since. All trichomoniasis tests on Wyoming cattle, no matter whether they are performed in diagnostic laboratories or conducted in-house by the veterinarians are required to be reported to WLSB by law. Nevertheless, not all bulls are routinely tested. State laws mandate only bulls grazing on open/public allotments or being traded or leased for reproductive purposes be tested prior to breeding or change of ownership. Prevalence of individual cattle and county distribution were collectively derived from data generated from these bulls. A linear regression was performed to analyze the effect of state regulations on individual prevalence. In the analysis data from 1999 were included as a baseline prior to the enforcement of state regulations. Data collected from WSVL between January 1, 2007 and December 31, 2010 were analyzed for herd prevalence and bull numbers per herd. All data analyses were performed using Sigma Plot® 11 (Systat Software, Inc., Chicago, IL) or Microsoft Excel® 2010 (Microsoft Cooperation, Redmond, WA) software.

Trichomoniasis tests

Standard testing of bovine trichomoniasis consists of detecting *T. foetus* in the bull battery. Common tests in laboratories throughout the USA include traditional cell culture, conventional gel-PCR and quantitative PCR (qPCR). Samples of smegma or saline preputial scrapings are collected from bulls that have had a minimum of one week of sexual rest by certified veterinarians. By law, every veterinarian in Wyoming has to be certified every five years for sample collection and/or performance of bovine trichomoniasis testing. Veterinarians designated diagnostic methods, i.e., cell culture or gel-PCR when samples were submitted to WSVL by express carriers at the ambient temperature. Samples were cultured for 48-72 hours in Diamond's medium at 37°C as previously described [28] and checked for live *T. foetus* using dark field microscopy [29]. Characteristic features of *T. foetus* include three anterior and one posterior flagellum, an undulating membrane [29] and a rolling motion of live organisms [30]. In PCR primers TFR3 and TFR4 were used to amplify a 347 base-pair DNA fragment of the 5.8S ribosomal RNA and the internal transcribed spacer region of the genome. PCR significantly increases sensitivity and specificity compared to cell culture [31,32]. It is often used for confirmation of the microscopic positive samples since no amplification of this product is yielded in other trichomonads including fecal contaminants. This is due to the primers' uniqueness to *T. foetus* genomic DNA sequence [31]. However PCR is significantly more expensive than cell culture. Pooled PCR of up to five samples dramatically reduces cost and at the same time keeps sensitivity and specificity comparable to individual PCRs [15]. Furthermore, a combination of cell culture and PCR is widely used in many laboratories. Samples are cultured, and then examined by PCR [17,32,33]. At WSVL the samples designated for PCR per clients' request were routinely cultured in Diamond's medium for 24 hours at 37°C and were microscopically examined before genomic DNA extraction. DNAs were extracted from 1 ml culture by following a well-established protocol as described [34], which yielded 200 µl DNA extracts. PCR was performed for 40 cycles, each consisting of 94°C 30s, 67°C 30s and 72°C 90s in 25µl with 5µl DNA extracts and 2.5µM of each of TFR3 and TFR4 primers. One positive and one negative control were included in each PCR test for quality control. PCR products were visualized by electrophoresis on 1.5% agarose gels.

Results and Discussion

Prevalence of trichomoniasis in Wyoming beef bulls

Individual prevalence: For many years WSVL tested only hundreds of samples annually until 2000 when state laws regulated *T. foetus* as a notifiable disease. Numbers of bulls tested yearly increased steadily from 2000 to 2003, and reached a plateau of over 7,000 in 2004 (Table 1). Annual prevalence ranged from 0.21% to 2.69% in individual beef bulls in the state between 1997 and 2010 (Table 1 & Figure 2). Whether or not the data were truly reflective of the overall prevalence could not be clearly defined since required submission was indistinguishable from non-required samples. It was felt that they were somewhat biased since the majority of samples were most likely from the required bulls. Nevertheless, this opportunistic sampling over a 14-year period on prevalence not only provided useful information for the disease in Wyoming beef cattle but also represented the longest longitudinal study published so far in literature on bovine trichomoniasis throughout the USA.

The data showed a higher prevalence in the years prior to enforcement of the state laws on the disease, which could mainly be explained by biased samples. The likelihood of voluntarily submitted samples from ranchers who were affected was almost certainly greater than those who were not. A steady and progressive decline in the

Year	Bulls tested	Bulls positive	Prevalence (%)	Predicted prevalence F(x)=261.020047-0.129685x
1997	433	5	1.15	
1998	919	18	1.96	
1999	1525	41	2.69	1.78
2000	4604	76	1.65	1.65
2001	6025	78	1.29	1.52
2002	5767	73	1.27	1.39
2003	6855	43	0.63	1.26
2004	7515	44	0.59	1.13
2005	7450	79	1.06	1.00
2006	7270	57	0.78	0.87
2007	7080	50	0.71	0.74
2008	7275	45	0.62	0.61
2009	7597	98	1.29	0.48
2010	8222	17	0.21	0.35

Table 1: Trichomoniasis prevalence of individual bulls in Wyoming beef cattle between 1997 and 2010.

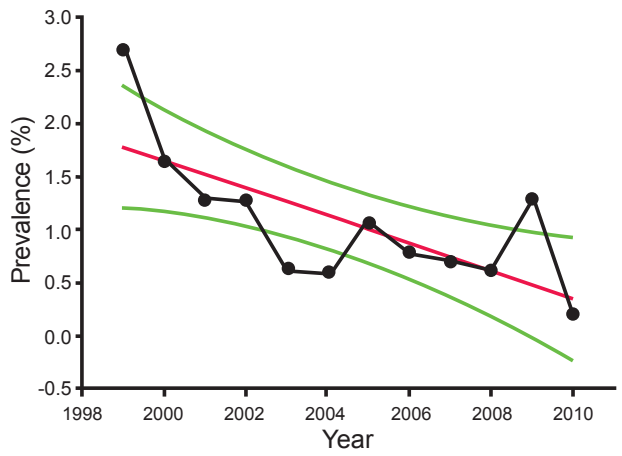

Figure 2: Trichomoniasis prevalence of individual bulls in Wyoming beef cattle between 1999 and 2010. Red line is the linear regression: F(x) =261.020047-0.129685x. Green lines define the upper and lower thresholds of 95% confidence intervals of the regression.

annual prevalence occurred in the first five years of enforcement of trichomoniasis regulations between 2000 and 2004. However, an 80% increase occurred in 2005, followed by a three-year decline. In 2009 a surge recurred with an over 100% increase from 0.62% in 2008 to 1.29% in 2009. In 2010, an individual prevalence of 0.21% was achieved, which was the lowest recorded prevalence of over a decade for which data had been collected.

We next analyzed the effect of state regulations on annual prevalence by regression. A linear regression was formulated: Prevalence F(x) = 261.020047-0.129685*Year(x); R=0.717; F=10.584; P=0.009. Annual prevalence was predicted by this regression as shown in Table 1 & Figure 2 (red line). It was not surprising that the observed prevalence of the year 1999, prior to the enforcement of state laws, was above the upper 95% threshold. Since regulation enforcement in 2000, only prevalence observed in 2009 was above the upper 95% threshold, suggesting a true recurrence. At this time, reason(s) for this recurrence could not been determined. It is plausible that multiple positive bulls of a few herds may account for this recurrence. As a matter of fact that the herd prevalence of 2009 was even lower than that of 2008 as detailed in the next section supports this notion. Prevalence of 2003 and 2004 was below the lower 95% threshold. Among the remaining years, the regression almost precisely predicted prevalence. If the trend holds, the regression predicts that bovine trichomoniasis be eliminated in 2013 in Wyoming. This very optimistic prediction should be taken with extreme caution since it is risky to predict an outcome using a regression beyond the data range between 1999 and 2010. The authors strongly believe that the data indicate that the current trichomoniasis control strategy of culling positive bulls is effective in keeping prevalence low, but is not eradicating the disease, at least not as soon as the model predicts.

Herd prevalence: Data presented in Table 1 & Figure 2 do not illustrate what percentage of ranches were affected by the disease across Wyoming. Herd prevalence accounts for this, and is more indicative of endemic status than individual prevalence. We then performed analysis of herd prevalence on available data from 2007 to 2010. In this analysis, a positive herd was defined as a herd with at least one positive bull. Multiple positive bulls would not change the statistical analysis for a herd. Data showed that 0.76% to 3.21% of herds with an average of 2.17% (SD 1.04%) were positive between 2007 and 2010 (Table 2). These were several fold higher than individual prevalence described above (Table 1), suggesting the disease was more widespread spread among herds than individual prevalence indicated. These are consistent with data in at least two other states, Florida and Nevada [8,21]. Herd prevalence is more predictive than individual prevalence in bovine trichomoniasis and therefore should be used whenever it is possible.

Wyoming Counties with positive herds: Geographical distribution is a good indicator of whether or not an infectious disease is under control. A reduction in distribution often signals control whereas expansion indicates the spread of a disease. Between 2007 and 2010, both Uinta and Lincoln counties, in southwest Wyoming, had positive herds identified in consecutive years; Sweetwater, Fremont, and Hot Springs had positive herds in three years; Big Horn and Washakie in two years; Sublette, Sheridan, Carbon, Albany, Laramie, Niobrara, Weston and Crook in one year. A total of 15 of 23 Wyoming counties had positive herds in the last four years (Figure 3). Furthermore, three (Sublette, Albany and Weston) and one county (Crook) were found positive in 2009 and 2010, respectively, suggesting the disease was spreading out, even though individual prevalence was decreasing. The data affirm the above conclusion that eradication of the disease in the state in 2013 is very unlikely.

Bull numbers per herd: We next analyzed bull numbers per herd from 2007 to 2010. This information is useful in estimating operation sizes since a bull:cow ratio of 1:25 is a standard practice in cattle operations. Bull numbers in Wyoming cattle herds ranged from 1 to 145, 1 to 157, 1 to 132 and 1 to 208 in these four years. As shown in Figure 4, the majority of ranches had less than five bulls, which defied a normal bell shape distribution. Medians were 4, 4, 4 and 3, respectively. The data indicated that cattle operations decreased in size in 2010, compared to the three previous years, which could be an

Year	Herd tested	Herd positive	Prevalence (%)
2007	705	18	2.55
2008	717	23	3.21
2009	788	17	2.16
2010	922	7	0.76
Total	3132	65	2.17

Table 2: Herd prevalence of trichomoniasis in Wyoming beef cattle between 2007 and 2010.

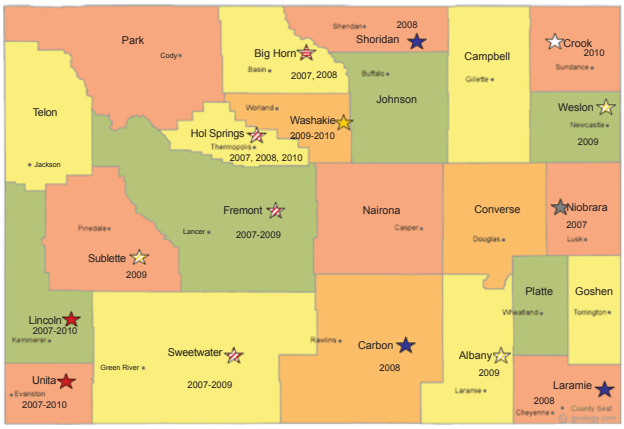

Figure 3: Trichomoniasis distribution in WY counties between 2007 and 2010. The counties with a minimum of one positive herd annually are labeled by color-coded stars and years when the disease is found.

Diagnostic methods		Culture		Total
		Negative	Positive	
PCR	Negative	3081	0	3081
	Positive	2	7	9
Total		3083	7	3090

Table 3: Degree of agreement between cell culture and gel-PCR analyses performed at the Wyoming State Veterinary Laboratorybetween 2009 and 2010.

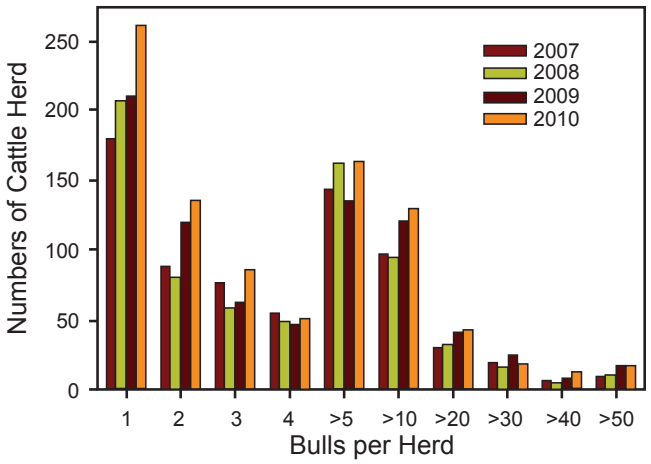

Figure 4: Numbers of bulls per beef cattle herd in Wyoming between 2007 and 2010.

outcome of the latest recession spanned between 2008 and 2010. The data also suggested that half of the cattle operations in Wyoming had 100 or fewer cows, consistent with the most recent agricultural statistics (Wyoming Agricultural Statistics 2010).

Laboratory diagnosis

Multiple sampling increases sensitivity: Due to preputial organism population fluctuations, two more samples are recommended in addition to the first one, i.e., each bull is tested three times in total. These additional tests post significantly extra expenses and inconvenience on ranchers, although they moderately increase sensitivity. Between 2007 and 2010, 7.23%, 7.38%, 7.28% and 3.31% of >7,000 bulls had undergone three tests, respectively. As shown in Figure 5, the 2nd and the 3rd test increased sensitivity by up to 0.20% and 0.09%, respectively. Assuming that 100% sensitivity was reached after three tests, the average sensitivity for the 1st and the 2nd test during the period of time was 81.4±3.1% and 93.6±1.8%, respectively. These would certainly be overestimates considering that less than 8% bulls underwent three tests. There might be more positive bulls if all the bulls would have been subjected to three tests. Nevertheless, the data are consistent with published work of many laboratories and support the recommendation of three samples for trichomoniasis testing in bulls [23,26,35-37].

Close agreement of gel-PCR and cell culture: As stated in materials and methods, all samples designated for PCR analysis by veterinarians were cultured for 24 hours and were examined microscopically before DNA extraction. This provided us with an opportunity to examine how well cell culture and PCR analysis agree with one another. As shown in Table 3, we tabulated a total of 3090 samples in the two year period of 2009 and 2010. All 3081 PCR negative samples were culture negative; all seven PCR positive ones were culture positive. However, two PCR positive samples were culture negative. Both diagnostic methods were 99.9% agreement with each other, indicating the more expensive PCR showed no overall advantage over the conventional cell culture. These data agree with the results of both field collected samples [26,36] and experimental infections [35]. That had been said, it should be emphasized that this increase in sensitivity in PCR over culture is significant, especially when overall prevalence is low. This might be the single most important factor in determining whether or not the disease is eliminated from a herd. Another routine diagnostic method qPCR targeted internal transcribed spacer region-1 of the genome is 2,500 and 500 time more sensitive than cell culture and gel-PCR, respectively [38]. However, qPCR is prone to higher false positive than culture and gel-PCR assays [26].

Although the data presented here showed very good agreement between the gel-PCR and cell culture, we recommend gel-PCR as a routine confirmatory test for bovine trichomoniasis due to its specificity. Mutto and colleagues found that 9 culture-positive samples are PCR

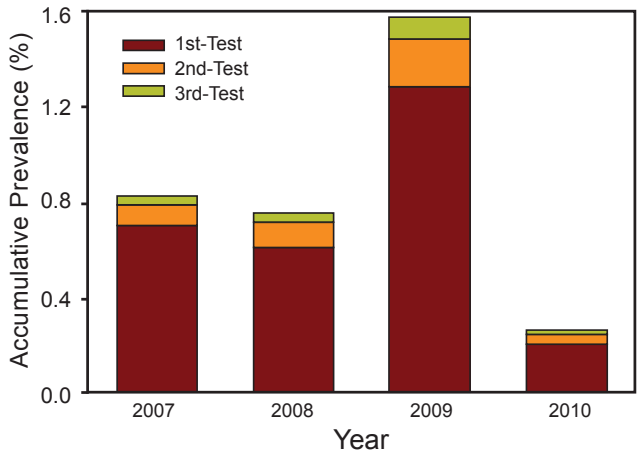

Figure 5: Accumulative prevalence of individual bulls after three tests in Wyoming between 2007 and 2010.

negative out of 203 preputial samples. The authors attribute this to the presence of contaminated trichomonads, which cannot be easily distinguished by the culture method [39]. Furthermore, virgin bulls are found culture positive with protozoa resembling *T. foetus* but being confirmed as non-*T. foetus* by four anterior flagella in fixed samples and no specific PCR product [33]. Once again, this is most likely due to contamination with fecal trichomonads [17,33]. Several non-*T. foetus* protozoa have been found in the preputial cavity of bulls. These include *Monocercomonas ruminantium*, *Pentatrichomonas hominis*, and *Pseudotrichomonas* spp. and *Tetratrichomonas* spp. [10,40,41]. Consequently, it should be a standard practice for all culture-positive bulls be confirmed by PCR to avoid unnecessary culling of potential *T. foetus*-negative bulls.

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