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Tritrichomonas foetus is not located in the accessory glands, epididymis, or testicles of infected bulls, and post mortem changes influence recovery of organism

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OBJECTIVE
To determine the presence of *Tritrichomonas foetus* in the accessory sex glands, epididymis, and testicular tissue of *T foetus*–positive bulls and to assess the impact of post mortem tissue acidity on quantitative reverse-transcription PCR results.

METHODS
8 bulls were euthanized from June 12 through June 15, 2023, and samples from their accessory sex glands, testicles, and epididymis were collected for *T foetus* testing. Preputial smegma was obtained before and after euthanasia and tested for *T foetus* by quantitative reverse-transcription PCR. The pH of the penile and preputial epithelial surfaces was measured.

RESULTS
Tritrichomonas foetus was not detected in samples from the testicle, epididymis, or epididymal semen. A post mortem decrease in the pH of the penis and prepuce correlated with a rise in cycle threshold values.

CONCLUSIONS
Tritrichomonas foetus was not present in samples taken, suggesting its absence in these specific reproductive tissues. A decrease in the pH of the penis and prepuce over time post mortem correlated with an increase in cycle threshold values in the samples. This relationship indicates that changes in pH may impact the detectability or stability of genetic material in these tissues, possibly affecting diagnostic outcomes as time from death progresses.

CLINICAL RELEVANCE
This research enhances the understanding of *T foetus* pathology in bulls and suggests that epididymal semen recovery could be a viable method for preserving genetic material from valuable bulls that test positive for *T foetus*. It also emphasizes the need for timely post mortem sampling to ensure accurate detection and management of *T foetus* in the cattle industry.

Keywords: bull, *Tritrichomonas foetus*, testicle, semen, accessory sex glands

Tritrichomonas foetus is an obligate parasite of the bovine reproductive tract. The trophozoite is a motile microaerophilic organism that prefers a neutral pH of 7.4 to 7.8, similar to that reported in the reproductive tracts of both male and female bovines.[1–3] A sexually transmitted pathogen in cattle, *T foetus* remains a significant challenge for cattle industries worldwide despite extensive control efforts.[4] Infected bulls, who are asymptomatic carriers, retain this carrier status throughout their lives and are the primary vectors for transmitting the disease.[5,6] During mating, these bulls pass the protozoan from the glans penis to the vagina of the female. Once transmitted, the motile organism moves through the cranial vagina and cervix, causing inflammation in the uterus and oviducts. This often leads to embryonic death and abortion, leaving cows unable to successfully conceive until they have developed short-term immunity and cleared the organism.[4]

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Reduced calf crops, extended calving intervals, and the necessity to cull infected animals due to lack of treatment can lead to severe economic losses for affected herds.[7,8] In the US, there are no legal treatments or preventative measures for *T foetus*.[9] Consequently, the main strategies for managing this disease involve testing and culling infected animals, along with implementing stringent biosecurity measures. The control of bovine trichomonosis is not coordinated at the federal level in the US, resulting in a patchwork of regulations across states as each sets its own cattle entry requirements.[10]

The reported prevalence of *T foetus* is highly variable, varying from state to state, with numbers ranging from less than 0.01% to 11.25%.[7,8,11–25] Other countries, such as Australia, have prevalence rates as high as 15.4%.[26] The prevalence of *T foetus* has been reported to be 3.5% in Argentina,[27] 3.7% in Brazil,[28] and 10.4% in Africa.[29] Most European countries are considered to be free of *T foetus*; however, reports of positive animals in Spain[30] and France[31] have been made in the last decade. Many of these studies are significantly outdated, and the varying methodologies used for sampling the population make direct comparisons between states difficult. Several of these studies[14,19,32] used abattoir samples to determine the prevalence in their states or countries.

Tritrichomonas foetus is known to reside on the epithelial surface of the penis, prepuce, and distal urethra of the bull.[33–35] However, there is limited evidence that the organism infects other reproductive organs outside of the penis, prepuce, and distal urethra. Lovelady[36] conducted a study of 22 bulls known to be positive by preputial scraping to determine the presence of *T foetus* in the accessory sex glands. In that study,[36] the accessory glands collected from known positive bulls resulted in no positives by culture, and only 1 bull was found to be weakly positive on PCR analysis of the prostate. Older manuscripts also describe the rare finding of *T foetus* in the accessory sex glands.[33,37,38]

In efforts to add to the depth of knowledge of *T foetus*, there were 4 objectives of this study: (1) to determine the presence or absence of *T foetus* in the accessory sex glands of *T foetus*–positive bulls by quantitative reverse-transcription PCR (RT-qPCR) testing, (2) to determine the presence or absence of *T foetus* in semen harvested from the epididymis of *T foetus*–positive bulls by RT-qPCR testing, (3) to determine the presence or absence of the organism in testicular and epididymal tissue from *T foetus*–positive bulls by RT-qPCR testing, and (4) to assess the impact of increased tissue acidity in post mortem bulls and track the resulting quantification cycle (Cq) values of *T foetus* preputial samples via RT-qPCR testing over time.

Methods

Eight sexually mature bulls previously diagnosed as naturally infected with *T foetus* as determined by a preputial smegma sample submitted for RT-qPCR to a state diagnostic laboratory were purchased. Bulls ranged in age from 2 to 6 years old, and all were of English or Continental breeding (Angus, Charolais, Hereford). All bulls were reconfirmed positive for *T foetus* by RT-qPCR upon arrival at the research facility.

Bulls were housed in a paddock and fed a balanced ration at the Bushland Research Facility in Bushland, TX. This project was approved by IACUC 2022–117. This project adheres to Animal Research: Reporting of In Vivo Experiments guidelines.

A preputial smegma sample for *T foetus* testing was collected 24 hours prior to euthanasia. Samples were obtained by scraping the preputial epithelium 10 times with the Pizzle Stick Trich (Lane Manufacturing) testing device attached to a sterile 20-mL syringe. The Pizzle Stick was inserted into the sheath and directed caudal to just cranial to the preputial fornix. Negative pressure was maintained on the syringe, and 10 back-and-forth searching motions focused on the approximate location of the midshaft and caudal portion of the free penis. Once obtained, samples were transferred to a sterile cryovial containing 2 mL of sterile PBS and submitted the same day of collection to our in-house infectious disease diagnostic lab for RT-qPCR testing. Bulls were euthanized from June 12 through June 15, 2023, by gunshot according to AVMA guidelines.[39] All other samples were collected post mortem on the necropsy floor at Texas Tech School of Veterinary Medicine.

Testicular harvest and epididymal semen extraction

Immediately following euthanasia, each bull was castrated, leaving the testicle and epididymis intact after ligation of the vas deferens to prevent semen loss. The testicles were rinsed of any blood with sterile saline, placed in sterile sleeves bathed in sterile saline, and taken to the lab for the epididymal harvesting procedure. The epididymis and vas deferens were dissected from the testicle, and a retrograde flushing of the epididymis was performed with sterile saline. The extracted semen was collected in a sterile cryovial. The volume of semen and sterile saline combined following the flush ranged from 1.5 to 3 mL per flush. Following extraction, the sample was submitted for RT-qPCR testing. Each epididymal flush was considered a single sample, resulting in 2 samples from each bull (left and right epididymal flush). Testicular and epididymal (corpus, body, and cauda) tissue was also harvested for RT-qPCR testing. For the testicular sample, a section including the rete testis was chosen.

Post mortem sampling of penis and prepuce

A preputial smegma sample was taken immediately posteuthanasia and labeled time 0 utilizing the same method as described above. The sample was placed in 1.5 mL of PBS for RT-qPCR testing and was considered time 0. Following collection of the preputial smegma sample, the penis was exteriorized, and a pH meter was utilized to determine the pH of the glans penis (Hanna Instruments; H19128). Following

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data collection, the penis was returned into the sheath. After collection of all samples, bulls were held a minimum of 8 hours in a walk-in necropsy cooler held at 1.67 °C. The sampling process was repeated at 8 and 24 hours posteuthanasia. Due to space constraints, only 4 of 8 bulls were held for sampling 24 hours posteuthanasia **(Supplementary Table S1)**.

Harvesting of accessory sex glands

Finally, the internal reproductive tract was harvested in total, making sure to take wide margins as to not contaminate the tract due to incision of an organ. Careful consideration was taken to prevent contamination from the penis and prepuce. Once the reproductive tract was isolated, each accessory sex gland (ampullae, vesicular glands, prostate, bulbourethral glands) was isolated, and tissue samples were taken. Paired structures were treated as individual specimens (ampullae, vesicular glands, bulbourethral glands), and careful dissection of the prostate was performed to allow for samples from both the corpus and disseminate portions of the prostate. Strict precautions were taken during tissue dissection to avoid cross contamination between samples by changing gloves and surgical instruments between the harvesting of each sample from each bull. Tissues were placed in cryovials containing PBS for identification and transportation to the in-house lab. A piece of liver was also harvested from each animal to act as a known negative tissue control. Following completion of necropsy, each bull was placed in the walk-in necropsy cooler, which is kept at 1.67 °C, for further testing.

Quantitative reverse-transcription PCR testing

Following collection, all samples were immediately taken to our in-house infectious disease diagnostic lab for RT-qPCR testing. This diagnostic testing included automated nucleic acid extraction and purification and RT-qPCR following all procedures and controls indicated by Ginter Summarell et al.[40]

Data analysis

The relationship between pH and average Cq values was evaluated over time using repeated-measures data from the same bulls at 4 different time points. Initially, a population-averaged model was found inadequate due to the high variability in Cq values, leading to the consideration of fixed-effects and random-effects models. A Hausman test indicated that a random-effects model was appropriate.

Given the strong correlation between time and pH, a direct inclusion of both variables as explanatory factors would introduce endogeneity. To address this, a generalized 2-stage least squares random-effects instrumental variables regression model was used, with time and its square as instruments for pH.

Results

Epididymal semen extraction

A total of 16 samples (8 left and 8 right) were evaluated by RT-qPCR. With all samples there was no detection of *T foetus*. Two positive control semen samples that were spiked with preputial smegma from 2 infected bulls containing a minimum of 10 organisms were utilized to ensure that the RT-qPCR procedure used would indeed produce a positive Cq value in line with preputial samples in PBS harvested from the same bulls.

Accessory sex glands, epididymis, and testicular tissue

Of the 64 samples submitted for evaluation by RT-qPCR, all samples resulted in no detection of *T foetus*. Positive and negative control tissue samples were utilized to confirm results. Positive tissue samples were created by spiking samples with preputial

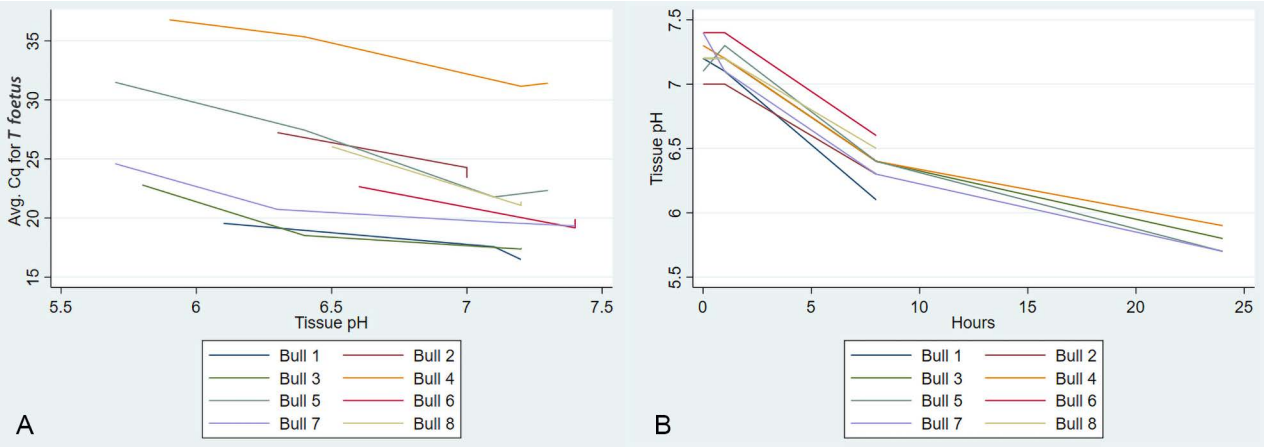

Figure 1—The average quantification cycle (Cq) values for *Tritrichomonas foetus* testing (A) and corresponding pH values (B) over time for 8 euthanized bulls naturally infected with *T foetus*. Bulls were euthanized from June 12 through June 15, 2023. After euthanasia, bulls were held a minimum of 8 hours in a walk-in necropsy cooler held at 1.67 °C. As tissue pH decreases, the average Cq for *T foetus* significantly rises. A correlating pattern is noted that as hours post mortem increase, the pH of the penis and preputial tissue decreases.

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smegma from 2 infected bulls with a minimum of 10 organisms as described above.

Correlation of sampling Cq values and pH of tissue

As time increases post mortem, on average the pH of the glans penis decreases from a neutral pH of 7.2 to more acidic values of 6.5 at 8 hours post mortem to 5.7 at 24 hours post mortem. This decrease in pH overtime is significantly correlated ($P < .001$; 95% CI, −5.024 to −3.500) with an increase of Cq values over time. A 1-U decrease in pH results in a 4.26-U increase in Cq values (**Figure 1**; Supplementary Table S1).

Discussion

This series of post mortem studies aims to address unresolved questions about the presence of *T foetus* organisms in various parts of the male reproductive tract beyond the penis, prepuce, and distal urethra. The project employed RT-qPCR testing to detect the presence or absence of *T foetus* in the bulbourethral glands, prostate, vesicular glands, ampullae, testicles, epididymis, and epididymal sperm of naturally infected bulls. Identifying *T foetus* in these accessory sex glands is essential for understanding the pathophysiology of chronic infections in bulls, which could impact future treatment protocols and prevention strategies. Furthermore, the presence or absence of *T foetus* in the testicle and epididymis has implications not only from a disease standpoint but also for the opportunity to save genetic material from economically valuable animals who unfortunately become infected with *T foetus* during natural mating situations.

Trichomonas vaginalis is a sexually transmitted protozoan that infects humans, exhibiting a life cycle and disease pathophysiology comparable to *T foetus* in cattle. *Trichomonas vaginalis* typically inhabits the male urethra but has also been identified in other parts of the male reproductive tract, including the prostate, epididymis, and testicle.[41–46] As the human male has a full complement of accessory sex glands similar to the bull, infection of these other locations with *T vaginalis* has led to speculation that *T foetus* may reside in similar locations in bulls.

Reports regarding *T foetus* being isolated from the accessory sex glands in bulls have been sporadic at best. Hammond and Bartlett[33] reported retrograde infections of the ampullae, seminal vesicles, and epididymides by *T foetus*. Their conclusion was supported by several isolated cases, each involving an infection in one of these organs, thereby demonstrating the potential for *T foetus* to ascend within the urogenital tract of bulls. Lovelady[36] further explored the possibility of *T foetus* in the accessory sex glands of bulls. Of the 20 *T foetus*–positive bulls that they followed to slaughter, only 1 bull was found to have a positive reaction on PCR testing of the prostate. Comparatively, none of the bulls in this study were found to be positive in any organ or gland tested by RT-qPCR. The advanced testing methods in this trial,[40] along with the controlled conditions of necropsy that cannot be mimicked in slaughterhouse settings, suggest that the likelihood of disease in the accessory sex glands, testicles, or epididymides is unlikely and may only occur in rare cases if at all.

Sixteen epididymal seminal flushes from the 8 bulls were all found to be negative for *T foetus*. Combined with the negative results from the testicular and epididymal tissue samples, this suggests that *T foetus* is unlikely to be present in this part of the reproductive tract. Epididymal semen recovery is a common method for preserving genetic material from valuable animals following catastrophic injury or illness that necessitates euthanasia. Currently, there is no approved treatment for *T foetus* in the US. Consequently, all bulls that test positive for *T foetus* are culled and sent to slaughter, leading to the loss of some genetically valuable bulls from herds upon a positive diagnosis. This results in significant economic losses for the producer, not only due to the disease itself including loss of calf crop and culling of positive animals but also from the loss of potential future economic gains from an animal's superior genetics. Since *T foetus* is commonly found in the distal urethra of positive bulls, electroejaculation or collection of semen by artificial vagina for semen cryopreservation is not a viable option. The results of this study suggest that semen harvested from the epididymides following castration may be an alternative method for cryopreserving the genetic material of valuable animals. While extending the semen collected and completing the process of cryopreservation from the epididymal flushes was beyond the scope of this study, further investigation is warranted. This should include testing extended cryopreserved semen straws from positive animals and conducting breeding trials before this method can be recommended as a general practice and accepted by regulatory officials.

Finally, the last objective of the study was to determine the correlation between time, pH, and Cq values of preputial samples submitted for RT-qPCR testing for *T foetus*. Post mortem, the pH of bovine tissue decreases due to the shift from aerobic to anaerobic metabolism. Upon death, blood circulation and oxygen supply cease, leading to a lack of oxygen in tissues. This anaerobic environment triggers glycolysis, where glycogen is converted into lactic acid. The accumulation of lactic acid causes the tissue pH to drop from approximately 7.0 in living tissue to about 5.5 within the first 24 hours post mortem.[47] When *T foetus* encounters more acidic pH conditions, it has been shown to have reduced viability.[3] As a fastidious organism that is susceptible to DNA degradation in the face of imperfect environmental conditions,[48–50] this reduction in DNA would explain the rise in Cq values over time demonstrated in this study. These findings are important as we strategically evaluate abattoir-derived *T foetus* prevalence studies. In none of the available studies is the time from death to sampling mentioned. Consequently, some bulls may have been falsely identified as negative based on our data and an increase in Cq over time as pH decreased. While post mortem sampling

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is valid, based on data presented in this paper, samples need to be taken within the first few hours following death to maximize the chances of producing a true negative or positive. This is exemplified by animals such as bull 4, who had a positive but higher Cq value than some of his positive contemporaries. By 8 hours post mortem, bull 4 would have been deemed negative by some diagnostic labs (> 35 Cq).

In conclusion, this series of projects supports the thought that *T foetus* is unlikely to be present in the accessory sex glands, testicles, or epididymides of bulls based on the advanced RT-qPCR testing methods and controlled necropsy conditions used in the study. This finding contrasts with sporadic reports and isolated cases of *T foetus* in these organs and suggests that such infections are rare if they occur at all. Furthermore, the study indicates that epididymal semen recovery might be a viable method for preserving the genetic material of valuable bulls that test positive for *T foetus* as the epididymal seminal flushes tested negative for the organism. However, further research is needed to confirm that cryopreservation of semen from animals that test positive for *T foetus* is safe for the public and will be accepted by regulatory officials. Lastly, the study highlights the importance of timely post mortem sampling to avoid false negatives due to the degradation of *T foetus* DNA in the more acidic pH conditions post mortem.

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Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

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Supplementary Materials

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