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# Effects of Biological Materials and Collection Media on PCR Detection of *Tritrichomonas foetus*

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## Abstract

*Tritrichomonas foetus* is an important pathogen of the bovine reproductive tract causing early embryonic death and abortion in cows and persistent, asymptomatic infection in bulls. PCR detection methods have greatly enhanced diagnostic accuracy over culture; however, pre-analytical sample handling is just as critical as technical performance in detecting this pathogen and is not well studied. The purpose of this study was to investigate the effects of biological materials present in the prepuce on PCR detection of *T. foetus* in a variety of collection media. Simulated preputial samples were created using InPouch™ (IP) media, lactated ringers solution (LRS), or sterile saline (SAL); inoculated with low numbers of one of three *T. foetus* strains; and spiked with either blood, semen, urine, or sham treatment. Samples were transported to the lab, placed in growth media (LRS and SAL samples), incubated, and tested for *T. foetus* by PCR. Samples containing urine had statistically significantly greater mean Ct values ($P = 0.008$) than samples containing other materials, seen most dramatically in IP ($P < 0.0001$.) Urine contamination resulted in significantly ($P = 0.037$) fewer samples being identified as "positive" for *T. foetus.* Overall, SAL collections also had significantly higher mean Ct than IP or LRS ($P < 0.001$), and were less likely ($P = 0.018$) to results in classification as a "positive" sample. Results of this study indicate that collection media and biological materials can affect *T. foetus* PCR detection. The presence of urine in preputial samples can result in false negative results, while blood had no detrimental effects.

## Keywords

*Tritrichomonas foetus*, PCR Detection, Urine, Semen, Blood

## 1. Introduction

*Tritrichomonas foetus* is an important pathogen of the bovine reproductive tract causing early embryonic death and abortion [1] [2]. This protozoal organism colonizes the epithelium of the penis and prepuce in the bull and has been identified in the distal urethra; however, not in the penile or prostatic urethra, accessory sex glands, nor epididymis [1] [3]. Attachment to urogenital epithelium is thought to occur via glycoprotein adhesins which results in minimal immune recognition or site-specific inflammation; consequently, clearance of the organism is rare, particularly in older bulls [1] [3] [4] [5].

Transmission of *T. foetus* to cows primarily occurs during breeding and is highly efficient, with up to 95% of females becoming infected after a single mating [6] [7] [8]. Economic impacts of *T. foetus* on a herd include loss of income from fewer and lighter-weight calves to market at weaning, loss of genetic potential from culled animals, costs of replacing infected bulls, laboratory fees for diagnostic testing, and feed costs for non-productive animals [1] [7] [9]. No approved treatments exist to clear the infection; consequently, identification and removal of infected bulls is the best way to minimize *T. foetus* impacts [8] [9].

Molecular diagnostic methods with high sensitivity and specificity have greatly improved the analytical detection of *T. foetus* [10] [11]. Pre-analytical sample handling, however, can account for up to 62% of diagnostic errors and it is critical to address sample collection and transport when evaluating diagnostic accuracy [9] [12]. The purpose of the present study was to evaluate the presence of biological materials that are commonly present in bull preputial samples on the PCR detection accuracy of *T. foetus* utilizing three different collection media.

## 2. Materials and Methods

### 2.1. Sample Preparation

The collection media used in this study included InPouch$^{TM}$ (IP) media (individual pouch), 1.5 mL of LRS, or 1.5 mL sterile saline (SAL); the three *T. foetus* strains used were the laboratory quality control strain, a clinical isolate from 1986 and confirmed by phenotypic and genotypic methods [PCR & RFLP] as *T. foetus* (QC) and two field strains. (FS1 recovered from smegma cultures submitted to the U.C. Davis Veterinary Medical Teaching Hospital and FS2 recovered from smegma cultures submitted to the California Animal Health and Food Safety Laboratory System.) Three replicates for each treatment group were created in the field and transported to the lab to mimic natural collection conditions.

Each sample was inoculated with approximately 150 organisms of an individual strain to simulate low levels of *T. foetus* colonization followed by one type of biological material (0.1 mL freshly-collected whole blood [BLOOD], 0.5 mL of freshly collected urine [URINE], 0.1 mL of freshly-collected semen [SEMEN], or 0.1 mL sterile saline [SHAM].) Volumes of biological materials were chosen to simulate the quantities that are consistent with what has been obtained during a collection of a preputial scraping or wash, particularly at the time of a breeding

soundness exam. The bull used for collection of biological samples had a complete blood count (CBC) and urinalysis that were within normal limits and was tested negative for *T. foetus* by PCR at the time of biological material collection.

Samples were transported to the lab within three hours of creation. The IP samples were incubated at 35˚C for 24 hours; mixed well; and 1mL aliquots were collected for testing. SAL and LRS samples were held at room temperature for one hour and were then inoculated into 10 mL tubes of modified Diamonds Plastridge media (DPM) [13] which contained 1.0% peptone-based broth, 1.0% dextrose, 0.1% sodium chloride, 0.3% beef extract, 5000 units of penicillin/mL, 0.1% of streptomycin, 0.3% ovine serum, and 0.07% agar. Inoculated DPM tubes were incubated at 35˚C for 48 hours, after which 1-mL aliquots were collected from the base of the tube for PCR testing. Due to the time *T. foetus* spent in non-growth media (SAL or LRS) before inoculation, DPM media is incubated for 48 hours per laboratory protocol to better support organism recovery.

## 2.2. Real-Time PCR Assay

Testing on IP was conducted as previously described [11]. Briefly, a 1 mL volume of media was centrifuged at 3500 rpm at room temperature for 3 min, decanted, and re-suspended in 300 µL of 1 × PBS. Due to the presence of agar in DPM that can interfere with PCR reactions [14], testing performance in this medium was optimized using centrifugation of the 1mL aliquot at 3500 rpm at room temperature for 3 min followed by decanting of supernatant, and re-suspension of the pellet in 200 µL of 1 × PBS.

DNA extraction was performed using MagMAX 96 Viral RNA Isolation Kit for total nucleic acid from low-cellularity biological samples (Life Technologies, Grand Island, NY) modified for *T. foetus* extraction with a final volume of 85 µL. Real time PCR was performed in a 33 µL reaction mixture containing 8 µL of template DNA; 1 µL of VetMAX *T. foetus* primer-probe reagent (Life Technologies, Grand Island, NY); 12.5 µL of VetMAX Plus qPCR Master Mix, (Life Technologies, Grand Island, NY); 3.5 µL of sterile water, and 8 µL of internal amplification control (IAC) DNA (Life Technologies, Grand Island, NY). PCR was conducted on the ABI 7500 Fast (Sunnyvale, CA); reactions included initial polymerase activation at 95˚C for 10 min, followed by 40 cycles of DNA dissociation at 97˚C for 5 s and annealing/extension at 55˚C for 40 s. Results were expressed as cycles to threshold (Ct) values, and simulated samples were PCR-tested in triplicate to obtain mean Ct values for each. Non-*T. foetus*, internal amplification control (IAC) DNA was included with all reactions to ensure that the PCR reactions performed as expected and no PCR inhibition was present in tested samples. Positive and negative extraction and amplification controls were tested with each run to ensure consistent performance of the assay.

## 2.3. Data Analysis

Statistical analyses were performed using SAS Version 9.4 (SAS Institute, Inc., Cary, NC, USA.) Mean Ct values were assessed using a least squares means pro-

cedure between *T. foetus* strain, collection media, and biological treatments. A Ct value of 40.0 was used for calculation of summary statistics of samples with no DNA detected. Comparisons of percent positive samples between treatment and media using the laboratory cut-off of 37.0 were performed using Fisher's exact test.

## 3. Results

### 3.1. Evaluation of Biological Materials and Collection Media on *T. foetus* Detection by PCR

The effects of the biological material on *T. foetus* PCR detection are shown in Figure 1. Across all media and *T. foetus* strains, samples containing urine had statistically significantly higher mean Ct values (36.6, $P = 0.008$) than all other treatments (34.6 - 34.9). The effects were most evident in samples inoculated into IP media, in which mean Ct values in urine-containing samples were five cycles greater (mean Ct = 38.5) than samples containing blood, semen, or no biological treatment (mean Ct 33.1 - 33.9, $P < 0.0001$.) Urine (mean Ct = 37.2) also significantly inhibited detection ($P = 0.002$) of *T. foetus* in SAL samples ($P = 0.002$) compared with other biological materials (mean Ct = 34.8.)

Figure 2 shows that across all treatments, samples collected in IP and LRS (mean Ct = 34.7) had mean Ct values which were significantly lower than samples collected into SAL (mean Ct = 36.3, $P < 0.001$.) Biological materials had no effect on samples collected into LRS. Samples inoculated into IP (mean Ct = 33.1) had significantly lower Ct values than LRS- (mean Ct = 35.3) or SAL-inoculated samples (mean Ct = 36.4) when spiked with semen ($P < 0.001$) but not samples spiked with blood (mean Ct 33.9 - 35.1, $P = 0.22$.) SAL had significantly higher mean Ct values for samples containing semen (mean Ct = 36.4) or urine mean Ct = 37.4) than for samples containing blood (mean Ct = 35.1, $P = 0.046$.)

### 3.2. Assessment of Biological Material and Collection Media on *T. foetus* Classification

Percentage of positive samples detected using the laboratory cut-off is shown in

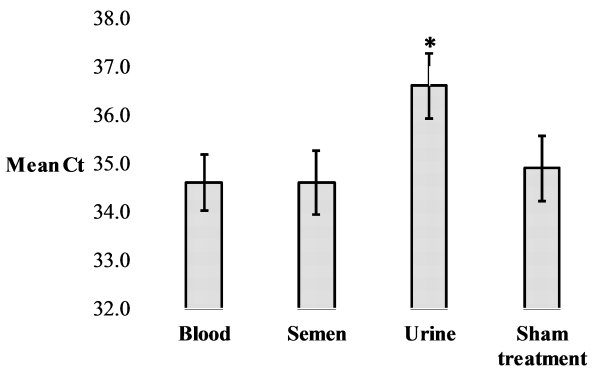


Figure 1. Mean Ct values for *T. foetus* DNA in simulated preputial samples spiked with blood, semen, urine, or sham treatment. *Mean Ct was significantly greater than other treatments ($P = 0.008$).

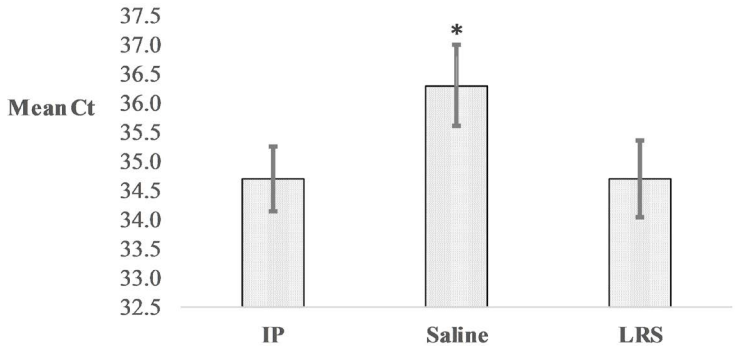


**Figure 2.** Mean Ct values for *T. foetus* DNA in simulated preputial samples collected in InPouch™ media (IP), sterile saline (SAL), or lactated Ringer's solution (LRS.) *Mean Ct was significantly greater than for other media ($P < 0.001$).

Figure 3(a) for biological media and Figure 3(b) for collection media. Overall, samples containing urine were significantly less likely ($P = 0.037$) to be classified as "positive" than any of the other treatments. Nearly 19% of *T. foetus*-spiked samples containing urine had no DNA detected at all during PCR testing, with 33% of FS1 and 55.5% of FS2 samples with urine yielding false-negative results. Regardless of the type of biological material, samples which were inoculated into SAL also had significantly fewer classified positive results ($P = 0.018$) with 26.0% having no DNA detected.

## 4. Discussion

Identification of bulls, which are colonized with *T. foetus*, continues to be a costly and frustrating problem to the cattle industry [1] [3]. As a fastidious organism which is susceptible to DNA degradation in the face of imperfect environmental conditions, *T. foetus* detection is critical to any control program [15] [16]. The implementation of diagnostic methods with high sensitivity and specificity such as real-time PCR can dramatically improve identification of infected bulls, particularly those that are colonized with low numbers of organisms expected during natural infection [15] [17]. In the present study, samples were created with a low number of *T. foetus* (50 - 60 organisms/ml in IP system) to assess how sample conditions affect DNA detection in these challenging samples.

This study demonstrates the importance of mimicking true sample conditions, including the use of field isolates, when evaluating diagnostic procedures for accuracy. The presence of urine interfered with DNA detection, resulting in higher mean Ct values for all three *T. foetus* strains evaluated in this study; however, the effects were seen most dramatically in field strains, with one-third (FS1) to one-half (FS2) of samples producing false negative results. *T. foetus* has been shown to be susceptible to extremes in pH, with optimal survival and DNA detection at 6.8 - 7.0, and can tolerate ranges of 6.0 - 8.5 [16] [18]. The urinalysis on this bull showed his urine pH to be 9.0, which may account for the greater Ct values and lower detection in SAL samples compared to IP and LRS which are designed to maintain a neutral pH. Additionally, our laboratory classifies bulls

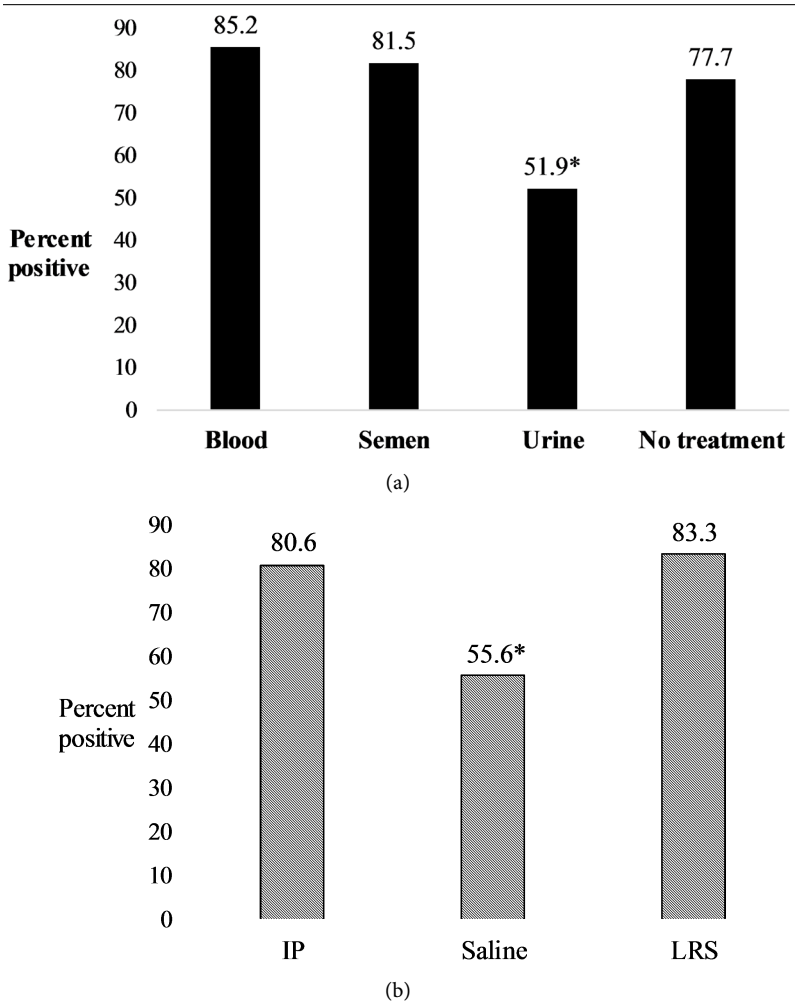


**Figure 3.** Percent positive for *T. foetus* DNA in simulated preputial samples (a) spiked with blood, semen, urine, or sham treatment (*percent positive was significantly lower than other treatments [$P = 0.037$]) and (b) collected in InPouch™ media (IP), sterile saline (SAL), or lactated Ringer's solution (LRS) (*percent positive was significantly lower than other media [$P = 0.018$.])

with high Ct values (37 - 39) as "inconclusive" since these values are near the limit of detection for the assay. PCR testing in this study identified two samples each spiked with blood or semen but seven samples spiked with urine fell into in this category.

Overall, samples created in SAL had significantly greater mean Ct values for *T. foetus*, even though samples were inoculated into DPM culture media within four hours of collection. Isotonic saline appears to do a poor job of maintaining conditions that preserve *T. foetus* for detection. LRS provides superior buffering capacities under physiologic conditions and was shown to maintain pH significantly better than isotonic saline even within the first 2 hours of administration [19].

There were several limitations to the present study. The *T. foetus* spiking concentration was selected to mimic bulls colonized with low numbers of organ-

isms but does not provide data on how these conditions would affect detection of larger numbers of organisms. Biological materials were collected from a single bull and volumes were chosen to simulate what might be obtained during preputial scraping; the effects of different animals and different volumes of these materials were not evaluated and may impact the extent of their presence on *T. foetus* detection. Testing was performed on incubated media to enhance the survival of *T. foetus* and preservation of DNA for detection; however, LRS and SAL samples inoculated into DPM were incubated for 48 hours compared with IP incubated for 24 hours per laboratory protocol which would be expected to produce lower Ct values and affect comparison between collection media.

The present study demonstrates that to maximize *T. foetus* detection, particularly from bulls harboring low numbers of organisms, collection procedures should include use of a media which provides an environment simulating physiologic conditions and avoiding urine collection at the time of preputial sampling. Even though *T. foetus* strains collected into SAL had only four hours of contact before DPM inoculation, DNA detection was significantly impaired over IP and LRS collections, which may indicate that a more complex and balanced support media is needed to support *T. foetus* prior to arrival at a diagnostic facility.

## Conflicts of Interest

The authors declared no potential conflict of interest with respect to the research, authorship and/or publication of this article.

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