Estimation of the Trichomonad Concentration in Beef Bull Preputial Samples and Resultant Population Effect of Pooling on Detection of *Tritrichomonas foetus* Using qPCR

David J Wilson*, Thomas J Baldwin, Amanda Wilhelm, Chelsea H Whitehouse, Jennifer L Bunnell, Kerry A Rood and Arnaud Van Wettere J

School of Veterinary Medicine, Department of Animal, Dairy and Veterinary Sciences, Utah State University, 950 East 1400 North, Logan, UT 84341, USA

*Corresponding author: David JW, School of Veterinary Medicine, Department of Animal, Dairy and Veterinary Sciences, Utah State University, 950 East 1400 North, Logan, UT 84341, USA, Tel: (435) 760-3731; Fax: (435) 797-2805; E-mail: David.Wilson@usu.edu

Abstract

The primary objective was assessment of effect of sample pooling on *Tritrichomonas foetus* detection using qPCR in beef bulls within 5 logs of trichomonad concentration in spiked preputial samples. The secondary objective was to use qPCR Ct values to estimate the percentage of beef bulls in the field population within each log of trichomonad concentration. Solutions containing none, 1,10,100,1000 and 10,000 trichomonads (T)/ml were made and tested by qPCR. Test sensitivities for single positive samples were: 1 T/ml, 20/40 (50%); 10 T/ml, 40/40 (100%); 100 T/ml, 40/40 (100%); 1000 T/ml, 40/40 (100%); 10,000 T/ml, 40/40 (100%). For 5-sample pools (1 positive and 4 negative), sensitivities were: 1 T/ml, 6/40 (15%); 10 T/ml, 26/40 (65%); 100 T/ml 40/40 (100%); 1000 T/ml, 40/40 (100%); 10,000 T/ml, 40/40 (100%). Specificity for both single and pooled negative samples was 40/40 negative (100%). Diagnostic field samples (n=130) in which *T. foetus* was detected by qPCR were classified by Ct to estimate the proportion of positive bulls within each trichomonad concentration: 1 T/ml 10 (7.7%); 10 T/ml 17 (13.1%); ≥ 100 T/ml 103 (79.2%). Accounting for the above sensitivities and proportions of bulls, sensitivity for trichomoniasis detection in the bull population was 96.2% using single preputial samples and 88.9% using 5-sample pools. Therefore 7.3% of all *T. foetus*-positive bulls were not detected using 5-sample pools compared with using single samples. Detection of bovine trichomoniasis by qPCR is minimally affected by using 5-sample pools, considering the trichomonad concentration of most positive bulls.

Keywords: Trichomoniasis; Bovine; Bulls; Individual samples; Pooled samples

Introduction

Bovine trichomoniasis is a venereal disease of cattle caused by the protozoan *Tritrichomonas foetus*. Infection with *T. foetus* results in decreased pregnancy rates and increased cases of abortion, stillbirth, endometritis and pyometra. In bulls, infection is subclinical [1-3]. Interestingly, no refereed publications regarding financial loss have been authored in the past 25 years; virtually all references to the cost of bovine trichomoniasis cite Rae in 1989 [4] who stated that productivity and profitability of cow-calf operations were reduced by 5% to 35% as measured by return per cow attempted to be impregnated. To date, the most effective control strategies for bovine trichomoniasis focus on identification and elimination of infected carrier bulls [2,3].

Most states have trichomoniasis control programs that focus on testing of bulls and elimination of positive animals. As a result of such a program, prevalence of trichomoniasis in bulls in the state of Utah has declined from national historic levels of approximately 5% [5] to 0.06% in 2016 (Unpublished data, Utah Veterinary Diagnostic Laboratory [UVDL], 2016). Identification of carrier bulls is accomplished by collection of preputial scraping samples followed by either: 1) protozoal culture and microscopic identification, 2) protozoal culture followed by polymerase chain reaction (PCR)-based identification, or 3) PCR-based identification alone [3,6-8]. Although protozoal culture has been historically considered the gold-standard method of detection, the reported sensitivity of culture for

identification of *T. foetus* varies (67.8% to 98.4%), depending on factors such as sample collection, transport, and specific culture methods employed [7-9].

The development of PCR for detection of *T. foetus* has resulted in a lower limit of detection, the ability to detect lower concentrations of trichomonads (T)/ml in preputial samples, and increased sensitivity compared to culture methods [3,6,10]. However, PCR assays are more expensive. Because PCR testing is more sensitive, pooled testing of preputial samples is of interest to beef producers and veterinarians as a means to reduce costs.

The use of pooled samples from multiple bulls for trichomoniasis detection has been evaluated previously [11]. Preputial scrapings from 187 virgin bulls and 150 steers in Canada, defined as true negatives, were evaluated for false-positive results for *T. foetus* using both culture and qPCR. Specificity was 98.8% (333/337 correctly tested negative) for culture and 100% (no false positives) for qPCR. The same study evaluated sensitivity of qPCR on pooled samples, using negative preputial scrapings spiked with *T. foetus* obtained from a test-positive (naturally infected) bull. Samples were formulated with trichomonads (T)/ml of 1000, 10,000, 100,000 and 1,000,000. From each T concentration, pools were created with one *T. foetus*-positive sample in pools of 2, 3, 5, 10, 20 and 25 total samples. Sensitivities for detection of trichomonads (detecting the presence of the one positive bull) for the sample pool ratios were: 1:10, 90.3%; 1:2, 1:15, 1:20, and 1:25, 93.5%; and 1:3 and 1:5, 96.8%. Overall sensitivity of the qPCR for detection of *T. foetus* was 94%. Differences in sensitivity among the different sizes of sample pools were not significant (P=0.70) [11].

Preputial samples from 305 non-virgin bulls were used in a Colorado study [12]. Solutions of 3,000, 300, 30, 3 and 0.3 T/ml were made from PCR *T. foetus*-positive bulls. The "individual bull sample" solutions of 3,000, 300, and 30 T/ml were 100% classified as detected (each concentration had 14/14 samples positive) by PCR. The 3 and 0.3 T/ml solutions were 86% (12/14) and 50% (7/14) detected as positive by PCR. The 305 original preputial samples were used to create 61 pooled samples with 5 different bulls in each pool, with 16 pools having one positive bull with 4 negative bulls, and 45 pools having 5 negative bulls. The composition of the pools was blinded to the technicians doing PCR testing. The concentration of T/ml in the original samples used to make the pools was not determined. Sensitivity of the PCR on 5-sample pools was 100% (16/16). Specificity was also 100%, but this value was calculated using culture as the "gold standard", which resulted in 2 sample pools being detected as positive for *T. foetus* that were culture-negative, resulting in an alternative calculation of specificity as 95.7% (45/47) [12].

The main objective of the study reported here was to determine the sensitivity (% of true positives detected as positives) and specificity (% of true negatives tested as negatives) of a qPCR for the detection of *T. foetus* in single spiked samples and compare to those same test characteristics for 5-sample pools (1 positive and 4 negative). Five logs of trichomonad concentration (T/ml) were tested. A secondary objective was to use qPCR Ct values from the spiked samples to estimate the proportion of *T. foetus*-positive bulls that naturally occur within each log of T/ml concentration, based on the positive bulls' Ct values.

Materials and Methods

Spiked single and pooled sample preparation

Live *T. foetus* (Biomed Diagnostics Inc., White City, OR) and In-Pouch (IP) (TF Media, Biomed Diagnostics Inc., White City, OR) culture media were purchased and 60 ml of a stock solution of 10,000 trichomonads (T)/ml in culture IP media was made. The trichomonad concentration was estimated using a hemocytometer. Using this 10,000 T/ml stock solution, 60 ml of 10,000, 1000, 100, 10 and 1 T/ml solutions were made using 10-fold serial dilutions. In-Pouch culture media was used as a *T. foetus*-negative solution. Forty samples of each of the following were prepared and tested by qPCR: negative single samples and negative 5-sample pools, 1 T/ml single samples and 5-sample pools (all pools consisted of 1 positive and 4 negative), 10 T/ml single samples and 5-sample pools, 100 T/ml single samples and 5-sample pools, 1000 T/ml single samples and 5-sample pools, and 10,000 T/ml single samples and 5-sample pools. Each pool of 5 samples was prepared by pipetting 1000 µl from the negative solution or 200 µl from the 1, 10, 100, 1000, or 10,000 T/ml solution was added to 800 µl of the negative solution to create a pooled sample volume of 1 ml.

Trichomonad concentrations in naturally infected bulls' preputial samples

The spiked 1 ml samples of the 5 concentrations of T/ml described above were used to determine the ranges of qPCR Ct values for samples within each log of trichomonad concentration. Diagnostic preputial samples in culture IP media pouches or tubes were incubated for 24 h at 37°C following collection by field veterinarians, then frozen and sent to the UVDL for qPCR testing. The Ct values of all bulls naturally infected with *T. foetus* and classified as positive or suspect at the UVDL from 2010 to 2016 were used to assign them to a log of T/ml concentration.

DNA extraction and qPCR

DNA was extracted and purified from one ml of single or pooled samples using a commercial extraction kit (NucleoMag 96 Genomic DNA from Tissue Kit, Macherey-Nagel, Inc. Bethlehem, PA) according to the manufacturer's instructions. The primers, probe, and reaction conditions were the same as described by McMillen and Lew, 2006 [10]. The reactions were run for 38 cycles on an Applied Biosystems 7500 Fast Real-Time PCR System. For diagnostic samples, samples with a Ct<38 were classified as positive. Samples with a Ct>38 were classified as negative.

Statistical analysis

Trichomonad concentration group and whether samples were single samples or 5-sample pools were both categorical variables. Whether trichomoniasis was detected (positive) or not detected (negative) was also a categorical variable. Statistically significant differences in detection of *T. foetus* between concentrations within the single or pooled sample categories, and between single or pooled samples within each T/ml concentration category were tested using Chi-square. The level of significance was α=0.05.

Results

Results are summarized in Table 1. The 50% sensitivity of qPCR testing single samples with 1 T/ml for *T. foetus* was significantly lower than the 100% sensitivity for single samples within all higher trichomonad concentrations (P<0.0001). For samples with 1 T/ml, the 15% sensitivity when testing 5-sample pools (1 positive and 4 negative) was significantly lower than the 50% sensitivity when testing single samples (P=0.01). For samples with 10 T/ml, the 65% sensitivity when testing 5-sample pools was significantly lower than the 100% sensitivity when testing single samples (P<0.001).

Trichomonads/ml	Single or Pooled Samples	Sensitivity
1	Single	20/40 (50%)[a]
1	5-sample pool	6/40 (15%)[b]
10	Single	40/40 (100%)[c]
10	5-sample pool	26/40 (65%)[d]
100	Single	40/40 (100%)[c]
100	5-sample pool	40/40 (100%)[e]
1000	Single	40/40 (100%)[c]
1000	5-sample pool	40/40 (100%)[e]
10,000	Single	40/40 (100%)[c]
10,000	5-sample pool	40/40 (100%)[e]

Table 1: Sensitivity of qPCR for detection of Tritrichomonas fetus in single spiked samples or 5-sample pools (1 positive and 4 negative) by trichomonad concentration. [a,b]Significantly lower sensitivity for 5-sample pools (1 positive and 4 negative) than for single samples with 1

trichomonad (T)/ml, P=0.01. [a,c]Significantly lower sensitivity for single samples with 1 T/ml than for single samples with higher trichomonad concentrations, P<0.0001. [c,d]Significantly lower sensitivity for 5-sample pools than for single samples with 10 T/ml, P<0.001. [b,d,e]Significantly lower sensitivity for 5-sample pools with 1 T/ml or 10 T/ml than pooled samples with higher trichomonad concentrations, P<0.0001. All other sensitivity values of 100% were not different.

Considering only 5-sample pools, the 15% sensitivity for 1 T/ml (P<0.0001) and the 65% sensitivity for 10 T/ml (P<0.0001) were significantly lower than the 100% sensitivity for pooled samples within all higher trichomonad concentrations. Specificity of qPCR when tested on single (n=40) or 5-sample pooled (n=40) negative samples was 100% (all test results were "not detected").

The Ct values of the 130 naturally infected *T. foetus*-positive bulls detected at UVDL from 2010 - 2016 were used to classify each bull within a log of T/ml, based on the ranges of Ct values within each log of the spiked samples. The Ct ranges that defined each trichomonad concentration were: 1 T/ml, Ct 35.0 to 37.99; 10 T/ml, Ct 32.0 to 34.99; 100 T/ml, Ct 28.5 to 31.99; 1000 T/ml, Ct 25.5 to 28.49; 10,000 T/ml, Ct<25.5. Based on these criteria, the proportions of all bulls within the logs of T/ml were: 1 T/ml, 10 bulls (7.7%); 10 T/ml, 17 bulls (13.1%); ≥ 100 T/ml, 103 bulls (79.2%). The latter group of 103 bulls, all of which had 100% test sensitivity by either method, consisted of: 100 T/ml, 27 bulls (20.7%); 1000 T/ml, 36 bulls (27.7%); 10,000 T/ml, 40 bulls (30.8%). The overall population sensitivity for qPCR detection of *T. foetus* in preputial scrapings from bulls was calculated, accounting for the proportions of bulls within trichomonad concentrations above. For single samples: 7.7% of positive bulls (1 T/ml) × 50% sensitivity (0.5 × 7.7%)=3.9% detected. All of the 92.3% remaining positive bulls (≥ 10 T/ml) were detected (100% sensitivity). Therefore the proportion of all positive bulls detected, the sensitivity of single sample qPCR testing=3.9%+92.3%=96.2%. For 5-sample pools (1 positive and 4 negative): 7.7% of positive bulls (1 T/ml) × 15% sensitivity (0.15 × 7.7%)=1.2% detected; 13.1% of positive bulls (10 T/ml) × 65% sensitivity (0.65 × 13.1%)=8.5% detected; all of the 79.2% remaining positive bulls (≥ 100 T/ml) were detected. Therefore the proportion of all positive bulls detected, the sensitivity of 5-sample pool qPCR testing=1.2%+8.5%+79.2%=88.9%. The proportion of all *T. foetus*-positive bulls not detected when using 5-sample pools compared with that using single samples=(96.2%-88.9%)=7.3%.

Discussion

There was some loss of overall sensitivity of qPCR for detection of *T. foetus* infected bulls when 5-sample pools were tested compared with single samples. The greatest loss of sensitivity associated with pooling (or when testing single samples, but less so) was for bulls with 1 T/ml. However, because these low trichomonad concentration bulls represented less than 8% of the total population, the overall sensitivity of qPCR for detection of trichomoniasis was high using either pooled (89%) or single (96%) samples.

Beef producers and veterinarians wishing to minimize the probability of failing to detect any bull infected with *T. foetus* should continue to test bulls using individual preputial scraping samples. If a group of bulls were tested that contained a higher proportion of bulls with 1 T/ml or 10 T/ml than the population mean, the loss of sensitivity with pooling would also increase. However, there is reasonable evidence for testing bulls for trichomoniasis with qPCR using pooled samples from 5 bulls.

Acknowledgements

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