Pooling of *Tritrichomonas foetus* Cultured Samples Followed by MagMAX™ Sample Preparation System and Amplification with Applied Biosystems qPCR Reagents.

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ABSTRACT

The objectives of this study were 1) to compare multiple sample preparation workflows (boiling, QIAGEN, MagMAX™) and real-time PCR assays currently used by diagnostic testing labs with MagMAX™ and Applied Biosystems VetMAX™ reagents for individual *T. foetus* testing, and (2) to assess the feasibility of pooling smegma samples followed by MagMAX™ sample preparation systems and amplification with VetMAX™ *T. foetus* reagents. Cultured smegma samples were collected from different regions of the United States with the collaboration of 5 diagnostic testing laboratories coded as A, B, C, D, and F. A total of 806 samples were collected and processed individually by their corresponding lab workflow systems. Additional aliquots of each sample were sent to the KSVDL study laboratory for individual DNA extraction with MagMAX™ Total Nucleic Acid Purification Kit and tested with Applied Biosystems VetMAX™ *T. foetus* reagents. Results from individual testing of all samples at the study laboratory showed 31 of 806 discrepant results between the diagnostic test laboratories and the study laboratory utilizing the Applied Biosystems workflow. Of the 31 discrepant results, 21 positives (C_T18.21-38.10) were detected with the Applied Biosystems workflow at the study laboratory but were not by A, B and D laboratories implementing boiling and homebrew qPCR. Only 1 positive sample was missed using the Applied Biosystems workflow by Lab C. No call differences were identified between the Applied Biosystems workflow at study laboratory and laboratory F using the Qiagen sample prep followed by homebrew real-time PCR. Conversely, a total of 9 discrepant negative samples were identified as positive by the boiling workflow method from laboratories A, B and D.

A total of 176 confirmed positives and 610 negative samples were tested using a pooling protocol. Positive pools of 1:5 (n=176) and negative pools of 5 (n=50) were constructed followed by MagMAX™ Total Nucleic Acid Purification and amplified with VetMAX™ *T. foetus* reagents. Results from pooling showed that individual suspect positive samples with Ct>38 were not detectable into 1:5 pools. At the same time, 4 out of 11 samples with individual Ct between 35.05, and 37.83 became negative calls in the 1:5 pools and 3 out of 11 samples became negative calls in the 1:3 pools.

INTRODUCTION

Bovine trichomoniasis is a sexually transmitted infection caused by *Tritrichomonas foetus* resulting in significant monetary losses to the cattle industry worldwide. *T. foetus* is a flagellated protozoan found in bovines that colonizes the uterine, vaginal and preputial epithelium, resulting in early embryonic death, abortion, and infertility. Although bulls are the main carriers of *T. foetus*, they remain asymptomatic for their entire life (Corbeil *et al.* 2010). Multiple sample preparation and real-time PCR workflows are currently used in diagnostic laboratories across the United States for the diagnosis of *T. foetus.* This may lead to discrepant calls, thus the need to standardize a single method is desired. Also, an increased interest in pooling *T. foetus* smegma samples is desired by private and government diagnostic labs to save time, labor, and reagent costs during mandatory government eradication programs or monitoring status in clean herds. The feasibility of pooling these samples for nucleic acid extraction and quantitative real real-time PCR has not been well researched, and the optimal number of pooled samples and volumes has not being determined. Robust studies needed to be conducted to determine the number of samples and volumes to be pooled that can accurately lead to positive and negative *T. foetus* calls. Little is known about the differences in individual sample testing between diagnostic testing labs and the effect of pooling smegma samples for the detection of *T. foetus*. A study to optimize the *T. foetus* detection and compare multiple workflows was proposed by the Parasitology Committee of AAVLD in 2010. Thus, in the present study we compared (1) the different sample preparation systems and homebrew real-time PCR with the MagMAX™ Total Nucleic Acid Purification kit and amplification with VetMAX™ *T. foetus* reagents and (2) the effect of pooling 1 positive sample with multiple Ct ranges and 4 negatives in a final input volume of 300 µL for MagMAX™ and amplification with VetMAX™ *T. foetus* reagents.

MATERIALS AND METHODS

Sample Collection

Eight hundred and six (806) smegma samples from multiple breeds were provided by 5 blinded diagnostic laboratories from across the United States as follows: Lab A contributed 374 samples, lab B contributed 100, lab C contributed 63, lab D contributed 50 and lab F 219 samples. Three procedures were followed for sample handling by the diagnostic testing laboratories: NF= In Pouch (IP) samples that have experienced one freeze/thaw cycle before shipping, FZ=IP samples that have experienced two freeze/thaw cycles before shipping, and FZ2=IP samples that have experienced three freeze/thaw cycles before shipping. Table 1 shows the summary of the different sample preparation and real-time workflows used from each laboratory.

Extraction Method

Three main sample preparation systems for the smegma samples were utilized. Lab A, B and D used boiling sample preparation methods. Laboratory C used the MagMAX™ sample preparation system, and Laboratory F used Qiagen columns. For all samples, the KSVDL study laboratory performed sample extraction with the MagMAX™ and amplified with VetMAX™ *T. foetus* reagents.

Real-Time PCR

Laboratories A and B ran McMillen's real-time PCR assay, Laboratory C and D used VetMAX™ *T. foetus* reagents. Laboratory F used a modified version of McMillen's qPCR assay and the study laboratory also tested the samples with the VetMAX™ *T. foetus* reagents. A total of 14 suspect samples were re-run in triplicate by real-time PCR in accordance with the following suspect workflow. Samples with 38 ≤ Ct <40 Ct were first identified as suspect and the extracted nucleic acid was re-tested in triplicate. If 1/3 replicates had a Ct <40 the sample was identified as suspect positive. If ≥ 2 replicates had a Ct of <40, the sample was called positive. Initial testing results by the diagnostic testing laboratories were not identified to the study laboratory but sent directly to the Study Coordinator, Dr. Marilyn Simunich, to be identified and unblinded for the development of a pooling strategy. Table 2 shows a summary of the individual sample testing by the diagnostic testing laboratories and the study laboratory with Applied Biosystems MagMAX™ and amplification with VetMAX™ *T. foetus* reagents.

Pooling Strategy

In the pooling workflow, both *T. foetus* positive and negative smegma samples were pooled to a final volume of 300 µL to be used for DNA extraction with the MagMAX™ and amplification with the VetMAX™ *T. foetus* reagents. 60 µL of individual smegma samples were added to a single well of a deep well extraction plate to create a pool of 5 animals consisting of 1 positive and 4 negative *T. foetus* samples. All positive and suspect positive samples identified at the study laboratory by individual testing were used to create 1:5 positive pools. The negative samples (n=629) were used to construct the positive (n=176) and negative (n=50) pools. The total number of pools from each testing laboratory was as follows:

Lab A: n=77, Lab B: n=31, Lab C: n=13 Lab D: n= 21 and Lab F: n= 34 each pool was constructed with positive and negative samples from the same laboratory. Table 4 shows the summary of the positive and negative samples provided by each of the diagnostic testing laboratories. For the negative samples, laboratory F was the only one having enough negative samples to complete 34 positive pools without reusing any samples. Laboratories A, B, C & D did not have enough negative samples, thus the negative samples were used multiple times to complete the construction of positive pools. The negative pools (n=50) were constructed using samples from Laboratories A and F, where the most number of negative samples were found. Figure 2 depicts the pooling strategy for each of the samples from the diagnostic testing laboratories. 1:3 Pooling was performed for samples with individual Ct>35 (n=34), where 100 µL of individual smegma samples were added to a single well of a deep well extraction plate to create a pool of 3 animals consisting of 1 positive and 2 negatives. Lastly, 1:3 negative pools were also constructed (n=34) where 100 µL of 3 negative samples where pooled.

RESULTS

Table 1. *T. foetus* testing methods at diagnostic testing and study laboratories.

Lab ID	Freeze/ Thaw	Sample Prep	InPouch Vol (uL)	PAC	PEC	Real-Time PCR	Cutoff Ct
A	NF	Boiling	400	No	No	McMillen's Assay	<35=pos, & 35-40=inconclusive
B	NF	Boiling	400	No	No	McMillen's Assay	<35=pos, & 35-40=inconclusive
C	FZ / Z2	MagMAX™	300	Yes	XENO	VetMAX™ *T. foetus* reagents	≤36.0=pos, >36.0 to ≤40=Inconclusive, & >40=negative
D	NF	Boiling	250	No	No	VetMAX™ *T. foetus* reagents	<38=pos, 38-39 suspect, & 40=negative
F	FZ	Qiagen	200	No	No	Modified McMillen's Assay	<37=Pos
Study lab	-	MagMAX™	300	T.foetus/ XENO	XENO	VetMAX™ *T. foetus* reagents	<37.9=Positive, suspect if value is 38 ≤ 40 & 40=negative

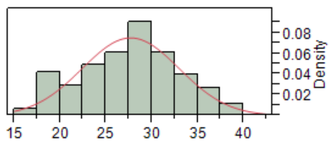
Table 1. Summary of the different sample preparation and real-time PCR methods utilized by diagnostic testing and study laboratory for sample testing. Three different methods were used by the diagnostic testing laboratories for sample collection and handling, sample preparation, and real-time PCR. Also, 4 different input volumes for various sample preparation systems were used. PEC= Positive extraction control to monitor for extraction efficiency and PCR inhibition. PAC= Positive added control to set up threshold. NF= In Pouch (IP) samples that have experienced one freeze/thaw cycle before shipping, FZ=IP samples that have experienced two freeze/thaw cycles before shipping, and FZ2=IP samples that have experienced three freeze/thaw cycles before shipping.

Table 2. Summary of *T. foetus* testing of individual samples at the diagnostic testing laboratories and study laboratory.

Lab ID	Sample Prep	Pos/Pos	Pos/Neg	Pos/Sus	Neg/Pos	Neg/Sus	Sus/Pos	Sus/Sus	Sus/Neg	Neg/Neg	Total
A	Boiling	67	9	0	2	0	1	0	3	292	374
B	Boiling	21	8	2	7	4	0	0	0	58	100
C	MagMAX™	9	1	1	0	0	0	3	3	46	63
D	Boiling	17	4	0	0	0	0	0	0	29	50
F	Qiagen	34	0	0	0	0	0	0	0	185	219
	Total	148	22	3	9	4	1	3	6	610	806

Table 2. Results from individual testing of all samples (n=806). Thirty one samples were discrepant between the diagnostic test laboratories and the study laboratory utilizing the Applied Biosystems workflow.
21 positive samples (C_T 18.21-38.10) were detected with VetMAX™ *T. foetus* reagents but were not detected by diagnostic testing laboratories A, B and D implementing boiling and homebrew real-time PCR.
One positive sample amplified at the study laboratory was missed by Lab C although both used the same Applied Biosystems workflow. No call differences were detected between the Applied Biosystems workflow at the study laboratory and lab F using Qiagen columns and homebrew real-time PCR (modified McMillen). 9 samples positive at the testing laboratory using the boiling workflow and homebrew qPCR were missed by the study laboratory.
All suspect samples were re-run in triplicate with the suspect workflow. Order of the call = i.e. Pos/Pos (1) study laboratory/ (2) diagnostic testing laboratory. Pos = Positive, Neg = Negative, and Sus = Suspect.
Suspect = value is 38 ≤ Ct <40. In the suspect workflow the samples were re-run in triplicate by real-time PCR and if Ct <40 = 1 is a suspect positive and Ct <40 = 2 of 3 = positive.

Figure 1 and Table 3. Distribution of all positive samples tested with the Applied Biosystems workflow.



Statistics of Positive Samples	Ct
Mean	27.65
Std Dev	5.28
Std Err Mean	0.39
Upper 95% Mean	26.86
Lower 95% Mean	28.43
Maximum	39.95
Minimum	15.62
N	176

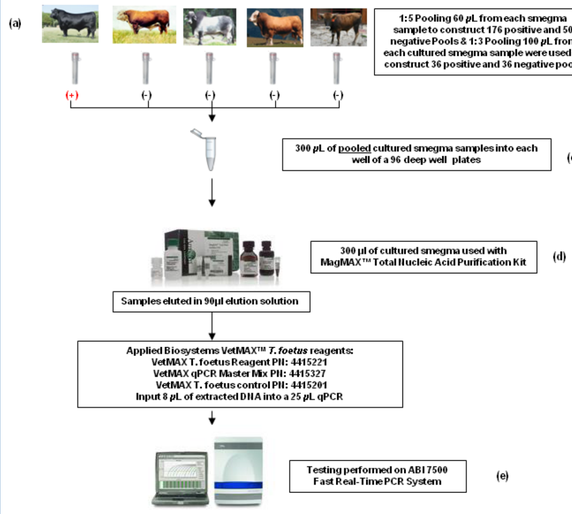
Figure 1. Distribution of positive samples from the 5 diagnostic testing laboratories. The mean Ct value found in the positive samples processed with the Applied Biosystems workflow was 27.65.

Table 4. Positive, suspect positive and negative samples used from each lab for pooling.

Laboratory ID	Positives Available	Neg Available	Negatives Needed	Surplus/Deficit
A	77	292	308	-16
B	31	58	124	-66
C	13	46	52	-6
D	21	29	84	-55
F	34	185	136	+49
Total	176	610	704	

Table 4. Total number of positive and suspect/positive samples used for pooling from the 5 diagnostic testing laboratories (n=176). Laboratories A, B, D did not have enough negative samples for 1:5 pools, therefore re-use of the negative samples was necessary to complete the positive pools from each laboratory.

Figure 2. Pooling Strategy



(a) One positive and 4 negative bull smegma samples were pooled into a deep well plate.
(b) 176 positive and 50 negative pools were constructed for the 1:5 pooling, and 36 positive and 36 negative pools for the 1:3 pooling.
(c) 300 uL of pooled input were used for MagMAX™ Total Nucleic Acid Purification Kit.
(d&e) 8 uL of the 90 uL of eluted DNA were used for real-time PCR using Applied Biosystems VetMAX™ *T. foetus* reagents.

Figure 3. 1:5 pooling results by Ct range

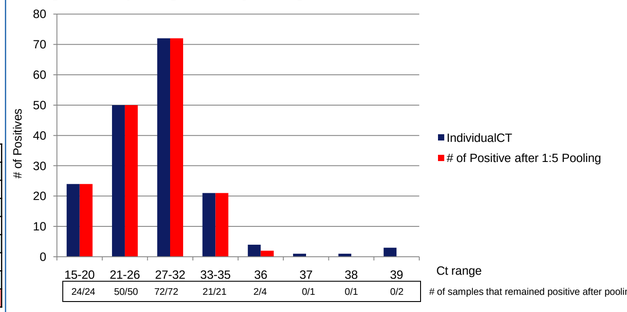


Figure 3. Ct ranges and number of positive samples shown in blue. Positive samples that remained positive in the pools (Ct<40) are shown in red.

Table 5. Effect of pooling for *T. foetus* samples with Ct>35 after individual testing.

Sample ID	Individual test Ct	Pooled1:5 Test Ct	Pooled1:3 Test Ct	Individual call	Pooled 1:5 call	Pooled 1:3 call
C-6-11	35.05	Undetected	Undetected	Positive	Negative	Negative
A-39-2	35.4	35.93	34.82	Positive	Positive	Positive
F-1-7	35.42	35.43	35.5281	Positive	Positive	Positive
F-18-1	35.52	36.16	34.7483	Positive	Positive	Positive
C-1-23	35.93	35.75	35.8208	Positive	Positive	Positive
B-8-4	36.01	34.91	35.5909	Positive	Positive	Positive
F-19-10	36.29	33.48	32.8247	Positive	Positive	Positive
B-4-1	36.44	Undetected	Undetected	Positive	Negative	Negative
A-27-9	38.1	Undetected	Undetected	Positive	Negative	Negative
C-6-10	37.2	Undetected	37.6866	Positive	Negative	Positive
A-40-5	37.83	37.83	37.859	Positive	Positive	Positive
C-4-5	38.81	Undetected	Undetected	Suspect Positive	Negative	Negative
A-24-10	38.85	Undetected	Undetected	Suspect Positive	Negative	Negative
C-6-15	39.53	Undetected	Undetected	Suspect Positive	Negative	Negative

Table 5. Number of samples with individual Ct of 35 and above that were called positive and negative after pooling. From the total number of positive samples individually detected by Applied Biosystems workflows, 4% were not able to be detected when pooling 1:5 and 3.5% when 1:3 pooling.

CONCLUSIONS

Individual testing

A total of 806 smegma samples were collected by 5 diagnostic laboratories testing for *T. foetus* for this study.
All the samples were tested by the laboratories with their test method and re-tested at KSVDL which served as the study laboratory for the pooling study.
Complete agreement was reached between the 5 diagnostic laboratories and the study laboratory for 758 samples (94%).
The study laboratory using the Applied Biosystems MagMAX™ and VetMAX™ *T. foetus* reagents amplified 21 samples that the testing diagnostic laboratories A, B & D missed, and missed 9 samples reported as positive by those laboratories.

Pooling testing

Positive results were obtained from all 1:5 pools with samples having a Ct <35 after individual testing.
7 of 14 positive and suspect positive samples with Cts between 35-39.53 were detected in 1:5 pools.
8 out of 14 positive samples with Cts between 35-39.53 were detected in 1:3 pools
Pooling at 1:5 dilution is therefore likely to miss 4% of *T. foetus* positive samples (7/176) and 1:3 pooling, 3.5% (6/176).

The results of this field study indicate that the Trichomonas Foetus DNA Test Kit produces acceptable results with respect to sensitivity and specificity on individual and pooled smegma samples isolated from culture.
Raw data and a report summarizing this study will be filed with USDA-CVB in support of a Veterinary Biological Product License application. These data support further validation activities for the Trichomonas Foetus DNA Test Kit.

REFERENCES

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TRADEMARKS/LICENSING