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Development and Validation of the VetMAX™-Gold MAP Detection Kit

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent for Johne's disease in cattle and causes severe economic losses in the cattle industry due to reduced productivity, reproductive losses, and the eventual death or culling of the infected animal.

The VetMAX™-Gold MAP Detection Kit is a real-time PCR assay for the rapid *in vitro* detection of MAP DNA purified from bovine feces. The assay targets a unique sequence element in the MAP genome to provide highly sensitive and specific results. The purpose of this study is to determine the performance characteristics of the VetMAX™-Gold MAP Detection Kit in detecting MAP DNA from nucleic acid extracted from individual and pooled bovine fecal samples.

The VetMAX™-Gold MAP Detection Kit was evaluated with both individual and pooled (n=5) bovine fecal samples from diverse geographic regions which represented a range of MAP infectivity including heavy, moderate, and light shedders to determine diagnostic sensitivity and specificity. The reproducibility and repeatability of the diagnostic results were also evaluated across multiple testing laboratories, kit lots, and testing days to ensure the diagnostic results were robust to common testing variations. Both studies were performed in support of USDA licensure.

The results of testing show the VetMAX™-Gold MAP Detection Kit produced diagnostic sensitivity and specificity values of 96.2% and 96.4%, respectively, when testing individual fecal samples, as compared to culture. Pooled sample tested with the VetMAX™-Gold MAP Detection Kit resulted in diagnostic sensitivity and specificity values of 96.2% and 100%, respectively, as compared to culture. There was also 100% concordance in diagnostic call during reproducibility and repeatability testing. The study indicates that the VetMAX™-Gold MAP Detection Kit, provides an economical and rapid solution for MAP detection from both individual and pooled fecal samples.

MATERIALS AND METHODS

Two studies were performed in support of USDA licensure. The results of this study are under review by APHIS' Center for Veterinary Biologics in support of a Biological Product License application. In the Sensitivity and Specificity (S&S) study, a panel of bovine fecal samples that had been previously characterized by culture were tested with the VetMAX™-Gold MAP Detection Kit workflow by a collaborator laboratory. Both individual as well as pooled fecal samples were tested. In a separate study, the Reproducibility and Repeatability (R&R) study, a panel of bovine fecal samples were sent to three collaborator laboratories for repeated testing under varying conditions to ensure repeatable diagnostic calls were achieved.

For the Sensitivity and Specificity (S&S) study, a collaborator laboratory purified nucleic acid from bovine fecal samples using the MagMAX™ Total Nucleic Acid Isolation Kit. MAP bacterium was physically and chemically lysed by homogenizing the fecal supernatant using the FastPrep®-24 homogenizer in the presence of lysis solution. 5,000 copies/reaction of Xeno™DNA Control was spiked into the lysis solution of each purification to monitor for inhibition. Samples were processed using the MagMAX™ Express-96 Deep Well Magnetic Particle Processor. 8 µL of extracted nucleic acid was tested with using the VetMAX™-Gold MAP Detection Kit on the 7500 Fast Real-Time PCR system according to the Instructions for Use.

Table 1. Individual Sample Culture Characterization

Characterization Method	Bacterial Titer/Shedding Status	Number of Samples (n)	% of Total MAP-Positive Pool Samples
TREK ESP™ Culture System II	Heavy Shedder	24	19%
	Moderate Shedder	17	13%
	Light Shedder	34	27%
	Negative by culture*	1	1%
PARACHECK™ 2 ELISA Kit	Strong Positive	26	21%
	Positive	12	10%
	Low Positive	12	10%

*Sample was later confirmed to by MAP-positive by an alternative qPCR assay.
The VetMAX™-Gold MAP Detection Kit was evaluated with 126 individual MAP-positive and 134 individual MAP-negative bovine fecal samples. The MAP status of each sample was confirmed with culture prior to the start of the study. MAP samples were sourced from diverse geographic regions and represented a range of MAP infectivity including heavy, moderate, and light shedders.

Table 2. Pooled Sample Culture Characterization

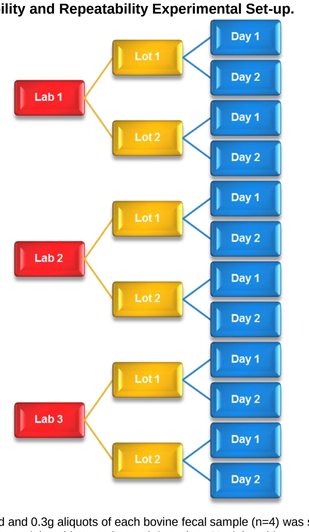
Characterization Method	Bacterial Titer/Shedding Status	Number of Samples (n)	% of Total MAP-Positive Pool Samples
TREK ESP® Culture System II	Heavy Shedder	7	14%
	Moderate Shedder	12	24%
	Light Shedder	31	61%
	Negative by culture*	1	2%

*Sample was later confirmed to by MAP-positive by an alternative qPCR assay.

The feasibility of pooling up to 5 bovine fecal samples into a single nucleic acid extraction and detection test was evaluated by testing 51 MAP-positive pools and 24 MAP-negative pools. All pools consisted of 5 individual fecal samples. 49 positive pools were created by combining 1 MAP-positive sample with 4 MAP-negative samples. 2 positive pools were created by combining 2 MAP-positive samples with 3 MAP-negative samples.. Positive samples within pools represented a range of infectivity with 61% coming from "light shedders". Negative pools consisted of 5 MAP-negative samples.

For the Reproducibility and Repeatability study, three collaborator laboratories were provided with a panel of 16 MAP-positive and 4 MAP-negative bovine fecal samples. Each laboratory purified nucleic acid from bovine fecal samples using the MagMAX™ Total Nucleic Acid Isolation Kit as described in the Sensitivity and Specificity test methods and 8 µL of extracted nucleic acid was tested with using the VetMAX™-Gold MAP Detection Kit on the 7500 Fast Real-Time PCR system according to the Instructions for Use. Each sample was extracted in duplicate and tested with two pre-license serial kit lots and two different days by each lab (Figure 1).

Figure 1. Reproducibility and Repeatability Experimental Set-up.



The R&R panel was blinded and 0.3g aliquots of each bovine fecal sample (n=4) was shipped to the three collaborating laboratories for nucleic acid extraction and detection. Nucleic acid was tested by three laboratories with two pre-license serials on two different days. Samples were obtained from four (4) different states and represented a range of shedding status including high positive (30%), medium positive (30%), low positive (20%) and negative (20%).

RESULTS

Table 3. Individual Sample S&S Results

True Sample Characterization	# of Samples	VetMAX™ Gold MAP Detection Kit Initial Call			VetMAX™ Gold MAP Detection Kit (Final Call)		
		Positive	Negative	Suspect	Positive	Negative	Presumptive Positive
Positive	126	116	5	5	121	5	0
Negative	134	2	121	11	5	128	1

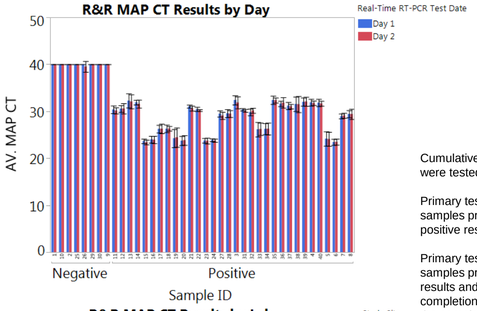
Primary testing of 126 MAP-positive samples yielded 116 initial positive results, 5 suspect results, and 5 discrepant results. Primary testing of 134 MAP-negative samples produced 120 initial negative results, 11 suspect results, and 3 discrepant results. All suspect and discrepant samples were run through their respective workflows. 12 of the 16 suspect samples produced a correct final call, 3 produced a false-positive call, and 1 produced a presumptive positive call. 1 initial discrepant call turned out to be correctly identified upon discrepant testing (false-negative for culture).

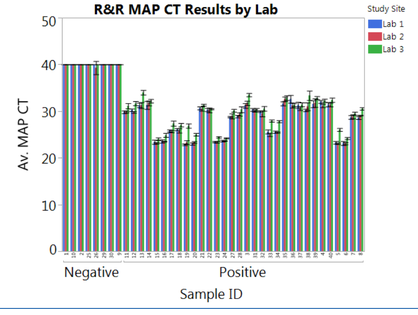
Table 4. Pooled Sample S&S Results

True Sample Characterization	# of Pools	VetMAX™ Gold MAP Detection Kit call after initial pool testing			VetMAX™ Gold MAP Detection Kit call after individual sample testing (Final Call)		
		Positive	Negative	Suspect	Positive	Negative	Presumptive Positive
Positive	51	46	2	3	48	2	0
Negative	24	0	23	1	0	24	0

Primary testing of 51 MAP-positive pools yielded 46 initial positive results, 3 suspect results, and 2 discrepant results. Primary testing of 24 MAP-negative pools produced 23 initial negative results and 1 suspect result. All suspect and discrepant samples were run through their respective workflows. All four suspect pools were confirmed correct (testing of individual samples). 2 samples produced a false-negative result.

Figures 2 and 3. R&R Study results by Day and Lab.





Cumulatively, 480 total samples were tested during the R&R study.

Primary testing of 384 MAP-positive samples produced 384 MAP-positive results.

Primary testing of 96 MAP-negative samples produced 95 MAP-negative results and 1 suspect result. Upon completion of the suspect workflow, the sample was confirmed to be MAP-negative.

100% concordance of diagnostic calls between all testing conditions was observed during R&R testing.

CONCLUSIONS

Results of field testing demonstrate that the VetMAX™-Gold MAP Detection Kit is a highly sensitive and specific kit for the detection of MAP DNA from bovine feces. The kit provides an economical and rapid solution for MAP detection from both individual and pooled fecal samples. The VetMAX™-Gold MAP Detection Kit demonstrated excellent sensitivity and specificity when testing DNA isolated from diagnostic bovine fecal samples (n=260)

The test kit demonstrated 96.2% sensitivity and 96.4% specificity when testing individual samples. The assay also demonstrated a 96.2% predictive value of a positive test and 96.4% predictive value of a negative test for individual bovine fecal samples. Samples that were missed contained very low titers of the MAP bacteria near the workflow's limit of detection. The test kit demonstrated 96.2% sensitivity and 100% specificity when testing pools containing five (5) bovine fecal supernatant samples. Pools that were missed contained individual samples with very low titers of the MAP bacteria (CT>35) near the workflow's limit of detection.

Table 5. Final S&S Results

Performance Characteristic	Individual Sample Result	Pooled Sample Result
% Sensitivity	96.2%	96.2%
% Specificity	96.4%	100%
Predictive value of a Positive Test	96.2%	100%
Predictive value of a Negative Test	96.4%	96.0%

The kit demonstrated 96.2% sensitivity for individual and pooled (n=5) samples. 96.4% specificity was seen with individual samples and 100% for pooled samples (n=5).

The VetMAX™-Gold MAP Detection Kit robustness was demonstrated with 100% diagnostic call concordance between three different testing labs with two kit lots on tested on two separate days. The repeatability and reproducibility of the VetMAXTM-Gold MAP Detection Kit is excellent for a qualitative assay and the results demonstrated high levels of precision for inter and intra-laboratory use.

Table 6. Final R&R Results

True Sample Characterization	# of Samples	VetMAX™-Gold MAP Detection Kit call after initial testing			VetMAX™-Gold MAP Detection Kit call after suspect workflow testing (Final Call)		
		Positive	Negative	Suspect	Positive	Negative	Presumptive Positive
Positive	384	384	0	0	384	0	0
Negative	96	0	95	1	0	96	0

100% agreement was seen in this study with R&R testing across 4 isolation replicates in three labs, two pre-license serials, and two days each.

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TRADEMARKS/LICENSING

The results of this study are under review by APHIS' Center for Veterinary Biologics in support of a Biological Product License application.

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