

# Correlation between a commercial real-time PCR assay and HEYM culture for MAP in bovine faecal samples



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## ABSTRACT

Disease control programmes for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) rely on accurate and sensitive tools for the detection of infected animals. Culture based detection of MAP takes many weeks whereas PCR enables rapid detection. Several commercial and many user designed real-time PCR assays exist for the detection of MAP in bovine faecal samples. We selected one commercial assay, the VetMAX<sup>TM</sup> MAP Real-Time PCR Screening Kit (Life Technologies), and calculated the correlation between real-time PCR threshold cycle (Ct) values and colony-forming units (CFU) on Herrold egg yolk medium (HEYM) culture, using different nucleic acid extraction kits. Results of HEYM culture of 40 faecal samples were negatively (inversely) correlated with their respective real-time PCR results. The Spearman's rank correlation between Ct and CFU ranged from good (0.67) to excellent (0.93), depending on which nucleic acid extraction kit was used. The MagMAX<sup>TM</sup> Total Nucleic Acid Isolation Kit (Life Technologies) and the InviMag<sup>®</sup> Stool DNA Kit (Invitex) produced the best correlations with HEYM culture. These results suggest that this real-time PCR assay is a useful alternative to culture on HEYM.

## INTRODUCTION

Numerous studies have been conducted to evaluate the accuracy (sensitivity and specificity) of the tests available to detect MAP.<sup>1</sup> Culture on HEYM has for a long time been considered the gold standard test for MAP because it is highly specific and more sensitive than either milk or serum ELISA testing.<sup>2</sup> However culture is a very slow process with results taking 6 weeks or more. Recent studies have shown that real time PCR is a highly accurate alternative to HEYM for the detection of MAP in bovine faeces.<sup>2,3</sup>

The VetMAX<sup>TM</sup> MAP Real-Time PCR Screening Kit is a complete set of reagents for a simple real-time PCR assay. This assay is intended for use in the rapid, in vitro detection of MAP DNA purified from bovine feces. The assay targets a sequence element in the MAP genome to provide highly sensitive and specific results. This study looked at comparing the performance of different nucleic acid extraction kits when coupled with this screening kit to traditional HEYM culture for the detection of MAP in bovine faeces.

## MATERIALS AND METHODS

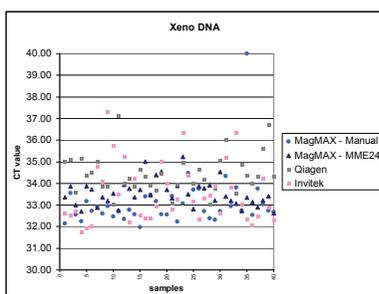
In the first part of this study, 40 bovine faecal samples were supplied by the Oregon Department of Agriculture Animal Health Laboratory. The MAP culture status of these samples had been determined in the Oregon laboratory using HEYM. Faecal samples were transported to Austin and stored at -20°C. For sample preparation, the following DNA isolation kits were used: MagMAX<sup>TM</sup> Total nucleic acid purification kit (Life Technologies), InviMag<sup>®</sup> Stool DNA Mini Kit (Invitex) and the QIAamp<sup>®</sup> DNA Stool Mini Kit (Qiagen). The kits were used according to the manufacturers' instructions.

The MagMAX<sup>TM</sup> purifications were performed manually and using an automated platform, the MagMAX<sup>TM</sup> Express - MME24 (Life Technologies). The InviMag purifications were performed on an automated platform, the KingFisher 96 instrument (Thermo Electron). The Qiagen purifications were performed manually.

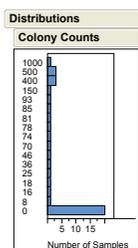
In order to monitor extraction efficiency and to enable detection of PCR inhibitors, 1 µL of Xeno<sup>TM</sup> DNA Control (5,000 copies/µL) was added per isolation to the lysis solution. Presence of PCR inhibitors would be seen by failure of both MAP Control DNA and Xeno<sup>TM</sup> DNA Control to amplify. The real-time PCR was set up, according to the manufacturer's instructions. Briefly, 25 µL reactions were prepared, and the assay was run on the Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies).

In the second part of this study, data gathered from the USDA John's disease proficiency panels from 2008 – 2011 was collated. The panels were assessed in the year of their issue using the MagMAX<sup>TM</sup> Total nucleic acid purification kit and the VetMAX<sup>TM</sup> MAP Real-Time PCR Screening Kit. Results of the testing, including the average CFU/tube for each sample which was determined by the National Veterinary Services Laboratory (NVSL) using HEYM, were collated. Colony counts were determined averaging results from 3 cultures for each cow. For high shedders the inoculum was diluted 10-x until colony counts were under 100 per tube. For statistical analyses, JMP<sup>®</sup> software was used. We used Spearman's rank correlation to assess the relationship between CT value and colony count. This statistic is a non-parametric measure of the strength and direction of association that exists between two variables measured. It has been used in the literature for similar comparisons.<sup>3</sup>

## RESULTS



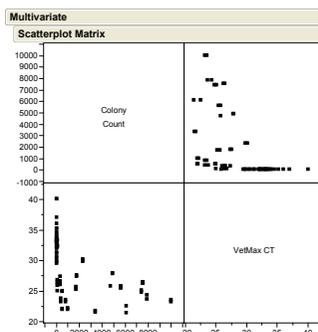
**Figure 1:** As a measure of extraction efficiency, Xeno<sup>TM</sup> DNA detection in samples was assessed. Sample 35 which is the only sample to give a negative Xeno<sup>TM</sup> DNA result on the MagMAX<sup>TM</sup> Manual extraction, was negative for MAP DNA across all of the extraction kits and platforms. The detection of Xeno<sup>TM</sup> DNA also provides a monitor for PCR inhibition.



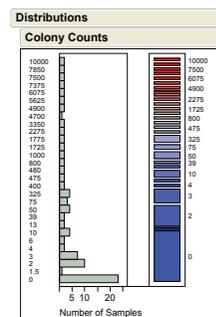
**Figure 2:** Histogram of the colony counts (CFU/tube) for 40 bovine faecal samples. There were 20 negative samples and 20 positive samples with colony counts ranging from 8 CFU/ml to 1000 CFU/ml.

**Table 1:** Spearman's rank correlation for colony count (CFU) and threshold cycle (CT) values of 40 samples tested for MAP using culture on HEYM and the VetMAX<sup>TM</sup> MAP Real-Time PCR Screening Kit, utilising different extraction kits. The criteria for interpreting the Spearman's rank correlation which ranges from -1 to +1 were as follows: greater than absolute value of 0.75 as excellent, less than absolute value of 0.40 as poor, and for absolute values between 0.40 and 0.75 as fair to good correlation.<sup>4</sup> \* all p values < 0.001

extraction kit	Spearman's rank correlation*	interpretation
Qiagen	- 0.676	good correlation
Invitex	-0.918	excellent correlation
MagMAX <sup>TM</sup> - Manual	-0.931	excellent correlation
MagMAX <sup>TM</sup> - MME24	-0.924	excellent correlation



**Figure 3:** Scatter plot matrix of colony count versus VetMAX<sup>TM</sup> CT value for 97 bovine faecal samples tested as part of the USDA John's disease proficiency panels from 2008 – 2011. Spearman's rank correlation between colony count and CT was -0.913 ( $p < 0.001$ ).



**Figure 4:** Histogram of the colony counts (CFU/tube) for 97 bovine faecal samples tested as part of the USDA John's disease proficiency panels from 2008 – 2011. There were 23 MAP negative samples and 64 samples with colony counts ranging from 1.5 CFU/tube to 10,000 CFU/tube.

## CONCLUSIONS

Results of the current study show that the correlation between quantitative MAP results from real-time qPCR (CT) and culture on HEYM (CFU) was excellent when using either the MagMAX<sup>TM</sup> or Invitex extraction kits, and good if using the Qiagen kit. The negative correlation estimates are the result of the inverse association between viable MAP DNA as measured by CFU on HEYM culture, and the CT of real-time qPCR. As the DNA content of a sample increased, CFU increased and less PCR cycles were required to reach the threshold. The excellent correlation between colony count and CT was illustrated again when analysing USDA proficiency testing results from 2008 – 2011 where the VetMAX<sup>TM</sup> kit was used following extraction by the MagMAX<sup>TM</sup> kit (Spearman's rank correlation was -0.913).

It is probable that the magnetic bead based extraction kits outperformed the column based kit because they were better suited to disrupting the tough bacterial cell wall of MAP. PCR inhibition was not an issue as the internal control (Xeno<sup>TM</sup> DNA) was detected in all MAP positive samples.

The MagMAX<sup>TM</sup> Total Nucleic Acid Isolation Kit employs mechanical disruption of samples with zirconia beads in a guanidinium thiocyanate-based solution that rapidly releases nucleic acids while simultaneously inactivating nucleases in the sample matrix.<sup>3</sup>

This study shows that the VetMAX<sup>TM</sup> MAP Real-Time PCR Screening Kit for the detection of MAP in bovine faeces is comparable to traditional culture methods, and gives rapid turn around time. For a complete workflow this kit can be coupled with either the MagMAX<sup>TM</sup> Total Nucleic Acid Isolation Kit or the InviMag<sup>®</sup> Stool DNA Mini Kit for superior results.

## REFERENCES

- Collins MT, Gardner IA, Garry FB, Rousseau AJ, Wells SJ (2006) Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. *J Am Vet Med Assoc.* 229(12):1912-9.
- Alinovi CA, Ward MP, Lin TL, Moore GE, Wu CC (2009) Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* ssp. *paratuberculosis*. *Vet Microbiol.* 136(1-2):177-9.
- Aly SS, Mangold BL, Whitlock RH, Sweeney RW, Anderson RJ, Jiang J, Schukken YH, Hovinge E, Wolfgang D, Van Kessel JA, Karns JS, Lombard JE, Smith JM, Gardner IA (2010) Correlation between Herrold egg yolk medium culture and real-time quantitative polymerase chain reaction results for *Mycobacterium avium* subspecies *paratuberculosis* in pooled fecal and environmental samples. *J Vet Diagn Invest.* 22(5):677-83.
- Fleiss JL, Levin BA, Paik MC (2003) Statistical methods for rates and proportions. 3rd edition. Wiley, Hoboken, NJ.
- Chirgwin J, Przybyla A, MacDonald A, Rutter W (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochem.* 18:5294.

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