Mycoplasma ovipneumoniae Diagnostic Testing Summary and Review

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Free-ranging bighorn sheep in southcentral British Columbia were tested for nasal shedding of *Mycoplasma ovipneumoniae* (M. ovi) in February and March 2020. Here we review and compare the protocols of the three methods used.

Methods: Triplicate samples were tested using Biomeme, a real-time polymerase chain reaction (RT-PCR) field system, and conventional PCR at two commercial laboratories: the BC Provincial diagnostic laboratory (Animal Health Centre, Abbotsford, BC; AHC) and Washington Animal Disease Diagnostic Laboratory (Pullman, Washington, USA; WADDL).

The PCR assays used at WADDL, AHC, and Biomeme use the same specific primers and probes to target the 16S (small ribosomal RNA subunit) region of the M. ovi genome (Ziegler et al 2014; Manlove et al 2019). The protocols for testing sheep and goat samples have minor variations. This method has been validated against the gold standard of culture and enumeration using colour changing unit assays.

The Biomeme system uses pre-aliquoted lyophilized reagents, primers, and probes to extract genetic material and identify target sequences. RNA is amplified in a hand-held thermocycler and results are displayed on a smartphone application. The process takes approximately one hour and numerical and graphical results are interpreted by the user on site. It is desirable and convenient to use this field unit as it is the only test able to be used remotely and that provides an "immediate result".The sensitivity of the Biomeme system is relatively poor (high false negatives) compared to the method used at WADDL (T. Besser *pers. comm.*). Shringi and Besser (2018) reported a sensitivity of 76% with an earlier version of the Biomeme system, versus 92% using conventional PCR at WADDL for duplicate nasal swab samples from domestic sheep. Further research at Dr. Besser's lab determined this to be at least partly due to inhibition within the DNA extracts due to the extraction method employed in the Biomeme system. AHC performs a sequencing step for 2 to 3 animals per group, if animals are from the same herd or geographical location.

WADDL and AHC use the Qiagen and MagMax extraction methods (depending on the number of samples to run); the Qiagen method also produces minor inhibitors. These extraction methods produce a much 'cleaner' DNA product than the Biomeme system as they incorporate steps to separate and remove debris from the sample. Dr. Besser suggests that dilution of the sample prior to extraction, or addition of bovine serum albumin (BSA; a well-known anti-inhibitor) may reduce inhibition of the DNA and improve sensitivity of the Biomeme system.

The graphical output of the Biomeme system is similar to conventional PCRs methods. The relative fluorescence units (RFU) are charted against the quantification cycle (Cq). A clear M. ovi-positive result will show an exponential increase (sigmoid-shaped) in RFU with a change of

greater than approximately 100 units on the raw data graph (Figure 1). A clear negative result will show no or very little change in RFU (<100 units). The baseline data graph gives an indication of the quality of the raw data; the line should remain relatively flat or show a small linear rise.

The cut-offs used for interpretation of Cq values may differ between labs (Table 1). This means that the reported result (i.e. positive, suspect, or negative) may vary for a given Cq. A difference in Cq of 1 to 3 between labs is common and acceptable (T. Joseph *pers. comm.*). This variation may be due to differing test protocols, sampling technique, sample handling, or biological variation.

For our field use of Biomeme, we used a Cq cut-off of 30 and above to rule out M. ovi (interpreted in light of raw and baseline data graphs). Dr. Joseph suggests that a Cq of 40 should be used as an absolute cut-off as repeatability decreases significantly beyond this range; animals with a Cq of between 30-40 should be considered 'suspect positives' if their graphs suggest a positive result (significant exponential rise in RFU). In consideration of the low sensitivity of the Biomeme system and the objective of removing all positive sheep, using a higher Cq cut-off is warranted and reduces the chance of missing positive animals (e.g. one animal was determined to be 'negative' with a Cq of 38.46 using Biomeme, but found to be positive from AHC and WADDL).

Occasionally a Cq of zero was shown accompanied with graphs that show a linear but significant early rise in RFU (Figure 2). Dr. Joseph sees these results with conventional PCR at AHC as well and suggest they may be due to a cross-reaction with another similar bacterial species, but we cannot rule out M. ovi. These animals should be retested if possible, or collared and released for further testing later.

Table 1. Interpretation of quantification cycle (Cq) M. ovi PCR results from Animal Health Centre (AHC), Washington Animal Disease Diagnostic Laboratory (WADDL), and Biomeme.

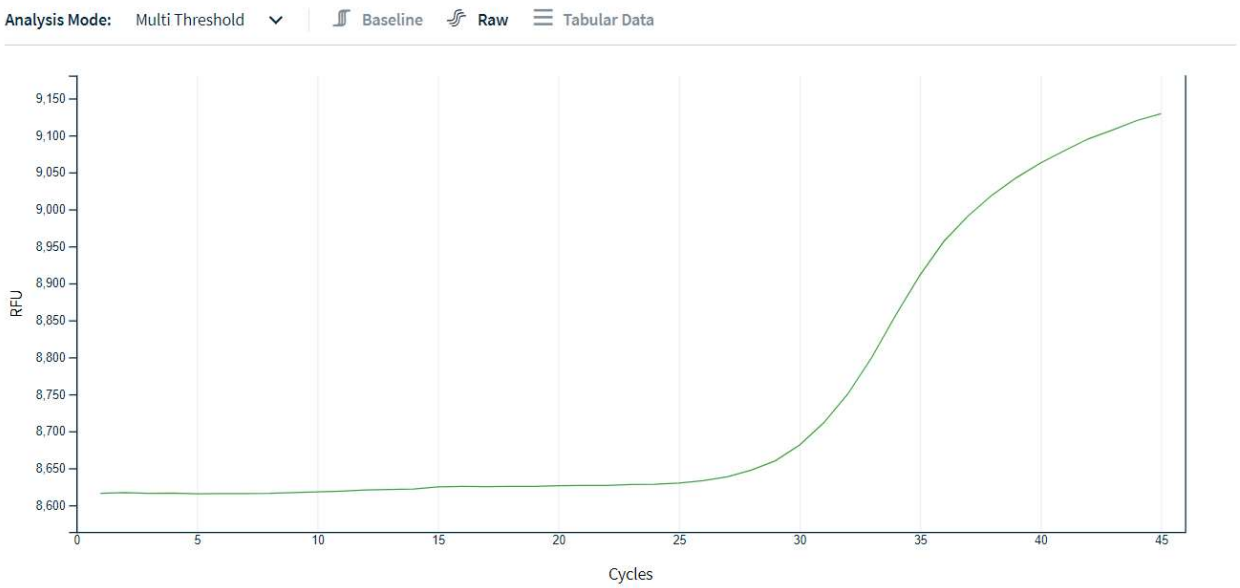
Interpretation	AHC	WADDL	Biomeme*
Strong Positive	< 30	-	< 30
Weak Positive	30 – 35.9	≤ 36 (= positive)	-
Suspect	36 – 39.9	> 36 (= indeterminant)	30 - 40
Negative	> 40	-	> 40

* Cq value must be interpreted with consideration of the graphical output of both the baseline and raw data

Table 2. Sources of error with the field use of the Biomeme system.

Source of Error	Effect
Temperature	Cold temperatures may reduce the quantity of DNA extracted due to the viscosity of the fluid and chemical properties of the reagents
Contamination	Dirt may reduce the quantity of DNA extracted or introduce possible cross-reactive bacterial species

Order of swab collection	The amount of DNA collected may decrease with subsequent swabs depending on the amount of bacteria being shed at the time of sampling
Personnel	Technique, such as depth swab is inserted into the nasal passage or contact with the nasal wall, may affect the amount of DNA collected



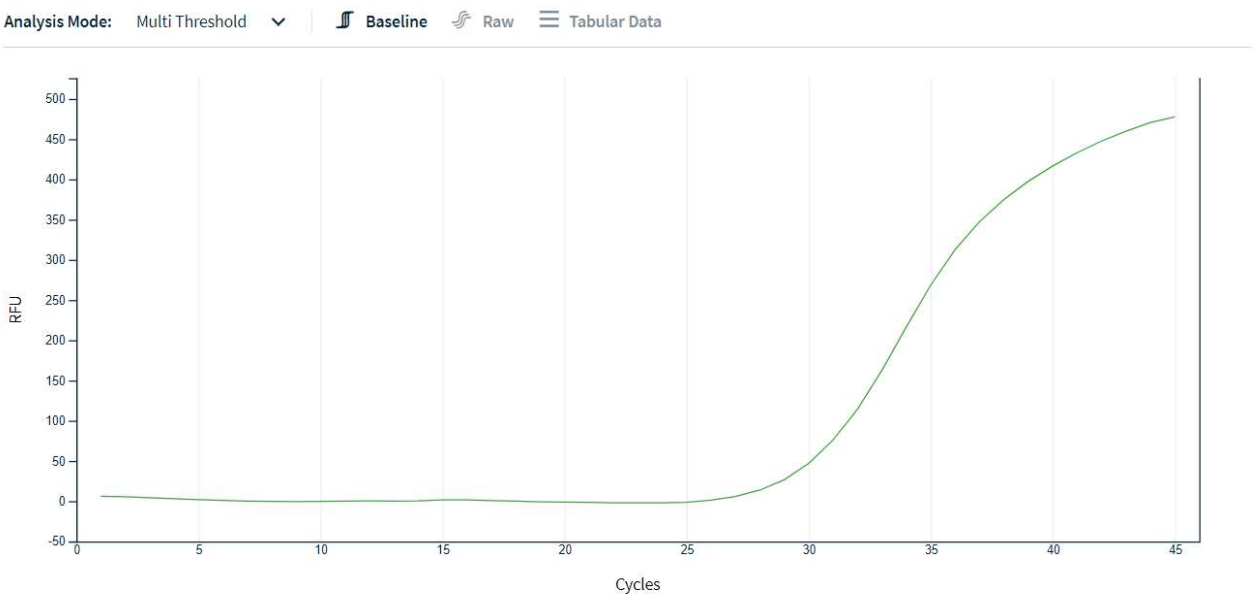
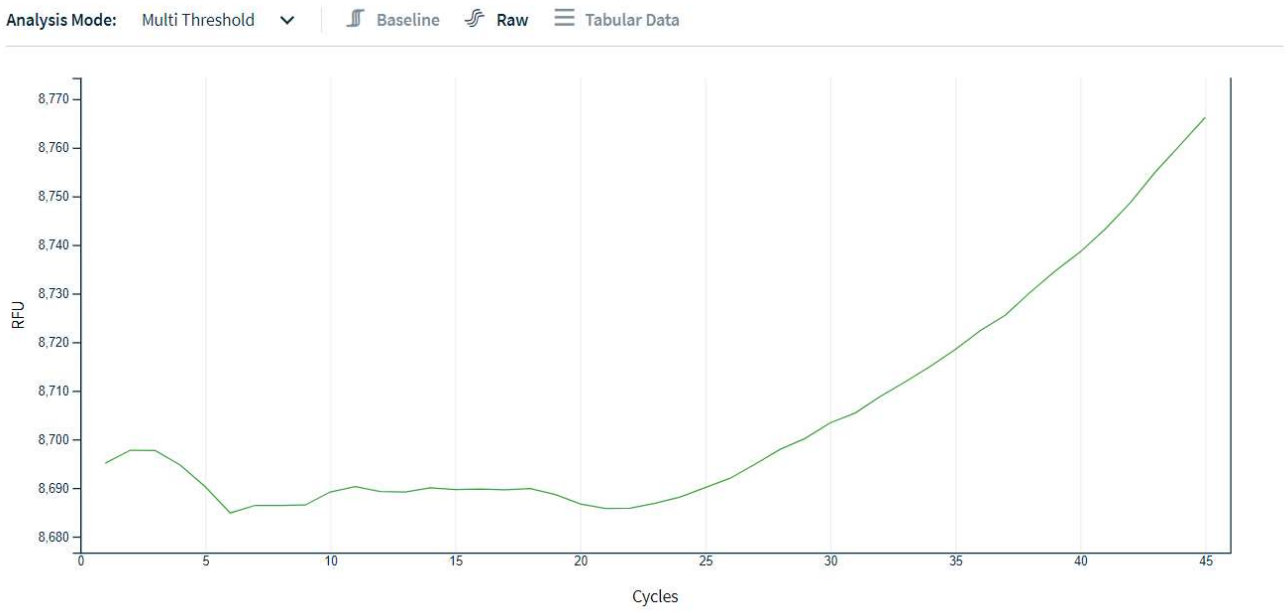


Figure 1. Raw and baseline data graphs for a clear *Mycoplasma ovipneumoniae*-positive nasal swab sample from a bighorn sheep using the Biomeme real-time PCR system.



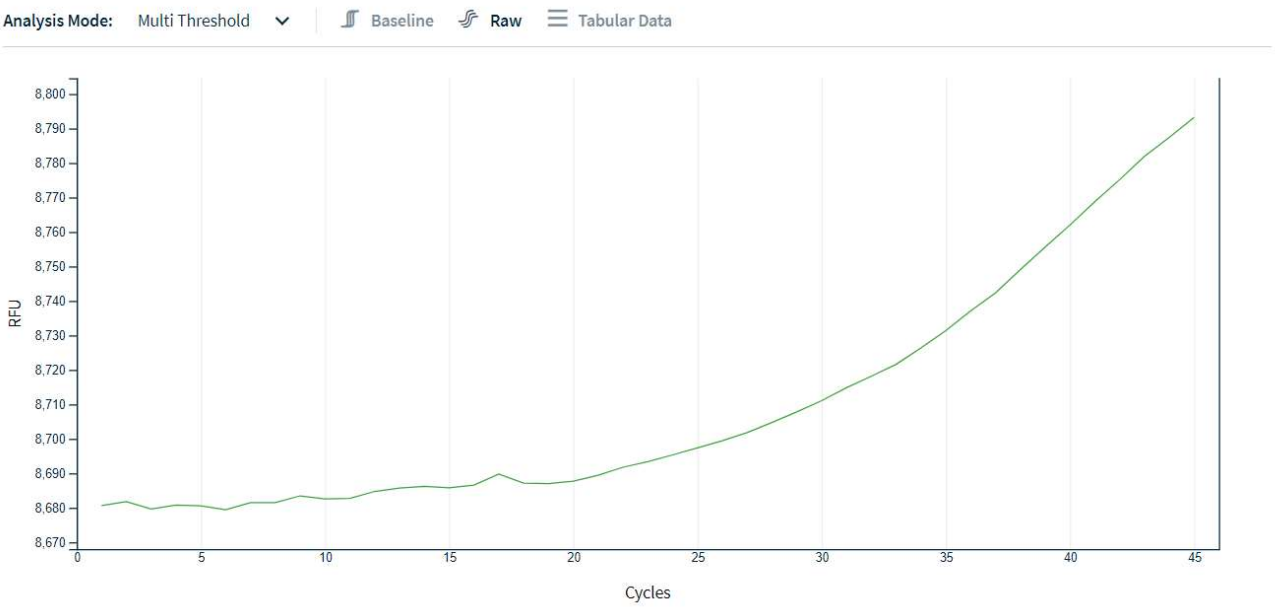


Figure 2. Raw data graphs for a *Mycoplasma ovipneumoniae* nasal swab sample from a bighorn sheep considered to be a 'suspect positive' using the Biomeme real-time PCR system.

<u>Recommendations for future use of the Biomeme system:</u>

- Collar and release sheep with a suspect positive results, such as:
    - those with a Cq between 30-40 (and a significant increase in RFU)
    - a Cq of zero, but raw data graph with a significant increase in RFU but not a clear sigmoid shape
- Maintain a consistent recommended temperature of cartridges and other materials (use hotpacks and cooler, and monitor with a thermometer)
- One person does all the swabbing
- Swab washing step:
    - submerge swab in VTM (RNA buffer solution) and agitate (Biomeme can provide pre-aliquoted vials with a ball-bearing in them for mixing)
    - use 200 µL of VTM sample solution (not swab itself) for Biomeme assay
    - the remainder of the VTM sample solution can be used for AHC and WADDL's PCR assays (this removes the issue of multiple swabs)
    - VTM sample solution can be stored at ambient temperature for up to 9 months
- Collect a 2nd swab and store dry/frozen for backup
- Use a pre-filter on the test syringe for drawing up sample (VTM sample solution)
- Record quality of sample (i.e. amount of dirt contamination, ease of extraction, etc.).
- If VTM or other swab-wash step is not used, consider increasing the time that the swab remains in the first well of the cartridge – this may help extract a larger quantity of DNA (the time should be consistent for all samples)

- Test swabs in the same order (i.e 1st swab used for Biomeme, 2nd swab send to AHC, 3rd swab sent to WADDL, 4th swab stored in PSB broth, etc.)
- Use Biomeme to test all animals in the field. Send only 'suspect' samples to AHC. As AHC and WADDL use the same protocol, variation between the labs is likely due to variation in the amount of genetic material on the swabs and acceptable differences in results. If strain-typing is desired and budge allows, send samples to WADDL instead of AHC.

REFERENCES

Ackermann MG, Schneider DA, Baker KN, Besser TE. 2019. Comparison of three methods of enumeration for *Mycoplasma ovipneumoniae*. *Journal of Microbiological Methods* 165:105700

Besser T. Microbiology & Epidemiology, Washington State University, Pullman, WA, USA

Joseph T. Veterinary Virology, Animal Health Centre, BC Ministry of Agriculture, Abbotsford, BC, Canada

Manlove K, Branan M, Baker K, Bradway D, Cassirer EF, Marshall KL, Miller RS, Sweeney S, Cross PC, Besser TE. 2019. Risk factors and productivity losses associated with *Mycoplasma ovipneumoniae* infection in United States domestic sheep operations. *Preventative Veterinary Medicine* 168: 30-38

Shringi S, Besser T. 2018. Use of rapid field-based PCR testing to detect *Mycoplasma ovipneumoniae* infection in bighorn sheep. Proceedings of the 21st Biennial Northern Wild Sheep & Goat Council Symposium. Whitefish, MT, USA.

Ziegler JC, Lahmers KK, Barrington GM, Parish SM, Kilzer K, Baker K, Besser TE. 2014. Safety and Immunogenicity of a *Mycoplasma ovipneumoniae* Bacterin for Domestic Sheep (*Ovis aries*). PLoS ONE 9(4): e95698