2016 JDRC PCR Screening Report

Herd Screening options for Johne's Disease: Progress towards a PCR screening test for effluent, as a dairy herd monitoring tool

1. Introduction

Options for screening individual animals for Johne's disease (JD) are either to detect *Mycobacterium avium ss. paratuberculosis* (MAP) bacteria directly via PCR or culture of faecal samples (de Lisle et al., 2006) or to assess their immune response using enzyme-linked immunosorbent assay (ELISA) technology on milk or serum. Herd level screens have been considered by LIC to see if a reliable indicator of infection status/risk could be identified. One option considered was ELISA screening of bulk tank milk; this has been employed for trial purposes as a method of identifying herds with JD-positive animals (discussed in 2016 JDRC Milk ELISA report). An alternative strategy is to use real-time PCR to monitor MAP levels in effluent as an indication of shedding in the herd. MAP bacteria are increasingly shed in the faeces as the disease progresses (JDRC vet guide). Given that the disease is spread via the faecal-oral route, monitoring the bacterial load in faeces and/or effluent could provide an indicator of infection risk.

To determine the feasibility of real-time PCR monitoring of effluent MAP levels, a three-stage approach was used; the first stage involved optimisation of the extraction and PCR method for detection of MAP bacteria in faecal and effluent samples. The second stage was to validate this method against other available assays and the final stage was to carry out an effluent sampling case study.

A sensitive PCR test for MAP bacteria relies on the optimisation of a robust disruption process to release bacterial DNA. Critical comparisons of bacterial extraction methods have been published and typically require mechanical and enzymatic disruption steps to disrupt the resilient bacterial cell wall (Sting et al., 2014; Park et al., 2014; Zhang and Zhang 2011). In line with our laboratory processes we required a suitable high-through-put magnetic bead-based extraction process in a 96-well plate handling format. Another requirement of the method was removal of substances from the sample that may be inhibitory to PCR.

A number of PCR-based methods for specific detection of MAP have been published (Wells et al., 2006) The target sequence chosen for this study was the IS*900* insertion sequence (Vary et al., 1990; Green et al., 1989; Bauerfeind et al., 1996). As there are 14-20 copies of IS*900* per genome this was thought to be a more sensitive target than a single copy gene such as the f57, which is also used as a MAP target (Ellingson et al., 1998; Tasara et al., 2005).

1.1 Aims – Method development

1) Develop an optimized real-time PCR method for detecting MAP in faecal and effluent samples.
2) Validate this optimized method against faecal culture and an alternative validated MAP-PCR test for faeces (inter-lab comparison).

1.2 Aims – Effluent sampling case study

1) To determine the most reliable location and sampling regime for detecting MAP within the effluent system of one farm with a history of JD.
2) To determine if environmental factors (e.g. weather) or management factors (e.g. emptying of effluent pond) have any effect on the level of MAP bacteria within effluent sampling points.
3) To determine if it is possible to detect changes in levels of MAP bacteria within the effluent system when known JD-positive cows are culled or dried off.

2. Materials & Methods

2.1. Identification of JD-positive animals

The IDEXX (previously Pourquier) *Mycobacterium avium ss. paratuberculosis* (MAP) ELISA screening kit was used by the LIC Animal Health lab to test herd test milk samples, enabling identification of animals with an antibody response to MAP infection (2016 JDRC Milk ELISA Report). There were four possible outcomes: 'High Positive', 'Positive', 'Suspect' (a group which may have background antibody levels) and 'No antibody detected'.

2.2 Farm selection

For validation of the PCR, multiple farms were used to provide faecal and effluent samples. For the effluent sampling case study one farm was selected which had a history of JD and with plans to cull JD-positive animals (as determined by milk antibody ELISA) during the period of the trial. As a split calving system with most JD cows in the autumn calving mob, these cows would be dried off during the trial thereby shedding less faecal matter into the effluent system. Toward the end of the trial, these cows would calve and be returned to the milking herd.

2.3 Sample collection

Individual faecal samples were collected from farms involved in the 2009 JDRC trial. Faecal and effluent samples were collected from 15 farms during the 2015 JDRC Genetic Study. Between one and four effluent samples were collected from sites such as feed pad, sump, sand trap, pond and yard.

For the case study, six sampling sites were identified within the sump, sand-trap and effluent pond for solid and/or liquid collection (Figure 1). Two replicate samples were collected from the sump and sand-trap sites and eight samples from the pond after it had been mixed for at least 15 minutes. Samples were collected every three days for the first month, every five days for the next six weeks and every seven days for the next seven weeks. All samples were collected by the farmer and stored

at room temperature until pick up (1-6 days after collection). After return to lab, samples were mixed, aliquoted and frozen at -20°C until testing.

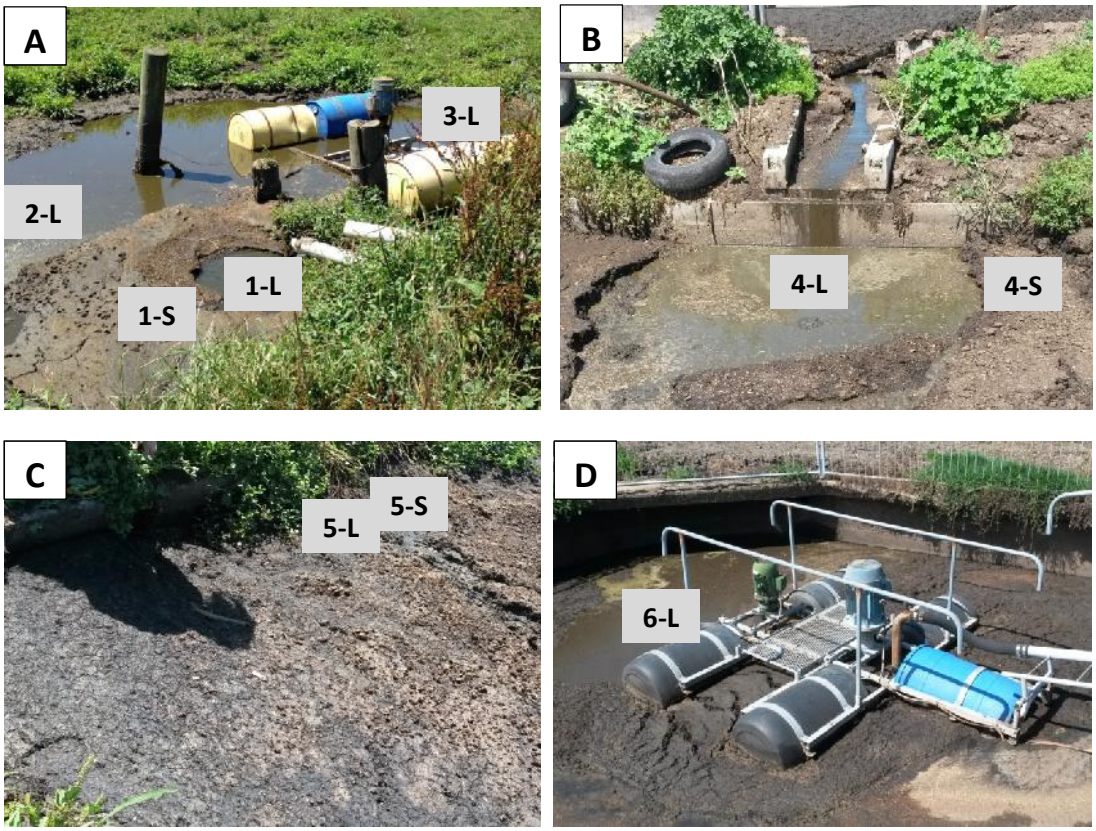

Figure 1: Two duplicate samples were collected from **(A)** sump - inlet liquid (1-L) and solid (1-S), left end of the sump liquid (2-L) and right end of the sump liquid (3-L); **(B)** sand-trap inlet - liquid (4-L) and solid (4-S); **(C)** sand-trap outlet - liquid (5-L) and solid (5-S). Up to 8 replicate samples were collected from **(D)** Pond - liquid (6-L).

2.4 Bacterial extraction from faecal and effluent samples

A three stage MAP extraction process was developed in-house and optimised to have comparable performance to the Magmax Total Nucleic Isolation Kit (Thermofisher Scientific; data not shown). Firstly, samples were treated with a proteinase enzyme and incubated in a denaturing solution. Samples were then mechanically disrupted using 0.1 mm Zirconia-silica beads using a Biospec bead beater. DNA was then extracted from a subsample using a magnetic bead-based high through-put extraction on a Kingfisher 96 unit (Thermofisher).

Faecal samples from a confirmed JD-positive animal and JD negative slurry were processed with each extraction plate, providing a positive and negative control for each experiment. A unique synthetic positive control DNA 'Xeno' (Thermofisher) was added to each magnetic bead extraction. This internal control DNA enabled monitoring of both the extraction process as well as any inhibitory effects in the PCR reaction.

2.5 Quantitative real-time PCR

For detection of MAP in DNA extracts, real-time PCR assays were performed using VetMAX MAP Real-Time PCR Screening reagents according to the manufacturer's recommendations (Thermofisher). These included PCR primers and Taqman probe for positive control Xeno DNA multiplexed with the IS*900* gene PCR primers and probe. A kit positive was included in each experiment (MAP plasmid at 3000 copies per reaction). All PCR reactions were performed on an Applied Biosystems 7500 machine. In Real Time PCR the accumulation of a fluorescent signal is detected and a plot generated for each target gene, as depicted in Figure 2. The Cycle Threshold (Ct) is the number of cycles required for the signal to cross a set threshold; the lower the Ct value, the greater the quantity of target in the PCR reaction.

MAP levels in faeces were classified as high, moderate, and low shedders or background and not detected depending on Ct values. MAP levels in effluent were classified as high, moderate and background. A reaction was deemed to 'fail' if the Xeno PCR Ct fell outside accepted limits.

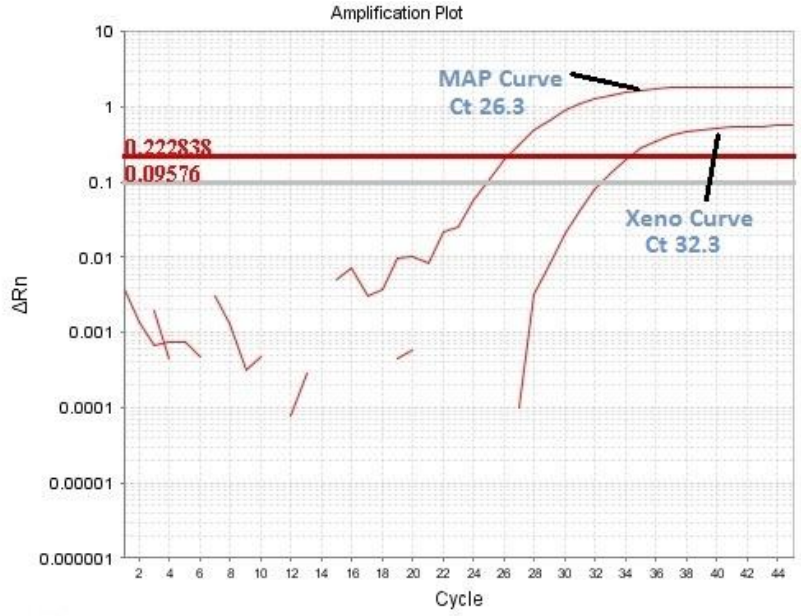

Figure 2: Graphical representation the Real-time PCR data for MAP and Xeno reactions; the ΔR (normalised fluorescence reporter signal) is plotted against the PCR cycle number

3. Results and Discussion

3.1 Extraction/PCR

PCR detection limits were determined by the creation of a standard curve based on a dilution series of MAP DNA of known copy number (Figure 3). There was a linear correlation between the Ct and the log copies per reaction with a detection limit of 6000 copies per ml, which is similar to other publications for this target (Sting et al., 2014). Although the IS*900* target is sensitive, there has been some comment that it is not as specific as the f57 gene (Tasara et al., 2005). The real-time PCR was therefore compared to MAP faecal culture.

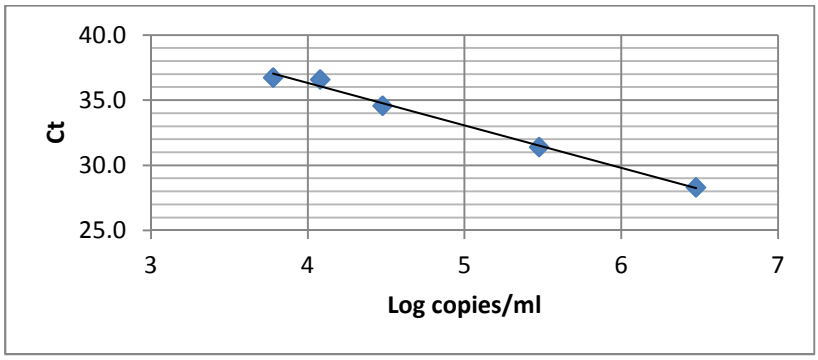

Figure 3: Detection limit of MAP Real time PCR: Ct values vs (Log) copies per ml

3.2 Faecal culture comparison

A total of 280 faecal samples from JD-high-positive or positive cows in the 2009 JDRC trial were tested using the in-house developed MAP PCR. This allowed confirmation of an animal's MAP shedding status.

From 233 JD-high-positive cows (as determined by milk ELISA), 91 % had a MAP PCR-positive faecal sample (combination of mod-heavy and low shedding; Table 1). However, only 83% of the JD-high-positive cows were found positive for MAP in faeces by culture methods. For the 47 JD-positive cows, only 58% and 47% were identified as having MAP in faeces by PCR and faecal culture, respectively. These results indicated that MAP-PCR appears to be a more sensitive detection method than faecal culture. Also, that JD-high-positive cows (as determined by milk ELISA) are more likely to be shedding MAP in the faeces than JD-positive cows. It must be noted that faecal culture requires the presence of viable bacteria within the faecal sample, whereas PCR only requires the presence of MAP DNA. A positive MAP result with PCR may not indicate that viable bacteria are present within the faeces.

Table 1. Percentage of JD ELISA-positive cows that tested positive by serum ELISA, faecal culture and faecal PCR.

	n	Serum ELISA	Faecal Culture	Faecal PCR (quantitative)		
		Positive	Positive	mod-heavy	low shedding	not detected
Herd-Test Milk Johne's ELISA PPV:						
'High Positive Cows' only	233	**98%**	**83%**	**55%**	**36%**	9%
Remaining 'Positive Cows'	47	64%	47%	11%	47%	42%
All Milk ELISA Positive Cows	280	**92%**	**77%**	**48%**	**37%**	15%
Serum Johne's ELISA PPV:						
'High Positive Cows' only			**86%**	**56%**	**36%**	8%
Remaining 'Positive Cows'			60%	15%	55%	30%

Positive Predictive Values: % Johne's ELISA-positive cows that tested positive by confirmation tests
Data from a JDRC trial with 280 herd-test milk ELISA positive cows in 26 New Zealand dairy herds

3.3 DRL Benchmarking

A validated MAP-PCR test method was benchmarked with Disease Research Laboratory (DRL) via both labs testing a selection of faecal samples from JD-positive animals (as identified by milk and serum ELISA). A total of 43 faecal samples from ELISA positive animals were run by LIC and DRL using their own extraction and PCR processes. The challenge with this inter-laboratory comparison was that both labs employed different methods including variation in starting sample volumes, sample to reagent ratios, target genes with different number of copies (IS*900* and f57) and the number of categories used for ranking results (DRL used six categories from 'Not Detected' through to 'Super-shedders', but LIC only utilised five categories). Overall, data from both labs matched up very well despite the differences in methodology. To aid comparison DRL also repeated their testing using the same target gene as LIC (IS*900*) with comparable results (Figure 4). This positive result reinforced the suitability of the developed in-house extraction and PCR method for detecting MAP in faecal samples.

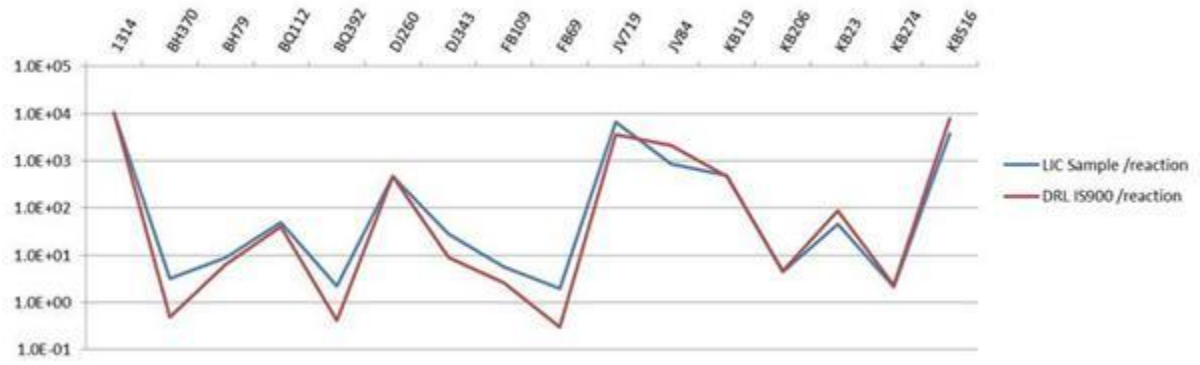

Figure 4: PCR detection of MAP (copies per ml) as performed either by LIC or DRL using the same target gene (IS*900*).

3.4 Application of MAP PCR to effluent samples

Effluent samples from 15 farms with JD-positive animals were also tested using the optimised MAP PCR method. MAP was detected in 30 out of the 31 samples collected (both solid and liquid), indicating that this PCR method was also capable of identifying MAP in environmental samples. The farm with the highest proportion of JD-high-positive and positive animals in the herd had the highest level of MAP detected in effluent samples (lower Ct is equivalent to higher MAP level; Figure 5). However, when 3-10% of the herd are JD-positive, the level of MAP in effluent varies between a 'high' and 'moderate' level and does not seem to follow any particular trend.

The level of MAP in effluent is a factor of both the proportion of animals in the herd that may be shedding MAP and also the numbers of bacteria being shed at any particular time. As noted in 3.2, not all JD-high-positive and positive cows are shedding MAP and shedding tends to be sporadic/intermittent (Mitchell et al., 2015). Therefore the lack of correlation between percentage JD-positive cows and level of MAP in the effluent may be due to variation in level of shedding in these herds. Alternatively, it is possible that different effluent collection systems or management factors may affect MAP detection in environmental sites. To rule out effects of management factors and/or weather on MAP detection, a case study was performed on one farm.

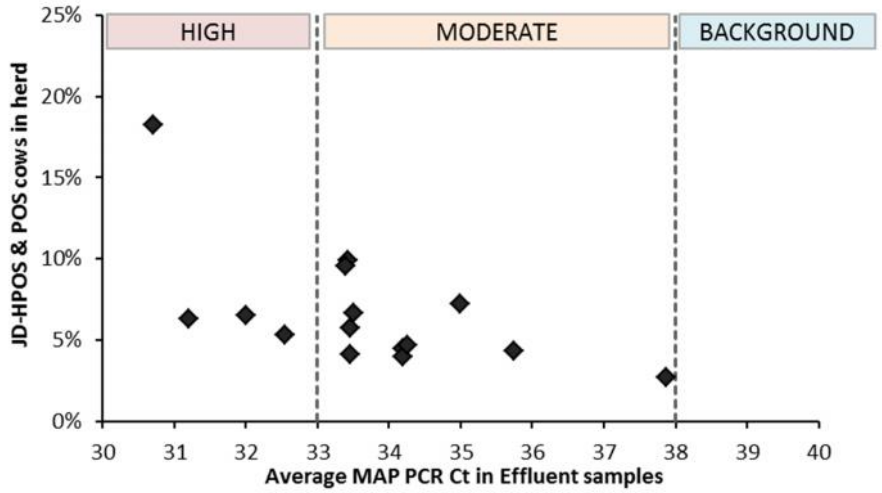

Figure 5. Average MAP PCR Ct in effluent samples from herds with JD-positive animals. Between one and four effluent samples were tested and PCR Ct averaged. Percentage of JD-high-positive and positive animals in the herd was determined using milk antibody ELISA (IDEXX). Increase in PCR Ct is indicative of reduced MAP in the sample tested. PCR Ct < 32 is defined as 'HIGH' MAP level, 33-37.9 is 'MODERATE' and ≥38 is background level.

3.5 Effluent sampling case study

MAP was detected in all sampled sites through the first month of the trial, although not consistently in the sump liquid. Due to such variable results, after the first month of sampling sump liquid sites were removed from the sampling regime.

Initially eight pond samples were collected for the first three weeks of sampling; however results showed good reproducibility between replicate samples. For subsequent collections, only four replicate samples were collected to reduce sampling effort for the farmer.

Rainfall, maximum temperature and sunshine hours per day appeared to have little effect on the level of MAP detected at each of the sites (data not shown). Likewise, shed washing volume and date and amount of pond pumping had no apparent effect on the level of MAP within the sampled sites. The sand trap and sump were cleaned out directly before the trial began and were not emptied again during the course of the trial. Therefore the effect of this on MAP levels within these sites is unknown.

A reduced level of MAP in the sand trap liquid samples was observed over the first 15 days of the trial (average of inlet and outlet samples; Figure 4A) with MAP PCR Ct moving from moderate to background range. Similarly, MAP levels also decreased in the pond liquid samples over this period (Figure 4B); however, the pond samples showed a return to the moderate range for the last sample date shown. The sand trap solid samples (average of inlet and outlet samples) were relatively constant over this period and remained at a moderate level (Figure 4A).

As weather and other effluent management factors appeared to have little effect on observed MAP levels, it is possible that the decrease in MAP in sand trap liquid samples was due to the removal of JD-positive cows from the herd. MAP has been shown to survive in faecal matter and soil for up to 12 weeks (Whittington et al., 2005) and up to 24 months in other environmental studies (Moravkova et al., 2012), therefore this may explain why the level of MAP remained more constant within solid samples over time. Whereas the constant flow of liquid through the sand trap may be reflective of the level of MAP being shed in faeces at any particular time. It must also be noted that cows which were dried off may still have been contributing faecal material to the effluent system due to their use of the feed pad during the dry period.

Further testing and analysis is required on these samples and the remaining collected samples but overall they tend to show a reduction in the level of MAP in effluent with removal of JD-positive cows from the milking herd.

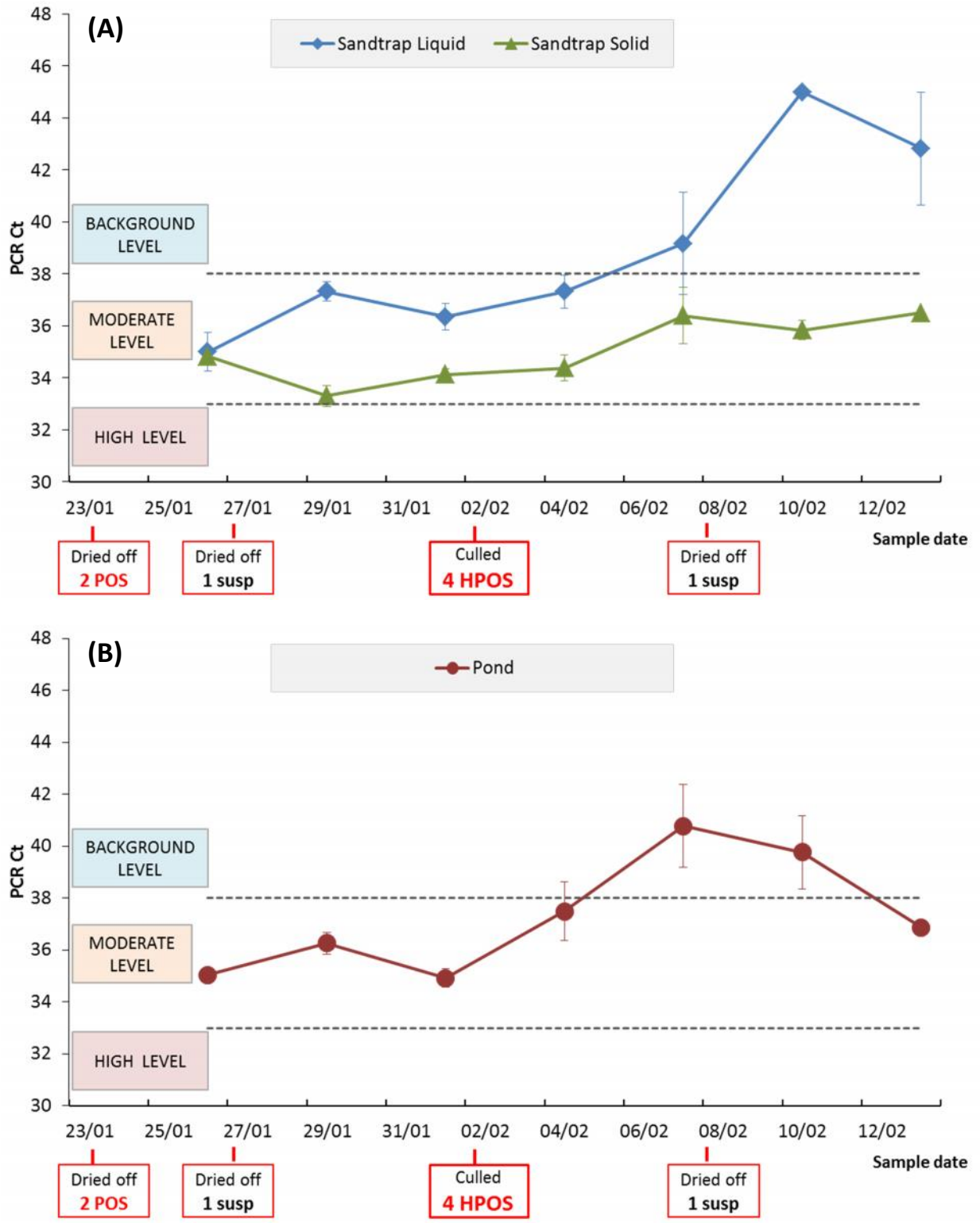

Figure 6. Average MAP PCR Ct (±SEM) across the first three weeks of sampling in **(A)** Sand trap liquid (blue diamond; inlet and outlet, n=4) and sand trap solid samples (green triangle; inlet and outlet, n=4) and **(B)** Pond liquid (n=8). Increase in PCR Ct is indicative of reduced MAP in the sample tested; Ct 33-37.9 is defined as moderate MAP levels, whereas ≥38 is background level. Known JD-suspect (susp), positive (POS) or high positive (HPOS) cows were culled or dried off during this period as indicated along the sample date axis.

4. Conclusion

The MAP extraction and real time PCR assay presented in this report have been developed and validated for the purpose of monitoring MAP bacterial levels in faecal and effluent systems. The case study presented suggests that levels of MAP in the environment may decline when JD positive animals are removed from the herd. Further on-farm case studies are required to better understand sampling sites on a variety of different effluent management systems. This type of testing option could potentially be used across seasons in conjunction with herd test milk ELISA to alert farmers to increased MAP shedding and therefore possible infection risk for young stock.

4. References

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