MILESTONE DESCRIPTION

Objective Title:	Milestone 3.2.12 Determining if environmental sampling can be used as a tool tor identification of super-shedding in dairy herds
Milestone Number & Title:	3.2.12.4 VNTR typing and analysis of samples to determine the efficacy of environmental sampling for detecting MAP subtypes in problem dairy herds
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Introduction

Although it is presently unclear whether herds with a more uniform type distribution are more at risk, given the array of types present on some farms, strain type monotony might indicate that a significant amount of super shedding is occurring. Subtyping of individual animals is costly, especially when investigating larger herds that are becoming increasingly prevalent in NZ as farming intensifies. A major goal of the recent dairy study was to provide a toolset that cost effectively identified high risk, high prevalence Johne's herds. As part of this study, environmental samples were analysed to determine how efficiently bacteria could be detected at the herd level. During the course of our previous subtyping analyses, samples containing more than one subtype were repeatedly obtained from dairy farms. Although it is possible to observe multiple subtypes in a given isolate by PCR it was not clear how sensitive this method would be when relative levels of the different types vary, especially for detecting types shed at lower levels or by a small fraction of the animals on the farm. Our aim here was to enhance the outcomes of the dairy intervention study by determining the different subtypes present in these environmental samples and comparing the distribution to that seen from analysing samples from animals with a positive serum or milk ELISA response in order to determine how accurately environmental samples reflect the diversity of subtypes being shed by individual animals. If it were possible to acquire knowledge of the prevalent sub-types in a given herd from environmental samples, then subtyping could become more cost effective and potentially more effectively utilised in control strategies. Results of culture and type distribution from farms with a diverse array of types, and the efficacy of this method for reflecting MAP sub-type diversity on high risk farms are described.

Methods

For culture, faeces and environmental samples were decontaminated using the double-incubation method described by Whitlock and Rosenberger {Whitlock, 1990 #3225}. Approximately 2 g of faeces

was added to 40 mL sterile distilled water, vigorously shaken, and allowed to stand for 30 minutes. A 5-mL aliquot off the top of the liquid was decontaminated in to 1% cetylpyridinium chloride (CPC). After decontaminating overnight, the samples were centrifuged, the supernatants discarded, and the pellets reconstituted in 1 mL of an antibiotic cocktail {Whittington, 1998 #2294}. After a further incubation period of 3 days, 0.5ml of the sample was inoculated into supplemented 7H9 (OADC glycerol tween mycobactin and egg yolk. The vials were incubated at 37°C, and examined every two to three weeks. Positive cultures were identified by acid-fast staining of aliquots of the culture samples.

DNA was extracted as described by de Lisle et al. (2006). In this method, 0.2 ml of a culture was added to 0.5 ml silica beads (FastPrep-24 Lysing Matrix B, MP Biomedicals, Solon, OH) and 1 ml of proprietary ASL buffer (QIAamp DNA stool kit) and shaken vigorously in a 2 ml micro-centrifuge tube for two periods of 20 s in a Ribolyser (FastPrep Cell disrupter; ThermoSavant, Holbrook, NY) set on 6.5, with 1 min cooling on ice between each period. The suspension was heated in a water bath at 95◦C for 10 min, vortexed for 15 s and then centrifuged at 20,000×g for 1 min. For each sample, 1 ml of supernatant was placed into a 2 ml tube, half an inhibitEX tablet was added and the mixture was vortexed until the tablet was completely dissolved. DNA was extracted as described in the manufacturer's protocol (QIAamp DNA stool kit) and stored at 4 ◦C. For PCR analysis of the five chosen variable number tandem repeat (VNTR) loci and the short sequence repeat (SSR) locus SSR8, approximately 15 ng of MAP DNA was subjected to PCR using the forward (F) and reverse (R) primers in Table 1. PCR reaction conditions were optimized for each VNTR locus. For all but SSR8, 25 ul PCR reactions contained 1×standard reaction buffer, 1–2 mM MgCl2, 0.2 mM dNTPs, 1 uM primer mix, 1.25 U Amplitaq (Roche), 0.1 mg/ml bovine serum albumin (BSA), 0–12% DMSO and 2ul MAP DNA. SSR8 reactions were as above but with appropriately scaled 50 ul reactions and no added BSA. PCR reaction conditions were: denaturation at 94 ◦C for 10 min, 35 cycles of denaturation at 94 ◦C for 45 s, annealing at 58 ◦C for 1 min for all but SSR8 PCR reactions, or annealing at 60 ◦C for 1 min for SSR8 PCR reactions, followed by extension at 72 ◦C for 1 min and a final extension at 72 ◦C for 10 min. The variable conditions of the PCR reactions optimized for each VNTR are summarized in Table 2. The un-purified SSR8 PCR products were outsourced for purification and sequencing to Macrogen.

Table 1 Primers for VNTR/SSR8 typing of MAP.

VNTR	Primer name	Sequence
292-F	DMC1468	CTTGAGCAGCTCGTAAAGCGT
292-R	DMC1469	GCTGTATGAGGAAGTCTATTCATGG
25-F	DMC1470	GTCAAGGGATCGGCGAGG
25-R	DMC1756	TGGTGTAAACGGTGAGCGG
X3-F	DMC1478	AACGAGAGGAAGAACTAAGCCG
X3-R	DMC1479	TTACGGAGCAGGAAGGCCAGCGGG
7-F	DMC1476	GACAACGAAACCTACCTCGTC
7-R	DMC1477	GTGAGCTGGCGGCCTAAC
3-F	DMC1474	CATATCTGGCATGGCTCCAG
3-R	DMC1475	ATCGTGTTGACCCCAAAGAAAT
SSR8-F	DMC1753	GCTACCCGGTGCTGACCTA
SSR8-R	DMC1754	GAGATGTCCAGCCCTGTCTC

Table 2. PCR reaction mixtures and % agarose gels used for analysis of the size of the PCR product.

VNTR	[Mg] mM	% DMSO	% agarose gel
292	2	12	2.5
25	1. 5	0	2.5
X3	1.25	0	2.5
7	1.5	0	4
3	2	10	4
SSR8	2	5	2.5
SSR2	1.5	10	2.5

Results

Our goal was to determine how well environmental samples reflected the distribution of types in individual animals by culturing and VNTR typing samples from individual animals with high milk ELISAs and samples from various environments likely to be contaminated by MAP. Unfortunately animals on 4 of the 5 farms that were analysed in 2013 all shed the most common cattle type and thus were not suitable for addressing this question. In 2014 we received individual animal and environmental samples from 7 different farms which were shown previously (Milestones 3.2.7 and 3.2.10) to harbour multiple MAP subtypes. These had more diverse type distributions and were thus suitable for addressing the feasibility of surveying MAP types via environmental sampling. Samples from the one farm from the 2013 survey and 6 of the 7 different farms in 2014 that had mixed types are described here.

MAP culture

MAP was successfully cultured from both individual animals and from environmental samples from 5 of the 8 farms characterised in 2013 and from each of the 7 farms characterised in 2014. For 5 of the 7 farms that were characterised in 2014, greater than 80% of the samples from individual animals gave rise to positive cultures. Samples from the other two farms were less fruitful. Only 60% of the samples from the individual animals on these farms gave rise to positive cultures. Analysis of ZN stains of samples from these farms indicated that they were contaminated with fungus which may have inhibited the growth of MAP. Anti-fungal drugs are added during the decontamination process used to prepare these cultures, but some fungi and other organisms are obviously resistant to these drugs. In the one instance where contaminated samples were VNTR typed, this contamination was not found to inhibit the VNTR analysis of the MAP that was present in these contaminated cultures. There were no sheep isolates were cultured in the current survey. We have recently used the same procedure at the Hopkirk to successfully culture this organism from sheep faeces, so may reflect a change in type distribution is likely to have occurred and the number of dairy herds increased in this time period.

It was possible to culture MAP from environmental samples from all of the farms that gave rise to cultures from individual animals. There were a variety of environmental sources sampled, and in most cases it was possible to culture MAP from these sources. It is unclear from our results whether any of these would be a superior source in terms of the ability to obtain a positive culture. The effluent pond samples were less practical simply because they were quite dilute and perhaps more likely to leak in transit than some of the other types.

VNTR/SSR8 type distributions.

Table 4 illustrates the VNTR types observed in samples from the different farms analysed in both the previous (Milestone 3.2.6) and present (Milestone 3.2.12) studies and also the types observed in environmental samples. Because this assay relies on PCR, it is only possible to rigorously determine mixtures of two types, since additional PCR products may arise erroneously when a mixture two different sized PCR products are amplified at the same time. The SSR8 locus was assayed for this analysis because it provides relevant distinction of unmixed types, but because samples must be sequenced to determine their SSR8 size, mixed samples are not reliably determined at this locus.

GGWD- the most frequently isolated type on this Hokitika farm in 2009/2010 was the (332(0.5,2,2.5)25) mixture, which was isolated from 3 animals that harboured both the common cattle type (332225) and the (332(0.5)25) variant. Each of the two components of this mixture was also isolated in pure culture from other animals on this farm. The distribution of types observed in 2014 varied from this earlier distribution in several ways. There were no isolates with the (332(0.5)25) type, or the (332(0.5,2,2.5)25) mixture of types. Instead most isolates were the common cattle type (332225) and there were several isolates with the second most common cattle type (322225). This striking difference in the observed type distribution likely reflects the large influx of cattle that was known to occur on this farm and suggests that the MAP population on the farm was dynamic. In 2014, the common cattle type (332225) predominanted (78%) in the samples that were isolated from individual animals, and was cultured from two different ponds, and from a sump. Both of the pond samples were mixed, but the second types in these mixtures (432225) in pond 1 and (332224) in pond 2, were not isolated from individual animals. The second most common cattle type (322225) which was cultured from 2 of the 9 animals that were sampled, was not cultured from these environmental samples. Thus these environmental samples were reflective of the predominant type on this farm, and indicated that there were other types being shed into the environment, but were not completely reflective of the types that were isolated from individual animals. This discrepancy may be the result of the small sample size for this analysis, or perhaps that the second most common cattle type does not survive for long in the environment. There was also no sign of the previously prevalent type (332(0.5)25) which might be expected if it was no longer being shed at high levels.

KDYG The common cattle type (332225) was isolated from 3 of the 4 animals that were sampled on this Dobson farm in 2009/2010. The other isolate from this farm at that time was from a cow that was shedding a sheep type. (332225) was also the most frequently isolated from individual animals in 2014, being shed by 8 of the 9 animals that were sampled. The remaining sample was from an animal that appeared to be shedding both the common cattle (332225) and the common deer (432224) types. Only the common cattle type (332225) was isolated from the sand trap sample thus reflected the predominant type being shed by this herd.

HBCF In 2009/10 the both common cattle type (332225) and the common deer type (432224) were cultured from cows in this Greymouth herd. In addition, mixed samples from animals likely to be shedding both of these types and perhaps others were observed. The 2014 distribution of types was similar, in that both the common dairy and the common deer type were being shed. The common dairy type was most frequently isolated and was isolated from animals shedding other types. The mixture of types cultured from the exit race and the side yard samples reflect the diversity of types present in the herd.

DHJT The second most common cattle type predominated on this Taranaki farm in the samples that were analysed both in 2009/10 and 2014, and this was reflected in the effluent pond and sand trap sample that were analysed. Although there were a larger number of animals sampled in 2014, the sample size is still relatively small and there may be animals shedding the less frequently isolated (332(0.5)25) type.

HKXW The common dairy type comprised 50% of the samples from 2009/10, but several other types were isolated as well including a sheep type. The types and mixtures of types isolated from individual animals in 2014 indicate that the common cattle type still predominates (now 72% rather than 50%), but that the second most common cattle type (322225) and (332(0.5)25) are still being shed. The single common dairy type isolated from the sandtrap sample was reflective of the predominance common cattle type being shed on this farm.

BVY and BOP Animals on this farm previously shed multiple types. All of the animals that were assayed harboured the same mixture of types and this mixture was also observed in the sand trap sample.

Conclusions

Farms that were known to previously harbour a mixture of VNTR/SSR8 types were a superior source of samples for the current analysis than those characterised in 2013. In all but one case these farms also harboured more than one type when reanalysed in 2014. In most cases the same mixtures that were previously determined were detected again, but for some farms there was a shift in the type distribution, perhaps reflecting the flux of cattle on these farms. Although it did not accurately reflect all the types that were harboured by individual animals in a herd, environmental sampling provided some indication of whether multiple types were harboured on a farm. If one type was isolated from more than 70% of the individual animals that were sampled from a farm, then the environmental samples were likely to resemble this type rather than a mixture of different types.

Table 3. Culture of MAP samples

Farm	Number of cows	# positive cultures from individual animals	Environmental Source (#)	culture
2014 samples				
BQFD	10	6 (60%) fungal contamination	foot bath effluent pond	+ -
GGWD	10	9 (90%)	pond (2) sump (2)	both + one +
BVY	12	10 (83%) bacterial contamination	sand trap effluent filter effluent pond	+ - -
DHJT	11	11 (100%)	yard effluent pond sand trap	+ + +
HKXW	11	7 (64%) fungal contamination	sand trap	+
KDYG	10	9 (90%)	sump sand trap	- +
HBCF	12	10 (83%)	exit race side yard	+ +
2013 samples				
NCJY	12	12 (100%)	sand trap (2) effluent pond (2) yard entrance (2)	both + both + both +
BQCY	8	1 (12.5%)	exit race A (2) exit race B (2) stone trap (2)	both + both + both +
GGDD	10	10 (100%)	Sand trap (2) effluent (2)	both + -
BYMK	5	3 (60%)	start of pit end of pit yard entrance	+ + +
MJCN	18	15 (83%)	Sand trap (2) yard end (2) sump (2)	both + one + both +
PQKN	10	0 (0%)	Sand trap (2) yard	- -
NCFQ	10	0 (0%)	setting pond	-
FGXN	10	0 (0%)	Feed pad (2) Sand trap (2) Yard entrance (2)	- - -

Table 2 VNTR/SSR8 types of previous and current isolates from the sampled dairy farms

Farm	Strain type distribution-2009/2010								Strain type distribution 2014 - this study							
	# animals	%	292	25	X1	7	3	SSR8	# animals / enviriron. Source	%	292	25	X1	7	3	SSR8
GGWD	3	60	3	3	2	0.5 2 2.5	2	5	7	78	3	3	2	2	2	5
(Hokitika WC)	1	20	3	3	2	0.5	2	5	2	22	3	2	2	2	2	5
	1	20	3	3	2	2	2	5								
									pond 1		3,4	3	2	2	2	5
									pond 2		3	3	2	2	2	4,5
									sump 2		3	3	2	2	2	5
									sump 1							
KDYG	3	75	3	3	2	2	2	5	8	89	3	3	2	2	2	5
(Dobson WC)	1	25	3	3	1	1	1	3	1	11	2,3,4	3	2	2	2	4
									sand trap		3	3	2	2	2	5
HBCF	2	33	3	3	2	2	2	5	4	57	3	3	2	2	2	5
(Greymouth WC)	2	33	4	3	2	2	2	4	1	14	3	3	2	2	2	4
	1	17	3	234	2	2	2	5	1	14	3,4	3	2	2	2	4,5
	1	17	34	234	2	2	2	5	1	14	3,4	3	2	2	2	4
									exitrace		3,4	3	2	2	2	5
									sideyard		3,4	3	2	2	2	5
									effluent pond							
DHJT	5	83	3	2	2	2	2	5	12	100	3	2	2	2	2	5
(Opunake Trnki)	1	17	3	3	2	0.5 2 2.5	2	5								
									effluent pond		3	2	2	2	2	5
									sand trap		3	2	2	2	2	5
									no sample							
HKXW	3	50	3	3	2	2	2	5	5	72	3	3	2	2	2	5
(Inglewood Trnki)	1	17	3	2	2	2	2	5	1	14	3	2	2	2	2	
	1	17	3	3	1	1	1	3	1	14	3	2,3,4	2	0.5 2 2.	2	5
	1	17	3	3	2	0.5 2 2.5	2	5								
									sand trap		3	3	2	2	2	5
BVY	2	29	3	3	2	2	2	5	8	100	1,3	3,2	2	2	2	5
(Murupa BOP)	2	29	3	234	2	2	2	5								
	1	14	3	2	2	2	2	5	sand trap		1,3	3,2	2	2	2	5
	1	14	4	3	1	1	1	3	effluent filter							
	1	14	5	3	1	1	1	3	effluent pond							
NCJY									8		3	3	2	2	2	5
Paeroa WKTO									1		3	2	2	2	2	5
previous sample set									1		3	3	2	2,3,4	2	5
									1		5	3	1	1	1	3
									1		4	3	1	1	1	3
									sand trap 1		3	3	2	2,3,4	2	5
									sand trap 2		3	3	2	2,3,4	2	5
									effluent pond 1		3	3	2	2,3,4	2	5
									effluent pond 2		3	3	2	2,3,4	2	5
									yard entrance 1		3	3	2	2,3,4	2	5
									yard entrance 2		3	3	2	2,3,4	2	5

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