Culturing and VNTR subtyping of selected MAP isolates from the Deer and Sheep studies.

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Executive Summary

Molecular typing of isolates of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has provided insights into Johne's disease epidemiology in New Zealand (NZ). Recent VNTR/SSR subtyping analyses indicated that different ruminants often harbour different MAP subtypes, but that when co-grazed they frequently carry the same subtypes. Dairy cattle harbour a variety of C subtypes with one predominant subtype (CDa1) accounting for 70% of the isolates, whereas most deer (76%) harbour a different C subtype (CDe). In contrast, 67% of sheep and 68% of beef cattle that are co-grazed carry the same S subtype (CSh). It is hypothesized that the virulence of MAP subtypes varies between ruminant hosts. If true, this could be exploited in Johne's disease control schemes. Here we took advantage of the well characterised samples from recent deer and sheep studies to further explore the relationship between MAP subtype and disease severity in deer and sheep. Because most of these isolates were found to be identical with our current typing assay and because overseas studies are showing that whole genome sequencing (WGS) is superior to VNTR/SSR typing for analysis of MAP subtypes, these studies were not carried to completion so that the remaining funds could be deployed to characterise NZ MAP by WGS. Results from the partially completed analysis are described.

Sheep isolates from 19 different Merino and non-Merino sheep farms, from different regions of the country, assessed as likely to have varying levels of Johne's disease were cultured and a subset was typed. Samples from most of the animals with apparent clinical Johne's disease were culture positive, but samples from many of the animals that had potentially mild infection were culture negative either because MAP levels in these samples were very low or because these animals were of poor condition for reasons other than infection with MAP. Of the 49 isolates that were typed, the majority (92%) had the common sheep subtype. Only 2 of the 4 isolates that were not the common sheep type were a C subtype (CDe) and one of these had both CDe and CSh making it difficult to make any inferences about the correlation between disease severity of C and S subtypes in sheep. Although the robustness of subtyping with SSR2 is questionable, this assay was performed on all the sheep isolates. There was no correlation between SSR2 subtype and disease severity, but in some instances there were predominant SSR2 subtypes on different sheep farms suggesting as in our earlier studies that this assay could be useful in some epidemiological analyses.

Samples from 98 deer on 4 different farms in the Otago region from animals with varying amounts of MAP organisms shed into faeces were characterised. MAP isolates were obtained from all of the samples from animals that shed high or moderate levels of MAP as judged by qPCR, but also from most of the samples that had lower numbers of MAP as judged by qPCR, indicating that when compared to qPCR, our culture method is an sensitive means of identifying MAP from deer faeces. All of the 74 isolates that were typed had the common deer C subtype and were thus of no use for making inferences about the disease severity of C and S subtypes in deer. SSR2 subdivided these isolates into 7 groups. Three farms had one predominant SSR2 type (SSR2-10) and this type was isolated from animals that were shedding very high levels of MAP organisms as well as from animals that were shedding very low levels of MAP. Most of the other SSR2 subtypes that were isolated from these three farms were from animals that shed low or moderate levels of MAP. One possible explanation for this is that CDE SSR2-10 is being super shed and perhaps passively infecting other animals in the herd. Most (88%) of the animals on the fourth farm shed CDE SSR2-9, again indicating the usefulness of the SSR2 assay for MAP epidemiology. The strain type diversity in this dataset is compared to results from previous studies and the overall usefulness of this method for characterising sheep and deer MAP is discussed.

Introduction

Recent VNTR/SSR subtyping analyses indicated that different ruminants often harbour different MAP subtypes, but that when co-grazed they frequently share the same subtypes. It is hypothesized that the virulence of MAP subtypes varies between ruminant hosts. If true, this could be exploited in Johne's disease control schemes. Here we took advantage of the well characterised samples from the recent JDRC deer and sheep studies to further explore the relationship between MAP subtype and disease severity in deer and sheep. Because most of these isolates were identical with our current typing assay and because overseas studies are showing that whole genome sequencing (WGS) is superior to VNTR/SSR typing for analysis of MAP subtypes, these studies were not carried to completion so that the remaining funds could be deployed to characterise NZ MAP by WGS. Results from the partially completed analysis are described.

Background

The history and current methods of typing *M. avium* subsp. *paratuberculosis* (*MAP*) have been summarised in recent reviews [1], [2]. Restriction fragment length polymorphism analysis based on the insertion sequence IS*900* (Collins et al 1990) very clearly distinguished strains of *MAP* into three groups. Strains of one group called Type C (also called Type II) infect cattle and deer and are uncommon in sheep, while the other strains called Type S (also called Types I and III) were found to infect primarily sheep and occasionally deer but in most countries are rarely isolated from cattle [3], [4], [5]. In previous years of this programme, we established a subtyping system for New Zealand isolates of *MAP* based on five variable number tandem repeat sequences (VNTRs) and two short sequence repeats (SSRs) and used this system to subtype isolates from NZ dairy cattle, beef cattle, sheep and deer. In 2010-12, in collaboration with Livestock Improvement Corporation (LIC) and Massey University, we applied the VNTR/SSR subtyping system to 211 isolates obtained from dairy cattle and 154 isolates obtained from beef cattle, sheep and deer on mixed farms [6] [7]. Dairy cattle were found to harbour a variety of C subtypes with one predominant subtype (CDa1) accounting for 70% of the isolates. Most deer (76%) harboured a different C subtype (CDe). The majority of sheep (86%) harboured type S strains with most (67%) harbouring the predominant type (CSh). Results from this study indicated that although there were predominant types associated with different ruminant host, co-grazed animals shared the same MAP subtype, *e.g*. beef cattle that were co-grazed with sheep were more commonly infected with Type S (68%) than with Type C isolates.

There is some evidence that certain MAP subtypes are more virulent than others to farmed NZ red deer. If this were the case then future intervention may come from developing strategies for co-grazing with animals that harbour less virulent subtypes. Mackintosh and co-workers found that a type C MAP isolate was pathogenic to experimentally infected deer whereas a Type S strain was relatively non-pathogenic to these animals [8]. Two different typing surveys, one involving isolates from 65 deer faeces samples that were typed as part of the mixed farm analysis described above (Milestone 3.2.5), and another involving 93 isolates from deer lymph nodes that were collected by JML (Milestone 3.2.9), indicated that most of the MAP (>95%) isolated from deer in NZ is Type C. Typing results for Milestone 3.2.9 were correlated with MAP load in the infected lymph nodes, and acid fast organisms were seen in many of the lymph nodes infected with two common type C strains, some in very high number, whereas no acid fast organisms were detected in lymph nodes from which S strains and some less common C strains were isolated. VNTR/SSR subtyping of these samples has revealed that deer tend to more frequently harbour different C subtypes than dairy cattle, although both the most common deer and the most common dairy subtypes when present were sometimes carried in high loads. Although no firm conclusions could be drawn because these major types were also isolated from lymph nodes with no qPCR detectable or very low numbers of organisms, these results suggest that these common C subtypes are more virulent to deer than the type S strains.

The aim of the current JDRC deer study was to define the relationship between seroprevalence, faecal shedding and lymph node lesion status in NZ herds of farmed red deer with the intention of developing an internationally accepted serological screen for the inexpensive and reliable detection of highly infectious deer. As a result of this study there was a large and well documented set of faecal samples available from deer with varying levels of clinical Johne's disease, faecal shedding, and seroprevalence. For the current study (Milestone 3.2.11) isolates from animals with high and low levels of MAP shed into faeces were subtyped in order to enable us to more definitively identify virulent subtypes and to determine whether certain narrow or broad ranges of subtypes are more commonly associated with at-risk herds.

Before co-grazing intervention schemes can be seriously considered, it will also be important to understand the virulence of different MAP subtypes for sheep. It is not known if the merino flocks, which tend to develop more clinical Johne's disease, have different distributions of subtypes than other sheep breeds or whether the level of pathology in merino or other flocks correlates with the presence of certain MAP subtypes. The sheep intervention study determined the level of Johne's disease in merino and non-merino mixed sheep flocks. Samples for culture and typing were available from faeces and lymph nodes of merino and non-merino sheep with levels of disease ranging from negative (no ELISA or histopathology), varying amounts of subclinical infection, and from animals displaying signs of clinical Johne's disease. Although type C strains were previously isolated from sheep, most isolates from sheep are type S (87%). The diversity of S subtypes in NZ sheep flocks is much lower than that of MAP subtypes observed in other ruminants.

Although results obtained from comparing differences at poly G repeat regions are questionable and perhaps not robust enough for use in standard typing assays, [9] [10] our recent addition of one of these regions, the short sequence repeat 2 assay (SSR2) [11], to the panel used for typing isolates increased the number of possible deer and sheep subtypes and was analysed in this work to determine its ability to distinguish subtypes that are more frequently associated with advanced pathology.

Methods

Culture of MAP isolates. Faecal and lymph tissue from sheep that were analysed as part of the sheep study were collected and stored at -80^{o}C. Faecal samples from deer were collected within two weeks of slaughter, split into aliquots for qPCR analysis at the Disease Research Laboratory (DRL) and for culture and typing at AgResearch, and the aliquots were frozen and stored at -80^{o}C. For culture, both lymph tissue and faeces samples were decontaminated using the double-incubation method described by Whitlock and Rosenberger [12]. For lymph node tissue samples, approximately 1 g of tissue was homogenized in 20 ml of sterile water in a Stomacher (Colworth; Seaward, Norfolk, United Kingdom). The tissue homogenate was filtered through sterile cheesecloth and decontaminated with an equal volume of 0.75% cetylpyridinium chloride (CPC) for 40 min. The decontaminated tissue homogenate was centrifuged at 3,500 × *g* for 20 min. The pellet was re-suspended in 1 ml of sterile water, and 0.5 ml was inoculated into 5 ml of 7H9 medium that was supplemented with OADC, egg yolk (0.8ml), 5 µg mycobactin J and antibiotics (PANTA; Becton Dickinson) [13]. For faecal samples, 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 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 [14]. After a further incubation period of 3 days, 0.5ml of the sample was inoculated into supplemented 7H9. The vials were incubated at 37°C, and examined every two to three weeks. Positive cultures were identified by acid-fast staining of culture samples.

VNTR typing. DNA was extracted as described by de Lisle et al. [15]. In this method, 0.2 ml of a culture was added to 0.5 ml glass 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

microcentrifuge 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 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) loci SSR2 and 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, and SSR2, 25 µl PCR reactions contained 1×standard reaction buffer, 1–2 mM MgCl2, 0.2 mM dNTPs, 1 µM primer mix, 1.25 U Amplitaq (Roche), 0.1 mg/ml bovine serum albumin (BSA), 0–12% DMSO and 2ul MAP DNA. SSR8 and SSR2 reactions were as above but with appropriately scaled 50 µl 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 and SSR2 PCR reactions, or annealing at 60°C for 1 min for SSR8 and SSR2 PCR reactions, followed by extension at 72°C for 1 min and a final extension at 72°C for 7 min. The variable conditions of the PCR reactions optimized for each VNTR are summarized in Table 2. The un-purified SSR8 and SSR2 PCR products were outsourced for purification and sequencing to Macrogen.

Allelic diversity (h) was estimated for sheep samples in the previous and present studies using the following equation:

$$h = 1 - \sum (Xi/N)^2 (N/(N-1))$$

Where x is the number if isolates of type i, and N is the total number of samples.

Table 1 Primers for VNTR/SSR 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
SSR2-F	DMC1806	GTGACCAGTGTTTCCGTGTG
SSR2-R	DMC1807	TGCACTTGCACGACTCTAGG

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

VNTR	[Mg] mM	% DMSO	% agarose gel
292	2.0	12	2.5
25	1.5	0	2.5
X3	1.25	0	2.5
7	1.5	0	4.0
3	2 0	10	4.0
SSR8	2.0	5	2.5
SSR2	1.5	10	2.5

Results and Discussion

Culture

Deer. A total of 93 samples from 4 deer farms in the Otago region were received and MAP was isolated from 89 of these samples (96%) (Table 3 and Appendix 1). Each of these faecal samples were also analysed by qPCR at DRL as described in [16]. Corresponding qPCR levels ranged from below reliable detection limits (0-10^2 genome equivalents per gram of faeces) to very high (≥10^6) in super shedding animals. Each farm provided samples containing a broad range of shedding levels. In agreement with the findings in O'Brien et al in [16], the five samples that failed to give rise to positive MAP cultures were from samples that gave "no" (0-10^2) or "background" (≥10^2 - <10^4) qPCR signal. The remainder of the samples, including 7 of the 8 for which there was "no detected" MAP genomes and 25 of the 28 that were considered "background" level by qPCR were culture positive, indicating that our culture system is a sensitive means of detecting MAP in deer faeces.

Table 3 Culture versus qPCR count in deer faeces

	None	Background	Low	Moderate	High	Very High	
Culture	0	1	2	3	4	5	6
positive	7 (87%)	25 (89%)	13 (100%)	5 (100%)	12 (100%)	12 (100%)	17 (100%)
negative	1 (13%)	3 (11%)	0	0	0	0	0

Sheep. A total of 142 samples from 21 different merino and non-merino sheep farms from both the North and South Islands were cultured. When it was available, MAP was cultured from lymph tissue since it is known to be more efficiently cultured from this source than from faeces and isolation from lymph tissue most likely indicates that the animal was infected while culturing from faeces may indicate just passage through the gut (passive infection). Of the samples that were cultured, 71 (49%) produced MAP isolates (Table 4, Appendix 2). Histopathology was characterised for each of these animals and was classified based on the system of Perez et al [17]. Of the sampled animals, 77 of the 142 were class 1 (had a mild nonspecific infection). Because many of the class 1 and 2 samples from the first series of 93 samples that were processed for this work were culture negative, an additional set of 49 class1 and 2 samples were processed. Of the 77 class 1 samples that were analysed in total, only 16 (21%) were culture positive. Of the 9 samples from animals with class 2 lesions, considered to have a moderate infection, 3 (33%) were culture positive. The 15 samples from clinically affected animals and the 30 samples from severely clinically affected animals were culture positive in most cases (89% and 97% respectively). Most (77%) of the samples from animals with class 3c histology were culture positive. The slightly lower percentage of culture positive samples from 3c animals may reflect the lower numbers of MAP bacteria in animals with this type of lesion. Serum ELISA levels for these animals are shown in Appendix 2, and serology is compared to culture in Table 4 and in Figure 1 where the percentage of seropositive and seroequivocal animals are compared to the percentage of animals that were culture positive. These results indicate that animals that have very low levels of infection are difficult to detect with either serology or culture whereas animals with moderate infection are better detected by serology than by culture. These results also indicate that culture may be slightly more sensitive than serology for detecting animals with clinical and severe clinical infections.

Table 4 Culture Vs Histology in sheep samples

	Class I mild nonspecific	Class 2 moderate	Class 3a clinical	Class 3b severe clinical	Class 3c severe nonspecific
Culture positive negative	16 (21%) 61 (79%)	3 (6%) 6 (67%)	13 (87%) 2 (13%)	29 (97%) 1 (3%)	10 (77%) 3 (23%)
Serology positive equivocal negative	9 (13%) 0 63 (87%)	8 (89%) 1 (11%) 0	9 (69%) 0 4 (31%)	19 (63%) 6 (20%) 5 (17%)	9 (75%) 0 3 (25%)

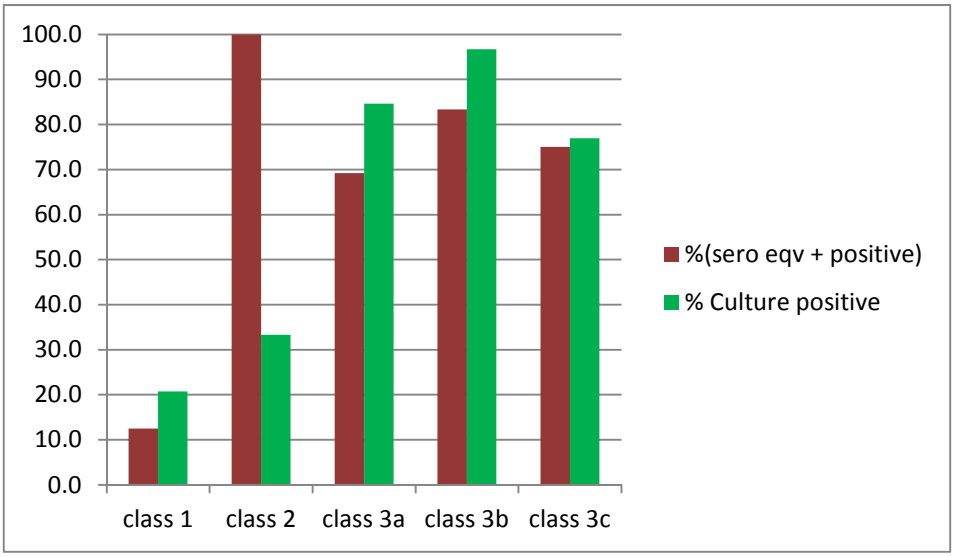

Figure 1 Histology class versus percent culture positive and (seroequivocal + seropositive) samples

In conclusion, since the well-established BACTEC system for growing and monitoring MAP is no longer available, we used a culturing system for these studies that is the basis of BACTEC (described in [18]) and monitored the cultures by ZN staining. As seen by others [19] [20], our results indicate that this procedure is effective for isolating both type C and S strains of MAP. Our culture system worked well for culturing MAP from deer samples and was also useful for culturing MAP from more severely infected sheep. As was seen by Perez et al [17] who analysed 166 Rasa and Rasa cross sheep in Spain, samples from sheep with lower levels of MAP were more often culture negative, presumably because of the difficulty of culturing the sheep strain, especially from samples with low numbers of bacteria as discussed in ([13]). These animals were selected for study because they were below average body weight and then MAP disease severity quantified in terms of the number and types of histological lesions that could be observed at necropsy. In some instances, the poor condition of class 1 animals that were surveyed was probably not due to MAP infection. This latter explanation is certainly the most plausible for Farm S where there was no seroprevalence or culturable MAP detected in any of the 11 animals that were surveyed (see Appendix 2). These results suggest that using histology as a gold standard for MAP infection in sheep has limited value.

VNTR

Deer. All the deer samples that were characterised for this Milestone had the common deer type (432224) and were thus not useful for comparisons of disease severity vs VNTR/SSR8 subtype. This lack of variation at first seemed surprising since there were multiple types detected in the mixed farm subtyping survey conducted in collaboration with Massey scientists (Milestone 3.2.5) and in the

subtyping survey of lymph nodes from NZ slaughter houses (milestone 3.2.9) (See Table 5 below). In Appendix 3, the lymph node isolates from the slaughterhouse survey are grouped by regional source, and this shows that deer slaughtered in the North Island were more likely than those from the South Island, especially Otago and Southland, to harbour different types including the common dairy cattle subtype (332225). Thus, the observed lack of diversity in the samples analysed here is at least in part because all were sourced from farms in the Otago region.

Although the robustness of subtyping with SSR2 is questionable (Milestone 3.2.9 and 3.2.10, [9] [10]), the deer isolates from the current study were assayed for variability at the SSR2 locus. The ability of SSR2 to subdivide these samples, and the associated qPCR levels of the animals from which the different subtypes were isolated are illustrated in Appendix 1. SSR2-10 was the predominant type on three deer farms, and was isolated from animals that were shedding very high levels of MAP as well as from animals that were shedding barely detectable levels of MAP, whereas most other subtypes (SSR2-7,9,11,12) were isolated from animals shedding very low to moderate levels of MAP. Although the number of each subtype are too low for statistical analysis, a possible explanation for this observed trend is that SSR2-10 is being super-shed by some animals on these farms and is passively infecting other animals in the herd as discussed in ([21]). Farm 4 was the source of the majority (50 of the 74) of the samples that were analysed for this study. In contrast to the SSR2 type distributions observed on the other 3 farms, the majority of the animals on farm 4 were shedding SSR2-9. The recent comparative analysis of dairy cattle types in Canada [22] indicated that subtyping by counting the number of repeats in a miniscule part of the genomes of these bacteria is likely to misrepresent the true level of diversity and relatedness of different MAP subtypes on these farms. If the cattle type that is frequently isolated from NZ red deer behaves in a similar fashion to the cattle strains infecting Canadian dairy cattle, there will likely be a multitude of genomic differences detected when SSR2-9 types from different farms are characterised by WGS. This comparison is a planned part of the revised milestones for this project, and may identify genomic differences (SNPs) that enable the SSR2-9 subtype to predominate on farm 4. The ability of SSR2 to distinguish farm 4 from the other three in terms of subtype and subtype diversity indicates that this assay is useful for some limited types of epidemiological questions.

Sheep. Only 2 of the 4 isolates that were not the common sheep type were a C subtype (CDe) and one of these had both CDe and CSh making it difficult to make any inferences about the correlation between disease severity of C and S subtypes in sheep. The distribution of subtypes from this current study (3.2.13) is compared to the mixed farm survey (Milestone 3.2.11) in Table 5. As seen previously, the majority of sheep isolates from the current sheep study had the common sheep subtype (CSh also called S3 and 431113 in Table 5). Two of the other subtypes that were previously isolated from sheep were also seen in this study, 1 isolate had the common deer subtype (CDe also called C12, and 432224 in Table 5) and 2 isolates were a variant of the common sheep type (S1, 331113 see Table 5). The diversity index for this current sample set was 0.10 versus the 0.52 index observed for the previous set indicating that even though this is a smaller sample set, the level of variability is lower in the present study and many of the types that were seen in the previous study were not seen this time. This lack of VNTR/SSR8 diversity may reflect the types of farms that were sampled. The previous set were from farms where sheep were co-grazed with other farm stock and the co-grazing status of the currently studied flocks is not clear from the data supplied with the samples. Appendix 2 shows the subtypes found on different sheep farms and reveals that the VNTR/SSR8 subtype distribution for most of the farms analysed in this study is uniformly the common sheep type. Most of the observed MAP VNTR/SSR8 subtype diversity is from samples taken from animals on Farm A. The cattle subtype isolated from this farm was isolated from an animal that had no lesions but any general conclusion about the virulence of this subtype in sheep would require further isolates of this type from animals with the same level of disease severity. As was true of the deer samples, SSR2 subtyping divided the uniform set of sheep samples into 4 different subgroups (SSR2-9,10,11 and 12). As discussed above, there are limitations on the use of this type of assay for

distinguishing MAP isolates with the same SSR2 subtype from different farms as they are often likely to be from quite distinct MAP lineages when analysed by WGS. Even so, the fact that there were instances where there was a predominant SSR2 subtype on certain farms, and a different predominant type on others, indicates that this assay may provide some limited aid in certain epidemiological analyses.

Subtype	Alias	Subtype 292-25-X3-7-3-SSR8	Dairy	Beef	Sheep		Deer		
					Mixed farm	This work	Mixed Farm	Deer LN	This work
					diversity (h)	diversity (h)			
					0.52	0.1			
C1		2-3-2-2-2-5	1						
C3	CDa2	3-2-2-2-2-5	25						
C4		3-2-2-3-2-5	2						
C5		3-3-2-0.5-2-5	6				3		
C6		3-3-2-2-2-3	1						
C7		3-3-2-2-2-4	4		2		1	4	
C8	CDa1	3-3-2-2-2-5	147	2	2		8	14	
C9		3-3-2-3-2-5	1						
C12	Cde	4-3-2-2-2-4	5	4	6	1	51	70	74
C13		4-3-2-2-2-5	1				1		
C16		5-2-2-2-2-5	1						
S1		3-3-1-1-1-3	3	1	5	2	1		
S2		3-3-1-2-1-3			1				
S3	CSh	4-3-1-1-1-3	11	12	50	45	2	3	
S5		4-3-1-2-1-3			5				
S6		5-3-1-1-1-3	1		2				
S10		7-3-1-1-1-3			1				
		Total	209	19	74	48	67	91	74
			mixed 30		mixed 8	mixed 1	mixed 2	mixed 3	

Table 5 Diversity of MAP sub-types in mixed farm samples (Milestone 3.2.5) and the survey of deer lymph nodes from NZ slaughterhouses (Milestone 3.2.9) with types observed in the current sheep and deer studies (Milestone 3.2.11, 3.2.13). Mixed indicates the number of samples in the indicated survey that had more than one detectable type. Diversity is calculated as described in the methods section.

Recent reports [23] [24] [25] [22] indicate that WGS and specific assays developed from WGS studies [26] [27] are the best approaches for studying MAP epidemiology and virulence. Our preliminary WGS data suggest that there is likely to be more variation when entire genomes of the isolates from these studies are compared. Representatives of the four major groups (CDa1, CDa2, CDe and CSh) were genome sequenced in collaboration with Karen Stevenson along with isolates from other parts of the world. Comparisons of the single nucleotide polymorphisms (SNPs) that were identified suggest multiple introductions of MAP into NZ. Our predominant dairy cattle type (CDa1) most closely resembles K10 from USA (35 SNPs differ between these two genomes) whereas the second most common dairy cattle type (CDa2) more closely resembles isolates from the UK than it does CDa1. CDa1 and CDa2 differ at only one locus when compared by our VNTR/SSR8 assay but there were approximately 80 SNPs that differed when their whole genomes were compared. The Common Deer type CDe more closely resembles European types, with approximately 250 SNPs that distinguish it from CDa1 and CDa2. As might be expected from the fact that there were 4 differences detected by VNTR/SSR8, there are over 3000 SNPs that differ when the genomes of the NZ Cattle types are compared to our common sheep type (CSh). Thus there are likely to be more distinctions when the SNP content in the genomes of isolates from the current work are analysed providing an excellent opportunity to achieve better MAP characterisation and further insights from studies that have already been funded by JDRC.

In Conclusion, this work has provided isolates from animals for which there is a very good assessment of disease status and a wide range of disease severity. Surveys of this kind are unlikely to be repeated in NZ in the near to medium future so it was advantageous to have the opportunity to

culture representative sample sets. There was not much variability in this set when analysed by VNTR/SSR. The ultimate resolution possible from analysing entire genomes will help to determine if this lack of variability was due to the shortfalls of VNTR typing or because the range of disease severities observed in farmed livestock is from differences in the ruminant hosts rather than from differences in the MAP. We have been developing expertise in processing and interpreting WGS data for sourcing *Mycobacterium bovis* breakdowns and we intend to implement this technology as part of our routine *M. bovis* typing system in the near future. Collaboration established between Cord Heuer, Jereon De Buck and AgResearch and JDRC will provide an opportunity to define the lineages of the important MAP subgroups. WGS analysis will more clearly correlate the tendencies of different lineages to be isolated from animals with more severe disease because of the higher resolution, and may also identify possible physiological explanations for differences in virulence, since it will enable us to understand the differences in terms of the gene functions that get altered by the changes. The intended practical outcomes of these additional WGS studies will be assays that can be developed to very specifically determine the source of MAP isolates and also to rapidly determine if herds/flocks harbour more virulent forms of MAP.

Acknowledgements

We would like to thank Cord Heuer and his helpers at Massey University, as well as Peter Anderson and the other vets who helped collect sheep samples, and also Solis Norton (JML), Peter Fennessy (Abicuss Bio), Simon Ligget and Rory O'Brien (DRL) for organising, sending and advising on deer samples and data.

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