3.3.2 Milestone 3.2 Strain Typing

Description	During the first two years, develop a typing system for New Zealand isolates of M. paratuberculosis based on repetitive DNA sequences. This will provide a tool for use over the following years of the consortium to answer epidemiological questions about sources of infection within and between farmed animals and the potential involvement of wild life species. This knowledge would be used to design better disease control strategies for use by farmers and will also contribute to other objectives.
Alignment with JDRC Strategy	Tool: Diagnostic providing evidence to support Herd/Flock management techniques to control JD
Status	On track, ongoing program. Years 1-5

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Molecular Strain Typing

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EXECUTIVE SUMMARY

DNA sub-typing of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) is an important general tool because of its ability to answer crucial epidemiological questions, and its usefulness in infection, vaccination and pathogenicity studies. Typing of *MAP* isolates from moderately large LIC and Massey studies into the two major subdivisions of *MAP* called Type C and Type S showed that infection of cattle with Type S isolates was much more prevalent than previously believed. To what extent these Type S isolates are causing progressive disease in New Zealand cattle is presently unknown. The frequency of the different sub-types found for the Type C isolates from dairy cattle was substantially different to that found for the Type C isolates from the Massey mixed farm project which included many deer isolates. These findings raise issues about the pathogenicity of different types and sub-types of *MAP* for different ruminant species that have a potential impact on most other JRDC milestones. Infection of individual cattle with more than one sub-type was frequent in the LIC study, and this probably reflects a situation where levels of infection are high on some dairy farms. There was the same predominant sub-type for Type S isolates in both the LIC and Massey study and this sub-type also predominated in the archived Type S isolates. Sub-typing of Type S isolates is substantially less than optimal and might be improved by the use of additional SSR and possibly VNTR sequences.

1. INTRODUCTION

The ability to distinguish between different strains of a pathogenic bacterial species provides a basis for answering important epidemiological questions about sources of infection and spread of disease and enables potential variation in pathogenicity of different strains to be more easily investigated. Since 1980, this process of distinguishing or typing strains has increasingly relied on the direct detection of DNA differences between strains. The history and current methods of

typing *M. avium* subsp. *paratuberculosis* (*MAP*) are summarised in a recent review [1]. The first DNA typing system based on restriction endonuclease analysis [2] was quickly superseded by restriction fragment length polymorphism analysis based on the insertion sequence IS*900* [3]. IS*900* typing 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) infect primarily sheep and occasionally deer but in most countries are rarely isolated from cattle [4-7], although exceptions to this have been reported from Iceland [6] and Spain [8]. While IS*900* has been very helpful in distinguishing *MAP* into strains that preferentially infect sheep and cattle, the technique is cumbersome and relatively few differences have been identified within each group. Until six years ago, other DNA based approaches were also cumbersome [9] or relatively non-discriminating [7, 10]. This lack of discrimination has made it difficult to answer questions about sources of infection when one group of strains overwhelmingly predominates. This has been the situation for Type C strains in cattle in many countries including New Zealand. The recent availability of large amounts of DNA sequence from mycobacterial genome projects has led to the identification of many repetitive DNA sequences; often referred to as variable number tandem repeats (VNTRs). The recent application of VNTR typing to *MAP* [7, 11-13] indicates that some VNTRs [7, 11, 13], including two very short sequence repeats (SSRs) [13], appeared to have a better ability to type strains of *MAP* than IS*900* and other methods. Not all VNTRs have yet been tested, and those used so far have only been applied to modest numbers of strains from a few regions.

In 2008 – 2010, we selected eight VNTRs and two SSRs that were reported from overseas studies to be particularly useful for sub-typing and applied them to archived New Zealand isolates from cattle, deer and sheep. A combination of the best 5 of these VNTRs gave good discrimination of 65 Type C isolates, resulting in a Simpson's index of diversity (h) of 0.69, and this was increased to 0.83 by including results from one of the short sequence repeats, SSR8. By also including the results from SSR1, the diversity index was improved to 0.92. Satisfaction with this last result was short-lived, as a preliminary stability study indicated that the SSR1 results may not be reliable. The other notable finding to emerge from this first study of New Zealand Type C isolates was that one sub-type that was common in deer was not found in any of the cattle isolates. The five VNTRs that showed some discrimination among the Type C isolates together with SSR1 and SSR8 were applied to 58 archived Type S isolates. Combining the results from all the VNTRs and the two SSRs resulted in a diversity index of 0.47, indicating that there was much less diversity among New Zealand Type S isolates than Type C isolates. The other major finding from the VNTR and SSR sub-typing of Type C and Type S isolates was that in all cases the VNTR/SSR types of Type C isolates were different from those of Type S isolates.

The three objectives of this current year of the project were to apply the VNTR/SSR sub-typing system established during the previous two years to: 1) 200 isolates obtained from an LIC study of dairy cattle; 2) 150 isolates obtained from the Massey University study which is investigating farms that have two or more farmed animal species in contact; and 3) to obtain improved information on the stability of VNTR/SSR sub-types, particularly SSR1, in Type C isolates.

2. METHODOLOGY

2.1. *MAP* samples

Two large groups of *MAP* isolates that had been cultured at Wallaceville from faecal samples submitted from other projects were used for typing. The first group of approximately 200 *MAP* cultures came from dairy cattle samples selected by LIC on the basis of serological results. The second group of approximately 150 isolates was cultured from beef cattle, sheep and deer samples submitted from a Massey University study of farms that had two or all three of these animal species in contact. These samples were not selected on the basis of prior testing. For the stability study, four Type C isolates from the previous year's study were continually sub-cultured

and three other Type C isolates were cultured and subjected to cloning by sub-culturing on solid medium and also by serial dilution in liquid culture.

2.2. DNA extraction methods

Isolates of *MAP* from the LIC and Massey studies were cultured in BACTEC medium containing egg yolk as described by Whittington [14]. DNA was extracted as described by de Lisle et al. [15]. In this method, 0.2 ml of a BACTEC culture was added to 0.5ml glass beads and 1 ml of proprietary ASL buffer (QIAamp DNA stool kit) and shaken strongly in a 2 ml microcentrifuge tube for 2 periods of 20 sec each in a Ribolyser (FastPrep Cell disrupter; ThermoSavant, Holbrook, New York, USA) set on 6.5, with 1 min cooling on ice between each period. The suspension was heated in a water bath at 95ºC for 10 min, vortexed for 15 sec and then centrifuged at 20,000g for 1 min. For each sample, 1 ml supernatant was removed and placed into a fresh 2 ml tube, half an inhibitEX tablet was added and the mixture was vortexed until the tablet was completely dissolved. Suspensions were incubated at room temperature for 1 min and then centrifuged at 15,000 g for 3 min. The supernatant was centrifuged for another 3 min and then 0.2 ml supernatant was treated with 15 µl of Proteinase K and 0.2 ml of AL buffer and incubated at 70ºC for 10 min. To precipitate the DNA, 4 µl of glycogen and 0.2 ml of ethanol were added to the suspension and vortexed. The suspension was then added to a Qiagen column and centrifuged for 1 min at 15,000 g. The column was washed with 0.5 ml AW1 buffer, centrifuged, washed a second time with 0.5 ml of AW2 buffer and centrifuged. Residual washing buffer was removed by centrifugation for 3 min and then DNA was eluted with 0.1 ml of AE buffer by centrifugation at 15,000 g for 1 min.

2.3. Determination of Type C/Type S status

MAP is divided into two major groups (Type C and Type S) and these groups can be clearly distinguished by a PCR assay developed for this purpose by Collins et al., [16]. All isolates were subjected to this PCR analysis to determine whether they were Type C or Type S isolates.

2.4. PCR amplification conditions for VNTR assays

For PCR analysis of the five chosen VNTR loci, 20 ng of *MAP* DNA was subjected to PCR using the forward (F) and reverse (R) primers in Table 1.

Table 1. Primers for VNTR analysis.

VNTR	Primer name	Sequence
292-F	DMC1468	CTTGAGCAGCTCGTAAAGCGT
292-R	DMC1469	GCTGTATGAGGAAGTCTATTCATGG
25-F	DMC1470	GTCAAGGGATCGGCGAGG
25-R	DMC1471	TGGACTTGAGCACGGTCAT
X3-F	DMC1478	AACGAGAGGAAGAACTAAGCCG
X3-R	DMC1479	TTACGGAGCAGGAAGGCCAGCGGG
7-F	DMC1476	GACAACGAAACCTACCTCGTC
7-R	DMC1477	GTGAGCTGGCGGCCTAAC
3-F	DMC1474	CATATCTGGCATGGCTCCAG
3-R	DMC1475	ATCGTGTTGACCCCAAAGAAAT

PCR reaction conditions were optimized for each VNTR locus. The 25 µl PCR reactions contained 1 X standard reaction buffer, 1-2 mM $MgCl_2$, 0.2mM dNTPs, 1 µM primer mix, 1.25 U Amplitaq (Roche) 1mg/ml bovine serum albumin, 0 - 12% DMSO and 2 µl *MAP* DNA. PCR reaction conditions were denaturation at 94ºC for 10 minutes, then 35 cycles of denaturation at 94 ºC for 45 sec, annealing at 58ºC for 1 minute, extension at 72ºC for 1 minute and a final extension at 72ºC for 7 min. The variable conditions of the PCR reactions optimized for each VNTR are summarized in Table 2.

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

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

2.5. PCR amplification conditions for SSR assays

The primers for SSR1 and SSR8 are listed in Table 3. The 50 µl reaction for both SSR1 and SSR8 contained 1 X standard PCR buffer, 2 mM $MgCL_2$, 250 µM dNTPs, 0.4 µM primers, 5% DMSO and 0.5 U of TaqGold, as outlined by Amonsin et al. [13]. PCR conditions were denaturation at 94ºC for 15 minutes, then 35 cycles of denaturation at 94ºC for 45 sec, annealing at 60ºC for 1 min, extension at 72ºC for 2.5 min and a final extension at 72ºC for 7 min. The PCR products were then outsourced for purification and sequencing using the corresponding primers in Table 3.

Table 3. Forward (F) and reverse (R) primers used for SSR sequencing.

SSR	Primer name	Sequence
SSR1-F	DMC1505	TCAGACTGTGCGGTATGGAA
SSR1-R	DMC1506	GTGTTCGGCAAAGTCGTTGT
SSR8-F	DMC1507	AGATGTCGACCATCCTGACC
SSR8-R	DMC1508	AAGTAGGCGTAACCCCGTTC

2.6. VNTR stability study

In the previous year, three clones each of four Type C cultures were isolated and sub-cloned successively into three liquid sub-cultures of Middlebrook 7H11 supplemented medium. These clones have continued to be serially sub-cultured in the same medium during the current year. DNA from these cultures will be extracted by the CTAB method [17] at the end of the experiment in two months time and will be sub-typed using VNTRs: 292, 25, X3, 7 and 3; as well as SSR1 and SSR8.

Three Type C isolates of *MAP* were grown on solid Middlebrook 7H11 supplemented medium and ten colonies of each are being sub-cultured in liquid medium. As a back-up in case of contamination, three type C isolates of *MAP* are also being cloned by making serial dilutions in liquid medium. At the end of the experiment in two months time, DNA from these cultures will be extracted by the CTAB method [17] and will be sub-typed using VNTRs: 292, 25, X3, 7 and 3; as well as SSR1 and SSR8.

3. RESULTS

3.1. Comparison of Type C/Type S status to VNTR/SSR8 type

All LIC and Massey isolates gave unequivocal results for Type C/Type S determination. In all cases, Type S isolates had VNTR/SSR8 sub-types that were different from those of Type C isolates. The clearest single VNTR difference between Type S and Type C isolates was seen with the VNTR3 assay; all Type S isolates had one copy of this VNTR sequence, whereas all Type C isolates had two copies. The same result was found for the archived Type C and Type S isolates in 2008-2010.

3.2. Sub-typing of LIC isolates

At the time this report was prepared, sub-typing had been completed for 103 of the planned 200 isolates; the remaining isolates should be completed by mid-April. Almost a quarter (24; 23%) of these 103 isolates yielded organisms that gave multiple alleles for one or, in the case of four samples, two of the VNTR assays. This indicates that at least two different sub-types of *MAP* were present in those samples. A comparison of the number of these multiple-allele samples found to date in the LIC study to those found in the Massey study is given in Section 3.5. The situation with regard to the sub-types found for the 79 LIC samples that gave single alleles for all VNTRs are shown in Table 4.

Table 4. LIC study; sub-types for 79 isolates

Group	Total	VNTR results					SSR8
		292	25	X3	7	3	
Type S (n=10)*	1	3	3	1	1	1	3
	9	4	3	1	1	1	3
Type C (n=69)*	10	3	2	2	2	2	5
	2	3	2	2	3	2	5
	1	3	3	2	0.5	2	5
	2	3	3	2	2	2	4
	51	3	3	2	2	2	5
	1	3	3	2	3	2	5
	2	4	3	2	2	2	4

* Each row is a different sub-type (a different combination of VNTR/SSR8 results)

The most surprising result was that approximately 13% (10/79) of these isolates, the majority of them (6) from a single farm in Takapau, were Type S isolates that are common in sheep. It was not expected before the start of this project that this many Type S isolates would be found in dairy cattle. Other interesting features of the LIC results in comparison to results from typing 142 Massey samples from sheep, deer and beef cattle (Table 5) and 121 archived isolates in 2008-2010 (Table 6) are presented in Section 3.4 below.

3.3. Sub-typing of Massey isolates

Approximately two thirds of the 154 samples containing *MAP* from the Massey University study that were sub-typed were pooled samples. Twelve of the samples gave multiple alleles for one of the VNTRs (VNTR 292 in all but one case) and in most cases (83%; 10/12) these came from pooled samples. A comparison of the number of these multiple-allele samples found in the Massey study to those found to date in the LIC study is given in Section 3.5. Results of the remaining 142 samples are given in Table 5 below.

Table 5. Massey study; sub-types and host distribution for 142 isolates

Group	Number of animal isolates				VNTR results					SSR8
	Total	Cattle	Deer	Sheep	292	25	X3	7	3	
Type S * (n=68)	3	1	1	1	3	3	1	1	1	3
	1	–	–	1	3	3	1	2	1	3
	56	12	2	42	4	3	1	1	1	3
	5	–	–	5	4	3	1	2	1	3
	2	–	–	2	5	3	1	1	1	3
	1	–	–	1	7	3	1	1	1	3
Type C * (n=74)	3	–	3	–	3	3	2	0.5	2	5
	1	1	–	–	3	3	2	2	2	3
	14	3	9	2	3	3	2	2	2	5
	56	5	48	3	4	3	2	2	2	4

* Each row is a different sub-type (a different combination of VNTR/SSR8 results)

The most surprising result was that more cattle were infected with Type S isolates (13) than with Type C isolates (9). In all but one case, the particular sub-type of Type S infecting these cattle was the most frequent sub-type found in sheep. There were also five sheep infected with common Type C sub-types. Other features that stand out in the Massey results are that the majority (76%) of the Type C isolates have a single sub-type and the majority (82%) of the Type S isolates have a single sub-type. Comparison of these results to those of the LIC and archived samples is given in Section 3.4.

Further analysis of these typing results will be carried out by Massey University who were responsible for obtaining the samples and are in a position to correlate these results with farm and herd/flock information. This overall analysis should reveal to what extent the same or different sub-types infect different host species on the same farm.

3.4. Comparison of all New Zealand isolates so far sub-typed by VNTR/SSR8

Results from sub-typing of the archived isolates in 2008-2010 are given in Table 6. For comparison purposes, similar formats have been used for Tables 4-6.

Table 6. Archived isolates; sub-types and host distribution for 121 isolates

	Number of animal isolates				VNTR combinations found					
Group	Total	Cattle	Deer	Sheep	292	25	X3	7	3	SSR8
Type S* (n=57)	1	–	–	1	3	3	1	1	1	3
	1	–	–	1	3	3	1	1	1	4
	52	–	–	52	4	3	1	1	1	3
	1	–	–	1	4	3	1	1	1	4
	1	–	–	1	5	3	1	2	1	3
	1	–	–	1	6	3	1	2	1	3
Type C* (n=64)	2	–	2	–	3	2	2	2	2	4
	6	4	2	–	3	2	2	2	2	5
	14	–	14	–	3	3	2	2	2	4
	15	–	15	1 (goat)	3	3	2	2	2	5
	1	1	–	–	3	3	2	3	2	5
	1	1	–	–	4	2	2	2	2	5
	1	–	1	–	4	3	1	2	2	5
	3	–	3	–	4	3	1	3	2	5
	2	–	2	–	4	3	2	2	2	4
	15	12	3	–	4	3	2	2	2	5
	1	1	–	–	4	3	2	3	2	5
	1	–	1	–	5	3	2	2	2	4
	2	–	2	–	5	3	2	3	2	4

* Each row is a different sub-type (a different combination of VNTR/SSR8 results)

A number of important features that emerge from comparison of the results given in Tables 4-6 for the LIC, Massey and archived isolates are outlined below.

A. The frequency of the different sub-types found for the Type C isolates from dairy cattle was substantially different to that found for the Type C isolates from the Massey mixed farm project which included many deer isolates. The most common sub-type in dairy cattle accounted for 74% (51/69) of the Type C isolates, whereas this sub-type accounted for only 12% (9/74) of the Type C isolates from the Massey project. In contrast, the most common sub-type among the Massey Type C isolates, which accounted for 76% (56/74) of the isolates, was found in only 3% (2/69) of the LIC Type C isolates. This sub-type is clearly very common in deer but rare in dairy cattle.

B. The second most common LIC sub-type representing 14% of the LIC Type C isolates (10/69) was present at a fairly similar frequency (9%; 6/64) in the archived Type C isolates but was not found for any of the Massey isolates. This likely reflects the inclusion of a number of isolates from dairy cattle in the archived isolates and the absence of dairy samples from the Massey study.

C. One sub-type of the Type S isolates predominated in all three studies. While the reason for this is unknown, the continued presence of such a dominant sub-type does limit the potential epidemiological usefulness of the current sub-typing for Type S isolates in New Zealand. The use of other VNTR or SSR sequences may enable this subtype to be further subdivided.

D. A wider range of different sub-types was found among the archived Type C isolates that had been collected over the last 25 years than among the LIC and Massey isolates that were

collected over 2009, 2010. As well, one of the common sub-types among the archived isolates that came from 12 cattle and three deer was not found among any of the LIC or Massey isolates. One possible explanation for these findings is that some sub-types are being preferentially selected because they are more pathogenic than others for some hosts.

3.5. Samples containing multiple sub-types

A complicating issue in analyzing the results from the LIC and Massey studies was the presence of multiple sub-types in some samples. These results were not included in Tables 4 and 5, in order to enable some important emerging trends to be presented in a way that was not obscured by less important details. The number of samples showing the presence of multiple sub-types is summarized in Table 7.

Table 7. LIC and Massey samples containing multiple sub-types

	Total samples	Samples with multiple sub-types	Samples with a VNTR giving 2 PCR products	Samples with a VNTR giving 3 PCR products
LIC	103	24 (23%)	7	17
Massey	154	12 (8%)	10	2

Overall, there was a higher frequency of samples with multiple sub-types among the LIC samples, than the Massey samples. These results show that not only is more than one subtype frequently present in individual dairy herds but that the levels of infection in some of these herds are sufficiently high for individual animals to be infected on more than one occasion.

Samples with multiple types fell clearly into two groups; those that gave two PCR products for one of the VNTR assays, representing two different copy numbers or alleles of one of the VNTR sequences, and those that gave three PCR products. Samples that gave two PCR products contained two different sub-types. In the case of the Massey isolates, most (10/12) of the samples with multiple sub-types had two PCR products and all but one of these came from pooled samples. In these cases, either one animal was infected with two sub-types or two animals in the pool were infected with different sub-types. These situations could be resolved by culturing and sub-typing separately all the animal samples that were combined to make the pools, although this would require a large amount of work and this might not be justified by the relatively small gain in knowledge that would be achieved. In the case of the one Massey and seven LIC samples that gave two PCR products and came from single animals, this indicates that these particular animals are infected with two sub-types. One of the Massey samples and seventeen of the LIC samples gave three PCR products. Whether these animals were infected with two or three sub-types cannot be stated with certainty. This is because while the finding of three different size PCR products might be because of the presence of three different sub-types, it is also possible that one of the three products is a PCR artefact arising from the hybridisation of products of two different lengths in the same PCR reaction. Distinguishing between these two possibilities would require considerable additional work involving isolation and typing of many individual clones from each sample. It must also be noted that while the reasons given above appear to be the most likely explanations for the presence of multiple PCR products in samples, other possible explanations include sampling cross-contamination, laboratory cross-contamination and genetic instability of the VNTR sequences.

3.6. VNTR stability study

Results of this study will not be available until early June. Sufficient evidence was obtained of the unreliability of the SSR1 results to discontinue the use of SSR1 for standard sub-typing.

4. DISCUSSION

The most important finding from the sub-typing of *MAP* isolates carried out in the current year has been the discovery that the range of types presently infecting sheep, cattle and deer in New Zealand is more complex than was previously revealed by the small amount of sporadic DNA typing carried out in previous years. Previously, it appeared that Type S strains of *MAP* were predominantly present in sheep and occasionally in deer, but that they caused few if any infections in cattle; and that Type C strains accounted for all the *MAP* infections in cattle, most of the *MAP* infections in deer and few if any of the *MAP* infections in sheep. This situation is largely reflected in the results shown for the archived isolates in Table 6. In contrast, the results of sub-typing recent isolates from the LIC and Massey studies show: a) that while Type C strains predominate in dairy cattle this is not the case for beef cattle in mixed farming operations; b) that while Type S strains do predominate in sheep they are also present in significant numbers of cattle including some dairy cattle; and c) that some sheep are infected with Type C strains. Even though such large numbers of *MAP* isolates have not been typed before in New Zealand, the absence of any Type S isolates in cattle in the archived isolates suggest these may be recent trends. More importantly, these findings raise a number of questions about host pathogenicity of different types and sub-types of *MAP* that require further investigation. First, to what extent do the Type S strains present in cattle and deer cause significant or progressive disease in those animals? There is some evidence that Type S strains are less pathogenic than Type C strains for deer [18] but the situation with regard to cattle is unknown. Second, it is clear that deer in mixed farming operations are predominantly infected with a single VNTR/SSR8 sub-type that is much less common in cattle. Is this situation due to increased pathogenicity of this strain for deer? Alternatively, does the predominance of this strain reflect the fact that numerous deer were imported into New Zealand in the 1980s to improve breeding stock and this may have led to the importation of a *MAP* sub-type that has not yet had time or opportunity to disseminate widely to dairy cattle? Third, to what extent do the Type C isolates found in sheep cause significant or progressive disease in those animals? The answers to these questions are important as they impact on most other JDRC milestones. For example, if some strains are less pathogenic for some animal hosts, then great care must be taken in measuring genetic resistance or vaccine effectiveness as these could be highly dependent on the type or sub-type of *MAP* to which the animals are exposed. Other important research areas that may also be impacted include the determination of markers of disease or protection, and the estimation of disease costs based on prevalence of infection. If infection with some types or sub-types of *MAP* has a significantly different outcome, then conclusions reached on the basis of results from ELISA or PCR tests for infection could be substantially inaccurate. Resolution of these questions will require appropriate investigations that include or are directed at determination of the pathogenicity of particular *MAP* types and sub-types for cattle, sheep and deer. Some guidance on these issues may be obtained from analysis of available animal and herd data that has yet to be collated with the current typing results, and provisional answers could be obtained by designing longitudinal and intervention studies to produce data that addresses these questions. However, if definitive answers to these questions are required, this will be achieved only by performing appropriate animal infection studies.

Among the LIC samples, those giving multiple sub-types were particularly common. This was not expected when the project was started, as these samples came from single animals and overseas studies found multiple sub-types in single animals much less frequently. The frequency of multiple sub-types in the LIC samples is consistent with there being a higher level of infection with Type C isolates on dairy farms than on mixed sheep, beef and deer farms. Many of the LIC samples gave three PCR products for one of the VNTR assays, and there was no simple way of interpreting whether this represented the presence of two or three different sub-types in the same

animal. Additional sampling and sub-typing work should be carried out on these farms if it is important to know the full range of infection that is present.

While the study on stability of the individual VNTRs and SSRs will not be completed for at least two more months, it has become very clear that meaningful results cannot be obtained for SSR1, because repeat assays of the same sample give different results. The use of SSR1 as part of the typing panel was therefore discontinued.

5. CONCLUSIONS

- **A.** Type S isolates are much more common in cattle in New Zealand than was previously believed.
- **B.** The frequency of the different sub-types found for the Type C isolates from dairy cattle was substantially different to that found for the Type C isolates from the Massey mixed farm project which included many deer isolates.
- **C.** Conclusions A and B raise a number of questions about host pathogenicity of different types and sub-types of *MAP* for different host species that require further investigation because of their potential effect on the results of most other JDRC milestones.
- **D.** Most of the Type S isolates in the Massey and LIC studies as well as the archived isolates had the same sub-type, leading to less than optimal discrimination of these isolates.
- **E.** Further subdivision of the predominant Type C subtype in dairy cattle and the common Type S sub-type might be achieved by using additional SSR or VNTR sequences for sub-typing.
- **F.** Further benefit from the typing results reported here will be achieved when these results have been correlated and analyzed together with the matching farm, herd and animal data that were gathered by LIC and Massey University.

6. RECOMMENDATIONS FOR FUTURE DIRECTION

There are many *MAP* DNA typing projects that can be carried out to answer a range of important questions. Many of these projects can be carried out on samples already held at AgResearch Wallaceville that have come from other investigations. Projects A – C itemized below have been proposed for year 4.

A. Determine the ability of other SSRs and, if necessary, other VNTRs to discriminate between the predominant New Zealand sub-types of Type C and Type S. By analogy from our work on VNTR analysis of *Mycobacterium bovis* isolates [21], it is apparent that while some of these DNA sequences are very useful for typing isolates from both New Zealand and many other countries, other DNA sequences are useful for typing New Zealand isolates but are of little or no use for isolates from other countries. So far we have only investigated two SSRs, one of which relies on copies of a single nucleotide and appears to be genetically too unstable for practical use. There are a number of other multinucleotide SSRs that have been found useful overseas and these should be investigated first.

B. Investigate the role of wildlife as a host for *MAP*. As part of a study initiated by Landcare with support from Massey University, we have approximately 200 *MAP* isolates from a number of different wildlife species associated with three heavily infected deer farms. We also have *MAP* isolates from deer samples acquired from these deer farms in the years before during and after the wildlife sampling. The question that needs to be addressed is whether the sub-types in wildlife are the same as those present in domesticated animals.

C. Carry out a more comprehensive investigation of the types and sub-types of *MAP* that have infected deer over the last 20 years and correlate this information with herd information on disease prevalence to identify the type (Type S or Type C) and the VNTR/SSR sub-types

that are associated with high prevalence. This would provide an indication of the pathogenicity of these types and sub-types for deer. The initial study by de Lisle et al. [5] revealed three Type S isolates in a group of 20 cases of *MAP* infection in farmed deer. Only one of these Type S cases was from an animal with clinical disease. A subsequent study [19] found cases of Type S isolates in deer from four different herds compared with 91 herds with Type C isolates. In the Massey study, three of the 63 isolates from deer were Type S isolates but the disease status of the animals was unknown. Recent experimental infection studies by Mackintosh et al. [18] showed that a Type C isolate was significantly more pathogenic for deer than a Type S isolate. O'Brien et al. [20] did not find any cases of Type S isolates in 74 animals with clinical paratuberculosis. The indications therefore are that Type S isolates are much less pathogenic for deer but this needs confirmation, and we have no information as yet on whether some sub-types of Type C isolate are more pathogenic for deer than others. The manifestation of clinical disease depends on the interaction of the host, the pathogen and the environment and without *MAP* typing it is very difficult to establish which is most important in any particular case. The evidence from the LIC study is highly indicative that at least two sub-types of Type C isolates cause significant levels of disease in some dairy herds. A comparison of the Massey and LIC data raises the possibility that one of these sub-types may not be as pathogenic for deer. Wallaceville holds large numbers of deer isolates of *MAP* that have come from our earlier investigations and a careful selection of these would be used to address these questions.

D. Investigate the longitudinal changes of VNTR/SSR types of *MAP* in farmed deer. Most of these will be Type C isolates. The Wallaceville laboratory has in their culture collections large numbers of isolates of *MAP* from deer dating back twenty years. This covers much of the period when Johne's disease developed into an endemic disease in farmed deer. An investigation of the strain types over this period would reveal which types have predominated, in which localities the types come from (North versus South Islands) and how they compare with the types present in cattle.

E. Investigate the longitudinal changes of VNTR/SSR8 sub-types of *MAP* in any new longitudinal or intervention studies carried out by the JRDC. These studies should be designed to enable this sub-typing to take place so that better conclusions can be drawn from the results with particular regard to the infectivity and pathogenicity of individual sub-types and the identification of the major sources of infection within and between herds and flocks.

F. Sub-type isolates of *MAP* obtained over the last year from the project carried out by Solis Norton who sampled both normal and enlarged lymph nodes of deer being slaughtered. Then determine the Type S / Type C status of the isolates and sub-type them using the same VNTR/SSR8 assays described earlier in this report. If enlarged lymph nodes are used as a proxy of progressive disease, then this project has the potential to identify the sub-types of *MAP* that more often cause progressive disease in deer. Since the data on these deer is owned by JML, this project would require permission from JML to proceed.

G. Investigate the *MAP* sub-types occurring in "outbreaks" of clinical disease in young deer. In some infected deer herds, multiple cases of clinical disease have been observed in young deer, approximately 1 year old. Sub-typing would determine whether or not these "outbreaks" are due to a restricted set of Type C sub-types that are more pathogenic in deer than non-outbreak associated Type C sub-types. An alternative hypothesis is that all Type C sub-types present in deer have the potential to cause "outbreaks" in young animals provided a given set of environmental/host factors are present.

H. Investigate selected cultures that have given three PCR products for a single VNTR sequence in order to determine if all three products have arisen because of the presence of three different isolates with different allele copy numbers in the culture or whether there are only two different isolates in the culture and the third PCR product line is a PCR artifact.

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