Within-farm strain dynamics of *Mycobacterium avium* subsp *paratuberculosis*: Evidence for limited vertical transmission

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Abstract

MAP-infected dairy cattle are assumed to be a high risk for transmitting infection to their daughters. Alternatively, if both dam and daughter are genetically more susceptible to MAP, they may be both infected but not necessarily due to vertical transmission. Using strain typing techniques including multi locus short sequence repeat (MLSSR) typing allows a potential distinction between vertical transmission and genetic susceptibility. Analyzing strain diversity in longitudinal datasets provides additional insight into within-herd infection dynamics, including the transmission of MAP from dams to daughters.

To investigate the importance of vertical transmission, we identified 12 pairs of dams and daughters for which both animals are known MAP infected from the Regional Dairy Quality Management Alliance (RDQMA) study herd in NY. All adult animals on the farm were tested for MAP via fecal culture semi-annually for seven years. Tissue samples were available on a subset of cull animals. Animals were considered MAP-infected if they ever cultured positive or if any of their tissues cultured positive at slaughter. Cultures were performed at University of Pennsylvania on HEYM solid media. Positive cultures were substreaked and processed for MLSSR typing. Following genotyping, isolates from each dam-daughter pair were compared to determine whether they shared the same MAP genotype. Environmental MAP burden at birth was assessed via typing of MAP-positive environmental samples (collected four times a year) and known MAP-infected animals present on the farm during the high-risk first year of life.

Of the 12 infected dam-daughter pairs, 9 had identical strains shared between the dams and daughters. In addition, 2 daughters had the dam's strain as well as another circulating strain. Overall, there were 8 strains represented in the daughters that did not come from dams (2 daughters had multiple strains which did not originate from the dam). These results lend additional importance to the impact of genetics on susceptibility, as 5 of 12 daughters carried different strains of MAP than their dams, even when concurrently infected with the dam's strain.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of an incurable, infectious, chronic, granulomatous enteritis in ruminants, called Johne's disease or paratuberculosis. The direct effects of Johne's disease on animal welfare and productivity may appear insignificant and are often masked by the largely subclinical character of the disease in domesticated livestock. The incubation period prior to the onset of clinical signs ranges from 2 to 10 years (Lepper, et al., 1989)(Chiodini, et al., 1984). However, the impact of Johne´s disease on the US dairy cattle industry is substantial, including decreased milk production (Smith, et al., 2009), increased risk of being culled and a decreased calving rate in high shedding animals (Smith, et al., 2010). Estimated costs of decreased weight gain, milk production loss, premature culling and reduced carcass value add up to an annual amount of $250 million (USD) in the United States industry alone (Ott, et al., 1999). In addition to the proven economic impact of MAP infection on ruminants, on-going controversy concerning the possible role of MAP in Crohn's disease (human inflammatory bowel disease) suggests that MAP itself might be of public health significance (Hermon-Taylor, et al., 2000).

Cows are most susceptible to infection as calves from birth to a few months of age (Taylor, 1953; Windsor and Whittington, 2010), but can also be infected as adults (Doyle and Spears, 1951; Rankin, 1961). Evidence of intrauterine transmission and shedding of MAP in milk and colostrum has been reported (Seitz, et al., 1989; Sweeney, et al., 1992; Nielsen, et al., 2008). Although calf-to-calf transmission has been demonstrated in several experimental settings where calves were being housed together (Rankin, 1961; van Roermund, et al., 2007) horizontal infection from cows to calves and vertical infection from dam to calf are believed to be the most common and most important infection routes in dairy cattle (Marce, et al., 2010). Previous work also described that calves born from latent dams who would go on to shed culturable levels of MAP or dams which were currently shedding MAP in their feces were more likely to shed MAP than calves without this risk-profile (Benedictus, et al., 2008). Calves growing up with a future high shedder and calves being born shortly following the freshening of a MAP infected dam were at higher risk of being infected than those not exposed to future high shedders or possibly contaminated calving environments (Benedictus, et al., 2008).

There is no satisfactory treatment for MAP infection. No antimicrobials are approved for the treatment of Johne's disease because of their lack of efficiency and their failure to provide bacteriological cure. A vaccine for MAP exists but is not commonly used because it offers only partial protection and interferes with interpretation of tuberculosis tests (Muskens, et al., 2002). Consequently Johne´s disease is currently controlled via improving management practices and test-and-cull programs. The most cost-effective option for reducing the prevalence of MAP infection in herds is application of management measures to disrupt known transmission routes (Groenendaal and Galligan, 2003; Kudahl and Nielsen, 2009). Specific management practice recommendations for reducing within-herd MAP transmission are focusing on removing high-risk animals (Nielsen and Toft, 2007) and minimizing the exposure of newborn calves to potential MAP sources (Pillars, et al., 2011). Currently, none of these control measures address genetic susceptibility to Johne's disease.

Heritability of susceptibility to infection with MAP has been estimated to be between 0.06 and 0.159 (Koets, et al., 2000; Mortensen, et al., 2004; Hinger, et al., 2008; Attalla, et al., 2010). These heritability estimates are affected by the definition of infection, diagnostic methods used in the studies (blood or milk ELISA, fecal cultures, tissue cultures), study sample sizes, study population (breed) and statistical models (mixed animal model, sire model) used in these studies. It is therefore difficult to provide a precise estimate of

heritability, other than that it appears to be greater than zero. Population-based heritability estimates have resulted in host genome based studies to identify genetic markers for MAP susceptibility. Two genome-wide studies identified loci for MAP resistance or conversely susceptibility to *MAP* infection using Illumina Bovine SNP50 assay (Settles, et al., 2009; Minozzi, et al., 2010). A SNP on chromosome 9 (BTA9) is associated with shedding (being fecal culture positive) when truly infected (tissue culture positive) (Settles, et al., 2009) and being ELISA positive (Minozzi, et al., 2010). Zanella et al. performed an association study on the same dataset as Settles et al. and found BTA6 to be associated with tolerance to paratuberculosis (Zanella, et al., 2011). Several candidate genes identified by these genome-wide studies have been examined for further association with infection. However, attempts to locate genes specifically associated with susceptibility or resistance to paratuberculosis have had limited success. Caspase recruitment domain 15 (CARD15) variants were associated with infection (Pinedo, et al., 2009a) in cattle, but not with clinical disease as reported in a comparative analysis of bovine, murine, and human CARD15 transcripts (Taylor, et al., 2006). Toll-like receptor 4 (TLR4) SNPs were associated with infection in one of two recent genomic analyses (Mucha, et al., 2009; Pinedo, et al., 2009b). The risk of MAP infection was associated with alleles of the SLC11A1gene (formerly NRAMP1) in two (Pinedo, et al., 2009a)(Ruiz-Larranaga, et al., 2010) but not in a third (Hinger, et al., 2007) recent study. Differences in diagnostic methods utilized in these studies may have contributed to the conflicting findings.

To investigate the importance of vertical transmission, studies have reported on the prevalence of fetal infection in cattle and thereby estimated the incidence of calves infected via *in utero* transmission. Based on a meta-analysis of the published literature, it was estimated that 9% of fetuses from subclinically, MAP infected cows (fecal culture positive) and 39% of fetuses from MAP infected cows with clinical signs of Johne's disease were infected with MAP prior to birth (Whittington and Windsor, 2009). True prevalence of fetal MAP infection could be higher than reported in these studies due to low test sensitivity (Whittington and Windsor, 2009)(Eamens, et al., 2000). Incidence of *in utero* transmission therefore depends on within-herd prevalence and the ratio of sub-clinical to clinical cases among infected cows (Whittington and Windsor, 2009).

Current methods in molecular genetics make it possible to discriminate between different bovine-specific MAP strains (Motiwala, et al., 2006). These molecular genetics techniques can also be used as a tool to investigate the possibility of vertical transmission by comparing MAP strains in samples from the dam with strains in samples from the daughter. When comparing different MAP fingerprinting techniques, the multi locus short sequence repeat (MLSSR) technique using all 11 loci selected by Amonsin et al. (2004) appeared to have the highest discriminatory power with a Simpsons diversity index of 0.967 (Motiwala, et al., 2006) . Four of these identified repeats (locus 1, locus 2, locus 8 and locus 9) were used to describe MAP strains in a US-wide collection (Harris, et al., 2006) and to study within-herd transmission dynamics of MAP (Pradhan, et al., 2011).

Although it remains difficult to distinguish between the effects of heredity of susceptibility and vertical transmission in a dataset, modern strain typing techniques applied to precise longitudinal studies can serve as a precise method to address this distinction. The objectives of this study were to assess the most likely route of transmission in positive dam daughter pairs and the importance of vertical transmission, through analysis of precise MAP strain typing results.

2. Materials and methods

2.1. Sampling

MAP isolates used in this study were part of a longitudinal dataset gathered by the Regional Dairy Quality Milk Alliance (RDMQA) on one of three commercial dairy farms in the northeast US (Pradhan, et al., 2009). Complete details of the longitudinal study including methods of sample collection and preliminary microbial analyses are available in previous work (Pradhan, et al., 2011). Briefly, fecal samples were collected from all cows semiannually and cultured for MAP on Herrold's Egg Yolk Media (HEYM) with Mycobactin J at the University of Pennsylvania using their standard sample processing methods. Environmental samples were collected four times a year and also cultured for MAP using the same method. In addition, when animals were sold for slaughter, four intestinal tissue samples (lymph nodes and ileum) and a slaughter fecal sample were collected by USDA Food Safety Inspection Service personnel at the slaughterhouse and shipped to University of Pennsylvania on ice for culture.

MAP shedding levels were quantified based on growth of colonies on four HEYM slants (multiplied by a correction factor of 4.3) (Pradhan, et al., 2011). For a subset of samples that were initially qualified as too numerous to count (TNTC) additional dilutions of original fecal material were performed to obtain an accurate count. Individual positive samples were substeaked onto HEYM slants containing mycobactin J and were shipped to Quality Milk Production Services (QMPS) at Cornell University for molecular typing. Because samples often had many colonies per unit, the majority of samples were shipped as substreaks of multiple colonies, rather than representing a pure culture of only one colony per animal.

2.2. Selection of samples

The evaluation of the most likely route of transmission was assessed by strain-typing isolates from dam-daughter pairs. A dam-daughter pair was selected if both a dam and daughter that had at least one of the following: (A) any MAP positive fecal sample (B) any MAP positive tissue or fecal sample collected at the slaughterhouse. To evaluate the horizontal transmission probability, all MAP positive samples identified on Farm A were further evaluated and any identified MAP strain was assigned a strain type. At least one tissue from every tissue-culture positive animal was strain-typed.

2.3. DNA extraction

DNA was extracted from the substreaked HEYM slants as described by Pradhan et al. (Pradhan, et al., 2011). Briefly, a sterile plastic 10 ul loop was used to remove a portion of the bacterial lawn from the substreaked samples. The bacteria were resuspended in sterile water (Invitrogen Corporation, Carlsbad, CA USA) and 250 ul of sterile 0.1 mm zirconia/silica beads (BioSpec Products, Inc. Bartlesville, OK USA) in a 2 ml beadbeater screwcap vial (Biospec Products, Inc., Bartlesville, OK USA). Cells were homogenized for 5 minutes at maximum speed in a Beadbeater-8 homogenizer (Biospec Products, Bartlesville, OK, USA). Following homogenization, DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA USA) following the manufacturer protocol with a few modifications. DNA was homogenized for 30 minutes at 70 °C prior to removing 600 µl to combine with 600 µl AL buffer and 60 µl of proteinase K (Qiagen, Valencia, CA, USA). This aliquot was mixed thoroughly and incubated for a further 30 minutes at 70°C. The remainder of the manufacturer protocol was followed exactly until elution in 150 µl sterile water. Eluted samples were stored at -20°C until used for PCR typing. Samples were extracted in small batches from 4 to 10 samples and an extraction negative was always processed alongside to ensure no contamination.

2.4. MLSSR

Initially the four loci described by Harris et al. (Harris, et al., 2006) were selected because of their diversity index and the existence of previous work using these loci to which we could compare our findings. We elected to add five loci also described by Amonsin *et al.* (locus 3, locus 5, locus 6, locus 10 and locus 11) to increase specificity of our strain typing methodology. The additional discriminatory value of these additional loci was evaluated after processing all isolates from the identified dam-daughter pairs and a subset of the all isolates available from farm A. When we did not observe diversity in this subset of samples (minimum of 60 samples/locus), the locus was not processed for the remainder of the samples. Locus 2 was not used in this analysis due to the difficulty in interpreting sequence reads when more than nine G repeats were detected.

PCR amplification was carried out with the extracted DNA for all isolates using the primers described in Amonsin *et al.* 2004 (Amonsin, et al., 2004). Alternate primers for locus 1 were used on a subset of samples: 5'-GTG TTC GGC AAA GTC GTT GT-3' and 5'-GCG GTA CAC CTG CAA G-3'. Information about modified primers for locus 1 was obtained from the Center for Genomics and Veterinary Population Medicine Department, University of Minnesota, St. Paul, Minnesota. The 25-µl PCR amplification reaction mixture for each SSR contained: 2X GoTaq Green Master Mix (Promega Corporation, Madison, Wisc.), 0.625 µl of 10 µM upstream and downstream primers (Integrated DNA Technologies, Coralville, Iowa), 9 µl of distilled water, 1.25 µl of DMSO (Dimethylsulfoxide) and 1 µl of genomic DNA. As in our previous work, PCR amplification was performed using the following conditions: an initial denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 7 min. The PCR amplification for the modified locus 1 primer set was performed using the same conditions as all other primer pairs, but for 35 cycles instead of 40. A PCR master mix blank was included as a negative control for each amplification and the extraction negative was processed for at least one locus for all extraction batches. Two µl of PCR product was electrophoresed at 105 V for 30 min on a 1.5% (wt/vol) agarose gel with 1.25 ul EtBr in 0.5X TBE buffer (0.45 M Tris-Borate, 0.01 M EDTA, pH 8.3). PCR products were then visualized through UV transillumination. Amplicons were purified with either a PureLink PCR purification kit (Invitrogen, Carlsbad, CA USA) or a QIAquick PCR spin column (Qiagen, Valencia, CA USA) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware). PCR amplicons were sequenced using standard dye terminator chemistry with a 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, Calif.) at the Cornell University Life Sciences Core Laboratories Center (http://cores.lifesciences.cornell.edu/brcinfo/).

2.5. Genotyping

Chromatograms were read in SeqMan (DNASTAR Inc., Madison, WI). Number of short sequence repeats were assigned for each locus, and each isolate was assigned a strain-type based on the pattern of repeats across all loci. All isolates with greater than or equal to 11 G repeats at locus 1 were considered equivalent (marked >11) (Clarke, et al., 2001; Amonsin, et al., 2004; Fazekas, et al., 2010). When there were multiple strains in one MAP sample (confirmed by homopolymer forward and reverse sequencing), isolates were assigned both potential strain types, and CFUs per gram were equally divided between those different strains. When we were unable to distinguish the combinations of these different strains due to multiple loci with ambiguous designation, the isolate was assigned a "mixed" designation. Complete MLSSR types were assigned after determining the allele combinations on locus 1, locus 6, locus 8, locus 9 and locus 10. Following genotyping, strain types of all isolates from

each dam-daughter pair were compared to determine whether they shared the same MAP genotype.

2.6. Statistical analysis

SAS v9.2 was used for statistical analysis (SAS Corporation, Cary NC). In order to estimate the horizontal MAP transmission pressure, all available environmental samples and all MAP-positive fecal samples collected within 12 months after birth of the daughter and the most recent fecal sampling before birth were analyzed. Samples were weighted using a semi-quantitative logarithmic scale by CFUs per gram of feces. Weighting factors were: 10 for samples with 1-50 CFU's per gram of feces, 20 for samples with 51-2000 CFU's per gram of feces and 30 for strains found in samples with >2000 CFU's per gram of feces. Independently, unweighted categories were created for cows that were ever-positive by fecal and/or tissue culture, with each cow contributing all strains that were cultured at any point throughout its entire lifetime.

The source of all daughter strains can be subdivided into three categories: "Horizontal": the daughter's strain does not match any of the dam's strains but is found on the farm (defined as circulating strains, based on fecal samples of currently-shedding animals present on the farm and contemporary environmental samples), "Dam Strain": the daughter's strain matches one of the dam strains or "Introduced": the daughter's strain does not reflect strains found on the farm or any of the strains found in her dam.

To determine the probability that an infected daughter carries the same strain as her dam by horizontal transmission, strain prevalence was calculated by summing the total number of strains in individual cows over the course of the study. The expected probability of horizontal transmission of specific strains (independent of the potential vertical transmission) was calculated using the above defined unweighted strain prevalence. In addition, the number of expected dam-daughter MAP-positive pairs was calculated by evaluating the probability of testing positive by each test and the probability of being tested. The probability of vertical transmission was then defined as the observed strain identity between dam and daughter, above and beyond the expected identity from horizontal transmission from either method.

3. Results

3.1 Data analysis

On farm A, 572 dam-daughter pairs were identified with fecal-culture information on both dam and daughter and 20 dam daughter pairs were identified with tissue-culture information for both dam and daughter, for a total of 574 dam-daughter pairs with at least one MAP test for each animal (Table 5.1). MAP prevalence among dams was 17% (101/574 MAP-positive dams, 51/572 by FC, 63/105 by TC) and MAP prevalence among daughters was 8% (48/574 MAP-positive daughters, 16/573 by FC, 35/91 by TC) on Farm A (Table 5.1).

The expected number of MAP-positive dam-daughter pairs on Farm A, assuming no vertical transmission between dam and daughter, calculated based on the MAP prevalence amongst daughters and dams was 1.5% (expect 8.4 dam-daughter MAP-positive pairs from 574 total pairs). The observed number of MAP-positive dam-daughter pairs on Farm A is 13, which is higher than expected given random mixing (Poisson distribution, P=0.05).

There was a trend toward being MAP test-positive when born to a dam with a positive fecal and/or tissue culture (Any+ vs Any-) (chi-square value 3.25, P=0.07, Figure 5.1). Daughter MAP tissue-infection-status was not influenced by dam MAP tissue-status (Fisher's exact test, P value =0.43, Figure 5.1), although sample size was small (n=18). Daughters with fecal-culture-positive dams were at more than 5 times increased risk of becoming fecal-

culture-positive as daughters of fecal-culture-negative dams (Relative risk 5.6, CI 1.7-12.8, P=0.01).

Table 5.1. Dam and daughter test status by fecal culture (FC), tissue culture (TC) or by either method ("Any"). Latent daughters are tissue-culture positive but fecal-culture negative throughout their lifetimes. Negative dams are animals that did not test positive by all method listed. Slow-latents are animals which never shed but are tissue-culture positive. Fast latents are not shedding prior to or up to the first fecal test following calving, but shed after the birth of the daughter classified. Shedding dams are fecal-culture positive before the calving of the identified daughter or at the first fecal culture post-parturition.

	Daughter FC			Daughter TC			Daughter Any			Latent
	+	-	sum	+	-	sum	+	-	sum	FC- TC+
Dam Fecals										
FC+	5	46	51	4	6	10	8	43	51	3
FC-	11	510	521	31	42	73	40	482	522	29
Sum	16	556	572	35	48	93	48	525	573	32
Dam Tissues										
TC+	2	61	63	5	9	14	7	56	63	1
TC-	0	42	42	1	3	4	1	41	42	5
Sum	2	103	105	6	12	18	8	97	105	6
Dam Any										
Any+	6	95	101	8	14	22	13	88	101	7
Any-	10	462	472	27	42	69	35	438	473	25
Sum	16	557	573	35	56	91	48	536	574	32
Potential Negatives										
Lifetime FC-	3	127	130	32	44	76	33	97	130	30
Lifetime FC- TC-	0	36	36	1	2	3	1	35	36	1
FC- before birth	1	24	25	0	2	2	2	23	25	1
Slow Latents (untested before birth)										
FC-TC+ after birth	1	33	34	4	7	11	5	29	34	4
FC-TC+ ever	1	47	48	4	7	11	5	43	48	4
Fast Latents (FC- before birth)										
FC+ after birth	0	11	11	3	5	8	3	11	14	3
Shedding Dams										
FC+ before birth	0	1	1	0	0	0	0	1	1	0

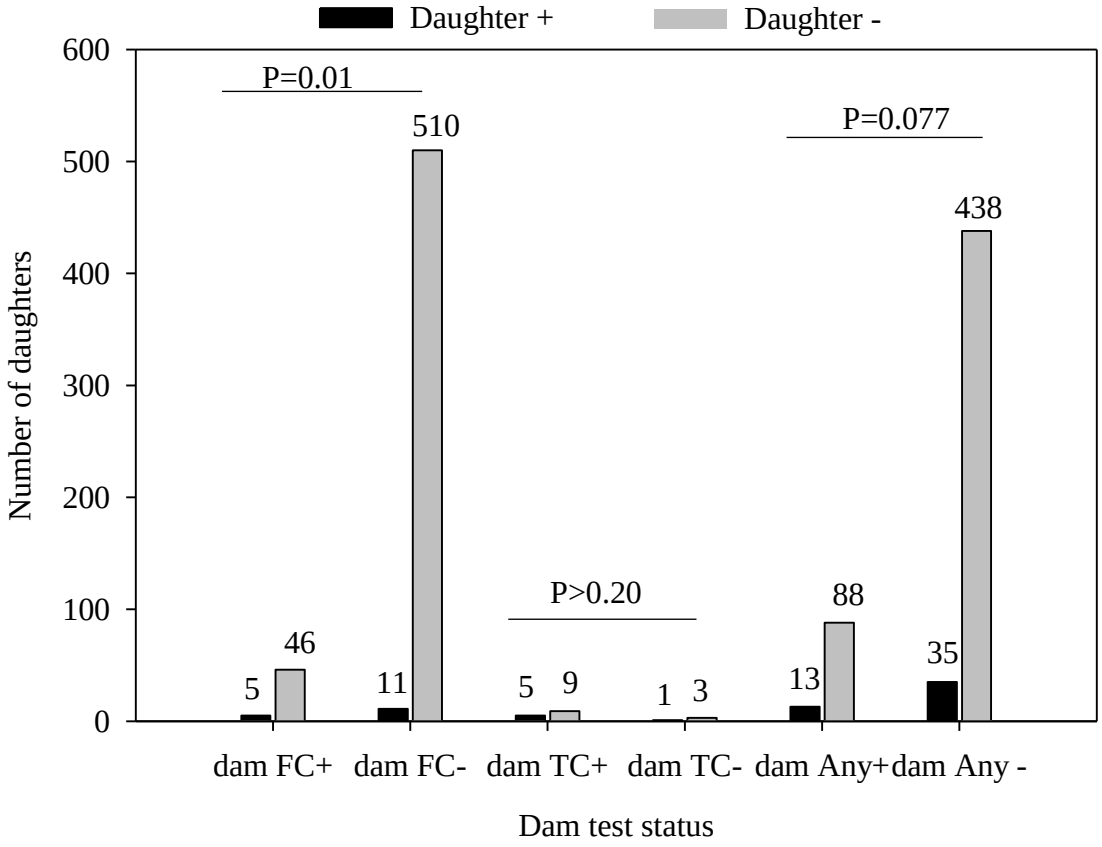

Figure 5.1. Relationship between dam fecal and tissue culture status and dam fecal and tissue culture status. FC= Fecal culture, TC= Tissue culture, Any = parallel testing using all available test results (so that any positive will classify an animal as infected). Black bars indicate daughters with positive tests, grey bars indicate daughters with negative tests. Dam FC status is compared to daughter FC status, dam TC to daughter TC and dam any-test status to daughter any-test status. Number of animals in each category is noted on the graph.

3.2 Genotyping

Diversity was observed at loci 1,6,8,9 and 10 of the 8 loci analyzed. All fecal and environmental isolated from Farm A were MLSSR-typed using the 5 loci for which there was diversity in the 12 dam-daughter pairs. Nine (75%) of dam-daughter pairs had identical MAP strains between dams and daughters (Table 5.2). Two of these nine daughters also carried additional MAP strains. In three pairs, daughter strains did not match any of the strains identified in the dam. Among all the 19 strains identified in the 12 daughters, eleven of these strains (58%) matched a strain present in the dam and eight (42%) present in the daughters did not originate from the dam.

Strains of dominant horizontal MAP exposure could be estimated for four daughters who were born within one year of the initiation of the RDQMA whole-herd samplings (Figure 5.4). The remaining eight daughters were born greater than one year prior to any of the sample dates. Therefore most of the birth events were not associated with contemporary

environmental or fecal culture samples and consequently exposure from on-farm sources other than dams could not be measured directly.

There was more diversity in MAP strains in tissue cultures relative to fecal cultures despite the larger number of animals tested by fecal culture (Figure 5.5, Panels A and B). Seven strains which were in tissues were never recovered on the farm, indicating low prevalence of fecal shedding. MAP strain 7-5-6-5-5 was dominant in both fecal-positive and any-positive animals.

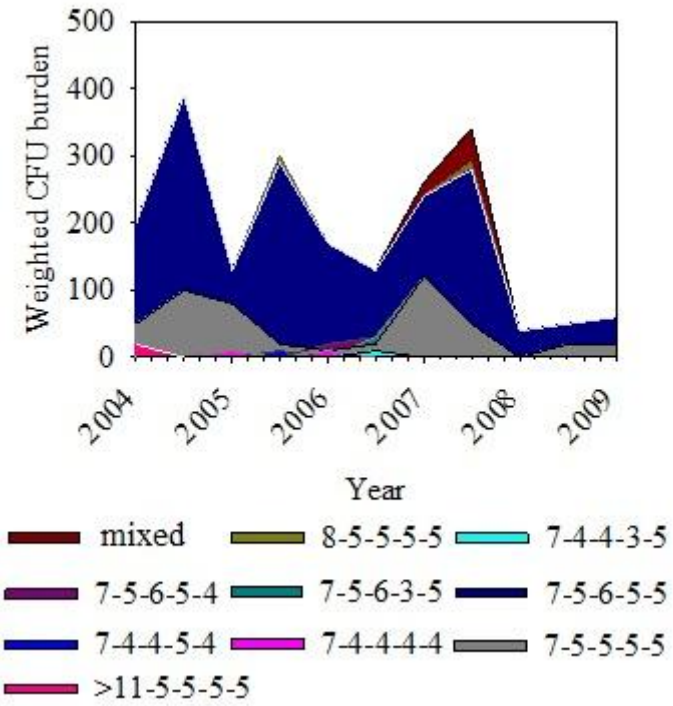

Figure 5.4. On-farm MAP burden at each sampling time-point based on fecal samples of currently shedding animals present on the farm and contemporary environmental samples weighted by CFU/g categories. Tissue culture data is not reflected in this graph.

Table 5.2. Strain types in daughters, dams, and the farm environment/herdmates which are shedding within one year following the birth of an infected daughter. Each pair is assigned an ID. All strains present in any of the three sources (dam, daughter, environment/herd) are listed for each pair. Each row represents samples of the same strain. Sample type (tissue or fecal) is indicated for samples from daughters and dams. Any strain that is common to dam and daughter is highlighted in grey, and any strain shared by daughters and the environment is italicised. Herd and environmental samples are weighted as described in the methods section. Mixed strains recovered from single samples which could not be differentiated are listed as mixed strains and any combination of alleles which is represented at least once on the farm is considered a possible strain from these samples. Because the majority of daughters were born previous to the initiation of the study, these animals do not have an environmental burden assigned.

	Daughter					Dam			Herd and Environment
Pair	Strain	ID	Birthdate	Sample	Isolates	ID	Sample	Isolates	Weighted contribution
1	7-4-6-3-5	1751	Aug 4, 2004	Tissue	2				
1	7-4-4-3-5	1751	Aug 4, 2004	Tissue	2				
1	7-5-5-5-5					1255	Fecal	2	22.4%
1	7-5-6-5-5								64.2%
1	>11-5-5-5-5								7.5%
1	7-(5/6)-(5/6)-5-5								4.5%
1	7-4-4-4-4								1.5%
2	7-4-4-3-5	1683	Oct 10, 2003	Fecal, Tissue	3				
2	7-4-5-3-5	1683	Oct 10, 2003	Tissue	1				
2	*7-5-6-5-5*	*1683*	*Oct 10, 2003*	*Tissue*	*1*				*72.5%*
2	8-5-5-5-5					774	Tissue	1	
2	7-4-4-4-4					774	Tissue	1	
2	7-4-5-5-5					774	Tissue	1	
2	7-5-5-5-5								15%
2	>11-5-5-5-5								12.5%
3	*7-5-6-5-5*	*1645*	*Oct 25, 2003*	*Fecal*	*1*	*1342*	*Fecal*	*1*	*72.5%*
3	7-5-5-5-5								15%
3	>11-5-5-5-5								12.5%
4	*7-5-6-5-5*	*1628*	*Sept 20, 2003*	*Fecal*	*2*	*1127*	*Fecal, Tissue*	*4*	*80%*
4	7-5-5-5-5					1127	Tissue	1	12%
4	>11-5-5-5-5								8%
5	7-5-6-5-5	1494	Oct 11, 2002	Fecal, Tissue	8	1099	Fecal	2	

5	7-5-5-5-5					1099	Fecal	2
6	7-5-5-5-5	1429	Apr 28, 2002	Tissue	1			
6	7-(4/5)-(5/6)-5-5					1091	Fecal	1
6	7-(4/5)-(5/6)-5-(4/5)					1091	Fecal	1
6	7-(4/5)-(4/5)-(5/4)-(4/5)					1091	Fecal	1
7	7-5-6-5-5	1415	Apr 20, 2002	Tissue	1	1120	Tissue	1
8	7-5-6-5-5	1372	Jan 10, 2002	Fecal	1	2126	Fecal	1
8	7-5-5-5-5					2126	Fecal	1
9	7-5-5-5-5	1300	Jul 17, 2001	Fecal, Tissue	3	1127	Tissue	1
9	7-5-6-5-5	1300	Jul 17, 2001	Tissue	2	1127	Fecal, Tissue	4
9	7-5-6-5-4	1300	Jul 17, 2001	Tissue	2			
10	7-5-6-5-5	1257	Mar 12, 2001	Tissue	2			
10	7-5-4-4-4					711	Tissue	1
11	7-5-6-5-5	1189	Nov 27, 2000	Tissue	3	655	Tissue	2
11	7-5-5-5-5	1189	Nov 27, 2000	Tissue	1	655	Tissue	1
12	7-5-6-5-5	1099	Dec 11, 1999	Fecal	2	693	Fecal	1
12	7-5-5-5-5	1099	Dec 11, 1999	Fecal	2			

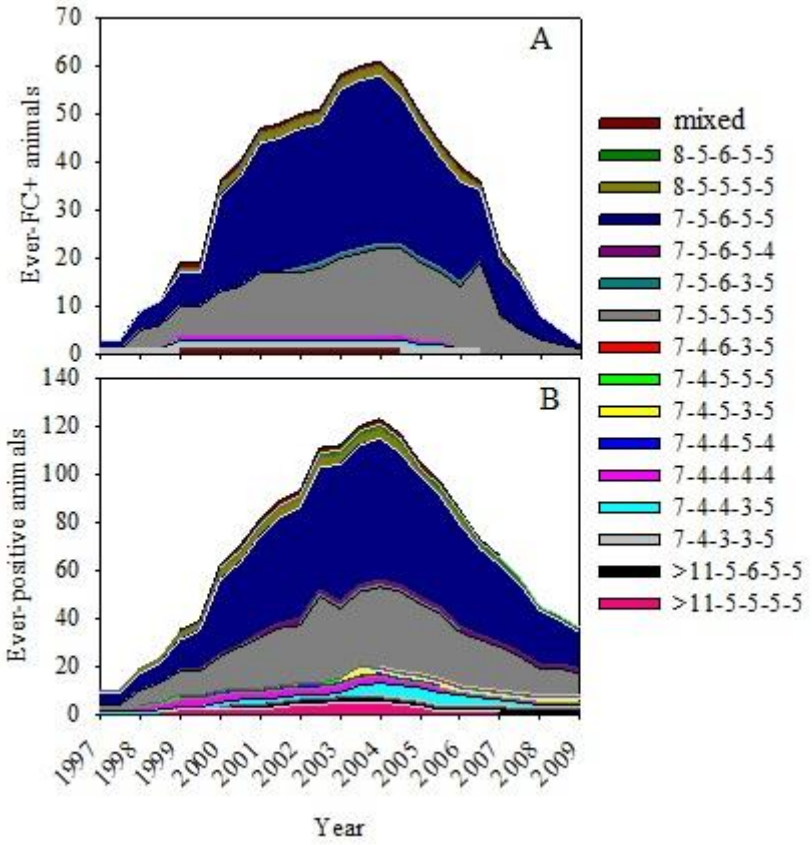

Figure 5.5. On-farm MAP burden assuming lifetime shedding of all fecal-culture positive animals (A) or fecal-culture and tissue-culture positive animals (B). Every animal that is FC+ or TC+ is represented for each strain identified in that animal for the duration of life. Each six month increment is calculated individually, and animals that are culled are included in the increment in which they exit the population.

3.3 Vertical transmission of MAP strains

All dam-daughter pairs which shared the same MAP strain shared one of the two dominant strains (7-5-6-5-5 and 7-5-5-5-5) which together consistently comprised more than half of the weighted CFU burden on the farm as well as more than half of all infected animals (Figures 5.4 and 5.5). Of the additional strains recovered in daughters of known-infected dams during the longitudinal study, three were not recovered from the environment during the calves' high-risk first year of life (7-4-4-3-5, 7-4-5-3-5, 7-4-6-3-5).

For the four daughters from the MAP-positive dam-daughter pairs born within one year of initiation of fecal sample collection, we evaluated the presence of the MAP strains in relation to the exposure of the daughter to these strains in her environment during her first year of life. The MAP strains in daughters, dams and on the farm are shown in Table 5.2. For example, for the 3rd daughter in Table 5.2, the conditional probability of uptake of strain 7-5-6-5-5 from her environment, conditional on that she was going to be MAP infected was 0.725 while uptake of any other strain circulating on the farm at that moment was 0.275. For the 4th daughter in Table 2, the conditional probability of uptake from the environment of strain 75655 was 0.80 while uptake of any other strain circulating on the farm at that moment was 0.20.

4. Discussion

Observed number of MAP-positive dam-daughter pairs on Farm A, exceeds the expected number of MAP-positive dam-daughter pairs. Vertical transmission can be responsible for the additional MAP-positive dam-daughter pairs observed; however, there is also substantial transmission of strains which are not commonly found in the farm environment and are not from the dam in the daughters. It is possible that the large number of strains in the daughters which are unique on the farm represent new introductions of MAP from external sources rather than within-farm transmission.

Increased prevalence of MAP-positive daughters among latent dams found in this study was consistent with previous work. Benedictus and colleagues described increased risk of infection for daughters born to infected dams versus daughters born to negative dams based on the same type of single-farm longitudinal data collection as this study. Daughters born to dams that tested positive by fecal culture within two years following birth (latent dams) were at increased risk of being infected versus daughters from test-negative dams. There was no difference in transmission rates between latent dams who shed shortly following calving or those that remained latent for at least two years after calving (Benedictus, et al., 2008). Aly and Thurmond studied the relevance of dam (serologic) status in a retrospective longitudinal study of 625 animals using ELISAs. Daughters of seropositive dams were 6.6 times more likely to be seropositive compared to daughters of seronegative dams (Aly and Thurmond, 2005), but as serology does not correlate well with low shedding, these could have been latent or shedding animals. Although the number of strains shared between dams and daughters suggest vertical transmission as transmission route of infection, genetic susceptibility could also account for the additional positive dam-daughter-pairs observed on farm A.

A concern with strain typing techniques that rely on genomic areas with high genetic variability is their stability over time. Mutation rates at these areas might be too high for usage in strain typing over several years. Evolutionary rates are unknown for *Mycobacterium avium* subspecies *paratuberculosis*, especially at sites of MLSSR analysis. Stability of short sequence repeats was investigated by Harris at al. by performing two experiments. In the first experiment , three MAP isolates were serially grown for ten passages *in vitro*. For every isolate, Locus 1, 2, 8 and 9 were sequenced for each serial passage. In the second experiment, Harris et al harvested DNA from ten colonies from a single isolation of each of the three isolates for SSR analysis. Both experiments showed no SSR genotype changes (Harris, et al., 2006). In studies of *Mycobacterium tuberculosis*, variable number tandem repeats (VNTR) methods are frequently used for strain typing. However, there is debate about the exact rate of evolution for VNTRs. Savine *et al.* examined the mutation rate for 12 VNTR loci, where 1.8% (1/56) of isolates from patients showed a change at one locus over an average period of time of 1.37 years, while 98.2% remained stable (Savine, et al., 2002). This finding reflects a mutation rate of 10^{-3} per locus per year. A mathematical modeling study estimated the average mutation rate of the same 12 VNTR's to be $2.3*10^{-8}$ (Grant, et al., 2008). Other estimates vary between $7*10^{-4}$ and $1.5*10^{-2}$ per locus per year (Reyes and Tanaka, 2010). Although the real mutation rate of MAP remains unknown, evolutionary rates close to those estimated for *Mycobacterium tuberculosis* inspire confidence in SSR stability. A more precise estimate of the MAP mutation in SSR's demands serial passages of several different isolates over a period of at least 10 years.

In *Mycobacterium tuberculosis* the use of molecular characterization of different strains with VNTR-methods demonstrated infections with multiple strains of *Mycobacterium tuberculosis* occurs in humans (Richardson, et al., 2002; Garcia de Viedma, et al., 2003; Garcia de Viedma, et al., 2004; Warren, et al., 2004; Garcia de Viedma, et al., 2005; Shamputa, et al., 2006; Dickman, et al., 2010). Several studies reported individuals

harbouring more than one strain in their sputum (Chaves, et al., 1999; Richardson, et al., 2002; Warren, et al., 2004; Garcia de Viedma, et al., 2005; Shamputa, et al., 2006). Multiple strains have also been found in different anatomical sites within an infected patient (Garcia de Viedma, et al., 2003; Garcia de Viedma, et al., 2004). Multiple infections were increasingly observed in high tuberculosis-incidence communities (Richardson, et al., 2002) and were even more evident in other very-high tuberculsis-incidence settings such as prisons (Nardell, et al., 1986; Chaves, et al., 1999; Chaves, et al., 1999; Sonnenberg, et al., 2001; Shamputa, et al., 2006), mines (Sonnenberg, et al., 2001), and homeless shelters (Nardell, et al., 1986). This suggests there could be a connection between crowded settings with high contacts between individuals and the prevalence of mixed infections in *Mycobacterium tuberculosis*. It must be mentioned that HIV prevalence which depresses immune function contributes to an increased number of mixed infections.

The similarity between conditions of these high prevalence settings in *Mycobacterium tuberculosis* and dense housing and high contact rate among dairy cows is evident. In this study multiple strains were observed in different samples from one cow but also within a single sample (confirmed by repeated culture, extraction and PCR amplification). Mixed infections will be further investigated in future work by determining the ratio/proportion of each strain within a sequence of the isolate and the result of amplifying the different strains within an isolate following dilutions. Of particular interest is whether having more than one MAP strain predisposes animals to become shedders or have clinical disease.

The completeness of this unique longitudinal dataset on a well-managed dairy herd with long follow-up (7 years of biannual sampling) combined with the additional value of tissue culture results on a subset of the culled animals provides us with the confidence that the correlations observed in this study reflect reality. In order to get a stronger estimate of MAP pressure of the different strains from on-farm sources, more frequent environmental samples should be taken around the freshening events. Additional discriminatory power of the MLSSR typing technique will be available with the addition of locus 2. Preliminary cloning experiments by the authors of this study show promising results on readability of locus 2 sequences. In addition to getting a better understanding of vertical transmission, a longer follow-up could provide us with a more complete display of MAP strain type diversity and evolutionary rates.

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