H.J.H. van Aaken

Supervisor Universiteit Utrecht:

Prof. Dr. T.J.G.M. Lam

Supervisors University of Calgary:

Prof. Dr. H.W. Barkema,
Assoc. Prof. Dr. J. De Buck
Amanda Mirto, MSc

A quantitative analysis of tissue samples collected from dairy calves infected with *Mycobacterium avium* subsp. *paratuberculosis*

Universiteit Utrecht

UNIVERSITY OF CALGARY
FACULTY OF VETERINARY MEDICINE

Summary

Johne's disease (JD) or paratuberculosis is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). JD is prevalent worldwide mainly infecting beef cattle and dairy cows. Animals are infected in the beginning of their life and after an incubation period typically ranging from three to five years become clinically affected. Animals with clinical JD are recognised by a chronic untreatable diarrhoea and subsequent weight loss. Most animals that become clinical are culled within weeks. *Mycobacterium avium* subsp. *paratuberculosis* is a slow growing mycobacterium that can persist in host cells for years without triggering clinical disease. The most important infection route is fecal-orally by the ingestion of MAP infected faeces. Programs to control JD are not only of significant importance because of the economic impact on the dairy and beef industry, but also because of the possible link between Crohn's disease in humans and MAP. Several ways to contain the spread of MAP on farm level include the development of national eradication programs utilizing culling, vaccination and education of farmers. However, there is a lack of commercially available vaccines that protect sufficiently without significant negative side-effects. Diagnosis of JD was traditionally done by assessing pathologic lesions and culturing faeces on solid media. However, new techniques including ELISA and qPCR have been developed and utilized to confirm infections with MAP in blood, milk and faeces more recently to confirm cases of JD.

In an infection trial 16 Holstein-Friesian bull calves were raised in a biosecurity level 2 facility at the University of Calgary. Calves were randomly allocated into two groups and euthanized at two and four months. In each group, six calves were infected with a library of Transposon (Tn) mutants created from a MAP strain named A1-157 located in the dominant clade of isolates obtained from Canadian farms. Two calves in each group received the wildtype (WT) A1-157 strain serving as positive infection 'controls'. After euthanasia, from each calf tissue samples from various anatomical locations were collected. For this study, the distal jejunum, distal jejunum lymph nodes, the spleen and inguinal lymph nodes were used. Using a self-developed protocol DNA from MAP was isolated from these samples in triplicates. A qPCR procedure targeting the F57 and IS900 loci was performed. qPCR results were then analysed with the help of a F57 plasmid standard curve to translate real time fluorescence measurements to bacterial log copy numbers.

All calves, library and WT euthanized at two or four months after inoculation, were successfully infected with MAP. F57 Ct values were all below 40 indicating that the tissues sampled were positive. Significant differences in log copy numbers between calves infected with the library of mutants euthanized at two and four months of age could not be detected in any of the tissues. As for the difference between wildtype calves euthanized at two and four months only in the spleen a significant difference in log copy numbers was noted with the four month old calves being less frequently positive (P=0,01). Furthermore, there was a significant difference in log copy numbers between library and wildtype calves euthanized at two and four months for the distal jejunum and jejunal lymph nodes. Log copy numbers were approximately equally divided for all four tissues in calves euthanized at two months, but in the calves euthanized at four months the distal jejunum and jejunum lymph nodes showed higher log copy numbers. CFU counts from MAP grown on agar were compared to CFU expected calculated from qPCR results of the same tissues. There was up to a 3-fold difference between the CFUs grown and expected in the jejunum and a 20-fold difference for the jejunal lymph nodes. We conclude, therefore, that both the WT and library calves were successfully infected and that the protocol to isolate and quantify MAP worked. Furthermore, more MAP was present in the tissues than grown on the agar plates.

Contents

Background

Johne's disease

Johne's disease (JD) or paratuberculosis is an important disease mainly affecting beef cattle and dairy cows. The causative agent of JD is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). JD affects animals worldwide and is endemic in most countries with bovine populations. The percentage of infected dairy herds in Canada ranges from 9.8 to 43% and in The Netherlands from 20 to 71%. Despite control programs being in place since the 1920's, control of this disease has proved difficult (Geraghty et al. 2014). MAP, while capable of infecting many different hosts, mainly infects ruminants and more specifically cows. Ruminants appear to be the natural host for MAP infections but non-ruminants like horses, pigs, camelids, dogs, non-human primates and humans can also be infected (Fecteau & Whitlock 2010).

Animals are infected at a young age and the incubation period can range from 2 to 10 years with the average being 3 to 5 years (Manning & Collins 2010). Clinically infected animals have a chronic, untreatable diarrhoea and significant weight loss (Tiwari et al. 2006). MAP is transmitted predominantly by the fecal-oral route. Uptake occurs through direct ingestion of contaminated faeces or indirectly via fecal-contaminated colostrum, milk, water or feed. MAP is being shed intermittently, making it challenging to diagnose infected animals and also making them infectious at different periods (Manning & Collins 2010).

It was long believed that calves older than 6 months are more resistant to an infection with MAP. Mortier *et al*. (2013) showed that calves up to 12 months of age also can be infected. The explanation behind the overall age dependency in MAP remains unclear, although studies suggest the higher amount of active Peyer's patches in neonates increases the susceptibility to MAP. Peyer's patches are small lymphoid organs which are situated in the ileum. Their function is to facilitate the immune response within this part of the intestines (Manning & Collins 2010).

JD has four different states of infection: silent infection, subclinical infection, clinical disease, and advanced clinical disease. The most important animals of these four in order to control JD are the subclinically infected animals, shedding MAP in their faeces thus continuing to spread the disease to other cows (Fecteau & Whitlock 2010). The Iceberg effect is a term used to describe the prevalence and spread of MAP on a farm. It is known that advanced clinical cases discovered within a herd are only a small portion of the infected cattle on a farm. Expressed in numbers this means that for every advanced clinical case diagnosed on a farm, 15 to 25 other cows are infected with MAP (Fecteau & Whitlock 2010)

Additionally, to the effect MAP has on animals, infections also pose a significant economic impact from losses primarily due to lower milk production, reduced slaughter value, increased involuntary culling, increased calving intervals, infertility and possible treatment costs (Behr & Collins 2010). Total annual losses for a 50-cow herd infected with MAP are estimated at $2,472 Canadian Dollars (Chi et al. 2002).

Mycobacterium avium subsp. *paratuberculosis*

Mycobacteria are Gram-positive, acid fast bacteria that include several human and animal pathogens. MAP is an intracellular bacterium which persists and multiplies in macrophages of the gastrointestinal tract. It is slow-growing and difficult to culture. MAP survives by invading the host, replicating, and avoiding detection by the host's immune system thus not being cleared from host cells (Harris & Barletta 2001).

When MAP is ingested it migrates through the gastrointestinal pathway until it reaches the mucosa of the small intestine. Microfold cells (M cells), present in Peyer's patches, facilitate the transport of organisms and particles from the gut lumen to immune cells. M cells are also capable of taking up MAP. MAP, similar to other enteroinvasive pathogens, utilizes specialized antigen-sampling cells which are present in the Peyer's patches of the small intestine (Secott et al. 2004). It has been suggested that M cells are the cells primarily infected with MAP at the early stages of infection. This is supported by electron microscopy showing the entry of MAP in bovine M cells (Momotani et al. 1988). In these early stages of infection the organism is found in phagocytic macrophages in the intestines prohibiting the maturation of these cells into phagolysosomes, a cellular body formed after the fusion of a macrophage with a lysosome. With this mechanism MAP escapes destruction. The hosts' immune system develops activated T cells including CD_4 T cells and cytotoxic CD8 cells in order to attack MAP infected macrophages. These cells interact with the infected macrophages through a network of cytokines and receptors. Despite this the infection is not cleared by the immune system and animals become subclinically infected (Radostits et al. 2006).

After infection of the small intestine, MAP spreads to the adjacent lymph nodes and eventually into a disseminated infection. Whether or not cows proceed to a subclinical state or clinical disease depends on the prevalence of MAP in the herd, suggesting that the rate of progression in JD is dependent on the dose of MAP that infected the calf and the age of infection (Behr & Collins 2010). Mechanisms behind the progression of cows from subclinical to clinical JD is associated with a decrease in the ability of mononuclear cells to produce IFN-gamma, a cytokine critical for the immune system. This leads to the loss of protective CD_4+ T cells and a lack of control of mycobacterial replications. What follows is a progressive granulomatous enteritis typical for JD (Koets et al. 2002).

Diagnostics

There are several ways to diagnose a MAP infection. The gold standard for identifying MAP from various sources has always been culture of faeces or tissue on solid media. When tested on 10 herds for a period of four years with repeated testing, the sensitivity of fecal culture was 33% and the specificity was 100% (Radostits et al. 2006). It is very difficult to isolate and culture MAP using tissue culture and because colonies do not normally appear for 6-8 weeks it is not a rapid test (Park et al. 2014). Other methods used are Enzyme-Linked ImmunoSorbent Assay (ELISA) for the detection of antibodies against MAP in milk and serum. The reported overall sensitivity of ELISA ranges from 40 to 55% (Radostits et al. 2006). However, animals usually do not build up a measurable antibody titre until clinical stages of JD, ELISA therefore does not detect subclinically infected animals (Logar et al. 2012). qPCR is an alternative method to bacterial culture and immunological methods of detecting MAP in tissues; it is a rapid test which can provide a higher throughput and sensitivity (Slana et al. 2008).

Quantitative real-time PCR

Quantitative real time PCR (qPCR) is different from conventional PCR because not only the amount of final DNA product is measured. During a qPCR procedure the reaction process is measured throughout every temperature cycle by measuring the light emitted from a fluorophore incorporated with the DNA product (Behr & Collins 2010). There are several advantages to qPCR compared to conventional PCR. qPCR has a higher sensitivity and because specific probes are used the confirmation of specificity is not required. Furthermore, the quantification of DNA is possible. Also larger numbers of samples can be processed and data handling is faster and easier (Rodríguez-Lázaro et al. 2005).

For the detection of MAP in this study two different loci were used named IS900 and F57. IS900 is the most commonly used target for MAP detection because it has the highest sensitivity. The reason for this is that the MAP genome contains 12 to 18 copies of IS900 (Bull et al. 2000). The downside of using this locus is the fact that other closely related mycobacterial species also possess IS900-like sequences thus negatively affecting the specificity of IS900 qPCR tests (Slana et al. 2008). The other locus F57 is a single copy element that is uniquely expressed in MAP, making it a better candidate for the identification and quantification of MAP.

Different probes can be used for qPCR, for this qPCR the most commonly used TaqMan® probe was used. On the 3' side of the TaqMan® probe a so-called quencher dye is attached, this dye prevents the fluorescent reporter dye on the 5' side from emitting light. The Taq polymerase is digested during the elongation and dyes are separated, allowing the reporter dye to emit light. As the amount of DNA product increases so does the fluorescent signal emitted by the liberated reporters.

The fluorescence will need a certain number of cycles to reach a specific threshold level. This value is referred to as the cycle threshold (Ct) value. A lower Ct value indicates a higher amount of target DNA is present in the sample. When these Ct values are correlated with known amounts of MAP DNA in the sample a standard curve can be used to determine the amounts of target DNA and absolute quantification can take place. A standard curve can be designed by using a known amount of plasmid DNA diluted down to several concentrations and then run through a qPCR protocol. The Ct values obtained correlate with the amount of DNA. Utilizing this technique the amount of colony forming units (CFU) per gram of tissue can be calculated.

Locations that are suitable for extracting MAP

The aim of this study was to quantify MAP directly out of tissue samples. The first step was to determine suitable spots to collect tissue from. This depends on the availability and amount of tissue present in the calves as well as data from papers suggesting the predominantly infected tissue locations. Looking at an infection trial done by Mortier et al. (2013) an impression of MAP infection by culture of tissues from different sites can be made. The top positive tissues in tissue culture are: mid ileum, ileocaecal valve, inguinal lymph nodes, ileal lymph nodes, and thereafter the spleen, distal jejunum, distal jejunum lymph nodes and the proximal ileum. Because of the limited amount of tissue available in this trial, the distal jejunum, distal jejunum lymph nodes, inguinal lymph nodes and spleen were used for this project.

Aim of the study

The aim of this study was to develop a method for quantifying the number of MAP bacteria present in different gastrointestinal and lymphoid tissues. The results from this study can be used to assess the success of this MAP isolation protocol for future quantification experiments.

Material and methods

Animals & study design

For this study 16 Friesian Holstein-Friesian bull calves were used. The calves all originated from farms across Alberta that had a MAP ELISA seroprevalence < 5%. Calves were raised in a quarantine barn classified as a biosecurity level 2 facility. This facility had 16 individual housing units with waterproof liners to contain any leakage. Taking care and feeding of the calves was done by

employees. The barn was outfitted with different pairs of coveralls ensuring cross contamination of calves could not occur. Animal care protocols were approved by the Animal Care Committee of the University of Calgary. Animals were randomly allocated to two groups.

Inoculum

A parent strain of MAP named A1-157 was chosen from more than 100 isolates collected from Canadian farms. This strain is located in the dominant clade including more than 80% of all Canadian isolates (Ahlstrom et al. 2015). The overall goal for the project was to find the essential genes in MAP pathogenesis using a genome-wide screen within this strain. A library of mutants was created with the mycobacteriophage MycoMarT7 which inserts a Mariner transposon at TA sites in the bacteria. This will cause mutations leading to the inability of some mutants to persist in host tissues. Using genome sequencing the pool of mutants present in the inoculum can be compared with those found in the tissues. The genes of interest are the most virulent genes that will be those that do not show up in the tissue because they have been disrupted.

The A1-157 library and the A1-157 strain were grown in an enriched environment to an OD_{600} of 1.5. The solution obtained was then spun down and frozen as 25% glycerol stocks. One day prior to inoculation the inoculum was resuspended in 7H9 broth enriched with Mycobactin J (2g/liter), glycerol (0.4%) and OADC (10%). Calves were orally inoculated with a dose of $1x10^{11}$ on two consecutive days ($5x10^{10}$ on each day). This amount was chosen in order to recover $1x10^{5}$ CFUs in the gastrointestinal tissues collected. The first group received an inoculum with a library of mutants and the second group received a 'control' inoculum containing the A1-157 wildtype strain. The calves were inoculated at two weeks of age by suckling the inoculum that was placed in a syringe and placed in the mouth of the calf.

Necropsies

During the trial there were two moments at which animals were euthanized. The first necropsy dates were between 9 and 29 October 2014, at which 6 animals that received the library, and 2 animals that received the wildtype dose were euthanized at the age of 2 months. The second set of euthanasia's and necropsies took place between 8 and 17 December 2014. Again, 6 library animals and 2 wildtype animals were euthanized at the age of 4 months.

The calves first received an intravenous injection with xylazine (Rompun®, Bayer Healthcare) adjusted to their estimated body weights. The calves euthanized at two months of age were shot with a bolt gun a few minutes after sedation. The calves at four months of age received a lethal injection with pentobarbital (Euthanyl Forte®, Bimeda-MTC Animal Health Inc) a few minutes after sedation. These procedures were approved by the Animal Care Committee of the University of Calgary. After that calves were transported to a facility at which the necropsies took place.

Samples

After opening the animal and removing the gastrointestinal tract the following samples were obtained: spleen, ileum, jejunum A section, jejunum B section, inguinal lymph nodes, ileal lymph nodes, jejunum A lymph nodes, and jejunum B lymph nodes. These samples were all stored at room temperature in Whirl-pak® bags containing a few millilitres of 1X PBS to maintain osmotic pressure.

The jejunum was divided into two different pieces called the A and B section. The A section is the most distal section closest to the large intestines and the ileocaecal valve. Consequently, the B section is the part more proximal following the A section. From both these parts about 50 cm of material was obtained, rinsed off and stored.

For the spleen a mid-section was taken of about 10x20 cm. Furthermore, both inguinal lymph nodes were collected. For the remaining lymph nodes, the lymph nodes that correlated to a certain part of the GI tract (Ileum, Jejunum A and Jejunum B) were taken and separately stored. All samples were then immediately transported to the lab where processing took place the same day according to the protocol.

Optimizing extraction protocol

To determine the ideal amount of tissue that would result in the best qPCR values, an initial protocol was tested on 25 and 100 mg of jejunum and spleen tissue.

Several steps were taken before a final DNA extraction protocol was ready. The first target of this project was to find a way to increase the amount of target DNA but decrease the amount of PCR inhibitors. PCR inhibitors are co purified components present in samples or reagents used during DNA extraction (Park et al. 2014). An article using a different pre-treatment method than commonly used was published by Park et al. (2014) and used to develop a protocol. Park et al. (2014) describe a pre-treatment method which was implemented in a protocol using the Qiagen DNeasy blood and tissue kit (DNeasy kit; Qiagen, MD). In the manuscript, a starting weight of 100 mg/ml of tissue was used. A pilot study was performed using this protocol to determine whether to proceed with a 25 or 100 mg/ml concentration of samples. To determine the sample concentration the amount of dsDNA was measured in duplicates using a spectrophotometer. Thereafter, the values were all diluted down to the lowest value (5.5 ng/µl) and another qPCR was performed. These results were compared to the outcome of the first qPCR. This revealed that in the 100 mg/ml samples the amount of extracted MAP DNA was lower compared to 25 mg/ml thus revealing that DNA extraction was more efficient in the 25 mg/ml samples.

A kit commonly used for extracting MAP DNA for the purpose of qPCR in fecal samples (MagMax™, Life Technologies) was compared to the developed protocol. The same tissue samples were taken for this extraction. Results from qPCR performed on these samples showed that DNA extraction was more effective using the Qiagen™ protocol.

The definitive protocol started with collecting 1g of each tissue sample and macerating it in an M-tube containing tissue lysis products (y-30 emulsion and Triton X-100) which is placed a GentleMACS machine. Thereafter, samples were diluted to a concentration of 25mg/ml in 2ml screw cap tubes containing 0.1 mm zirconia beads. These beads are necessary to break up the tough mycobacterial cell wall. After a 2-hour incubation step in animal tissue lysis buffer (ATL buffer; Qiagen) the sample was bead beated (Mini-Beadbeater ™, Biospec products inc.). Following this, the sample was incubated for 30 min in enzymatic lysis buffer (Tris-Cl, sodium EDTA, Triton X-100, and lysozyme). The sample was then bead beated again and then ethanol was added to precipitate the DNA. Finally, several washing steps using the DNeasy spin columns were done to purify DNA. The obtained DNA was then stored in -20°C. For more details on the extraction the complete DNA extraction protocol is attached as Appendix 1.

Data analysis

A plasmid standard curve was used for the data analysis of the F57 gene in MAP (Fig. 1). This standard curve was made for a fecal spiking experiment using plasmid DNA.

Using the statistics from this fecal spiking experiment an equation was determined to translate FAM F57 Ct values into log copy numbers. FAM is a fluorophore that excites the fluorescence in a qPCR reaction. The equation was $y = m(x)+b$, where y = Ct value, m = -3.628, b =42.4, and x= log copy number.

Theoretically it is possible to then translate these log copy numbers into amounts of bacteria expressed in colony forming units (CFU's). This was done by 10^x where x is the log copy number.

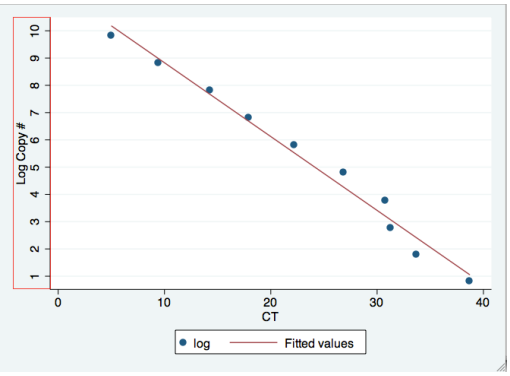

Fig 1. F57 plasmid curve edited from C. Corbett

Data analysis was performed using SPSS software (IBM SPSS Statistics software v22). Results obtained from qPCR were checked if normally divided by a Kolmogorov-Smirnov test and a Shapiro-Wilk test. After that results were compared using unpaired student's T-tests and one-way ANOVA tests.

Results

IS900 sequence and F57 sequence

All calves showed values for the presence of the IS900 sequence as well as the F57 sequence (Table 1). For each procedure a negative and positive control were included. Also an independent internal control probe (Q670) which is not shown here was used to check if the qPCR procedure succeeded and samples were redone in case this control did not respond

Table 1. Ct values for both the IS900 and F57 locus from all 16 calves and four tissues at two and four months.

		IS900 (Ct values)					F57 (Ct values)			
CALF#	Euthanasia (months)	Jejunum	Jejunum l.n.	Inguinal l.n.	Spleen		Jejunum	Jejunum l.n.	Inguinal l.nl	Spleen
2	2	30.84	32.07	32.44	30.56		36.07	34.46	37.58	33.94
4	2	31.40	31.09	34.62	34.09		35.61	34.54	37.86	35.54
5	2	33.36	33.90	33.07	33.72		37.34	37.29	37.28	37.32
9	2	29.27	30.77	30.95	29.92		33.62	35.45	33.62	32.80
11	2	30.35	30.76	32.48	31.73		34.28	34.65	35.68	36.07
12	2	32.75	33.32	32.91	32.08		35.96	37.65	35.93	35.33
15	2	28.64	28.98	28.02	29.74		32.16	32.78	31.14	33.31
16	2	28.96	28.71	29.81	29.43		32.70	32.67	33.33	33.43
1	4	30.89	30.59	31.43	30.79		37.01	33.96	33.70	33.77
3	4	34.12	30.20	32.09	33.50		39.02	34.22	37.69	36.27
6	4	33.04	32.11	33.63	33.58		36.61	35.63	38.73	38.07
7	4	33.49	33.93	34.98	33.53		37.48	39.21	38.09	37.53
8	4	34.21	34.85	34.07	32.89		36.88	36.68	38.46	37.81
10	4	30.80	29.85	30.15	31.01		35.36	33.80	33.64	34.56
13	4	29.98	29.00	32.31	32.23		33.33	32.99	34.95	36.13
14	4	25.66	24.44	32.50	32.59		29.04	28.02	34.75	36.74

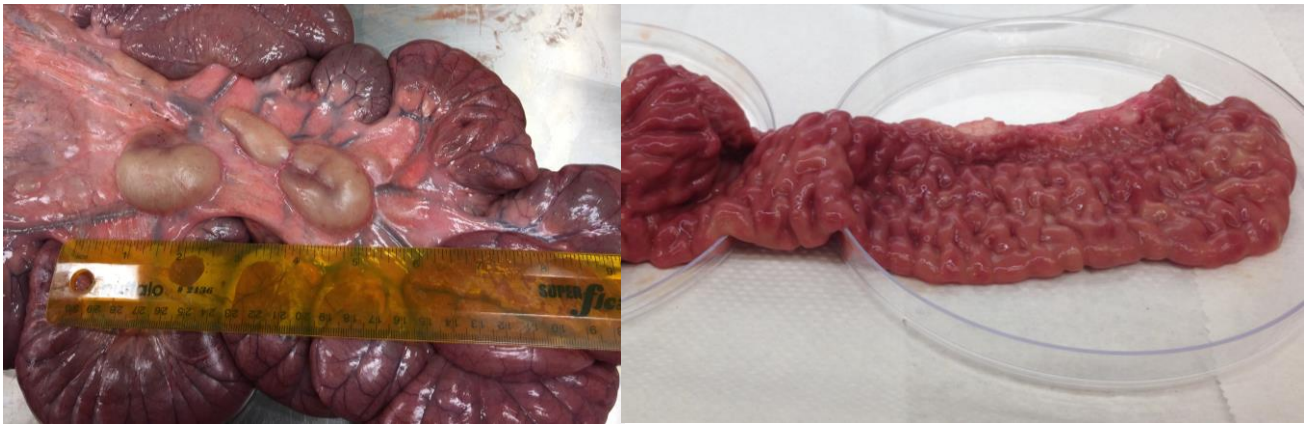

Figure 2&3. Jejunal lymph nodes and distal jejunum section from calf 14, a calf infected with the wildtype strain and euthanized at the age of four months.

Jejunum F57 FAM log copy numbers at two and four months

The FAM Ct values for F57 obtained from the qPCR were converted into log copy numbers using the previously mentioned standard curve. From now on results will be discussed using log copy numbers for the F57 locus.

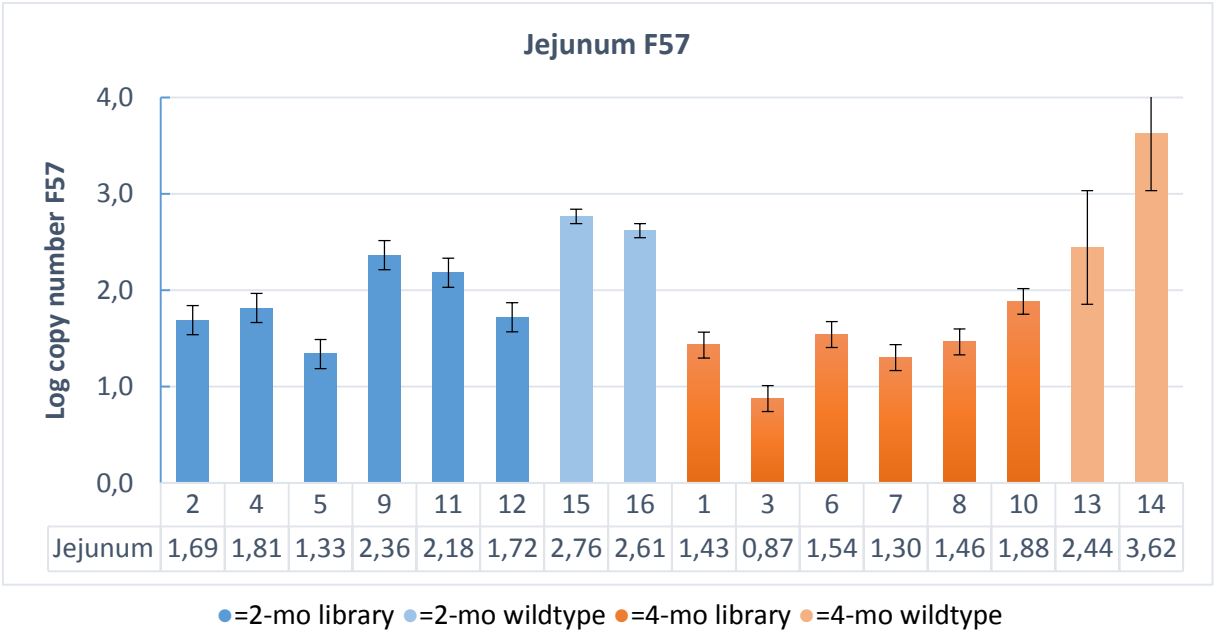

Figure 4. Log copy numbers for the distal jejunum of all calves.

The difference between log copy numbers for the jejunum from 2-month-old library and wildtype calves is significant (P=0.02). This was the same for calves euthanized at four months, showing a significant difference in log copy numbers between library and wildtype calves (P=0.005). Furthermore log copy numbers for jejunum tissue samples from library calves euthanized at two months tended to be significantly higher than those euthanized at four months of age (P=0.06). No significant difference between log copy numbers from two and four-month old wildtype calves was noticed (P=0.62).

Jejunum lymph nodes F57 FAM log copy numbers at two and four months

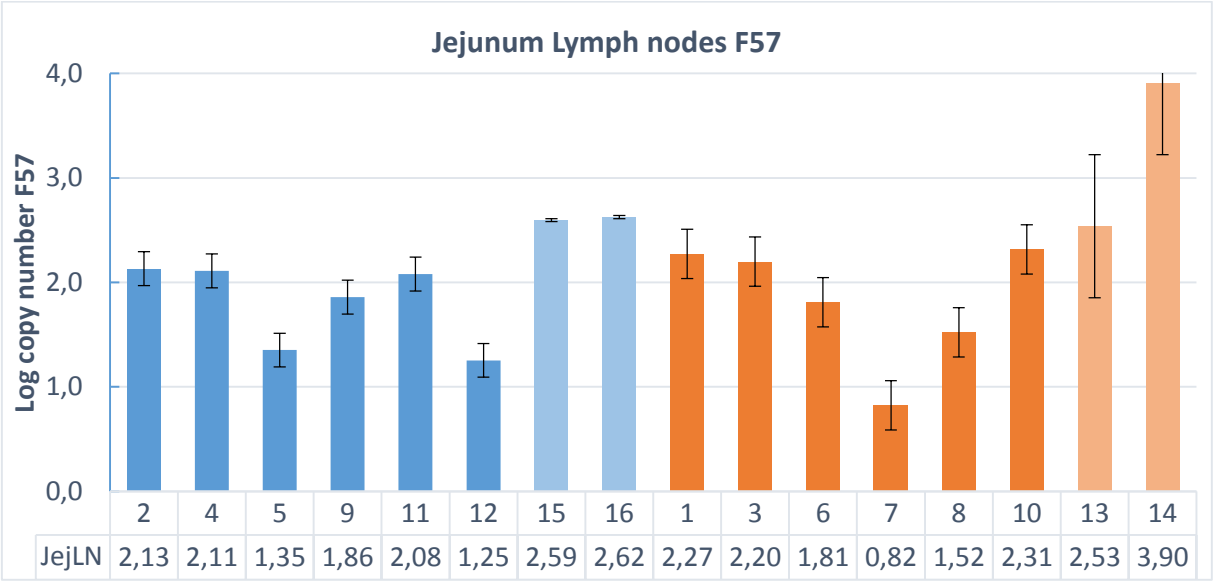

Figure 5. Log copy numbers for the jejunum lymph nodes.

A significant difference in log copy numbers for the jejunal lymph nodes was seen between 2-month-old library and wildtype calves (P=0.004). This was the same for the difference in log copy numbers between library and wildtype calves euthanized at four months (P=0.04). Between library calves euthanized at two and four months no significant difference in log copy numbers could be seen (P=0.93). The same applied to wildtype calves, showing no significant difference in log copy numbers between calves euthanized at two and four months (P=0.47).

Inguinal lymph nodes F57 FAM log copy numbers at two and four months

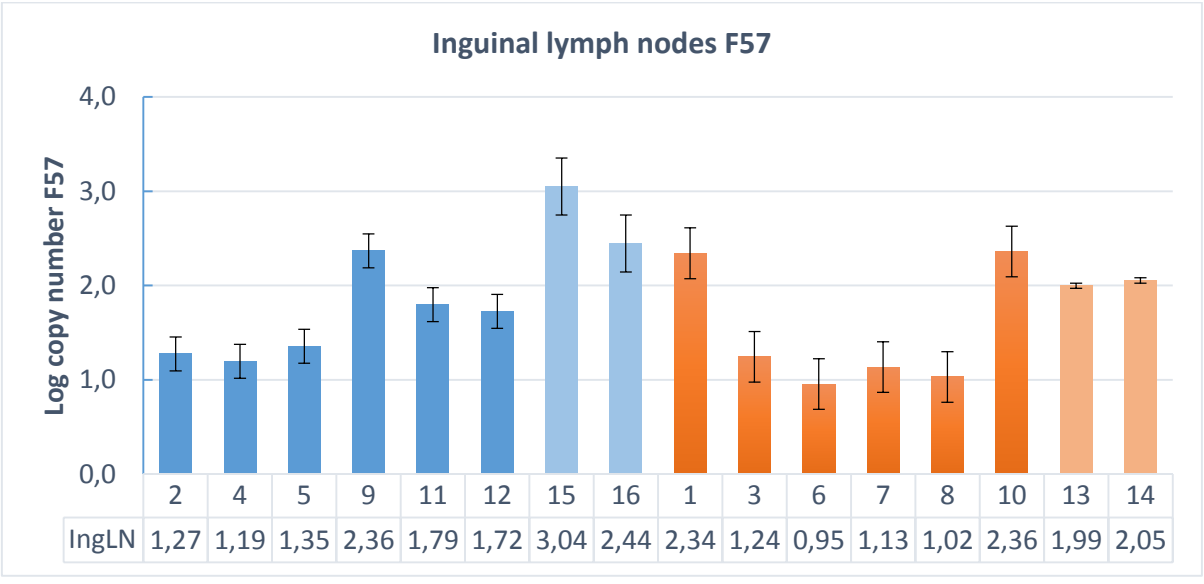

Figure 6. Log copy numbers for the inguinal lymph nodes.

A significant difference can be noted between log copy numbers for the inguinal lymph nodes from library and wildtype calves euthanized at two months of age (P=0.02). The library and wildtype

calves euthanized at four months of age did not show any significant difference in log copy numbers between each other (P=0.33). Between library calves euthanized at two and four months no significant difference in log copy numbers could be measured (P=0.75). This was the same for wildtype calves at two and four months, showing no significant difference in log copy numbers (P=0.14).

Spleen F57 FAM log copy numbers at two and four months

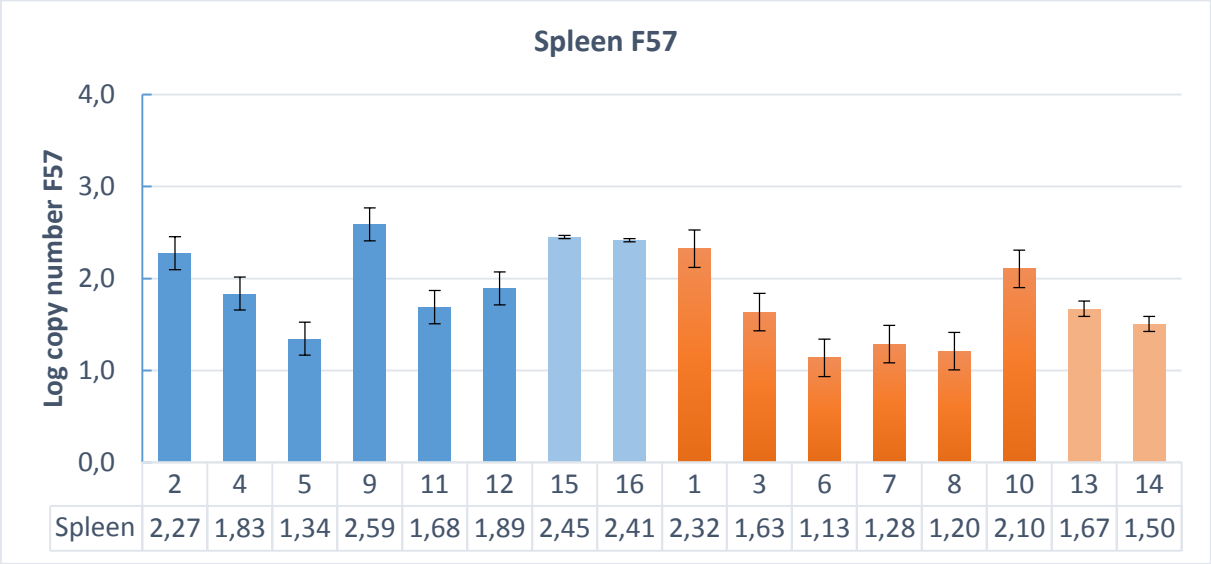

Figure 7. Logy copy numbers for the spleen.

Between library and wildtype calves euthanized at two months there was no significant difference in log copy numbers measured from the spleen (P=0.18). This was the same for library and wildtype calves euthanized at four months, showing no significant difference in log copy numbers (P=0.95). Between library calves euthanized at two and four months there was no significant difference in log copy numbers (P=0.26). For wildtype calves euthanized at two and four months there was a significant difference in log copy numbers (P=0.01).

CFU collected from tissues compared to CFU expected

MAP was isolated from tissues and plated out in order to obtain CFUs as part of the development of a vaccine. After an incubation period of 6 to 8 weeks colonies were counted on these plates. Tables 2 and 3 show the results from these countings, indicating the CFU per gram of tissue. This table also shows the potential amount of CFU present per gram of tissue when using the log copy numbers from this project in an obtained standard curve. Because the spleen and inguinal lymph nodes were not plated out no results are presented for those tissues.

Calf 14 was euthanized at four months of age and received the wildtype MAP strain. This calf had exceptionally high counts for the tissues plated and typical pathological findings (enlarged lymph nodes and thickened and corrugated mucosa) for MAP (Fig 2 and 3). The following averages are without the CFU count for calf 14. For the jejunum the average CFU collected from all calves euthanized at two months was 1209 and the expected amount of CFU was 3147, this almost a 3-fold difference. For calves euthanized at four months the average CFU count was 2627 and the expected CFU was 1082, a 2-fold difference.

For the jejunal lymph nodes the obtained CFU collected from calves euthanized at two months was on average 126, whereas the expected amount was 2632, which is a 20-fold difference. For calves euthanized at four months of age the counted amount of CFU was 1068 and the expected amount of CFU was 2289, a 2-fold difference.

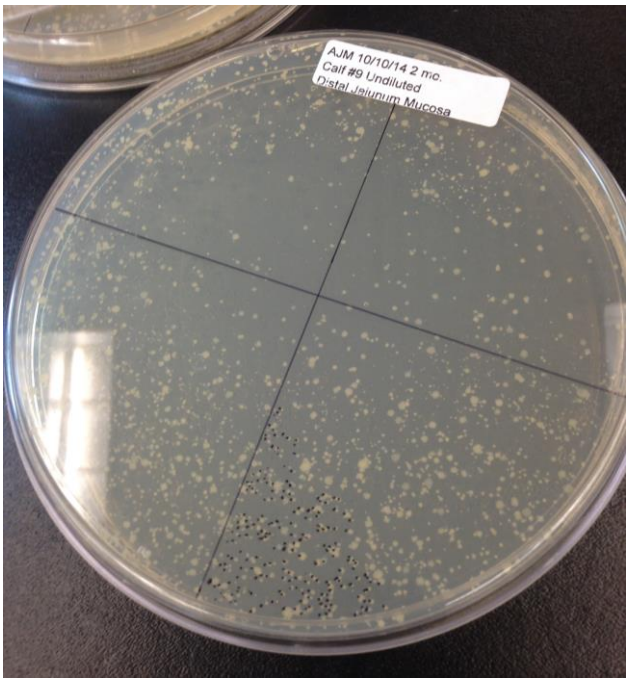

Figure 8. A undiluted agar plate from the distal jejunum of calf 9 six weeks after incubation

Table 2. CFU counts and expected CFUs for the jejunum from calves euthanized at two and four months. Due to contamination of plates from calf 16 no CFU data are known.

Jejunum			
Calf	Euthanasia	CFU collected from plates (per 1 g)	CFU expected by qPCR (per 1 g)
2	2	28	784
4	2	233	1048
5	2	373	349
9	2	4336	3706
11	2	380	2433
12	2	37	841
15	2	3080	9357
16	2	N/A	6656
1	4	3	432
3	4	56	121
6	4	55	556
7	4	2	321
8	4	65	467
10	4	207	1226
13	4	18000	4450
14	4	3160000	67701

Table 3. CFU counts and expected CFU for the jejunal lymph nodes from calves euthanized at two and four months.

Jejunum lymph nodes			
Calf	Euthanasia	CFU collected from plates (per 1 gram)	CFU expected by qPCR (per 1 gram)
2	2	19	2174
4	2	649	2064
5	2	6	360
9	2	112	1160
11	2	93	1927
12	2	46	287
15	2	0	6328
16	2	85	6756
1	4	56	2994
3	4	291	2537
6	4	227	1034
7	4	7	107
8	4	50	533
10	4	504	3300
13	4	6340	5516
14	4	413333	129200

Discussion

In this study, 16 Holstein-Friesian bull calves were orally inoculated with MAP. Twelve calves received a library of mutants created from a dominant strain and four calves received the same wildtype strain. These calves were euthanized at two and four months of age and necropsy was performed. DNA was extracted from four of the tissues collected; the amount of DNA were measured using qPCR. Thereafter, results were analysed using a F57 plasmid standard curve. The different parts of this study will be discussed separately.

Inoculation

All calves were obtained from farms with a MAP ELISA seroprevalence < 5%. These calves were separated from their dam as fast as possible after birth. It is possible that these calves came in contact with faeces containing MAP after birth. Because these farms were not completely negative for paratuberculosis testing it is possible that calves were infected before they arrived at the trial facility. Because the aim of this trial was to create a uniform pool of infected calves all infected within the same time period this could have caused calves to be infected earlier in the trial, leading to false results. Furthermore calves were infected with a library of mutants, the influence of calves being infected on the farm with a wildtype strain could ruin the goal of the experiment.

DNA extraction and measurement

The DNA extraction protocol was developed using the manuscript by Park et al. (2014). These authors compared several extraction techniques for extracting MAP from tissue samples. The protocol suggested was adjusted and rewritten in order to be useful for this project. Because qPCR extraction from tissue samples is a fairly new technique, not a huge amount of validated data and special kits are available for this purpose. It is possible using this protocol that DNA was lost during the extraction process. Several centrifugation and pipetting steps were included in the protocol, and

it is also possible that during these steps MAP DNA remained in either the pellet or pipette tip. In order to better validate the protocol, efforts could be made to start out with known amounts of DNA and adjust steps in the protocol one at a time to look at which steps have the most influence on the DNA yield.

Analysing results

All calves at both euthanized ages for all tissues gave Ct values for F57 lower than 40, which, from previous research can be considered as positive (Mortier et al. 2013). From this it can be concluded that all calves were successfully infected with MAP. Also, all calves gave positive results for the IS900 locus with Ct values low enough to consider calves being infected.

The distal jejunum samples taken from all calves at both ages were all positive. There was a difference in log copy numbers between the library calves at two months and four months, with the log copy numbers from the 2-month-old calves tending to be significantly higher. This is something we found in all four tissues, while it was not significant in any of them, given that the p-value is 0.06 the odds is 94% that this difference is not a coincidence. The same could be seen when looking at the difference in log copy numbers between wildtype calves at two and four months, a difference could be noted but not significant. A possible explanation for this finding could be that calves were still going through an initial infection following the inoculation, which faded away when time progresses, making tissues less positive at four months of age. However, no scientific literature is available to support this hypothesis.

When comparing the distal jejunum results of the calves infected with the library or wildtype strain the two month and four month age group differed in log copy numbers, with the wildtype calves being more positive than the library calves. Both these groups received the same dose ($1x10^{11}$) and were infected within the same time period. A possible explanation for this is that with the library inoculum certain genes were disabled that were crucial to establish a more severe infection. Another explanation would be that handling and manipulation of the strain in the mutagenesis process has affected the infectivity of the strain. This would correlate with the results obtained.

The jejunum lymph node samples taken from all calves at both ages were all MAP qPCR-positive. The same differences between age and inoculum groups were found as in the distal jejunum samples. Significant differences in log copy numbers could be noted between library and wildtype calves at two and four months. Also, no significant differences in log copy numbers were present between library calves at two and four months and wildtype calves at two and four months.

The inguinal lymph nodes of all calves at both ages were positive. There was a significant difference in log copy numbers between the library and control calves at the age of two months. At four months no significant difference in log copy numbers could be measured. This was the same for library calves euthanized at two and four months and wildtype calves euthanized at two and four months, showing no significant difference in log copy numbers. The inguinal lymph nodes clearly showed a different pattern than the jejunum and jejunum lymph nodes, with less difference in log copy numbers between library and wildtype calves. A possible explanation for this could be that because the wildtype strain still expresses every gene compared to the library these genes interact in a certain way in the GI tract that is necessary to establish a severe infection. In less favourable tissues like the inguinal lymph nodes and the spleen this might not be happening.

All calves showed a positive result for the spleen samples collected, both at two and four months. No difference in log copy numbers was detected between library and wildtype calves at two and four months. Library calves euthanized at two and four months also showed no significant difference in

log copy numbers for the spleen. Wildtype calves euthanized at two and four months were significantly different for the spleen, with wildtype calves at four months showing significantly lower low copy numbers than calves at two months. Potentially, an infection with MAP travels to the spleen before two months and is party cleared or reduced within two months. However, there is no literature available to support this hypothesis.

When considering the MAP pathogenesis the jejunum and jejunal lymph nodes are infected earlier than the inguinal lymph nodes and spleen. But when we look at the average log copy numbers at two months of age there was not a big difference (jejunum= 2.06, jejunum l.n.= 2.00 , inguinal l.n.= 1.90 and spleen= 2.06). At four months of age there was a slight difference, log copy numbers were on average lower but the jejunum and jejunal lymph nodes were quite a bit higher than the inguinal lymph nodes and spleen (jejunum= 1.82 , jejunum l.n.=2.17 , inguinal l.n.=1.64 and spleen= 1.61). An explanation for this could be that in the beginning the MAP infection travels through the body of the calf, but after four months the MAP bacteria stay at the place where they are most likely to be found and where clinical infection shows, being the gastrointestinal tract. In order to study this hypothesis animals should be infected with marked MAP bacteria that can be followed with for example fluorescence, however, these studies have never been performed.

The actual and expected CFU counts are presented in Tables 2 and 3. The majority of the calves had less CFUs on the plates than could be expected by qPCR results. For the jejunum there was a 3-fold difference in CFU at two months and a 2-fold difference in CFU at four months. For the jejunum lymph nodes this difference in CFU was a 20-fold difference at two months and a 2-fold difference in CFU at four months. There are several hypotheses for these differences in CFUs. In intestinal tissue samples a lot of host and environmental bacteria are present (Whittington 2010). In order to isolate MAP several incubation steps in bacterial lysing products including antibiotics are used (Whittington 2010). It is possible that during these steps MAP bacteria were killed, it has to be noted that this is a hypothesis and at this time is not backed up by scientific literature. Furthermore, MAP is a bacterium that depends on precise growing conditions as for temperature and humidity. For the expected CFU calculation a standard curve was used, this curve has not been validated yet. Finally, the difference between the amount of bacteria grown on the plates and calculated by qPCR could be explained by the fact that only the live bacteria grow on agar plates and for the qPCR DNA is measured, which is released by dead bacteria too. This DNA from dead bacteria was either still present in the tissue or released by killed bacteria during the extraction process.

The bigger picture

This research was a relatively small part of a larger trial with the aim of developing a marked, live attenuated JD vaccine. Calves were infected with either a library of mutants or the wildtype strain to differentiate between these two and to be able to review which genes were expressed in the tissues. This study assisted in understanding and analysing the success of a MAP isolation protocol from tissue and to grow these on agar. As presented in the results, this study concluded that with the protocol used only a small portion of the bacteria present in the tissue grew on plates. With this information it is possible to take a look at this protocol again to see which steps might inhibit MAP extraction. For the overall project of developing a vaccine targeting MAP infections, this research project helped in understanding MAP infection dynamics.

Further recommendations

For future research related to this project there are several things that would be interesting to look at. First, only four tissues were used for this analysis. Typical predilection sites for MAP infections that generated the highest positive results include the ileum, ileocaecal valve and ileal lymph nodes.

It would be interesting to perform the same DNA extraction procedure and qPCR on these tissues. These results could then be compared to the four tissues used to see if they indeed are more often positive as expected from previous trials (Mortier et al. 2013).

The main goal why these calves were infected was to culture MAP extracted from the tissues. For this extraction and plating process four tissues were used, the Ileum, ileal lymph nodes, the distal jejunum and distal jejunum lymph nodes. These tissues were chosen because it was expected that these tissues would come up as most positive. Following the previously mentioned extraction on the ileal tissue and lymph nodes it could be valuable to extract and culture MAP from the spleen. This project revealed that the spleen was positive and had the third highest log copy numbers from the four tissues used. Since there is more spleen tissue from calves compared to for example inguinal lymph nodes or ileal tissue this could be helpful in cultivating more CFU from plated tissue.

All calves were infected in this infection trial, either with the library or a wildtype strain of MAP. For the DNA extraction and following qPCR it was not possible to obtain control tissues from calves that were definitely not infected and in the same age group. I would recommend that in a future similar project it would be valuable to have negative control tissues from (non-infected) calves to serve as a negative control.

Acknowledgements

First of all I want to thank my supervisors Jeroen de Buck and Amanda Mirto from the University of Calgary for their hospitality and assistance during my research project. Without their help I would have never succeeded with finishing this project. Despite them being very busy with their own projects they always had time to help me out, give advice or talk about stuff that was going on. Furthermore, I want to thank Uliana Kanevets for all her help in the lab doing the extractions and interpreting the results. Last of all I want to thank Herman Barkema and Theo Lam for their efforts to set up this research project and make all the arrangements necessary.

Literature

Ahlstrom, C. et al., 2015. Limitations of variable number of tandem repeat typing identified through whole genome sequencing of Mycobacterium avium subsp. paratuberculosis on a national and herd level. *BMC genomics*, 16(1), p.161. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4356054&tool=pmcentrez&rendertype=abstract [Accessed March 19, 2015].

Behr, M.A. & Collins, D.M., 2010. Paratuberculosis: Organism, disease, control. In *Paratuberculosis: Organism, Disease, Control*. CABI Publishing, pp. 1–375. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84890719836&partnerID=tZOtx3y1.

Bull, T. et al., 2000. Characterization of IS900 loci in mycobacterium avium subsp. paratuberculosis and development of multiplex PCR typing. *Microbiology (Reading, England)*, 146 (Pt 12, p.3285. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11101687 [Accessed January 26, 2015].

Chi, J. et al., 2002. Direct production losses and treatment costs from bovine viral diarrhoea virus, bovine leukosis virus, Mycobacterium avium subspecies paratuberculosis, and Neospora caninum. *Preventive Veterinary Medicine*, 55(2), pp.137–153. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-0037201366&partnerID=40&md5=18e08e7d2e982c79e64b0f300c03d689.

Fecteau, M. & Whitlock, R.H., 2010. Paratuberculosis in Cattle. In *Paratuberculosis: Organism, Disease, Control*.

Geraghty, T. et al., 2014. A review of bovine Johne's disease control activities in 6 endemically infected countries. *Preventive Veterinary Medicine*, 116(1-2), pp.1–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24997766 [Accessed October 10, 2014].

Harris, N.B. & Barletta, R.G., 2001. paratuberculosis in Veterinary Medicine Mycobacterium avium subsp . paratuberculosis in Veterinary Medicine. , 14(3).

Koets, A. et al., 2002. Progressive bovine paratuberculosis is associated with local loss of CD4(+) T cells, increased frequency of gamma delta T cells, and related changes in T-cell function. *Infection and immunity*, 70(7), pp.3856–64. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=128076&tool=pmcentrez&rendertype=abstract [Accessed February 7, 2015].

Logar, K. et al., 2012. Evaluation of combined high-efficiency DNA extraction and real-time PCR for detection of Mycobacterium avium subsp. paratuberculosis in subclinically infected dairy cattle: comparison with faecal culture, milk real-time PCR and milk ELISA. *BMC veterinary research*, 8, p.49. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3423054&tool=pmcentrez&rendertype=abstract [Accessed February 7, 2015].

Manning, E.J.B. & Collins, M.T., 2010. Epidemiology of paratuberculosis. In *Paratuberculosis: Organism, Disease, Control*.

Momotani, E. et al., 1988. Role of M cells and macrophages in the entrance of Mycobacterium paratuberculosis into domes of ileal Peyer's patches in calves. *Veterinary pathology*, 25(2),

pp.131–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3363791 [Accessed January 26, 2015].

Mortier, R.A.R. et al., 2013. Evaluation of age-dependent susceptibility in calves infected with two doses of Mycobacterium avium subspecies paratuberculosis using pathology and tissue culture. *Veterinary Research*, 44(1). Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84885003049&partnerID=40&md5=a941beb7813261c8bf3e832f5cb60a76.

Park, K.T., Allen, A.J. & Davis, W.C., 2014. Development of a novel DNA extraction method for identification and quantification of Mycobacterium avium subsp. paratuberculosis from tissue samples by real-time PCR. *Journal of microbiological methods*, 99(1), pp.58–65. Available at: http://www.scopus.com/inward/record.url?eid=2-s2.0-84896845290&partnerID=tZOtx3y1 [Accessed January 23, 2015].

Radostits et al., 2006. Veterinary Medicine, 10th Edition. Available at: http://store.elsevier.com/product.jsp?locale=en_EU&isbn=9780702027772 [Accessed February 7, 2015].

Rodríguez-Lázaro, D. et al., 2005. Real-time PCR-based methods for detection of Mycobacterium avium subsp. paratuberculosis in water and milk. *International journal of food microbiology*, 101(1), pp.93–104. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15878410 [Accessed January 26, 2015].

Secott, T.E., Lin, T.L. & Wu, C.C., 2004. Mycobacterium avium subsp. paratuberculosis fibronectin attachment protein facilitates M-cell targeting and invasion through a fibronectin bridge with host integrins. *Infection and immunity*, 72(7), pp.3724–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15213112 [Accessed January 26, 2015].

Slana, I. et al., 2008. On-farm spread of Mycobacterium avium subsp. paratuberculosis in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. *International journal of food microbiology*, 128(2), pp.250–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18824269 [Accessed January 26, 2015].

Tiwari, A. et al., 2006. Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *The Canadian veterinary journal. La revue vétérinaire canadienne*, 47(9), pp.874–82. Available at: /pmc/articles/PMC1555680/?report=abstract [Accessed January 23, 2015].

Whittington, R., 2010. Cultivation of MAP. In *Paratuberculosis: Organism, Disease, Control*.

Appendix 1: DNA extraction protocol and qPCR protocol

Tissue samples:

-Slice of distal jejunum (A section)
-Jejunal lymph node (A section)
-Inguinal lymph node
-Spleen

Intestinal tissues:

1. Scrape the mucosa from the distal jejunum with a glass slide to separate mucosa from muscle layers and collect 1g in a gentle MACS tube.

   Lymphoid and spleen tissue:
2. Remove fat from the inguinal and jejunal LN, and cut with a scalpel into small pieces.
3. Collect all the pieces of LN tissue on a petri dish, homogenize, and collect 1g and put it in to separate gentle MACS tubes.
4. Cut off 1g of the spleen and put it into a gentle MACS tube.
5. Make a 1% Y-30 emulsion (Sigma Aldrich #A6457) solution in PBS enriched with 0.5% Triton X-100 to prevent foaming.
6. Add 10ml of the Y-30/Triton X-100 solution to the tubes.
7. Let the tubes stand for 1 hour.
8. Macerate the tissue in a Gentle MACS machine using protocol: Protein_01.01 for 55 seconds (2x for each tube).
9. Pour approximately 100µl of 0.1mm zirconia beads into 2mL screw cap tubes and autoclave them.
10. Pipet 250µL into 3 2mL autoclaved screw cap micro centrifuge tubes and add 750ul of 1X PBS solution (25mg/mL).
11. Centrifuge the tubes at 15,000 x g for 15 min.
12. Carefully remove and discard the supernatant with a 1ml pipet tip.
13. Resuspend in 360µl of tissue lysis buffer (ATL buffer; Qiagen) and 40µl of proteinase k and incubate for 2 hours with occasional vortexing (every 20 minutes) at 56°C until visible particles are digested.
14. Centrifuge the tubes at 15,000 x g for 15 min.
15. Carefully remove and discard the supernatant with a 1ml pipette tip.

    The pellet can now be processed for DNA extraction.
16. Add 1.2% Triton X-100 and 20mg/ml lysozyme to the pre-made enzymatic lysis buffer already containing the Tris-Cl and sodium EDTA.

17. Add 275µl of enzymatic lysis buffer (20 mM Tris–Cl (pH 8.0), 2 mM sodium EDTA, 1.2% Triton X-100, and 20 mg/ml lysozyme,).
18. Vortex and incubate the tubes for 30 min at 37°C.
19. Bead beat (Mini-Beadbeater™, Biospec products inc., Bartlesville USA) the samples for 5 minutes.
20. Centrifuge the tubes at 4000 x g for 30 seconds to spin the beads and foam down.
21. Add 25 µl of proteinase k (N600 mAU/ml; Qiagen) and 300 µl of AL buffer (Included in kit) and incubate for 2h at 56°C.
22. Repeat bead beating step for 5 minutes.
23. Centrifuge the tubes at 4000 x g for 30 seconds to spin the beads and foam down.
24. Add 100% ethanol at the ratio 1 to 2 (Ethanol: supernatant) and thoroughly mix by vortexing
25. Centrifuge the tubes at 12,000 x g for 10 min at room temperature.
26. Carefully transfer the supernatant to the mini spin column tubes.
27. Put the mini spin column into a 2ml collection tube and pipet the mixture onto the center of the filter. Centrifuge at ≥6000 x g for 1 min. Discard flow through and collection tube.
28. Place the DNeasy mini spin column in a new 2ml collection tube, add 500µl Buffer AW1, and centrifuge for 1 min at ≥6000x g. Discard flow through and collection tube.
29. Place the DNeasy mini spin column in a new 2ml collection tube, add 500µl Buffer AW2, and centrifuge for 3 min at 20,000 x g to dry the DNeasy membrane. Discard flow-through and collection tube.
30. Place the DNeasy mini spin column in a clean 1.5 ml or 2ml micro centrifuge tube, and pipet 200µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x g to elute.
31. Repeat previous step.
32. Measure the amount of DNA in all tubes using a spectrophotometer and record data.
33. Store the samples at -20°C

Protocol for qPCR

Take samples out of freezer to let them thaw out

Prepare the mastermix containing:

For IS900:

-TaqMan® Fast advanced mix (life technologies) (10µl per sample)

-Forward primer (1µl per sample)

-Reverse primer (1µl per sample)

-IS900 probe (1µl per sample)

-Internal control probe (1µl per sample)

-H2O (2µl per sample)

-Plasmid IS900 (2µl per sample)

For F57:

-TaqMan® Fast advanced mix (life technologies) (10µl per sample)

-Forward primer (1µl per sample)

-Reverse primer (1µl per sample)

-F57 probe (1µl per sample)

-Internal control probe (1µl per sample)

-H2O (2µl per sample)

-Plasmid F57 (2µl per sample)

Pipet 18µl of either the IS900 or F57 mix in a 0.2ml rtPCR tube.

Pipet 2µl of the sample in the rtPCR tube.

Make sure the PCR plate contains a positive and negative control and is performed for IS900 and F57

Put the lids on top of the tubes and make sure it is sealed tightly.

Place the tubes in the qPCR machine and close the lid.