Distribution of *Mycobacterium avium* subspecies *paratuberculosis* in tissues of experimentally infected steers

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Summary

Tissue culture was carried out on 30 experimentally MAP inoculated calves in 5 different age groups (2w, 3m, 6m, 9m and 12m) with two different doses and on 3 uninfected calves (control), after euthanasia at 17 months of age. In total 21 tissues per animal were taken from different locations: duodenum, jejunum (mid and distal part), ileum (proximal, mid and distal part), ileocaecal valve, caecum, colon (spiral colon and transverse colon), rectum, lymph nodes corresponding with the previously mentioned gastro-intestinal tract samples (except for the spiral colon, transverse colon and rectum), hepatic lymphnode, tonsil, retropharyngeal lymph node and the superficial inguinal lymph node. After decontamination and 48 days of incubation at 37°C a confirmatory identification of MAP with F57 qPCR was performed.
In total 19 of the 31 infected calves (61,3%) were tissue culture positive. The tissue with the highest probability for detection of an infected animal was the ileocaecal valve with 0.19, considered very low. In 45,2% of the infected animals MAP was found in the GI tract tissue, in 25,8% of the animals had positive tissues for the associated lymph nodes of the GI tract and for the other lymph nodes (hepatic, retropharyngeal and superficial inguinal) MAP was found in 12.9% of the animals. Out of the 19 tissue culture positive animals, MAP was detected in 73,7% of the animals with GI tract tissues.
Considering that all MAP inoculated animals are successfully infected, which is perhaps not true, the results suggest that tissue culture, considered to be the gold standard, detects a lower percentage of infected animals than expected, this leads to underestimation of test agreements. Tissue samples from multiple sites are necessary to detect 100% of the infected animals. The probability of detection of an infected animal per location is too low to use as single detection method of an infected animal. Dissemination of MAP takes already place in the subclinical stage of JD instead of the previous assumed clinical stage of JD.

1. Introduction

Johne's disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic contagious granulomatous enteritis mainly found in ruminants. JD is an endemic disease and causes significant economic losses to the agriculture industry. In Canada the annual losses are estimated to be $15 million in the dairy industry (1). The high costs of JD for farmers are primarily due to progressive weight loss, reduced fertility, reduced milk production, premature culling and reduced slaughter value (1-4). There is no cure available, and factors as the long incubation period, subclinical shedders, low sensitivity of the available tests and continued susceptibility after vaccination, make JD difficult to eradicate (5). The main focus of control programs is on management practices to prevent infection of susceptible cattle, calves less than 1 year, by reducing the chance of fecal-oral transmission (5-11). Other more difficult or impossible to prevent routes of transmission are through bio-aerosols or in utero respectively (12-16).
Of concern is that a correlation has been found between the presence of MAP in patients with Crohn's disease (CD), an inflammatory bowel disease in humans. MAP could have a zoonotic potential, however, an actual relationship between MAP and CD has not been demonstrated until now (2,6,7,17,18).

In four stages JD will lead to the death of an infected animal as described by Whitlock and Buergelt, 1996 (19). The silent infection is characterized by the absence of clinical signs and no diagnostic tests are able to detect MAP infection reliably. In the subclinical stage animals may start shedding MAP in their feces and have detectable amounts of antibodies against MAP. Decreased fertility is typical for this stage. Clinical infection starts when an animal shows clinical signs, mostly 2 to 10 years after infection, such as weight loss, diarrhea and decrease in milk production (up to 16%) (20). Most animals will be positive on available diagnostic tests. Animals not culled will progress to the advanced clinical infection. Animals are lethargic, weak, emaciated and their milk production stops (20), they have watery diarrhea and finally this will result in death (7,21).

On-farm different tests are used for ante mortem screening for MAP: milk and serum ELISA (milk: sensitivity 21-84%, specificity 83-100% and serum: sensitivity 7-95% and specificity 41-100%) (6,22,23) and (pooled) fecal culture (sensitivity 29-74%, specificity approaching 100% if confirmed with *IS*900 PCR). Dust culture is possible to screen if a herd is possible infected with Johne's disease (6,23). For individual ante mortem diagnostic tests serum ELISA and fecal culture are used (24). In general, all of these tests have a low sensitivity, especially during the first stages of JD (2,7,23).

Postmortem tissue culture is also available. MAP culture from a tissue sample confirmed by PCR is considered to be the gold standard for paratuberculosis infection (6). Tissue culture is seen as the most accurate method to estimate herd-prevalence (6) and it is believed that it can detect MAP before other diagnostics can (22,25), although low-concentrations of MAP can be missed (10). An important note has to be made as tissue culture is not useful for screening on farms or individual diagnostics, as culling or surgical procedures are required, this method can be used for (herd-)prevalence studies at slaughterhouses, research and for the individual animal when culled. Furthermore, culturing tissues is labor intensive, logically difficult and an expensive diagnostic test with a cost of $35 to $60 per sample (1,6,22). Serum ELISA is used in most prevalence studies as the costs are lower and easier to apply, disadvantage is the lower sensitivity (26) .

Cultivation of MAP is used for fecal, tissue and environmental samples. Cultivation of MAP at 37°C has a termination point of 8-12 weeks for liquid media and needs a exogenous source

of mycobactin for growth. In general, liquid culture methods have a greater sensitivity and shorter time till detection of growth than solid culture methods (6).
Identification of MAP can be done by PCR. The two mainly used gene targets are *IS*900 and F57. *IS*900 is an insertion sequence with multiple copies (14 to 20) in the MAP genome which makes detection of this target a sensitive method, but it is not highly specific as other bacteria have IS900-like sequences, thus can cause the reaction to be false positive (6,18,27-30). F57 on the other hand is a less sensitive target for detection as it has a single copy in the MAP genome, but is highly specific for MAP (31). One short run of *IS*900 conventional PCR was performed to see the difference in sensitivity.

In short, after ingestion of MAP bacteria MAP will enter the intestinal wall through the intestinal M cells, especially in the ileum. Macrophages will phagocytize MAP, generally MAP survives, and migrate to the local lymph nodes. The presence of MAP antigens leads to a cellular immune response in the area of the intestinal submucosa and mesenteric lymph nodes. As the cellular immune response is unable to control the infection, a humoral immune response will develop (20,22). With the progression of the infection diffuse granulomatous changes, mostly without necrosis or inflammation, will affect multiple (also extra intestinal) tissues (7,20,21,32).

The literature is conflicting about which and the amount of tissue samples need to be taken to detect an infected animal, increasing amounts tissue samples improve the likelihood of detection of an infected animal and the costs (33). The international guidelines (33) for experimental infections with MAP defined the following knowledge gap: What is the optimal-but-practical number of tissue samples needed for diagnosis of MAP infection?
To answer this question tissue samples were taken from 30 experimentally MAP inoculated Holstein Friesian steer calves, inoculated at different ages, and 3 non-inoculated control calves. At 17 months of age the animals were euthanized and at necropsy 21 different tissues samples were taken from the gastro-intestinal tract and corresponding lymph nodes under clean circumstances (see material and methods).

The goals of this study were 1) to determine the tissue location with the highest probability for detection of a positive animal and 2) to assess the best combination of tissues for a high probability outcome to detect MAP infected animals.

2. Material and Methods

This research project was part of a larger study containing 56 Holstein Friesian calves divided in two different runs. This research project was based on the tissues collected during the necropsies of the first run in August 2011.

1.1 Experimental Design

Newborn Holstein Friesian male calves (*n =33)* were obtained from 20 dairy farms with a within-herd seroprevalence of MAP infection lower than 5%. Calves were born supervised by researchers and directly separated from their dam. They were fed 6L of irradiated colostrum within 12 hours of birth. Till seven weeks of age they were fed High Performance Calf Milk Replacer. As of 2 weeks of age they were fed Calf Starter and high quality hay. They were weaned at 7 weeks of age. Calves were dehorned and castrated.
The calves were housed individually with their own drinking bucket, feeding pen and 'own' coveralls for entering the pen are supplied. The pens were 16 feet by 6 feet, and deep litter.

The calves were infected with MAP at five different ages (2w, 3-6-9 and 12 mo). In each age group half of the calves received a low (5×10^7 CFU) or high (5×10^9 CFU) bacterial inoculum dose, on 2 consecutive days. There were 3 uninfected control calves. During the trial all calves were sampled on regular basis for blood, urine, serum and feces.

1.2 Inoculum

For the inoculum a virulent cattle type MAP strain was isolated from a clinical case (Cow 69). Cow 69 had an identical IS900-RFLP profile as the reference strain K10, recommended by Hines et al 2007 (33). To eliminate clumps the inoculum was vortexed with 3-4 mm glass beads. The viability was checked by a fluorescent Live/Dead® BacLight™ Bacterial Viability Kit (Invitrogen, Burlington, Ontario, Canada). MAP was quantified using the 'pelleted wet weight method' as described by Hines et al 2007. The inoculum was not contaminated, viable and had the right CFU after analysis. The inoculum was mixed with a small volume of culture broth and the inoculum was given PO by using a syringe containing the mixture.

1.3 Necropsies

The calves were euthanized at 17 months of age by intravenous administration 120 ml of Euthanyl Forte (Bimeda-MTC, Ontario, Canada) per calf, and necropsies were performed immediately. Postmortem 21 tissue samples were collected with separate sterile instruments for each tissue site to prevent cross contamination. The location of the tissues were selected based upon the pathogenesis and previously published articles about tissue culture. Most authors found that tissue samples of the GI tract had the highest detection rate, with up to 90% of the animals, with the jejunum, ileum and ileocaecal valve as most sensitive locations (10,24,34-36). With the collection a probability of 80-90% of detecting infection was reached by the collection of three tissue sites – the proximal ileum, distal ileum and ileocaecal valve (33). Non-GI tract lymph nodes were taken based upon the experimental pathogenesis and MAP shedding in milk (8,20).
Samples of the lymph nodes were taken before any of the gastro-intestinal tract samples to prevent contamination. Gastro-intestinal tissue samples were taken from the duodenum, jejunum (mid and distal part), ileum (proximal, mid and distal part), ileocaecal valve, caecum, colon (spiral colon and transverse colon) and rectum. Lymphatic tissue samples were taken from the lymph nodes corresponding with the previously mentioned gastro-intestinal tract samples (except for the spiral colon, transverse colon and rectum). On top of that, following lymph nodes were also taken; hepatic lymphnode, tonsil, retropharyngeal lymph node and the superficial inguinal lymph node.
The tissue samples were rinsed with PBS till they were visually clean. The epithelium of the intestinal walls was scraped off using microscope slide, the lymph nodes were cleaned from

all other tissue, stomachered for 5 minutes, aliquotted in 3 bags and stored at -80 °C until culture could be performed.

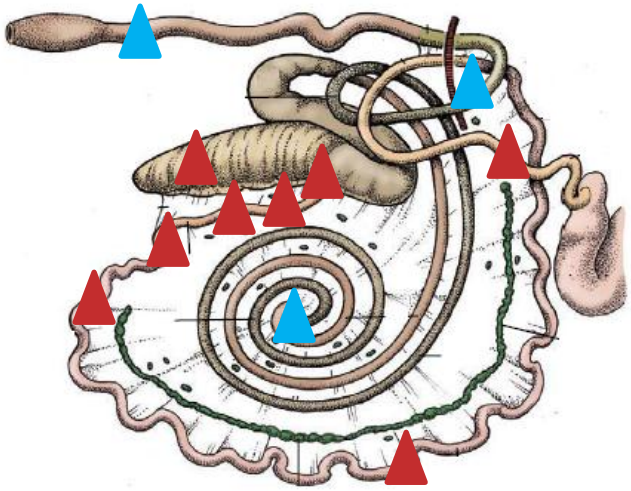

Figure 2-1 Locations of the taken gastro-intestinal tissue samples. The red triangles correspond to gastro-intestinal locations where also the corresponding lymph nodes were taken, and the blue triangles correspond to sites were only the intestinal tissue was taken. Picture by Dyce et al, 2010 (37).

1.4 Tissue Culture

For tissue culture 2.0 gram of each tissue sample was added in a sterile polyethylene stomacher bag with 5 mL ½ BHI (Difco Laboratories, Maryland, USA) + 0.6% HPC (Alfa Aesar®, USA), and stomached till homogenized (for simplified protocol see Appendix 1). If homogenized, the sample was added to 20 mL of ½ BHI + 0.6% HPC. After an incubation of 3 hour at 37°C the tubes were centrifuged at 1700G for 20 minutes. The supernatant was removed and the pellet was resuspended in 1 mL Para-JEM® antibiotic solution (TREK Diagnostic Systems, Ohio, USA), and incubated overnight at 37°C. The contents of the tube were added to a Para-JEM Broth bottle for TREK ESP® Culture System II containing 1 mL Para-JEM® EYS (TREK Diagnostic Systems, Ohio, USA), 1 mL Para-JEM® GS (TREK Diagnostic Systems, Ohio, USA), 0.5 mL Para-JEM® AS (TREK Diagnostic Systems, Ohio, USA) and 0.05 mL Para-JEM® Blue (TREK Diagnostic Systems, Ohio, USA), and the bottles were incubated for 48 days at 37°C.

1.5 DNA extraction

After incubation the DNA was extracted, for stepwise protocol see Appendix 2. Shortly, 200 µL of the vortexed culture broth was added to 800 µL of 100% ethanol. This was centrifuged at 7500 rpm for 9:00 minutes. The pellet then was washed twice with Gibco® DPBS (Invitrogen™, NY, USA). Then the pellet was resuspended in 100 µL Gibco® UltraPure™ Distilled Water (Invitrogen™, NY, USA) and boiled for 30 minutes. The supernatant, or lysate, was transferred in a new eppendorf cup and stored at -80°C.

1.6 Real-time qPCR –F57

The oligonucleotide primers used for the F57 real-time qPCR have been described by Slana et al. (38). In short, the forward primer designated F57qPCRF, the reverse primer F57qPCRR and the probe F57qPCRTM were used (table 2-1). The internal control plasmid was created by using PCR copy an artificially produced 99 base-pair single stranded oligonucleotide based on the *StTS1* gene (AF483209) from the potato, flanked by the reverse and forward primer of F57 into a Topo TA Plasmid (Invitrogen™, NY, USA).

The amplification was performed in a reaction with a total volume of 20 µL containing 10 µL of TaqMan® Fast Advanced Master Mix (Applied Biosystems™, California, USA), 1 µL of each forward and reverse F57 primer, 1 µL of each F57 probe and IC probe, 2 µL of Invitrogen™ UltraPure Distilled Water (Invitrogen™, NY, USA), 2 µL of Internal Control Plasmid (at an approximate concentration of 900 copies/ µL) and 2 µL of the samples DNA lysate. All real-time qPCR assays were carried out in a Bio-Rad CFX96™ Real Time System (Bio-Rad, Ontario, Canada) comprising a pre-incubation of 2:00 minutes at 50°C, and an initial denaturation step at 95°C for 0:20 min, followed by 40 cycles of 0:03 min at 95°C, 0:30 min at 61°C with a plate reading after each cycle. The final extension was performed with 1:00 min at 95°C and 5 min at 72°C.

Target gene	Name	Type	Sequence 5'-3'
F57	F57qPCRF	Forward	GCCCATTTCATCGATACCC
	F57qPCRR	Reverse	GTACCGAATGTTGTTGTCAC
	F57qPCRTM	Probe	6FAM–AATTCTCAGCTGCAACTCGAACACAC–BHQ
IAC-F57	IACqPCRTM	Probe	Cy5–GGCTCTTCTATGTTCTGACCTTGTTGGA–BHQ

Table 2-1 The primers and probes for F57 and the internal control with sequences as described by Slana et al. (38).

As positive controls, a positive tissue sample lysate of Cow 1 of the distal ileum (C1 IL3) and a DNA positive control extracted from a MAP culture were used. As negative control UltraPure Distilled Water (Invitrogen™, NY, USA) was used. The Bio-Rad CFX Manager™ 2.0 Software (Bio-Rad, Ontario, Canada) was used.

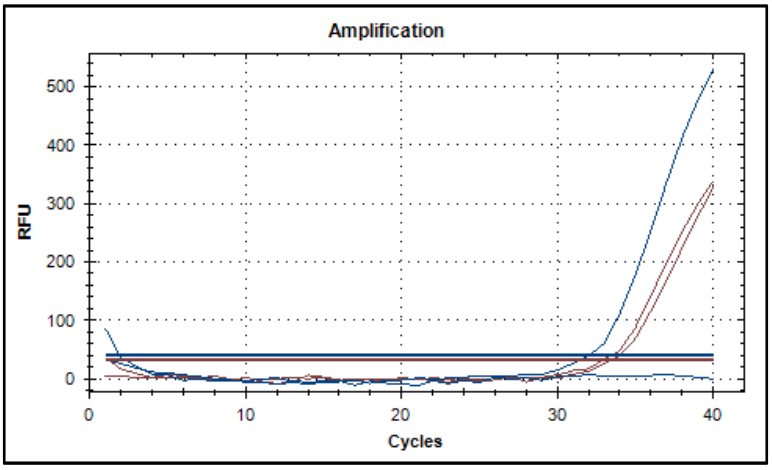

Figure 2-2 Example of a positive and negative sample for F57 qPCR. FAM (F57) is shown as a blue line and the Quasar 670 (IC) is shown as a brown line.

To determine if a sample was positive, negative of false negative (inhibited) Ct values of the F57 probe and the Internal Control probe were read. The blue line represented the color of the fluorophore of FAM, the indicator of the probe for F57, and the brown line the color of the fluorophore of Quasar 670, the indicator of the Internal Control probe (IC probe) as seen in figure 2-2. A sample was considered inhibited when the IC did not pass the threshold after 40 cycles, supposed to pass around a Ct value of 32-35, or when it did not form a sigmoidal curve. If the latter one happened a rerun of the sample was performed. Before considering if a sample is positive or negative, it was made sure there was no inhibition of the PCR by looking at the IC curve. A positive sample was recognized by a sigmoidal curve of FAM (F57) that passed the threshold. A negative sample on the other hand showed no sigmoidal curve and stayed below the threshold (see figure 2-2). No cut-off value for the Ct value was used as all the control samples were negative.

For the IC plasmid a standard curve was made to decide which concentration would be the best to use. The goal was to use a concentration with approximately 900 copies/µL which had a Ct value of around 34-35. The following concentrations of IC plasmid were used; 8 x 10^6, 8 x 10^5, 8 x 10^4, 8 x 10^3 and 8 x 10^2 copies/µL. In this run only Quasar 670 was used as there was no detection for F57 needed. In Figure 2-3 the standard curve showed a near linear regression (R^2=0.998). Concluding, the concentration of 8 x 10^2 was used for the IC plasmid as it had a Ct value of 34.

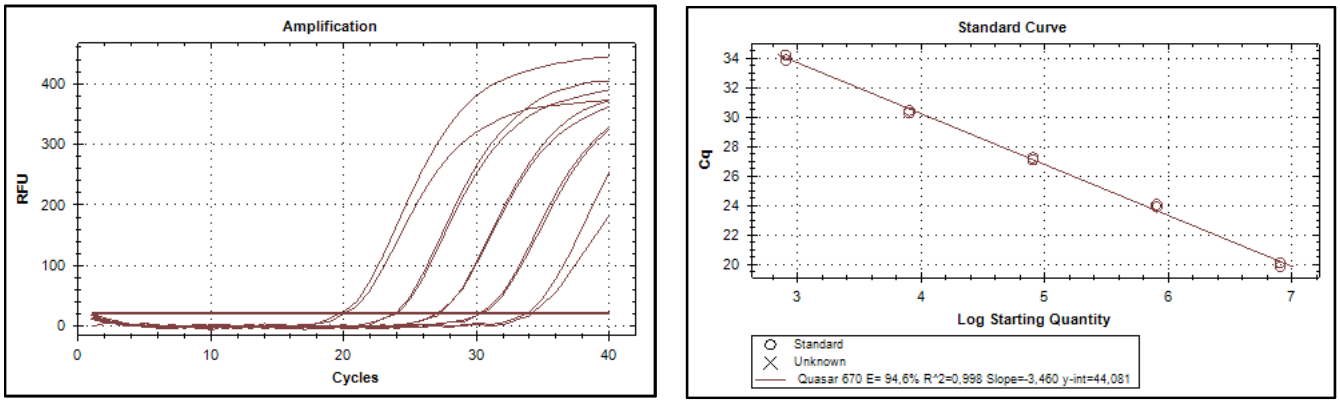

Figure 2-3 Amplification and standard curve for the different concentrations of the IC plasmid.

1.7 *IS*900 conventional PCR

The oligonucleotide primes used for the IS900 conventional PCR have been described by Vary et al. (27). The amplification was performed in a reaction with a total 50 µL containing TopTaq DNA Polymerase Kit (Qiagen, Ontario, Canada), 0,4 µM of each forward and reverse primer and 20 nmol of each dNTP (Invitrogen, Burlington, Ontario, Canada) and 5 µL template. The reactions were performed in a Bio-Rad C1000 Thermocycler (Bio-rad, Ontario, Canada) comprising an initial denaturation step at 95°C for 5:00 min, followed by 34 cycles of 1:00 min at 94°C, annealing of 1:00 min at 54°C and extension at 72°C for 1:00 min. The final extension was performed with 7:00 min at 72°C. In a 1% agarose electrophoresis gel (Bio-Rad, Ontario, Canada) the PCR amplification products were run and visualized with a BioDoc-It Imaging System (UVP, California, USA).

1.8 Statistical Analysis

For each calf, the number of tissue positive culture and Ct value were recorded. Analysis of the probability for the of detecting a positive animal with tissue culture overall and the probability of detecting a positive animal with tissue culture for each location was performed. For the probability overall the numerator were all tissue culture positive animals and the denominator was the number of tissue culture positive animals plus the number of false negative animals (infected animals but not detected with tissue culture). For the probability for each location the numerator were all tissue culture positive animals on that location and the denominator was the number of tissue culture positive animals for that location plus the number of false negative animals (infected animals but not detected with tissue culture on that location). The 95% confidence interval per tissue location and overall was established with the test agreement module of the program Win Episcope 2.0 (University of Edinburgh, United Kingdom).

As disease definition the assumption that all experimentally MAP challenged calves were successfully infected was used. To state a calf as tissue culture positive it needed at least one or more tissues that were culture positive after confirmation with F57 qPCR.

3. Results

F57 qPCR

In total 689 tissues were cultured from 33 animals, 45 tissues were culture positive in 19 animals. The following animals were positive on tissue culture for MAP: C1 (distal jejunum, distal ileum, ileocaecal valve), C2 (duodenal lymph node, ileocaecal valve, ileocaecal lymph node), C3 (distal jejunal lymph node), C5 (hepatic lymph node, duodenum, duodenal lymph node, distal jejunum, spiral colon), C6 (ileocaecal valve), C9 (duodenal lymph node), C12 (retropharyngeal lymph node, cecum), C13 (mid jejunum , distal jejunum), C14 (ileal lymph node, retropharyngeal lymph node), C16 (hepatic lymph node), C17 (cecum), C20 (proximal ileum, mid ileum, distal ileum, ileocaecal valve, ileocaecal lymph node), C21 (proximal ileum, mid ileum, ileocaecal valve), C24 (ileal lymph node), C26 (distal jejunum), C27 (ileocaecal valve), C30 (proximal ileum, ileal lymph node, spiral colon, transverse colon), C31 (tonsils, medial jejunal lymph node, distal jejunum, distal jejunal lymph node, spiral colon, superficial inguinal lymph node) and C36 (superficial inguinal lymphnode) (see table in Appendix 3). Control calf C24 had a positive tissue culture on the ileal lymph node and was included in the probability analysis as infected (table 4-1, Appendix 3).
There were 14 animals tissue culture negative, wherefrom 12 animals experimentally infected with MAP and 2 uninfected control calves. The following calves had negative tissue cultures: C4, C8, C10, C15, C19, C22, C23 (control calf), C25 (control calf), C28, C29, C32, C33, C34 and C35 (see table in Appendix 3). All negative control samples for tissue culture, DNA extraction and PCR were consistently negative for F57 qPCR.

The 2 week infection group had in total 10 positive tissue samples, the 3 month group had 13 positive tissue samples, the 6 month group had 7 positive tissue samples, the 9 month had 6 positive tissue samples, the twelve month had 8 positive tissue samples and the control group had 1 positive tissue sample. In all infection groups both the high and low dose were at least once positive for tissue culture. In both the high and low dose groups there were 9 calves positive for tissue culture, but comparing the amount of positive tissues for the high and low dose you see an difference, respectively 28 and 16 tissue culture positive samples per dose group (table 3-1).

Out of the 21 tissue sample locations 20 locations came up at least once as positive after culture. The caecal lymph node was the only tissue that was negative on tissue culture for all 33 animals. In table 4-1 the number of positive tissue cultures per tissue location out of 33 animals was described, also the probability of detecting a MAP positive animal with at least one positive tissue with 95% confidence interval was included in this table. The organism was most frequently found in the ileocaecal valve and distal jejunum with respectively 6 and 5 positive tissue cultures (table 4-1).

*IS*900 conventional PCR

*IS*900 conventional PCR was done to compare with F57 qPCR. 48 selected positive, negative, control and rerun samples were used. The results were that 41 of the 48 samples were positive. The negative samples were the PCR control, the DNA extraction control and the negative culture control samples, one F57 positive- and one F57 negative tissue samples (respectively, C30 spiral colon and C23 inguinal lymph node).

Table 3-1 Number of positive tissue samples per age- and dose group.

Age Group	Cow number	Dose	Number of Positive Tissue Cultures
2 wk	C16	High	1
	C17	High	1
	C20	High	5
	C19	Low	0
	C21	Low	3
	C22	Low	0
3 month	C1	High	3
	C4	High	0
	C5	High	5
	C2	Low	4
	C3	Low	1
	C7	Low	
6 month	C31	High	6
	C32	High	0
	C34	High	0
	C33	Low	0
	C35	Low	0
	C36	Low	1
9 month	C6	High	1
	C8	High	0
	C10	High	0
	C9	Low	1
	C12	Low	2
	C13	Low	2
12 month	C14	High	2
	C28	High	0
	C30	High	4
	C15	Low	0
	C26	Low	1
	C27	Low	1
	C29	Low	0
Control	C23	N/A	0
	C24	N/A	1
	C25	N/A	0

4. Statistical analysis

Of the 689 cultured tissue from 33 animals, 45 tissues (6.5%) were culture positive and they represented 19 tissue culture positive animals. The probability of detecting a positive animal in this study was 0.61, as 19 out of the 31 calves were positive for at least one tissue sample. Control calf 24 (C24) was included in the probability analysis as infected (table 4-1, Appendix 3).

The probability to detect a tissue culture positive animal per location is shown in figure 4-1 and table 4-1. In general the probability to find a positive tissue culture per location was very low, as most tissue locations had a probability to be positive of less than 0.10 (figure 4-1). The confidence intervals are not significant because zero is included, except for the ileocaecal valve and distal jejunum, and all show overlap (table 4-1). The ileocaecal valve had the highest probability to be positive with 0.19, which is low. The power might be low for the tissue locations and the confidence intervals can change in favor with the results of run 2.

To find a good combination for tissue sampling the tissues samples were organized according to their origin in three areas: gastrointestinal tract tissues (GIT – Tonsil, duodenum, medial jejunum, distal jejunum, proximal ileum, medial ileum, distal ileum, ileocaecal valve, caecum, spiral colon, transverse colon and rectum), corresponding lymph nodes of the GI tract tissues (GILN – duodenal lymph node, medial jejunal lymph node, distal jejunal lymph node, mid ileum lymph node, ileocaecal lymph node, caecal lymph node) and other lymph nodes, not associated with the GI tract (OLN - lymph node retropharyngealis, hepatic lymph node and inguinal lymph node). Based on the paper of Pavlik et al, 2000 (34).
MAP was detected in the gastrointestinal tract tissue of 14 calves (45,2%), MAP was detected in GI-associated lymph nodes were detected of 8 calves (25,8%) and MAP was detected in the other lymph nodes in 4 calves (12,9%). Of the 31 calves challenged calves, 8 calves (25,8%) were detected positive with only the GI-tract tissue. The second most frequent combination was the GI-tract tissue combined with their corresponding lymph nodes as 5 calves were detected tissue culture positive (16,1%). There was no detection of only other lymph nodes (OLN) (table 4-2). Out of the 19 calves with culture positive tissues 14 were detected with at

least 1 GI tract positive tissue sample (73,7%), adding the GI tract associated lymph nodes to compare it with the results of Pavlik et al. 15 out of the 31 animals were detected (48,9%) (34).

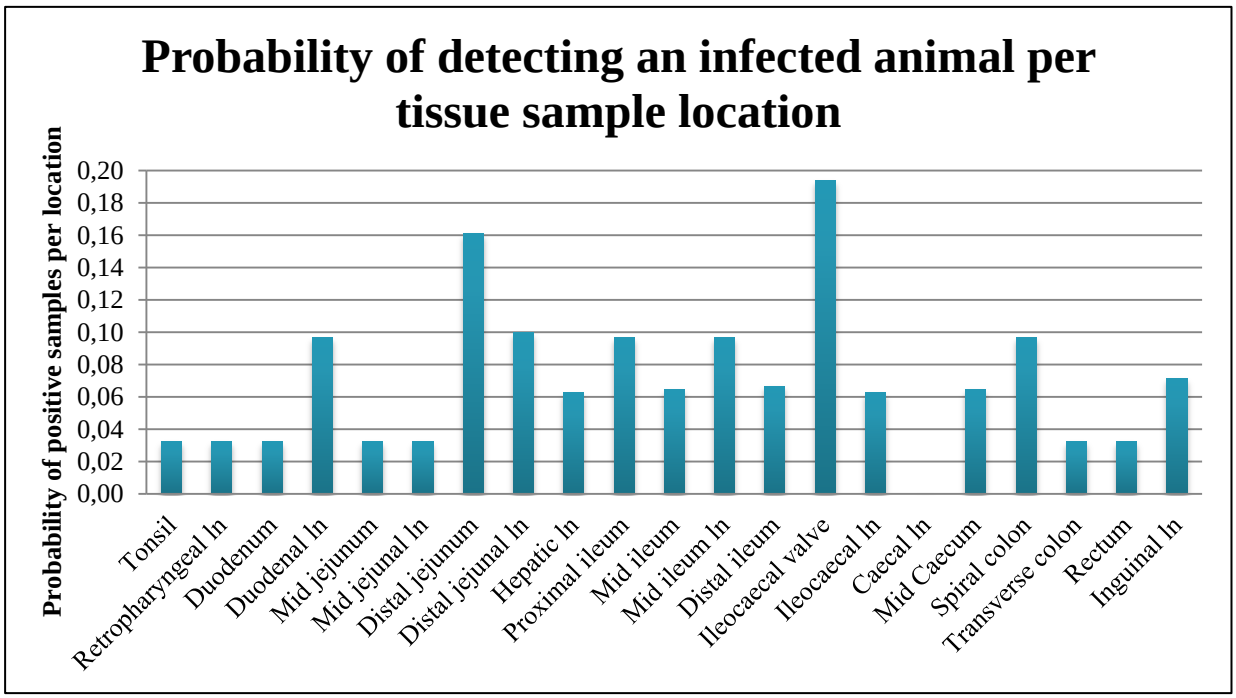

Figure 4-1 Probability to detect a positive animal per location of tissue culture positive samples, following experimental infection with MAP. Tissue sites are from proximal to distal. The bar height represents the probability per location.

Table 4-1 Probability of detecting MAP positive animals per location with at least one culture positive tissue.

Tissue location	Number of positive tissues per location	Probability	95% CI
Tonsil	1	0.032	0.000-0.095
Retropharyngeal ln	1	0.032	0.000-0.095
Hepatic ln	2	0.063	0.000-0.151
Duodenal ln	3	0.097	0.000-0.201
Mid jejunal ln	1	0.032	0.000-0.095
Distal jejunal ln	3	0.100	0.000-0.207
Mid ileum ln	3	0.097	0.000-0.201
Ceacal ln	0	0.000	0.000-0.000
Ileocaecal ln	2	0.063	0.000-0.151
Duodenum	1	0.032	0.000-0.095
Mid jejunum	1	0.032	0.000-0.095
Distal jejunum	5	0.161	0.032-0.291
Proximal ileum	3	0.097	0.000-0.201
Mid ileum	2	0.065	0.000-0.151
Distal ileum	2	0.067	0.000-0.156
Ileocaecal valve	6	0.193	0.054-0.333
Mid Caecum	2	0.065	0.000-0.151
Spiral colon	3	0.097	0.000-0.201
Transverse colon	1	0.032	0.000-0.095
Rectum	1	0.032	0.000-0.095
Inguinal ln	2	0.071	0.000-0.167

Table 4-2 Distribution of MAP in 3 different organ groups after tissue culture with their possible combinations and the percentage of total positive animals per combination[a]

GIT	GILN	OLN	Number of calves positive for tissue culture	Percentage
+	-	-	8	25,8%
-	+	-	2	6,5%
-	-	+	3	9,7%
+	-	+	0	0,0%
+	+	-	5	16,1%
-	+	+	0	0,0%
+	+	+	1	3,2%
-	-	-	12	38,7%
			31	100%

[a] **Explanations: GIT (gastrointestinal tract): tonsil, duodenum, medial jejunum, distal jejunum, proximal ileum, medial ileum, distal ileum, ileocaecal valve, caecum, spiral colon, transverse colon and rectum; GILN (corresponding lymphnodes of the gastrointestinal tract): duodenal lymph node, medial jejunal lymph node, distal jejunal lymph node, mid ileum lymph node, ileocaecal lymphnode, caecal lymph node; OLN (other lymph nodes): lymph node retropharyngealis, hepatic lymph node and inguinal lymph node**

5. Discussion

Tissue culture is assumed to be the gold standard for detection of MAP (6). In this study, 61.3% of the 31 experimentally with MAP infected calves were tissue culture positive. This low number will lead to underestimation of test agreements as most of the calculations are based upon tissue culture as gold standard. It has to be said that all these calves where in a subclinical stage and the results for tissue culture can change when concerning clinical cases, although detection in the subclinical cases with tissue culture is assumed to be possible (22,25).

The presence of MAP in other tissue than GI tract tissue and GI-tract associated lymph nodes (hepatic lymphnode, retropharyngeal lymph node and inguinal lymphnode) suggest that there is dissemination throughout the body. Expectations need to be adjusted as our animals were in a subclinical stage and dissemination throughout the body is expected to occur in the clinical stage (21). This finding is confirm literature where similar results have been described (10,34,35).

Detection of an infected animal based upon one tissue location was very low. This study found maximal 19.3% of the animals positive for the culture of one tissue location, the ileocaecal valve. Other authors had the highest detection of an infected animal based upon one tissue sample with tissue samples from the jejunum, ileum and ileocaecal valve as these were the most sensitive locations with up to 100% detection for the distal jejunum and proximal ileum (10,24,34-36). In our study the distal jejunum, distal jejunal lymph node and ileocaecal valve had the highest probability of detecting an infected calf with one tissue location. In general the probability to detect an infected animal based on one tissue sample was very low and not useful for diagnostics.

Most of the animals were detected with a GI-tract tissue, this makes the GI-tract an important area for tissue culture. Results up to 83,0% for the detection of assumed positive cattle for tissue culture of the GI-tract tissue with associated lymph nodes (19 tissue samples) were found by previous authors (34). Tissue culture of the GI-tract was recorded in more articles as more sensitive, as MAP enters the animal by the M-cells in the intestines, this makes it more obvious that experimentally infected animals will show positive tissue cultures over here (10,20,21,34-36). The same is true for the pathogenesis related to the associated lymph nodes.

The lower detection of infected animals for one tissue sample or all 21 tissue locations than in other studies (10,33-36) might be due to the use of F57 qPCR. F57 qPCR has a lower sensitivity for MAP than *IS*900 as conventional *IS*900 PCR is able to detect lower concentrations of MAP, due to multiple copies of *IS*900 (14 to 20) on the MAP genome. The choice to use F57 qPCR instead of conventional *IS*900 PCR for confirmation was that it has a 100% specificity whereas *IS*900 similar sequences were found in other bacteria (29) . The results of the 48 randomly chosen positive and negative tissue sample outcomes of F57 qPCR and controls showed that the detection of tissues with conventional *IS*900 PCR is higher, but confirmation that it was MAP could not be proven directly.

A positive tissue culture of the inguinal lymph node was found in 2 of the 31 infected animals. In other studies they cultured also positive inguinal lymph nodes from (lactating) cows and higher percentages of positive inguinal lymph nodes (8,10). In this study the samples were from steers, with generally a lower blood flow through to the inguinal lymph node, and therefore it was unexpected to find positive tissue cultures for the inguinal lymph node. However, presence of MAP in this particular location forces us to consider a dissemination of MAP throughout the body even in the early stages after infection. With these

results, dissemination in non-lactating heifers of MAP to the inguinal lymph node has to be considered.

As disease definition the assumption that all experimentally MAP infected calves were successfully infected was used, but it can be discussed if this was really the case as animals may have been able to clear the infection. For tissue culture there is no gold standard, as it is the preferred test itself, to work with probability analysis the assumption that all inoculated animals were truly infected was used. In this trial however, most animals negative for tissue culture were shedding or had positive serum ELISA results. There is thus a reason to believe that more calves were successfully infected than observed with tissue culture alone. Moreover, C24, a control calf, was included as infected in the statistical analysis as he had a positive tissue culture for the ileac lymph node. The reason why C24 was positive is not clear, as it can be a truly infected calf, a false-positive or the other control calves can be false negative. To prevent lowering of the specificity, in case of sensitivity analysis, C24 was considered infected.

The results suggest that to detect all the positive animals extensive tissue sampling is needed in subclinical calves up to 17 months of age. Tissue sampling based on one location will lead to underestimation of the number of truly infected animals. Other authors came to the same conclusion that in order to detect subclinical animals tissue culture from multiple locations is needed (10,21,34,35). Tissue culture is not useful as individual or screening diagnostic test on-farm as for the (extensive) collection of tissue samples culling of the animal is required. This test can be used in research, test agreement studies and prevalence studies with slaughterhouse material.

6. Conclusion

Tissue culture is not a preferred gold standard based on these results. If used as a gold standard test agreement may be underestimated. Less subclinical infected animals were detected with tissue culture as expected with the information of previously published papers, this was the case for all tissue samples together as well as for the separate tissue samples. Especially the chance to detect an infected animal with one tissue sample was disappointing. With the GI-tract combination of samples most of the infected animals positive on tissue culture were detected, but not all of them. Tissue samples of more organs are necessary to detect all infected animals.

Based on the findings of MAP in multiple tissue locations, dissemination of MAP over multiple organs seems to happen sooner than expected.

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Appendix

Appendix 1

Protocol for tissue culture

- Add 2g of a tissue sample to a stomacher bag, add 5 mL of ½ BHI + 0.6% HPC. Stomacher until homogenized.
- Add the contents of the stomacher bag to a 50 mL tube containing 20 mL of ½ BHI + 0,6% HPC
- Incubate for 3 hours at 37°C
- Centrifuge tubes at 1700g for 20 min.
- Remove the supernatant
- Resuspend the pellet in 1 mL Para-JEM® antibiotic solution (TREK Diagnostic Systems, Ohio, USA)
- Incubate tubes overnight at 37°C
- Add contents to Para-JEM® bottle containing 1 mL EYS, 1 mL GS, 0.5 mL AS and 0.05 mL Para-JEM® Blue (Trek Diagnostic Systems, Ohio, USA)
- Incubate 48 days at 37°C

Appendix 2

Protocol for DNA extraction after tissue culture of MAP in Para-Jem TREK ESP® bottles:

1. Vortex the Para-Jem bottle for 10 seconds
2. Extract from the Para-Jem bottle 2 x 600 µL culture broth and put this in a 1.5 mL Eppendorf cup
3. Pipet 200 µL out of the Eppendorf cup and add this in another Eppendorf cup, containing 800 µL of 100% Ethanol. Centrifuge the Eppendorf afterward for 9 minutes at 7500 rpm.
4. Resuspend the supernatant and dilute the pellet with 900 µL Distilled PBS (Gibco). Centrifuge Eppendorf again for 9 minutes at 7500 rpm.
5. Do step 4 again.
6. Resuspend the supernatant and dilute the pellet this time in 100 µL of UltraPure Distilled Water (Gibco)
7. Put the Eppendorf for 30 minutes in a hot water bath of ~ 99°C
8. Centrifuge the Eppendorf cup for 2:30 minutes at 7500 rpm after cooking.
9. Remove supernatant, lysate, and transfer this to a new eppendorf cup. Store it at -80°C.

Appendix 3

Data set of tissue culture on tissue of different locations as explained in material and methods. The Ct value is included as + with the value behind it. When there was no positive tissue culture it is included as -. See next two pages for the data.

C19	C17	C16	C15	C14	C13	C12	C10	C9	C8	C6	C5	C4	C3	C2	C1	Cow number
-	-	-	-	-	-	+36	-	-	-	-	-	-	-	-	-	Retropharyngeal ln
-	-	+37	-	-	-	-	-	-	-	-	+38	-	-	-	-	Hepatic ln
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Tonsil
-	-	-	-	-	-	-	-	-	-	-	+35	-	-	-	-	Duodenum
-	-	-	-	-	-	-	-	+39	-	-	+34	-	-	+33	-	Duodenal ln
-	-	-	-	-	+39	-	-	-	-	-	-	-	-	-	-	Mid jejunum
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Mid jejunal ln
-	-	-	-	-	+38	-	-	-	-	-	+36	-	-	-	+34	Distal jejunum
-	-	-	-	-	-	-	-	-	-	-	-	-	+37	+35	-	Distal jejunal ln
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Proximal ileum
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Medial ileum
-	-	-	-	-	-	-	-	-	-	-	-		-	-	+33	Distal ileum
-	-	-	-	+34	-	-	-	-	-	-	-	-	-	-	-	Ileal ln
-	-	-	-	-	-	-	-	-	-	+34	-	-	-	+36	+30	Ileocaecal valve
-	-	-	-	-	-	-	-	-	-	-	-	-	-	+39	-	Ileocaecal ln
-	+34	-	-	-	-	+37	-	-	-	-	-	-	-	-	-	Caecum
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Caecal ln
-	-	-	-	-	-	-	-	-	-	-	+35	-	-	-	-	Spiral Colon
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Transverse
-	-	-	-	+38	-	-	-	-	-	-	-	-	-	-	-	Rectum
-	-	-	-	-	-		-	-	-	-	-	-	-			Inguinal ln
0	1	1	0	2	2	2	0	1	0	1	5	0	1	4	3	Total number of positive tissue samples

Cow number	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36	Total
Retropharyngeal lm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Hepatic ln	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Tonsil	-	-	-	-	-	-	-	-	-	-	-	+36	-	-	-	-	-	1
Duodenum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Duodenal ln	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
Mid jejunum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Mid jejunal ln	-	-	-	-	-	-	-	-	-	-	-	+36	-	-	-	-	-	1
Distal jejunum	-	-	-	-	-	-	+37	-	-	-	-	+31	-	-	-	-	-	5
Distal jejunal ln	-	-	-	-	-	-	-	-	-	-	-	+35	-	-	-	-	-	3
Proximal ileum	+34	+33	-	-	-	-	-	-	-	-	+33	-	-	-	-	-	-	3
Medial ileum	+31	+35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Distal ileum	+34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Ileal ln	-	-	-	-	+35	-	-	-	-	-	+34	-	-	-	-	-	-	3
Ileocaecal valve	+29	+35	-	-	-	-	-	+37	-	-	-	-	-	-	-	-	-	6
Ileocaecal ln	+36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Caecum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Caecal ln	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Spiral Colon	-	-	-	-	-	-	-	-	-	-	+37	+37	-	-	-	-	-	3
Transverse colon	-	-	-	-	-	-	-	-	-	-	+36	-	-	-	-	-	-	1
Rectum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Inguinal ln	-	-	-	-	-	-	-	-	-	-	-	+36	-	-	-	-	+37	2
Total number of positive tissue samples	5	3	0	0	1	0	1	1	0	0	4	6	0	0	0	0	1	45

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