Severity of macroscopic lesions in 56 calves experimentally infected with *Mycobacterium avium* spp. *paratuberculosis* in relation to age at infection and infectiondose

Name

Studentnumber : 0353175 Rienske Mortier

Date: October 2012 – January 2013 Susanne Eisenberg

Content

Summary 3

Introduction 4

Materials and methods 6

Experimental design 6

Animals 6

Infection 6

Necropsies 7

Macroscopical evaluation 8

Histology 9

Microscopical evaluation 9

Tissue culture 10

Fecal culture and PCR 10

ELISA on serum 10

Statistical analysis 11

Results 12

Macroscopic evaluation 12

Microscopic evaluation 14

Tests 17

Statistical analysis 19

Conclusion 22

Acknowledgements 23

Appendix 1 26

Appendix 2 28

Appendix 3. 31

Summary

Johne's disease is a widely spread chronic wasting disease among cattle. Johne's disease or paratuberculosis is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and cows get infected at a young age. However, symptoms such as diarrhea and decreased milk production are usually developed after two to ten years. These symptoms are caused by a chronic granulomatous enteritis with regional lymphangitis and lymphadenitis. The aim of this study was to investigate the severity of macroscopic pathological changes in experimentally infected calves in relation to age at infection and infection dose. Therefore 56 calves were given an inoculum with a high dose or a low dose of MAP at either two weeks, 3, 6, 9 or 12 months of age. After 17 months necropsies on all the animals were performed. Macroscopic changes in the calves were ranked in different categories and a statistical analysis was performed on the outcome. Furthermore a large number of samples was collected and different screening tests were performed to confirm infection. Our results show a significant relation between infection dose and severity of macroscopical changes. Also a trend was seen in the relation between age of infection and severity of macroscopical changes.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP), an acid-fast bacterium, is the causative agent of Johne's disease in ruminants. The disease is widely spread among dairy cattle in the world. In 2003 the prevalence among dairy herds in Alberta, Canada, where this study takes place, was estimated to be 28-57 % [1].

Since no treatment or vaccination is available in North-America, disease control programs are focused on prevention of infection of susceptible animals. A calf or heifer gets infected with MAP through the fecal-oral route. Due to resistance at a later age, only calves up to 6 months get infected and develop the disease after an incubation period of two to ten years [2,3]. This resistance is the foundation for separation of calves and cows in numerous control programs [4].

Johne's disease develops over four stages. At the first stage (silent infection) the calf gets infected but clinical signs of Johne's disease are not yet observed. At stage two of the disease (carrier stage) the animal becomes a carrier and MAP can be spread into the environment through the feces of the infected animal, but critical concentrations of MAP to test positive in fecal culture may not be reached [5]. A positive test in this stage confirms the disease, although false negative test results may appear [6]. During the carrier stage, the animal still shows no clinical signs of Johne's disease, however, other signs might be visible, such as reduced milk production and fertility rates [6]. At stage three (clinical stage) the clinical signs are present as the animal develops diarrhea, loss of body weight and decreased milk production. At the clinical stage, large amounts of MAP can be found in the animal and the disease can be confirmed using an ELISA test or fecal culture. In the fourth and final stage of the disease (advanced clinical stage) the animal becomes emaciated, weak and has chronic diarrhea. Intermandibular edema due to hypoproteinemia is very characteristic for this stage. The hypoproteinemia is caused by the protein-losing enteropathy. As there is no cure for Johne's disease, the animal eventually dies of cachexia or has to be culled [5,6].

The pathological changes of Johne's disease are a chronic granulomatous enteritis with regional lymphangitis and lymphadenitis [7]. A few days after the animal has ingested the bacteria, it can be found in the tonsils and retropharyngeal lymph nodes [3]. At three months after infection the bacteria has spread to the intestines and can be found in the Peyer's patches and the mesenteric lymph nodes. The lymphatics in the mesenteric serosa are dilated and the mesenteric lymph nodes are enlarged [8]. At six months after infection the bacteria have caused extensive granulomatous lesions in the intestinal mucosa, especially in the ileum and it stops

abruptly at the ileocaecal valve [3,7]. At necropsy this can be seen macroscopically as thickening and corrugation of the ileal mucosa. Microscopical changes consist of granulomas in the intestinal villi and ileal and ileocaecal lymph nodes. Also lymphocytes and giant cells can be found within the lesions. Using a Ziehl-Neelsen stain, large numbers of acid-fast bacilli can be demonstrated inside macrophages [9]. The macroscopical changes are most visible in a clinical case of Johne's disease, which only develops after the incubation period of a few years. Since the necropsies in this project are done when the calves are 17 months old, macroscopical pathological changes might be visible in only a few cases. Microscopical changes might be seen in more cases and the bacteria can be shown using the Ziehl-Neelsen staining.

The MAP infection mechanism is not yet fully elucidated. Especially, the relation between age of the calf and dose of MAP needed to get infected is unknown. It has been described that young animals are at higher risk to get infected with a fixed dose of MAP than older animals [2,10]. However, this is based on multiple studies with a low number of animals and different doses and administration routes of MAP are used in the different studies. Therefore the University of Calgary, Canada, has designed a follow-up study to test age and dose-dependent susceptibility to MAP infection in dairy calves. Within this study, this project aims to describe the macroscopic and microscopic lesions observed during necropsy and relate this to the obtained tissue culture results.

Hypothesis

The hypothesis for the outcome of this study is that calves get infected at a young age and therefore more pathological changes will be seen in the calves infected at an early age than the calves infected at a later age. A second hypothesis is that a difference in the severity of the disease will be seen between calves infected with a low infection dose and a high infection dose. To proof these hypotheses two questions are formulated;

Do calves infected at a young age show more macroscopic changes compared to calves infected at an older age?

Do calves infected with a high dose show more macroscopic changes compared to calves infected with a low dose?

Materials and methods

Experimental design

This research project is part of a larger project involving 56 Holstein-Friesian steer calves experimentally infected with MAP at different ages and with two different doses. At the age of 17 months the calves are euthanized and a necropsy is performed on each of them to evaluate macroscopical pathological changes. At this necropsy tissue samples are collected for MAP culture and a selection of these samples is used for histology to evaluate microscopical pathological changes.

Animals

The calves used for this experiment are collected at birth from selected farms with a within herd seroprevalence of less than 5%. Directly after birth the calves are separated from the dam without them having been in contact with the dam or feces. The animals are then brought to the housing facility set up for this experiment. In this facility the calves are housed in loose housing in individual pens (6 by 8 feet) with their own manger for food and a waterbucket. In front of each pen is a changing area with a set of boots and coveralls, bootdip and gloves to prevent cross-contamination.

Within the first 12 hours after birth the calves are fed 6 liters of irradiated colostrum. After the colostrums, feeding with High Performance Calf Milk Replacer (Grober Nutrition, USA) is started and at two weeks of age calf starter (FeedRite, Canada) and high quality hay are added to the diet. At 7 weeks of age the calves are weaned, dehorned and surgically castrated. At 3-4 months of age the calves are moved to larger pens (6 by 14 feet).

Because the housing facility is not large enough to conduct the experiment with 56 calves at one time, the calves are divided into two groups. The first group consists of 33 calves and this experiment was executed in the period between January 2010 and August 2011. The second group of 23 calves was in trial between June 2011 and December 2012. In both groups all of the different ages and doses of infection as well as control animals are represented.

Infection

The calves are infected with MAP at two weeks, 3, 6, 9 or 12 months of age. Each cluster of age at time of infection consists of ten animals and a control group of non-infected animals consists of 6 animals. Within a cluster, half of the animals are infected with a low bacterial inoculum dose of MAP ($5 * 10^7$ Colony Forming Units (CFU)) on two consecutive days and

half of them with a high dose (5 * 10^9 CFU) on two consecutive days. These doses correspond with environmental contamination of high and low shedding MAP infected animals/ animals with Johne's disease.

The inoculum was obtained by using a MAP isolate from a clinical case. After analysis it appeared to have a similar genotype as the K-10 strain which is recommended for challenge models by Hines et al [11]. Our isolate was grown in BD Difco Middlebrook 7H9 broth (BD) supplemented with Mycobactin J (Allied Monitor, Missouri, USA), 2% glycerol and Middlebrook OADC Growth Supplement (Sigma Aldrich, St Louis, Missouri, USA) to mid-log growth phase. The inoculum was tested for contamination with Gram-staining, blood agar and RT-PCR. After testing the inoculum was vortexed with glass beads to eliminate clumps and was checked for viability with fluorescent Live/Dead® BacLight™ Bacterial Viability kit (Invitrogen, Burlington, ON, Canada). To acquire the right dosage the inoculum was quantified using the 'pelleted wet weight method' recommended by Hines et al [11]. The solution for administration to the calves was established by diluting the inoculum with culture broth. Because the natural route of infection is the fecal-oral route, the inoculum was administered to the calves by a syringe on the back of their tongue.

Necropsies

All of the calves, including the control animals, were euthanized at 17 months of age. Animals were secured in an hydraulic squeeze chute and a 120 ml intravenous dose of Euthanyl Forte® (Bimeda-MTC, Ontario, Canada) was administered by a veterinarian. Immediately after the euthanisation the animal was desanguinated and the necropsy was performed. The animal was placed lying on its left side and access to the organs was gained by abducting the right hindleg and removing the skin, abdominal muscles and peritoneum of the right abdomninal wall. Acces to the heart and lungs was gained by removing the ribs on the right side of the animal. After the duodenum and rectum were tied off and the mesenterium cut through at its base the intestines were taken out of the carcass and put on another table. Pathologic changes in the animals were assessed by a veterinary pathologist (Dr. Jan Bystrom, University of Calgary, Department of Veterinary Medicine, Calgary, Canada) with special attention to the digestive tract. During the necropsy samples were taken from the following sites to use for tissue culture; liver, hepatic lymph node, kidney, spleen, inguinal lymph node, retropharyngeal lymph nodes, tonsils, duodenum, duodenal lymph nodes, mid jejunum, mid jejunal lymph node, distal jejunum, distal jejunal lymph node, proximal ileum, mid ileum, distal ileum, ileal lymph node, ileocaecal valve, ileocaecal lymph

nodes, caecum, caecal lymph nodes, spiral colon, transverse colon and rectum. All of the samples from the intestinal lymph nodes were taken before opening the intestines to prevent cross contamination. Also a different set of scissors and tweezers and clean gloves was used for taking each separate sample.

Macroscopical evaluation

All of the calves were evaluated for macroscopical changes at the necropsy performed at 17 months of age. Together with veterinary pathologist Dr. Jan Bystrom we looked at pathological changes in the major organs; heart, lungs, liver, spleen and kidneys as well as the digestive tract. Possible Johne's disease related pathological changes would be endocardial or aortic calcifications and microgranulomas in the liver [12]. But the most important pathological changes associated with Johne's disease are seen in the digestive tract. Therefore we looked at the intestines with special attention.

Johne's disease causes a chronic granulomatous enteritis that is mainly focused in the ileum[8,13]. Also an intestinal lymphadenitis and lymphangitis develops in the infected animals[7,12]. Based on these described pathological changes, we developed six different categories to compare the pathological changes between the age groups. The categories represent increasing severity for the macroscopical changes seen in the digestive tract of the calves. Each calf was assigned to one of these categories to score the degree of pathological changes.

Categories for macroscopical changes:

0. No macroscopical changes
1. One enlarged or edematous lymph node of the small intestine or liver
2. Multiple enlarged and edematous lymph nodes of the small intestine and/or hyperemia of the ileocaecal valve
3. Enlarged lymph node(s) of the small intestine and/or mild thickening of one part of the ileal mucosa (proximal, mid or distal ileum)
4. Enlarged lymph node(s) of the small intestine and moderate thickening of one part of the ileal mucosa or thickening of a greater part of the ileal mucosa
5. Enlarged lymph node(s) of the small intestine and severe thickening and corrugation of the ileal mucosa

Enlargement of the lymph nodes was evaluated after the intestines were taken out of the carcass, so that a clear view of the lymph nodes and their proportion and natural anatomy within the digestive tract was given. The lymph nodes were cut through to evaluate if they were edematous. The lymph nodes that were assessed for pathological changes were the hepatic lymph node, jejunal lymph node, ileal lymph node and ileocaecal lymph node.

A part from the proximal ileum, the mid ileum and the distal ileum was evaluated for thickening and corrugation. The ileocaecal valve was evaluated as well, because it could show hyperemia as a sign of Johne's disease related inflammation.

Histology

From each calf a cut section of the distal ileum, ileal lymph node, ileocaecal valve and ileocaecal lymph node were evaluated for microscopical changes related to Johne's disease. A piece of about 1 cm^2 from each of these sites was taken during the necropsy and put into a cassette. To fix the tissue the cassette was immediately put into 10% neutral buffered formalin. The cassettes in formalin were send to Prairie Diagnostic Services, Saskatoon, Canada to be embedded in paraffin wax and cut into 4 μm sections. The slices were put onto microscopic glass slides and stained with Hematoxylin-Eosin and Ziehl-Neelsen staining.

Microscopical evaluation

Microscopical evaluation was done for the 33 calves in the first trial only, the slides of the second trial were still being evaluated and the results were not available before the end of this project. However the microscopical evaluation of one calf out of the second trial, cow 89, was available as the slides were evaluated by Dr. Jan Bystrom shortly after the necropsy of this calf. The slides of the first trial were evaluated for pathological changes as well as for the presence of acid-fast bacteria by another veterinary pathologist, Dr. Oscar Illanes (Ross University School of Veterinary Medicine, St. Kitts, USA). For the evaluation he used six categories to score the severity of the microscopical pathological changes proposed by Gonzales et al [9]. Each calf was assigned to one of these categories.

Categories for microscopical changes [9]:

0. No microscopical changes
1. Focal lesions; small granulomas in the ileal or ileocaecal lymph node
2. Multifocal lesions; small granulomas or scattered giant cells in the intestinal villi and lymph node

3. Diffuse multibacillary lesions; severe granulomatous enteritis affecting different intestinal locations and lymph nodes with macrophages containing a large number of acid-fast bacteria.
4. Diffuse lymphocytic lesions; inflammation with mainly lymphocytes and a few macrophages or giant cells with a few or no acid-fast bacteria.
5. Diffuse intermediate form; infiltration of a large amount of lymphocytes and macrophages with many acid-fast bacteria.

The slides of the ileal lymph node and the ileocaecal lymph node were evaluated for the type of inflammatory cells and the severity of the inflammation throughout the lymph node. In the slides of the ileum and the ileocaecal valve, we looked at the lamina propria as well as the gut associated lymphoid tissue (GALT) to see if the intestinal villi were still intact and what type of inflammation was going on. The type of inflammatory cells was then determined. A Ziehl-Neelsen stained slide of each sample was evaluated for the presence and amount of acid-fast bacteria.

Tissue culture

Tissue culture was done using the samples taken at necropsy. The samples were homogenized decontaminated and incubated for 48 days at 37 °C. After incubation MAP specific Polymerase Chain Reaction (PCR) was used to confirm presence of MAP. The results of the first trial were available for the evaluation performed in this report. Tissue culture results of the second trial however were still in progress and could not be finished before the end of this project.

Fecal culture and PCR

Fecal samples of each calf were collected routinely during the course of the trial. They were taken every 12 weeks before infection, weekly in the first month after infection and monthly further onwards until necropsy. Every sample was decontaminated and cultured for 48 days after which the TREK culture system named a culture positive or negative. From each sample DNA was extracted after culture. The DNA was used for MAP specific PCR to confirm and quantify MAP bacteria in the sample. The results from the fecal culture and the PCR were available for the evaluation performed in this report.

ELISA on serum

Serum samples were collected at the same times as fecal samples (see above). Antibody ELISA was performed on all of the samples using the Pourquier ELISA™ (Institut Pourquier,

Montpellier, France). The results of this test were available for the evaluation performed in this report.

Statistical analysis

To test the two hypotheses if more macroscopical changes occur in animals infected at a young age and in animals infected with a high dose of MAP we used the ordinal logistic regression method. This choice was based on the fact that our variable, the severity of macroscopical changes, is an ordinal categorical variable. For the first analysis, testing the dose effect on the severity score of macroscopic lesions, we have considered age at infection (2 weeks, 3, 6, 9 and 12 months) and the trial (2010 vs. 2011) as potential modifier or confounder. The model applied is:

$$\log \frac{p}{1-p} = \beta_0 + \beta_D + \beta_A + \beta_T + \beta_{AT} + \beta_{DA} + \beta_{DT} + \beta_{DAT}$$

D =dose, *A* =age, *T* =trial

The method applied was a backward selection, we assessed modification by the significance of the *p*-value ($p < 0.05$). The same model and method were applied for the second analysis. In this analysis for testing the effect of age at infection on the severity score of macroscopic lesions, we have considered the dose of infection (low or high) and the trial as potential modifier or confounder.

Because the used variable is ordinal categorical, we could use cut-offs for the model. Since we have six categories for the severity of macroscopic lesions, five cut-offs were established. Cut-off >0, meaning severity categories 1, 2, 3, 4 and 5. Cut-off>1, meaning categories 2, 3, 4 and 5. Cut-off>2, meaning categories 3, 4 and 5. Cut-off>3, meaning categories 4 and 5. Cut-off>4, meaning category 5. For each cut-off the modification and confounding was assessed separately. The outcome of the function is the odds of an animal in a specific age and infection group and trial having macroscopical lesions above the cut-off point compared to a lower cut-off. The generated function was used to calculate the odds for that specific cut-off.

Results

Macroscopic evaluation

No macroscopical changes were found in the control animals. In the infected calves no Johne's disease related pathological changes were found in any of the organs other than the intestines, except for one calf. Cow 89, infected with a high dose of MAP at two weeks of age, showed a mild hydroperitoneum and diffuse edema of the mesenteric serosa. The complete pathological report of cow 89 can be found in Appendix 2.

The severity of the macroscopical pathological changes in the intestines of each calf seen at necropsy was scored using the six categories described earlier in this report. A table with the combined results of the macroscopic and microscopic evaluation of all the calves can be found in Appendix 3.

The results of the macroscopical evaluation of the infected animals are summarized in tables 1 and 2. Table 1 contains the results of the calves infected with a low dose of MAP and table 2 contains the results of the calves infected with a high dose of MAP.

Age at infection	Cat. 0	Cat. 1	Cat. 2	Cat. 3	Cat. 4	Cat. 5	Total
2 weeks	1	1	0	3	0	0	5
3 months	1	1	1	2	0	0	5
6 months	3	1	0	1	0	0	5
9 months	4	1	0	0	0	0	5
12 months	2	0	0	2	1	0	5
Total	11	4	1	8	1	0	25

Table 1: Number of low dose infected calves in each category for the severity of macroscopical changes.

Age at infection	Cat. 0	Cat. 1	Cat. 2	Cat. 3	Cat. 4	Cat. 5	Total
2 weeks	0	0	1	1	1	2	5
3 months	1	0	0	4	0	0	5
6 months	2	1	0	2	0	0	5
9 months	3	0	0	2	0	0	5
12 months	1	0	0	3	1	0	5
Total	7	1	1	12	2	2	25

Table 2: Number of high dose infected calves in each category for the severity of macroscopical changes.

In 18 of the infected calves, comprised of 11 low dose calves and seven high dose calves, no macroscopical lesions were found, those animals represent category 0. In five animals, comprised of four low dose and one high dose calf, one lymph node of either the liver or the small intestine was enlarged, these animals represent category 1. In two animals, comprised of one low dose and one high dose calf, more than one lymph node was enlarged, these animals represent category 2. In 20 animals, comprised of eight low dose calves and 12 high dose calves, macroscopical changes were seen in the lymph nodes as well as in the ileum. One or more lymph nodes were enlarged and one part of the ileum was mildly thickened. In three animals, comprised of one low dose and two high dose calves, one or more lymph nodes were enlarged and the ileum was moderately thickened or mildly thickened over a larger part. These animals represent category 4. In two animals, both high dose calves of the two week infection group, the intestinal lymph nodes were enlarged and the ileum was severely thickened and corrugated (see figure 1). These animals represent category 5.

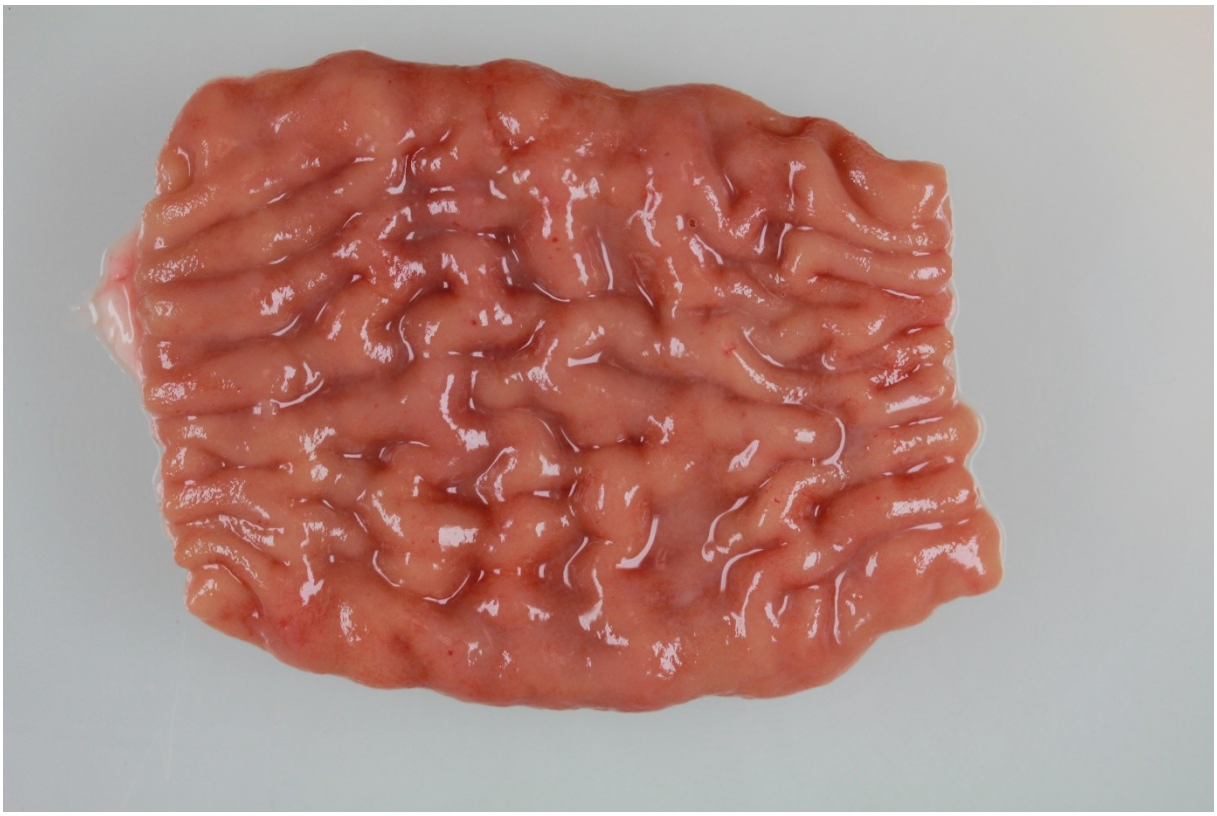

Figure 1: Severely thickened and corrugated proximal ileum of calf 75, infected with a high dose of MAP at two weeks of age.

Microscopic evaluation

The 33 calves of the first trial and cow 89 of the second trial were evaluated for microscopical changes using the six categories described earlier. Two of the three control animals in trial 1 showed no microscopical pathological changes. In one of the three control animals however a mild pathological change was seen. Cow 24 showed a few small granulomas in the ileal lymph node, therefore the calf was scored with category 1 of microscopical lesions. However no acid-fast bacteria were found with the Ziehl-Neelsen staining.

The results of the microscopical evaluation of the infected calves of trial 1 are shown in table 3 and 4. Table 3 shows the results of the calves infected with a low dose of MAP and table 4 shows the results of the high dose infected calves. The table also shows if any of the samples of the calf was found positive with the Ziehl-Neelsen staining.

Age at infection	Cat. 0	Cat. 1	Cat. 2	Cat. 3	Cat. 4	Cat. 5	Total	ZN positive
2 weeks	0	2	1	0	0	0	3	1
3 months	1	0	1	0	0	0	2	0
6 months	0	2	1	0	0	0	3	0
9 months	3	0	0	0	0	0	3	0
12 months	0	3	1	0	0	0	4	0
Total	4	7	4	0	0	0	15	1

Table 3: Number of low dose infected calves from trial 1 in each category for the severity of microscopical changes.

Age at infection	Cat. 0	Cat. 1	Cat. 2	Cat. 3	Cat. 4	Cat. 5	Total	ZN positive
2 weeks	0	2	1	0	0	0	3	1
3 months	0	0	3	0	0	0	3	2
6 months	0	1	2	0	0	0	3	0
9 months	1	1	1	0	0	0	3	0
12 months	0	1	2	0	0	0	3	0
Total	1	5	9	0	0	0	15	3

Table 4: Number of high dose infected calves in trial 1 in each category for the severity of microscopical changes.

As shown in table 3 and 4 only microscopical changes of category 0, 1 or 2 were found in the calves from the first trial. Five of the infected animals, of which four infected with a low dose and one with a high dose, showed no microscopical lesions (category 0). Twelve of the infected animals, of which seven low dose and five high dose infected calves, showed only focal lesions in the ileal or ileocaecal lymph node. These calves represent category 1. Thirteen infected animals, of which four low dose and nine high dose infected calves, showed multifocal lesions in the intestinal villi and a lymph node. These calves represent category 2. Only four animals in total were found positive with the Ziehl-Neelsen staining (see figure 2).

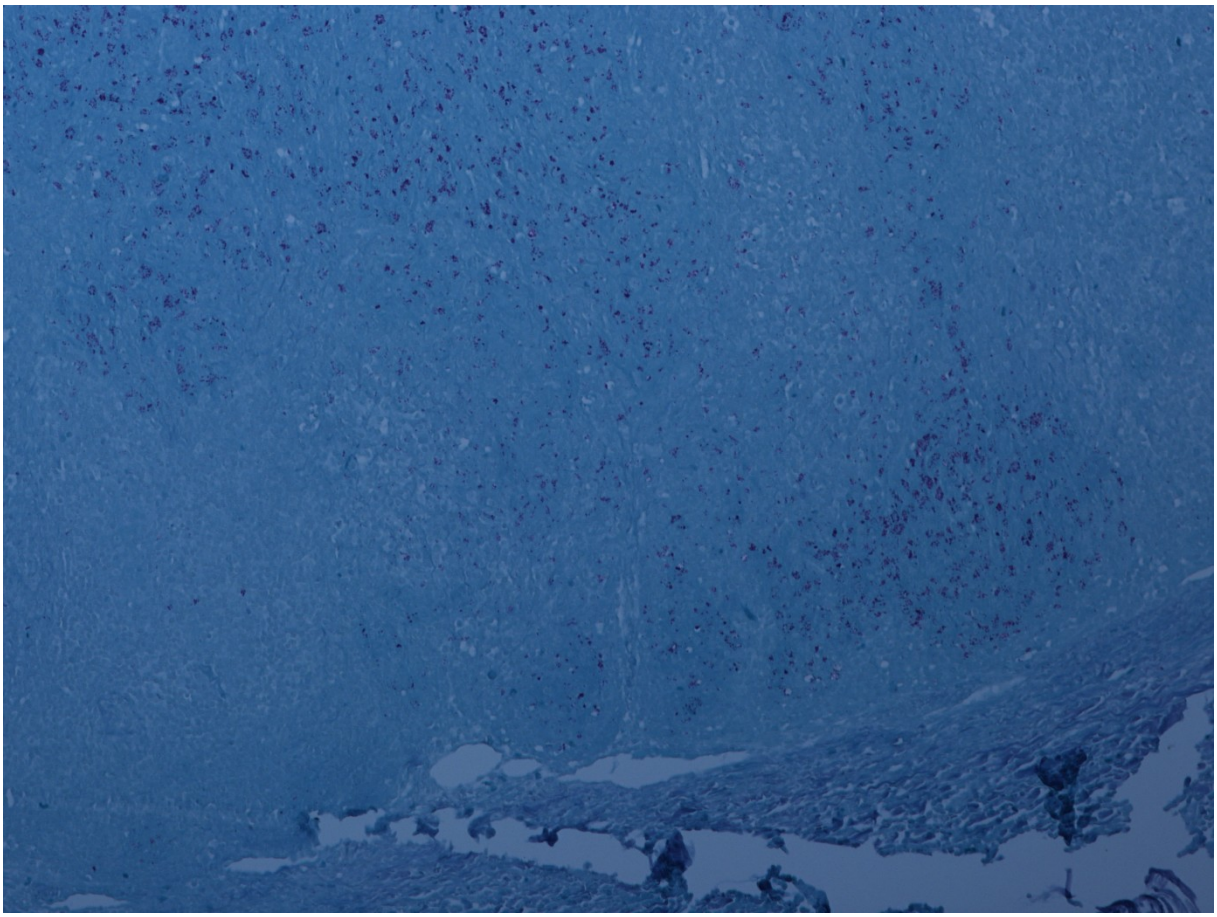

Figure 2: A section of a mesenteric lymph node stained with Ziehl-Neelsen staining shows acid-fast bacteria (magnification 100x)

The one calf of the second trial, cow 89, showed a severe granulomatous enteritis of the ileum (figure 3). The architecture of the villi and the crypts was severely altered, because the mucosa was heavily infiltrated by macrophages containing large numbers of acid-fast bacteria. The submucosa was infiltrated by macrophages as well as lymphocytes. A granulomatous lymphangitis was shown by the large amount of macrophages with acid-fast bacteria surrounding the small lymphatic vessels. The serosal connective tissue was severely edematous. The examined mesenterial lymph node showed a severe multifocal to coalescing granulomatous inflammation and the liver showed a multifocal granulomatous hepatitis. Because of the participation of large amounts of lymphocytes as well as macrophages that contained many acid-fast bacteria this calf was scored category 5 of the microscopic lesions.

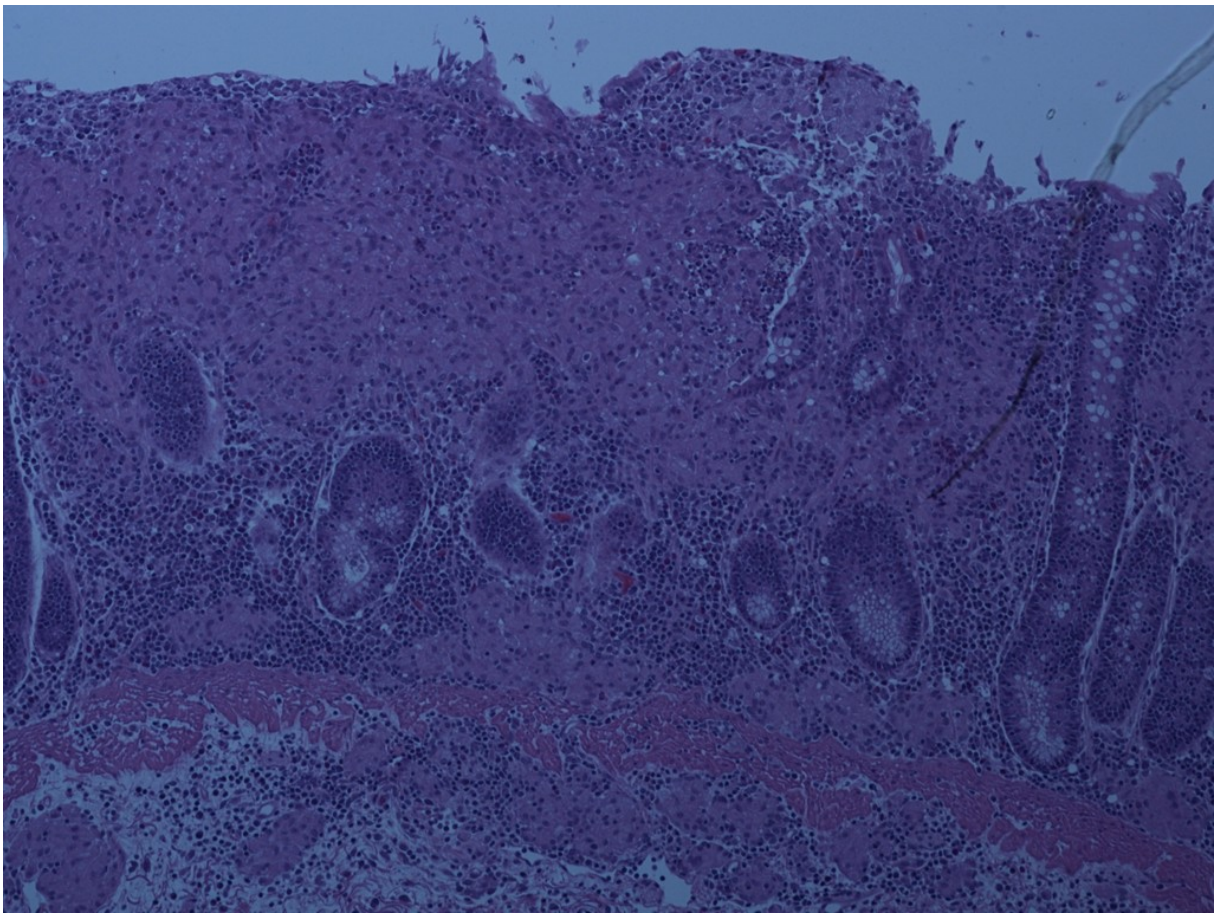

Figure 3: A cross section of part of the ileal wall from cow 89 showing a severe granulomatous enteritis. (magnification 100x)

Tests

A great number of tests was performed on different samples from each calf as described earlier. The results of these tests are summarized in Appendix 1. With these results we can determine if an animal was indeed infected if it tested positive on one of the tests.

Out of the six control animals four animals tested negative on all of the performed tests. Two control animals tested positive, cow 24 tested positive on tissue culture and cow 84 tested positive on MAP specific PCR on fecal culture. Out of the ten animals in the two week infection group nine animals tested positive on at least one of the tests. One animal, cow 88, tested negative on all of the tests performed so far. However the results of the tissue culture of this cow were not available yet. Out of the ten animals in the three month infection group nine animals tested positive on at least one of the tests. One animal, cow 4, tested negative on all of the performed tests. Out of the ten animals in the six months infection group eight animals tested positive on at least one of the tests. Two animals, cow 32 and 35, tested negative on all

of the performed tests. Out of the ten animals in the nine month infection group eight animals tested positive on at least one of the tests. Two animals, cow 8 and 80, tested negative on all of the performed tests. However the tissue culture results for cow 80 were not available yet. Out of the ten animals in the 12 months infection group seven animals tested positive on at least one of the tests. Three animals, cow 29, 82 and 91, tested negative on all of the performed tests. However the tissue culture results were not available yet for cow 82 and cow 91.

Out of all the 50 infected calves, 41 calves tested positive on at least one of the tests and nine calves tested negative on all of the tests. But not all test results of the second trial were available, because not all of these tests were completed before the end of this report.

The available test results were used to make a comparison between the macroscopic and microscopic evaluation and the other tests used; fecal culture, PCR on fecal culture, ELISA and tissue culture. Figure 4 shows the percentage of infected cows with a positive result in a specific test. A remark should be made that the results of the microscopical evaluation and the tissue culture results were only available from the first trial and therefore the percentage is out of 30 cows instead of out of 50 cows for the other tests.

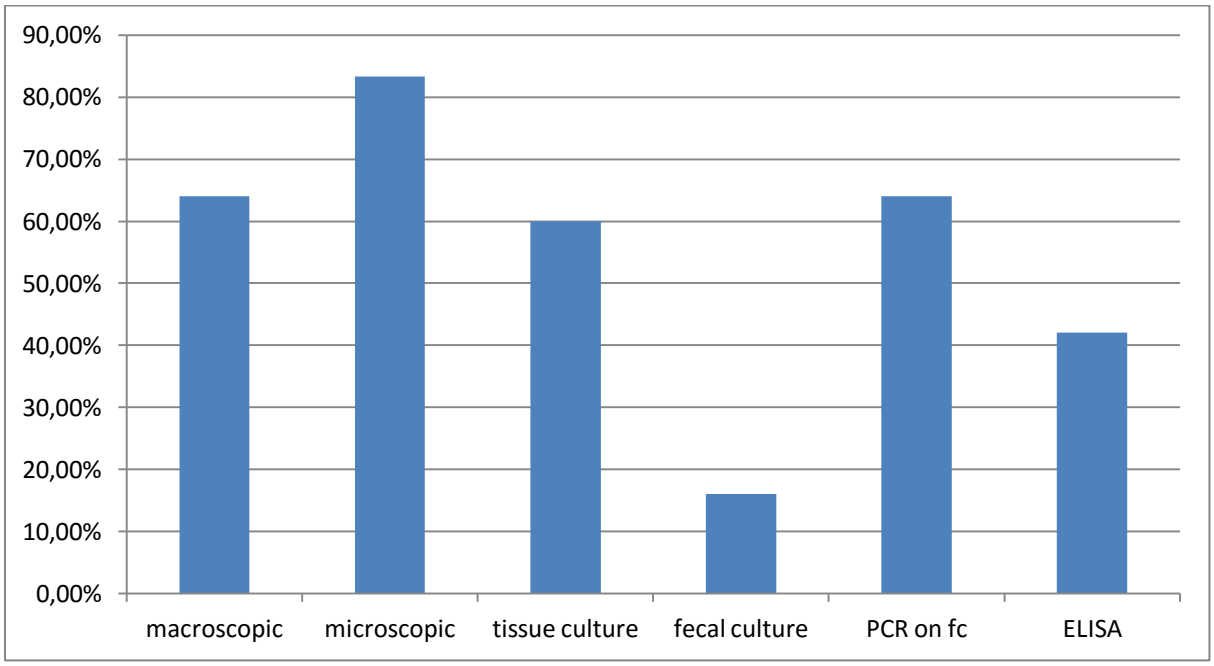

Figure 4. Percentage of positive tested infected calves on each test.

By looking at the height of each bar a comparison can be made between the macroscopical and microscopical evaluation and the other tests. Preferably a comparison would be made

with tissue culture since this is the gold standard. However no tissue culture results were available for the second trial. Therefore we also used the results of three other tests performed on samples of the animals to compare with macroscopical and microscopical evaluation. As shown in figure 4, these two forms of evaluation had a high positive outcome compared to tissue culture results and especially compared to the other three test forms. Macroscopic evaluation found 32 out of 50 infected animals positive, microscopic evaluation found 25 of 30 infected animals positive and tissue culture found 18 out of 30 animals positive. Fecal culture results were poor, since only 8 of the 50 infected animals were found positive. PCR on fecal culture found 32 of the 50 infected animals positive and ELISA on serum samples found 21 out of 50 infected animals positive. Microscopic evaluation had the highest positive outcome, so with this test the most infected animals were found positive. However this test included only the first trial, therefore no statistical analysis was performed to compare the different test used since not all results were available.

Statistical analysis

Using the ordinal logistic regression model we determined that there was no significant relationship between the age group and the severity of macroscopical lesions. However with increasing age the odds for presence of macroscopic lesions tended to decrease (p = 0.07).

We did find a significant relationship between the dose and two of the cut-offs for severity of macroscopic lesions. After using backward selection, the function used to calculate the odds for cut-off>1 was $\log \frac{p}{1-p} = \beta_0 + \beta_{dose} + \beta_{age}$. The function used for cut-off>2 was $\log \frac{p}{1-p} = \beta_0 + \beta_{dose} + \beta_{age} + \beta_{trial} + \beta_{age*trial}$, because age at the time of infection and trial number jointly confounded the relationship between dose and macroscopic lesions.

The odds for a calf of a certain age infected with a high dose of MAP having macroscopic lesions of category 2, 3 or 4 (cut-off>1) were higher than that same calf having lesions in category 1 or 2 (p =0.047). The odds for a calf of a certain age and in a certain trial infected with a high dose having macroscopic lesions in category 3, 4 or 5 (cut-off>2) were also higher than that calf having lesions in category 1, 2 or 3 (p= 0.037). The calculated odds for these cut-offs are listed in table 5 and 6. For example the odds for a calf in trial 2 infected at two weeks of age with a high infection dose for having macroscopic lesions of category 3, 4 or 5 are 3.12 times the odds of that same calf having macroscopic lesions in category 1 or 2.

	Age	odds
Low	0,5	1.18
dose	3	0.90
	6	0.66
	9	0.48
	12	0.35
High	0,5	3.95
dose	3	3.03
	6	2.21
	9	1.62
	12	1.18

Table 5: The odds for cut-off>1

	Age	Odds trial 1	Odds trial 2
Low	0,5	0.72	0.85
dose	3	0.71	0.54
	6	0.70	0.31
	9	0.69	0.18
	12	0.69	0.10
High	0,5	2.63	3.12
dose	3	2.61	1.97
	6	2.58	1.13
	9	2.54	0.65
	12	2.51	0.38

Table 6: The odds for cut-off>2

Discussion

The main goal for this project was to look at the severity of macroscopic lesions in MAP infected calves in relation to age at infection and infection dose. As described previously, calves get infected with MAP in the first six months of their life.[2,3] With this project, however, we have shown that calves can get infected at a later age as well. We found macroscopic as well as microscopic pathological changes in the calves infected at six, nine and twelve months of age. Furthermore, calves of these infection groups showed positive test results in multiple screening tests performed during this experiment.

The calves were infected at different ages, however necropsy of all of the calves took place when they were 17 months old. This means that the calves infected at two weeks of age were already infected for 16,5 months at the time of necropsy and the calves infected at 12 months of age were infected for only five months at the time of the necropsy. Because the incubation period of Johne's disease is two to ten years, this could mean that the two week infection group was already passing into the clinical stage, while the other infection groups were still in the subclinical or even silent stage. This could have had an effect on the test results as well as on the severity of macroscopic and microscopic lesions. For example most severe macroscopical changes were found in the two week infection group. Which could be explained by them being in the clinical stage as two of the animals in this group already showed some clinical signs during the last weeks before necropsy. However we also found quite a large number of cows with macroscopical and microscopical changes in the 12 week infection group. This could be explained by the pathogenesis of the disease. As described earlier the bacteria establishes infection of the intestines and intestinal lymph nodes in the first six months after ingestion by the animal. Since these calves were infected only five months before necropsy more bacteria could be found in the intestines and lymph nodes as compared to especially the six month and nine month infection group which were in the silent stage of the disease. Although the results show a decreased risk of macroscopical lesions for calves of increasing age at infection, the different stages of disease are potential confounders. The difference in calculated odds in higher age groups between trial 1 and trial 2 can be explained by a low number of animals in the higher age groups in trial 2. As a result of the shorter incubation period before necropsy in the higher age groups, the risk of false negative results is increased. Since the odds in trial 2 are based on a lower number of animals, one false negative result has a high impact on calculation of the odds.

The results show that a high dose results in an increased risk at macroscopic lesions. Since the increased risk is observed over all age groups, possible effects of a shorter incubation period before necropsy are negligible in this comparison. Based on these results, minimizing exposure in prevention programs might already reduce the risk of developing Johne's disease.

Interestingly, macroscopical evaluation showed a higher incidence of moderate to severe changes (category ≥3) in infected calves than microscopical evaluation. This can be explained by the fact that during macroscopical evaluation, the total gastrointestinal tract was assessed, while during microscopical evaluation, only the distal ileum, ileocaecal valve and corresponding lymph nodes were assessed.

To determine whether the calves were truly infected we used multiple screening tests and compared these with the golden standard test (tissue culture) [6]. However, at the time of report, the tissue culture results of the second trial were not yet available. Therefore, we defined a calf as being infected when at least one of the screening tests was positive. However, by using this statement not all infected calves were found positive. This could be explained by the poor sensitivity of the tests, including tissue culture [6]. Another explanation for negative test results of the MAP bacteria in the calves , could be e that the calves were in the silent or subclinical stage of the disease [5,6]. Therefore, we decided to use all of the experimentally infected calves for the statistic analysis.

Two of the six control animals were found positive in one of the screening tests. This could be explained as a false positive outcome or could be the result of infection of the calf during the duration of the experiment or contamination of the sample during processing. Notable in this case was control animal 24, which was positive at tissue culture of the ileal lymph nodes and had microscopical lesions in the ileal lymph nodes.

This experiment gives more insight in the pathological development of Johne's disease. Both macroscopical and microscopical changes could be observed in the gastro intestinal tract of the calves. This experiment also shows that infection can arise in calves older than 6 months, indicating that prevention programs should not be limited to calves younger than 6 months.

Conclusion

In this experiment we investigated the severity of macroscopical changes in calves infected with MAP in relation to age at infection and dose of infection. Our results show that calves infected with a high dose of MAP have a significantly higher chance at severe macroscopical

changes than calves infected with a low dose of MAP. The results also show a trend between the age of the calf at infection and the severity of macroscopical changes. It seems that calves infected at a young age have a slightly higher chance at severe macroscopical changes.

This experiment helped in further elucidating the epidemiology of Johne's disease. Future experiments however could focus on the incubation period by keeping the infected calves longer in the experiment before performing the necropsies.

Acknowledgements

I am grateful to dr Bystrom and dr Ilianes for their help in the pathological evaluations. I am also grateful to my supervisors of the University of Calgary for their hospitality and help during my internship and would like to thank them for giving me the opportunity to participate in this interesting project.

References

1. Tiwari, A., VanLeeuwen, J. A., McKenna, S. L. B., Keefe, G. P. & Barkema, H. W. Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. *Can. Vet. journal. La Rev. vétérinaire Can.* **47,** 874–82 (2006).

2. Windsor, P. A. & Whittington, R. J. Evidence for age susceptibility of cattle to Johne's disease. *Vet. J.* **184,** 37–44 (2010).

3. Payne, J. & Rankin, J. A comparison of the pathogenesis of experimental Johne's disease in calves and cows. *Res. Vet. Sci.* **2,** 175–179 (1961).

4. McKenna, S. L. B., Keefe, G. P., Tiwari, A., VanLeeuwen, J. & Barkema, H. W. Johne's disease in Canada part II: disease impacts, risk factors, and control programs for dairy producers. *Can. Vet. journal. La Rev. vétérinaire Can.* **47,** 1089–99 (2006).

5. Whitlock, R. H. & Buergelt, C. Preclinical and clinical manifestations of paratuberculosis (including pathology). *Vet. Clin. North Am. Food Anim. Pract.* **12,** 345–56 (1996).

6. Behr, M. & Collins, D. *Paratuberculosis: Organism, Disease, Control*. (2010).

7. Clarke, C. J. The pathology and pathogenesis of paratuberculosis in ruminants and other species. *J. Comp. Pathol.* **116,** 217–61 (1997).

8. Brady, C., O'Grady, D., O'Meara, F., Egan, J. & Bassett, H. Relationships between clinical signs, pathological changes and tissue distribution of Mycobacterium avium subspecies paratuberculosis in 21 cows from herds affected by Johne's disease. *Vet. Rec.* **162,** 147–52 (2008).

9. González, J. *et al.* Histopathological classification of lesions associated with natural paratuberculosis infection in cattle. *J. Comp. Pathol.* **133,** 184–96 (2005).

10. Larsen, A. B., Merkal, R. S. & Cutlip, R. C. Age of cattle as related to resistance to infection with Mycobacterium paratuberculosis. *Am. J. Vet. Res.* **36,** 255–7 (1975).

11. Hines, M. E. *et al.* Experimental challenge models for Johne's disease: a review and proposed international guidelines. *Vet. Microbiol.* **122,** 197–222 (2007).

12. Buergelt, C. D., Hall, C., McEntee, K. & Duncan, J. R. Pathological evaluation of paratuberculosis in naturally infected cattle. *Vet. Pathol.* **15,** 196–207 (1978).

13. Valentin-Weigand, P. & Goethe, R. Pathogenesis of Mycobacterium avium subspecies paratuberculosis infections in ruminants: still more questions than answers. *Microbes Infect.* **1,** 1121–7 (1999).

Appendix 1

Results from fecal culture, MAP specific PCR on fecal culture, ELISA on serum and tissue culture.

inf. group	trial	inf. dose	cow number	fecal culture	PCR op fc	ELISA	tissue culture
control	1		cow 23	negative	negative	negative	negative
control	1		cow 24	negative	negative	negative	positive
control	1		cow 25	negative	negative	negative	negative
control	2		cow 77	negative	negative	negative	
control	2		cow 83	negative	negative	negative	
control	2		cow 84	negative	positive	negative	
2 weeks	1	low	cow 19	negative	positive	positive	negative
2 weeks	1	low	cow 21	negative	positive	negative	positive
2 weeks	1	low	cow 22	negative	positive	negative	negative
2 weeks	2	low	cow 86	negative	positive	positive	
2 weeks	2	low	cow 88	negative	negative	negative	
2 weeks	1	high	cow 16	negative	positive	negative	positive
2 weeks	1	high	cow 17	positive	positive	positive	positive
2 weeks	1	high	cow 20	positive	positive	positive	positive
2 weeks	2	high	cow 75	positive	positive	positive	
2 weeks	2	high	cow 89	positive	positive	positive	
3 months	1	low	cow 2	negative	positive	negative	positive
3 months	1	low	cow 3	negative	negative	positive	positive
3 months	2	low	cow 92	negative	positive	negative	
3 months	2	low	cow 93	negative	positive	positive	
3 months	2	low	cow 95	negative	positive	negative	
3 months	1	high	cow 1	positive	positive	positive	positive
3 months	1	high	cow 4	negative	negative	negative	negative
3 months	1	high	cow 5	negative	positive	positive	positive
3 months	2	high	cow 94	negative	positive	negative	
3 months	2	high	cow 96	positive	positive	positive	
6 months	1	low	cow 33	negative	positive	negative	negative
6 months	1	low	cow 35	negative	negative	negative	negative
6 months	1	low	cow 36	negative	positive	negative	(positive)
6 months	2	low	cow 98	negative	positive	negative	
6 months	2	low	cow 100	negative	positive	negative	
6 months	1	high	cow 31	negative	positive	negative	positive
6 months	1	high	cow 32	negative	negative	negative	negative
6 months	1	high	cow 34	negative	negative	positive	negative
6 months	2	high	cow 97	negative	positive	negative	
6 months	2	high	cow 99	positive	positive	positive	
9 months	1	low	cow 9	negative	negative	positive	positive
9 months	1	low	cow 12	negative	negative	positive	positive
9 months	1	low	cow 13	negative	negative	negative	positive
9 months	2	low	cow 78	negative	positive	positive	

9 months	2	low	cow 81	negative	positive	negative	
9 months	1	high	cow 6	negative	negative	positive	positive
9 months	1	high	cow 8	negative	negative	negative	negative
9 months	1	high	cow 10	negative	negative	positive	negative
9 months	2	high	cow 79	negative	positive	negative	
9 months	2	high	cow 80	negative	negative	negative	
12 months	1	low	cow 15	negative	negative	positive	negative
12 months	1	low	cow 26	negative	negative	positive	positive
12 months	1	low	cow 27	negative	positive	negative	positive
12 months	1	low	cow 29	negative	negative	negative	negative
12 months	2	low	cow 91	negative	negative	negative	
12 months	1	high	cow 14	positive	positive	positive	positive
12 months	1	high	cow 28	negative	positive	negative	negative
12 months	1	high	cow 30	negative	positive	negative	positive
12 months	2	high	cow 82	negative	negative	negative	
12 months	2	high	cow 90	negative	positive	negative	

Appendix 2

Case History

Calf infected high dose of MAP at 2 weeks of age. Has never grown very well, now showing inappetance and poor water intake. This with dry hair coat and moderately depressed. Loose feces.

Diagnosis

SEVERE GRANULOMATOUS ENTERITIS (JOHNE'S DISEASE)

Comments

FINAL INTERPRETATION: October 24, 2012

This animal has a severe case of Johne's Disease affecting the mid to distal ileum and also the spiral colon - somewhat unusual to see it this severe in the later organ in an animal this young. Granulomas are also found in the liver and there is the classically described lymphangitis present. The severity of the condition at this age is surprising, presumably related to infective dose. With JD, it is unusual to see this much edema surrounding the entire gastrointestinal tract with this perhaps being reflective of two things, 1. the severity of infection with pronounced lymphangitis and dissemination to the liver and mesenteric lymph nodes and
2. hypoproteinemia. Reduced appetite demonstrated by this animal is not very typical of Johne's Disease, again perhaps due to severity and the marked mucosal and serosal edema seen in the abomasum. The gross suggestion that coccidiosis may have been at least partially resposible for this animal's clinical condition has not been corroborated on histology.

Necropsy

Report Status

GROSS NECROPSY FINDINGS: One castrated male Holstein calf presented alive, dangle tag #89, RFID 238 518 704, 394 kg. Euthanized by intravenous barbiturate immediately before necropsy; carcass was in moderate body condition with low internal fat stores and with prominent ribs and dry hair coat. Hind end was fouled with dried feces. There were no lesions found in the thoracic cavity. Abdominal cavity contained mildly excessive straw-colored serous fluid. Edema was noted around most gastrointestinal organs extending from the serosa into periserosal mesenteric tissue, most prominently around the abomasum and into spiral

colonic mesentery. Rumen content was mildly scant, content more scant in the abomasum. Abomasal mucosa contained a few small ulcers, less than 1 cm. in diameter, and also showed very severe edema of rugal folds markedly thickening them in a round, bulbous fashion and with edema fluid running from them upon cutting. Mesenteric lymph nodes adjacent to the distal ileum were large with a moderately wet and bulging appearance on cut section. Mid ileal mucosal surface was mildly roughened, mildly to moderately thickened, difficult to judge extent against serosal edema possibly obscuring some of the thickening visible at the mucosal surface. Spiral colonic mucosa was moderately roughened with a dull, ground glass appearance. Rectal mucosa was grossly normal with scant loose feces seen in the lumen.

GROSS MORPHOLOGICAL DIAGNOSES:
1. MILD HYDROPERITONEUM WITH DIFFUSE SEROSAL/MESENTERIC EDEMA MOST SEVERE AROUND ABOMASUM AND SPIRAL COLON
2. DISTAL ILEAL LYMPHADENOPATHY
3. MILD MIDILEAL MUCOSAL ROUGHENING AND THICKENING
4. SPIRAL COLON: SUSPECT COLITIS, MODERATE, SUBACUTE

HISTOLOGICAL FINDINGS: LUNG: There are several peribronchiolar accumulations of mononuclear cells, mostly not forming distinct follicles, and made up of lymphocytes and histiocytic cells with very occasional plasma cells. HEART: Papillary muscle has a few small accumulations of lymphohistiocytic cells in perivascular zones with occasional mild extension into adjacent perimysial interstitial tissue. LIVER: There are multifocal accumulations of mixed inflammatory cells made up of lymphocytes, fewer plasma cells and variable numbers of macrophages often in epithelioid clumps with occasional multinucleate forms, both within portal triad regions and seemingly randomly in sinusoids. KIDNEY: There are a few small perivascular accumulations of mixed inflammatory cells made up of lymphocytes with fewer plasma cells. MESENTERIC LYMPH NODE: Marked subcapsular mixed leucocytosis is seen with admixed lymphocytes, plasma cells and occasional epithelioid macrophages. Cortices are markedly infiltrated by irregular nodular to arborizing accumulations of epithelioid macrophages, many containing acid-fast organisms on Ziehl-Neelsen stain. Medullary cords are markedly edematous. Perinodal connective tissue is also heavily infiltrated by inflammatory cells, both outside the capsule in laminar arrays and arranged outside small muscular arteries as nodules of mixed lymphocytes, plasma cells and epithelioid macrophages. SKIN: Skin from the ear shows light to moderate accumulations of mixed

mononuclear cells (lymphocytes, plasma cells) around superficial dermal small vessels. ABOMASUM: Ulcers noted grossly have sharp edges at full-thickness loss of the mucosal surface. Ulcer bed is made up of fibrovascular connective (granulation) tissue deeply becoming less mature as approach the surface and with more mixed inflammatory cells, mostly mononuclear, admixed. There are variably sized foci of mineralization seen within granulation tissue. Small bits of feed material adhere to the surface. Tissue underlying the ulcer is lightly infiltrated by mononuclear cells around small vessels. Submucosal connective tissue underlying rugal folds is markedly expanded by edema giving the layer a rarefied appearance. ILEUM: There is marked shortening, blunting and fusion of villi with effacement of mucosal architecture giving it a smooth, flattened profile at the surface. This is caused by very heavy infiltration of the mucosa by mixed inflammatory cells, heavy complement of eosinophils, admixed throughout nodular to coalescing accumulations of large epithelioid macrophages that contain numerous acid-fast organism on Ziehl-Neelsen stain. Crypt numbers are markedly reduced and those remaining are spread apart by infiltrating macrophages. Macrophages breach the basement membrane to form a nodular to coalescent layer in the superficial submucosa with an underlying layer of edematous submucosa with extension into perimysial areas of the underlying muscular layer. Accompanying inflammatory infiltrate below the basement membrane becomes more mononuclear, largely lymphoplasmacytic, compared to that above the basement membrane. Inflammatory cells surround virtually every small vessel, arterial, venous or lymphocytic, with extension into walls of a few. Small lymphatic vessels often contain plugs of epithelioid macrophages containing acid-fast organisms on Ziehl-Neelsen stain. Serosal connective tissue is edematous. SPIRAL COLON: Lesion is similar to that seen in the ileum described above, also with numerous acid-fast organisms seen on Ziehl-Neelsen stain. It is less severe with accumulations of epithelioid macrophages remaining nodular versus coalescent and with less loss of deep crypts. Nodular clusters of macrophages breach the basement membrane to form nodular clusters in the submucosa. RECTUM: Rectal mucosa is moderately edematous with rather heavy complement of mixed lymphoplasmacytic cells in the lamina propria. SPLEEN, ADRENAL GLAND, GALL BLADDER, NASAL PLANUM, TONGUE, RUMEN: NVL.

HISTOMORPHOLOGICAL DIAGNOSES:

1. ILEUM, SPIRAL COLON: ENTERITIS, GRANULOMATOUS, SEVERE WITH INTRALESIONAL ACID-FAST ORGANISMS CONSISTENT WITH MYOBACTERIUM

SP. AND WITH GRANULOMATOUS LYMPHANGITIS AND SUBMUCOSAL/SEROSAL EDEMA, SEVERE

2. MESENTERIC LYMPH NODE: GRANULOMATOUS LYMPHADENITIS, MULTIFOCAL TO COALESCING, SEVERE, CHRONIC WITH INTRALESIONAL ACID-FAST ORGANISMS CONSISTENT WITH MYCOBACTERIUM SP.

3. LIVER: GRANULOMATOUS HEPATITIS, MULTIFOCAL DISSEMINATED, MODERATE, CHRONIC

4. LUNG: LYMPHOHISTIOCYTIC PERIBRONCHIOLITIS, NONSPECIFIC, VERY MILD, CHRONIC

5. HEART: LYMPHOHISTIOCYTIC MYOCARDITIS, NONSPECIFIC, VERY MILD, CHRONIC

6. EAR SKIN: SUPERFICIAL PERIVASCULAR DERMATITIS, NONSPECIFIC, MILD, CHRONIC

7. ABOMASUM: MULTIFOCAL SMALL ULCERS, CHRONIC AND SEVERE EDEMA OF RUGAL FOLDS

.

Appendix 3.

inf. group	trial	inf. dose	cow number	macroscopic	microscopic	Ziehl-Neelsen
control	1	control	cow 23	0	0	negative
control	1	control	cow 24	0	1	negative
control	1	control	cow 25	0	0	negative
control	2	control	cow 77	0		
control	2	control	cow 83	0		
control	2	control	cow 84	0		
2 weeks	1	low	cow 19	1	1	negative
2 weeks	1	low	cow 21	3	2	positive
2 weeks	1	low	cow 22	3	1	negative
2 weeks	2	low	cow 86	3		
2 weeks	2	low	cow 88	0		
2 weeks	1	high	cow 16	2	1	negative
2 weeks	1	high	cow 17	3	1	negative
2 weeks	1	high	cow 20	4	2	positive
2 weeks	2	high	cow 75	5		
2 weeks	2	high	cow 89	5		
3 months	1	low	cow 2	3	0	negative
3 months	1	low	cow 3	0	2	negative
3 months	2	low	cow 92	3		
3 months	2	low	cow 93	1		

3 months	2	low	cow 95	2		
3 months	1	high	cow 1	3	2	positive
3 months	1	high	cow 4	3	2	positive
3 months	1	high	cow 5	0	2	negative
3 months	2	high	cow 94	3		
3 months	2	high	cow 96	3		
6 months	1	low	cow 33	0	1	negative
6 months	1	low	cow 35	0	1	negative
6 months	1	low	cow 36	3	2	negative
6 months	2	low	cow 98	1		
6 months	2	low	cow 100	0		
6 months	1	high	cow 31	3	2	negative
6 months	1	high	cow 32	0	1	negative
6 months	1	high	cow 34	3	2	negative
6 months	2	high	cow 97	1		
6 months	2	high	cow 99	0		
9 months	1	low	cow 9	0	0	negative
9 months	1	low	cow 12	0	0	negative
9 months	1	low	cow 13	1	0	negative
9 months	2	low	cow 78	0		
9 months	2	low	cow 81	0		
9 months	1	high	cow 6	3	0	negative
9 months	1	high	cow 8	3	2	negative
9 months	1	high	cow 10	0	1	negative
9 months	2	high	cow 79	0		
9 months	2	high	cow 80	0		
12 months	1	low	cow 15	3	1	negative
12 months	1	low	cow 26	3	1	negative
12 months	1	low	cow 27	0	1	negative
12 months	1	low	cow 29	0	2	negative
12 months	2	low	cow 91	4		
12 months	1	high	cow 14	3	2	negative
12 months	1	high	cow 28	3	2	negative
12 months	1	high	cow 30	3	1	negative
12 months	2	high	cow 82	4		
12 months	2	high	cow 90	0		