JOHNE'S DISEASE IN SHEEP

Association between lymph node size and histologic characteristics

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Abstract

AIM: To describe if there is a relationship between mesenteric lymph node (MLN) size and histologic characteristics of *Mycobacterium avium* subsp. *Paratuberculosis* (*MAP*). Hunnam *et al.* (2010 submitted) found that in deer 94% of the enlarged lymph nodes were *MAP* positive. The aim of this study is to determine if there is a same relationship in sheep.

METHODS: 750 MLN were sampled from 250 slaughter lambs from 25 different farms (10 sheep/farm). *Mycobacterium avium* subsp. *Paratuberculosis* status of the farms was unknown. The LN were sampled at 25%, 50% and 75% of the MLN chain, where the 25% section was taken at the beginning of the ileum and the 75% section from the ileocecal LN. The circumference of the MLN was measured and abnormal coloration described. The samples were stored in 60 ml containers with formalin. At the 75% sample location an additional two samples were taken and stored fresh frozen at -80°C for PCR and culture. Fixed sections were stained with Haematoxyline and Eosin and if necessary with Ziehl Neelsen. The slides were examined for lesions typical for Johne's disease using an Olympus BX51 and XC50 microscope.

RESULTS: No LN were found histologically positive for Johne's disease. In one LN typical lesions for Johne's were found, but on the Ziehl Neelsen no acid fast organisms (AFO) were found. This LN was categorized as highly suspicious for Johne's disease. Most LN showed some hyperplasia and some worms and coccidia were found accompanied by eosinophils. LN size was not affected by abnormal coloration, but appeared to be significant affected ($P<0,001$) by sample location (25, 50 or 75%). The smallest circumferences were measured at the 75% sample location at the largest at the 50% sample location.

CONCLUSION: To determine if there is a relationship between LN size and histologic characteristics more LN will have to be examined preferable of older sheep from the same farms where the lambs for this study were sampled. This will provide useful information about LN characteristics and the relationship with age in *MAP* infected sheep. A culture and/or PCR must be done on the sampled lamb LN to determine if these lambs were *MAP* infected or not, even though no histological lesions were found.

KEY WORDS: *Johne's disease, Mycobacterium avium subsp. Paratuberculosis, sheep, mesenteric lymph nodes, histological characteristics*

Introduction

Johne's Disease (JD, paratuberculosis) is a chronic progressive enteritis due to infection from *Mycobacterium Avium* subspecies *Paratuberculosis (MAP)* (Turner *et al.,* 2005*). MAP* is a non-motile, short Gram positive, strongly acid fast bacullis, measuring approximately 1-2 μm x 0,5 μm. *MAP* is genetically related to other Mycobacterium avium spp. (Coetzer & Tustin, 2004). Johne's disease in New Zealand sheep was first described by Williamson and Salisbury in 1952 (Armstrong *et al.,* 1956). Since then the disease has been diagnosed and confirmed on a lot more farms in both South and North Islands (Armstrong *et al.,* 1956*,* Davidson, 1970).
Although *MAP* is inactivated by most disinfectants, it is relatively resistant to environmental factors and can survive for at least 270 days in water (Coetzer & Tustin, 2004). In ruminants Johne's has become a worldwide problem and it has been recognized in many species such as sheep, cattle, deer, goats and more (Turner *et al.,* 2005).

Animals may become infected by one of three ways:
1) In utero
2) Ingestion of infected milk from animals harboring the organism
3) Intake of *MAP* from contaminated objects or pasture
After ingestion, the bacilli penetrate the gastrointestinal mucosa and are taken up by macrophages, where they are resistant to digestion by phagolysosomes and can multiply. Intracellular they can survive for up to five weeks (Coetzer & Tustin, 2004).
Sheep infected with *MAP* may eventually develop some clinical signs. The sheep will lose condition, are weak and look emaciated. The wool pulls out easily and after the clinical symptoms appear, death can occur within a few weeks. The feces vary from diarrhea to firm pallets, but the diarrhea is less often seen compared to cattle (Armstrong, 1956).
The majority of sheep with clinical signs are 3 to 4-year old and clinical signs in sheep under an age of 2 years are rare (Armstrong, 1956). Cattle develop an age-dependent resistance to infection with MAP (Chiodine *et al.,* 1984), but little is known about the age that sheep are most susceptible to infection. Not all animals exposed to *MAP* become affected, this depends on immune status and the quantity of bacilli ingested (Wakelin, 1995). After ingesting an infective dose of the bacterium a long incubation period starts in cattle and sheep, which may explain why the clinical signs usually do not occur under 2 years of age (West, 2002). Some animals may never develop clinical signs and stay subclinically infected. These sheep appear to be normal, but are *MAP* infected and post mortal lesions may be present. These subclinical cases of Johne's

disease constitute the majority of infections and are a very important aspect of the disease, because these animals are much harder to detect and may lead to underestimation of the true prevalence (de Lisle, 1970).

Post mortem examination of sheep shows gross intestinal lesions compatible with those of Johne's disease in 70 per cent of clinical cases in sheep (Coetzer & Tustin, 2004). These lesions include macroscopic lesions in the distal ileum, the caecum, proximal colon and the associated lymph nodes (LN). The lymph nodes are oedematous and may be up to four times the normal size (Armstrong, 1956).
Histologically tuberculoid (caseating) granulomas may be seen in the intestines, mesenteric lymph nodes (MLN) and lymphatics. The granulomas can be mineralized and contain numerous epithelioid macrophages and may contain some Langerhans' type giant cells. Acid fast mycobacteria may be difficult to find (McGavin & Zachary, 2007). The finding of acid fast organisms (AFO) also depends on the histo-pathological form present, 'multibacillary' or 'paucibacillary'. The 'multibacillary' form is characterized by diffuse granulomas composed of epitheloid macrophages filled with large numbers of mycobacteria. The 'paucibacillary' form is characterized by a diffuse lymphocytic infiltrate with some epitheloid macrophages and few or sometimes no mycobacteria (Turner *et al.,* 2005).
Affected tissues can be stained with an acid fast stain, such as Ziehl Neelsen (ZN), to show the organisms in the macrophages. In cattle subclinically affected animals have lesions that contain few Langerhans' giant cells and a few epithelioid macrophages in the intestinal villi or mesenteric lymph nodes, and AFO are rarely found (Coetzer & Tustin, 2004). Subclinically infected sheep have not been studied as much as clinically infected animals (Turner *et al.,* 2005). In sheep Langerhans' giant cells seem to be rare. AFO are found in approximately 85 per cent of clinical cases of JD and in approximately only 30 per cent of the subclinical cases (Coetzer & Tustin, 2004).

Apart from histology other diagnostic tests are available. Unfortunately all tests have low sensitivity and specificity, for that they fail to detect all infected animals and cannot differentiate *MAP* from other mycobacterial related infections. Cultural isolation of feces or tissue samples is the standard test to detect JD and is considered the 'golden standard'. *MAP* is difficult to culture from sheep feces and tissue and takes at least 12 weeks. The advantage of the test is that it has a high specificity (100 %). A faster method to detect *MAP* is to use a Polymerase Chain Reaction (PCR), where probes containing repetitive DNA elements are used to measure genetic similarity. The specificity of the test is high (97 to 100%) and results can be achieved within three

days, but the sensitivity is low (35 %) en costs are high (Coetzer & Tustin, 2004). The PCR test might be particularly useful in sheep, where culture is not very sensitive and the bacteria grow slow (Worthington, 2004).

This project will look at histologic characteristics of *MAP* in sheep lymph nodes and the relationship with lymph node size. Hunnam *et al.* (2010 submitted) found that in deer, over 94% of 'abnormal' MLN (i.e. those with gross pathological lesions, and/or with a circumference of >55mm) were infected with *MAP*. 45% of the grossly 'normal' (<55mm, without gross pathological lesions) MLN in deer were found *MAP* positive by culture (Stringer *et al.,* 2010). This suggests that most animals that become infected will be subclinically infected and may not develop any clinical or subclinical pathological signs at all. Subclinical infection can be measured by fecal, gut and lymph node sampling. Tests used for this study detection include culture, PCR and histology (H&E and ZN staining). Similar studies have not been conducted in sheep in New Zealand. This project was the first stage of an investigation of the infection rate of *MAP* in sheep and the relationship with lymph node size. Additional sampling will be continued by a subsequent researcher to achieve the required sample size to achieve statistical validity. This report describes the collection and management of samples, measurements taken, and the histological characteristics.

The primary objective of this study will determine if there is a relationship between lymph node circumference and histological evidence of *MAP* infection. MLN were collected from slaughter sheep and the lymph node circumference has been measured. The slices of lymph nodes were taken from three different sites in the mesenteric lymph node chain. Histology has been performed on the MLN and samples were archived fresh frozen at -80°C for future culture and or PCR tests, based on the outcome of the histopathology test.
The secondary objective will determine if there is a difference between mean lymph node size between farms and between mean lymph node size per sample location in the mesenteric lymph node chain.

The hypotheses for this research are

1) that it is possible to detect evidence of MAP infection in sheep lymph nodes histologically

2) that there is a relationship between lymph node size and histological evidence of *MAP* infection.

Materials and methods

Sheep were sampled at a commercial sheep slaughterhouse at Feilding in the Manawatu district of New Zealand from October to December 2010. A flock was defined as sheep farmed at a single location. A line was defined as a shipment of sheep sent for slaughter from the same flock to the same slaughterhouse on the same day.
The sample size has been determined according to standard population sampling procedures, based on an estimated 16% prevalence of *MAP* infection at the individual animal level. A minimum of 172 sheep should be sampled, but to compensate the intra-chain correlation, this minimum was increased to a minimum of 480 sheep (10/line). With 10 samples per line, there should be an 80% chance of detecting a positive line to be positive. This report describes samples from 10 sheep in each of 25 lines from different farms.

All tissues were collected by a sterile technique using new equipment for each sample. There were five rat tooth forceps per package to keep the rest free from contamination during sampling. A cutting board was used to cut the LN's. A new piece of plastic was wrapped around the cutting board between each LN to avoid cross contamination. After the examination of the organs by a meat inspector the intestines were taken and stored individually in a plastic bag. After ten intestines were collected, the sampling of the first one was started. Fresh samples were kept chilled in an insulated container.

Area of origin, age, LN location and abnormal color (red, brown, green, yellow, white, other) was recorded at the time of sampling. Age was aggregated into young (lamb) and adult, but only young animals were sampled. All herds were mixed sex.

For the histology a 5 to 8 mm wide section was cut at 25%, 50% and 75% of the mesenteric LN chain length. The 25% section was taken at the beginning of the ileum, the 50% cut at the middle of the ileum and the 75% samples were taken from the ileocaecal lymph nodes located at the end of the ileum. The sections were stored individually in 60 ml containers filled with 10% buffered formalin. The circumference of each lymph node was measured using a flexible measuring tape.
The fixed samples were dehydrated, cleared and embedded in paraffin. Sections were stained with Haematoxylin and Eosin (H&E) according to Gill's (II). After finding typical JD lesions a Ziehl-Neelsen (ZN) was requested. Slides were examined under 4x, 10x and 40x magnification using a Olympus BX51 and XC50 microscoop.
N/25 is the 25% sample, N/50 is the 50% sample and N/75 is the 75% sample (ileocaecal).

Two samples were taken for PCR and culture. The culture and PCR sample were taken from the ileocecal LN, because this LN was the most likely to be affected. Sections taken for culture and PCR were stored at -80°C. The containers were labeled with a *N* P for PCR and *N* C for culture.

A sample record was used at the slaughterhouse with the following design:

Sample ref			**Diameter**	**Lymph vessel**	**Other**
LN 1/25					
LN 1/50					
LN 1/75					

Lymph vessel: The presence of enlarged lymph vessels on the serosa of the distal ileum. This is a very common sign when an animal is infected with *MAP*.

Other: space for comments like color, shape, lesions etc. Later they were categorized as 0 ('normal' LN), 1 (red coloration of the LN) or 2 (other coloration, like green and yellow) and referred to as PathScore.

Microsoft Word 2010, Microsoft Excel 2010 and SAS (Statsitical Analysis System version 9.2, Institute Inc., Cary, NC, USA), were used for data collecting, analysis and statistics.

Protocol for H & E staining as used in laboratory at Massey University

Haematoxylin and Eosin

XL Prgogramming Sheet

Program 1

STEP	STATION	REAGENT	TIME (min:sec)	EXACT
1		OVEN	10:00	Y
2	1	XYLENE	3:00	Y
3	2	XYLENE	2:00	N
4	3	ABSOLUTE ALCOHOL	1:30	Y
5	4	ABSOLUTE ALCOHOL	0:30	N
6	5	70% ALCOHOL	1:30	N
7		WASH 1	1:00	N
8	7	GILL'S HAEMATOXYLIN	4:00	Y
9		WASH 2	0:30	Y
10	6	SCOTTS TAPWATER	0:30	Y
11		WASH 3	1:00	Y
12	8	EOSIN/PHLOXINE	2:00	Y
13		WASH 4	0:30	Y
14	13	70% ALCOHOL	0:10	Y
15	14	955 ALCOHOL	0:15	Y
16	15	ABSOLUTE ALCOHOL	0:45	N
17	16	ABSOLUTE ALCOHOL	1:00	N
18	17	XYLENE	1:00	N
19	18	XYLENE	1:00	N
20		EXIT		

Protocol for Ziehl Neelsen as used in laboratory at Massey University

Ziehl Neelsen (for acid fast organisms)

Solutions Required

1. **Carbol-Fuchsin**

Basic fuchsin – 1gm
Absolute alcohol – 10mls
5% phenol aqueous – 100mls

2. **0,5% HCl in 70% Alcohol**

3. **0,1% Methylene Blue**

Method

1. Bring sections to water
2. Stain in hot carbol-fuchsin in coplin jar at 56 C for 30 mins
3. Decolourise in 0,5% HCl in 70% alcohol – 2 mins.
4. Wash in running tap water – 5 mins.
5. Counterstain in 0,1% methylene blue – approx. 10 seconds.
6. Dehydrate, clear and mount

Results

Acid fast organisms – bright red
Background – blue

Time required – 1 hour

Culling, Cellular Pathology Technique, 4th edn, p336 (1985)

Results:

Samples were taken from 250 lambs from 25 lines (10 lambs/ line). All sampled animals were lambs under an age of 1 year. No exact age was recorded. All lines were of mixed sex and from farms in the Manawatu district of New Zealand. From each lamb three LN were sampled for histology and one LN (ileoceacal LN) was sampled for culture and PCR. In total 750 LN were sampled for histology and 250 for culture and PCR.

Histology

Lines 1 to 7 were looked at with both H&E and ZN staining. In these samples representing 70 sheep all slides were negative histologically. From line 8-10 only H&E staining has been done. Additional ZN staining could be requested if granulomas were found on H&E staining. After line 10 (100 sheep) there were still no positives found with histology. From that moment on only the 50% LN cut was prepared for histology, since these were the LN with the largest circumference and therefore possibly more likely to be found positive for Johne's disease.

No MLN were found positive for Johne's disease. A ZN stain was requested for one slide (Nr. 171/50), but no AFO were found. With only granulomas present and no AFO this LN was categorized as highly suspicious for Johne's disease. It may also be possible that this animal had a 'paucibaccilary' form of Johne's, where often no AFO are found. During the sampling no lymphangitis was observed on the distal ileum, which would have been highly indicative for Johne's disease.

Histologically most LN were showing some hyperplasia and in some cases worms or coccidia were found accompanied by some eosinophils.

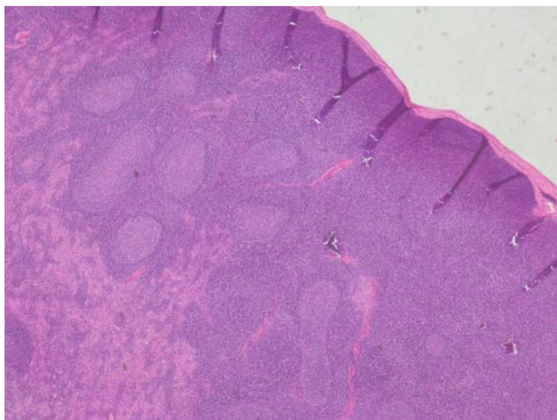

Figure 1 'Normal' LN showing follicles in the cortex 4x magnitude

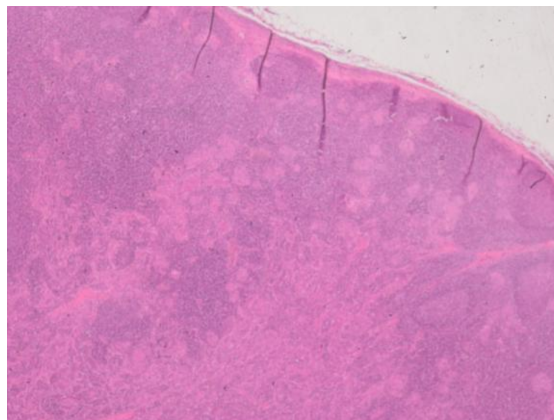

Figure 2: Affected LN with granulomas in the cortex 4x magnitude

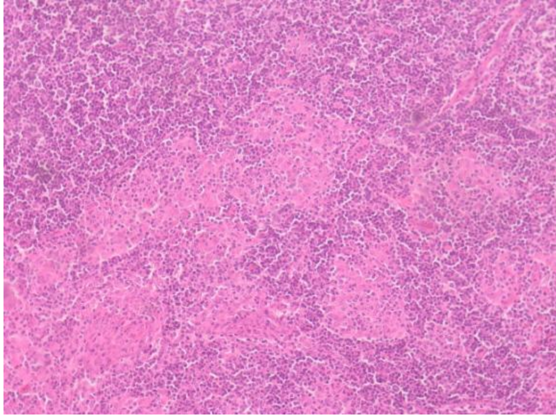

Figure 3: 20x magnitude of the affected LN showing a close-up of the granulomas

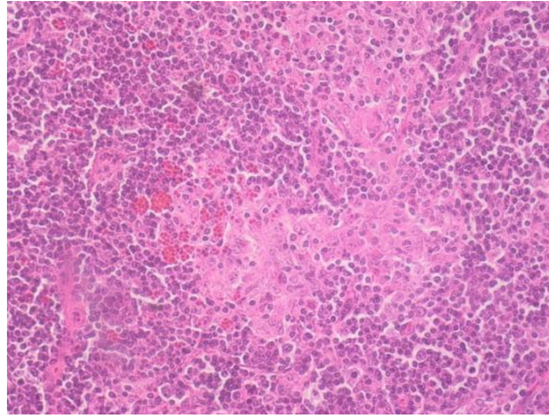

Figure 4: 40x magnitude of the affected LN showing epitheloid macrophages typical for Johne's disease lesions

LN size

Figure 5 shows the mean LN circumference per line, all LN sample locations included.

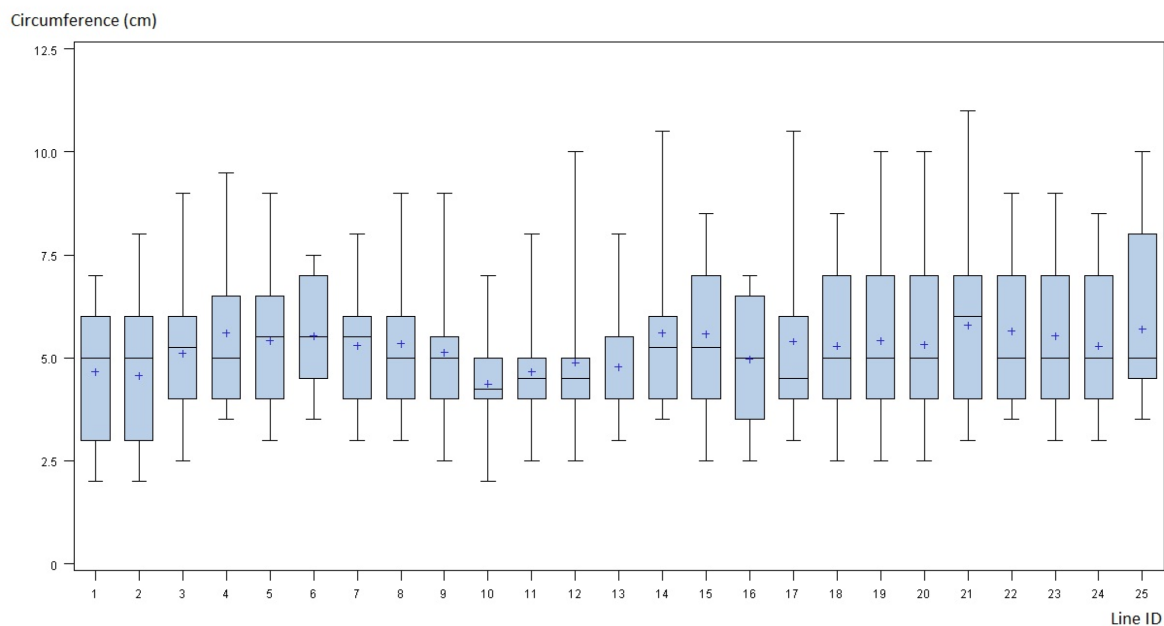

Figure 5: Box plot of the lymph node circumference (cm) of sheep (n= 10/line) from lines from 25 farms (The + represents the mean and the – the median, the blue box represents the central 50% of the data, and the bars represent the absolute range of the LN circumferences of that line).

LN size and location

Table 1 shows the mean circumference (in cm) of the LN per sample location with its standard error and 95% confidence interval. LN collected at the 75% sample location have the smallest circumference, while the LN sampled at the 50% location had the biggest circumference.

Effect	site along LN chain (%)	Mean	SE	95% Confidence Lower	interval Upper
LocationID	25	4,9	0,1	4,7	5,2
LocationID	50	6,7	0,1	6,4	7,0
LocationID	75	4,0	0,1	3,7	4,3

Table 1 Table of Lymph node circumference (cm) of sheep showing the mean circumference, the standard error and the 95% confidence interval per site along the LN chain (25, 50 and 75%).

Figure 6 presents the frequency of LN circumference categories (cm) at the three LN sampling locations. This figure shows clearly that the 75% LN had the smallest and the 50% LN the biggest circumferences. The circumference that had the highest frequency at the 25% sample location was 5 cm. At the 50% sample location the circumference that had the highest frequency was 7 cm and the circumference most frequently measured at the 75% sample location was 4 cm.

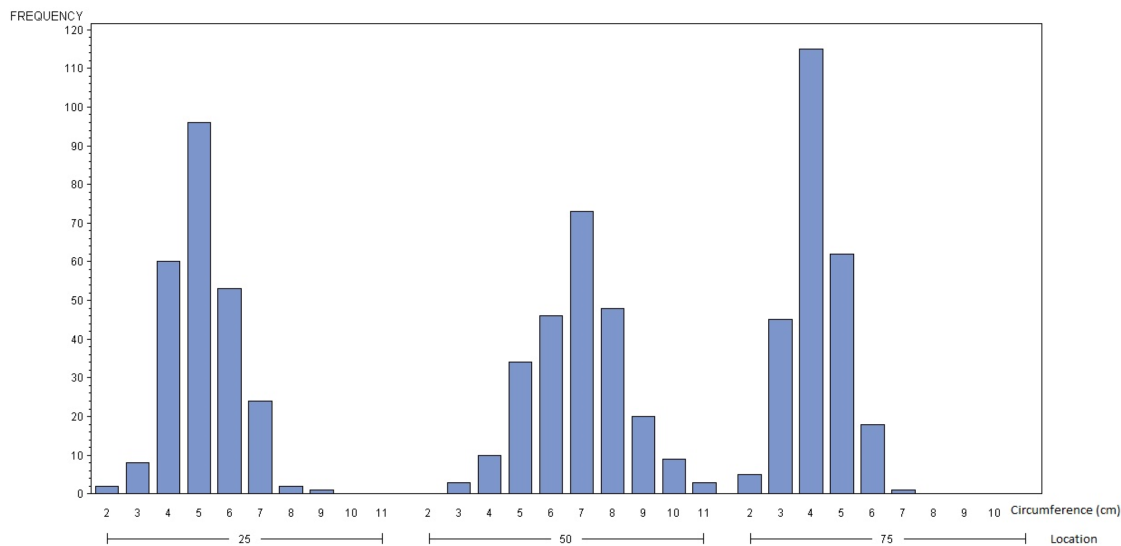

Figure 6. A frequency histogram of lymph node circumference (cm) in sheep, including three different sample locations (25, 50 and 75%) in the LN chain length.

Figure 7 shows the mean circumference per sample location with its absolute range in measured LN circumference. A circumference of 2 cm was the smallest measured and was found in both the 25% and 75% location. 3 cm was the smallest circumference found at the 50% location. The biggest circumference (11 cm) was found at the 50% location, where the 75% and 25% location had respectively 7 cm and 9 cm as biggest circumferences.

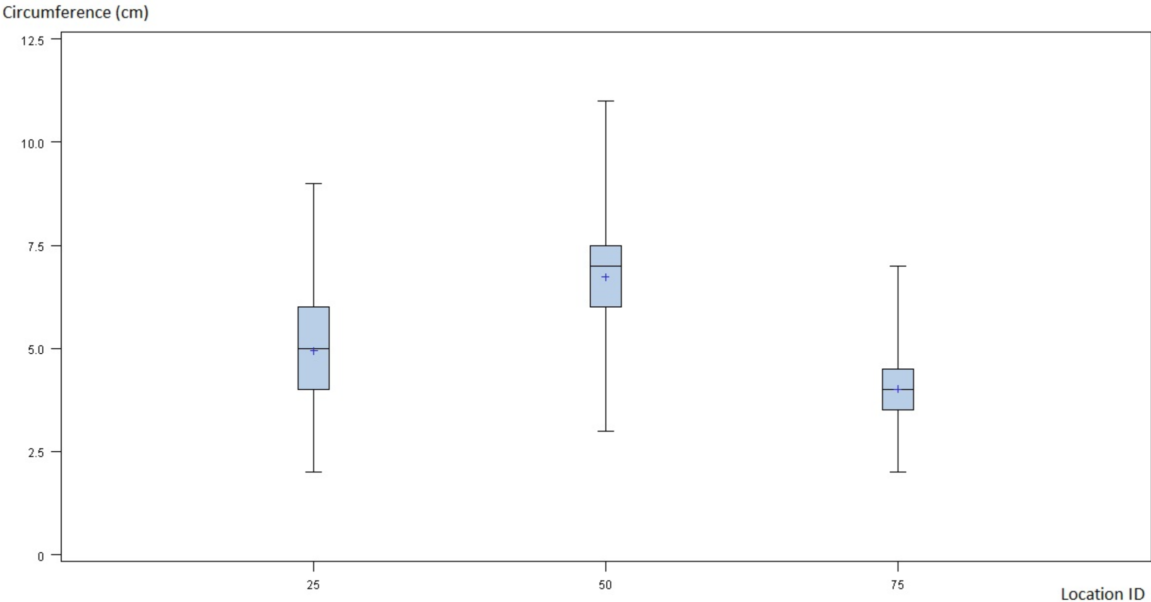

Figure 7. Box plot of lymph node circumference (cm) in sheep per sample location (25, 50 or 75%) The + represent the mean, the – the median of this lymph node location and the blue box the central 50% of the data. The bars represent the absolute range of lymph node circumference at that sample location.

During sampling, LN abnormalities were recorded such as red, green or yellow coloration and abnormalities on the cut surface. These abnormalities are listed as PathoScore in table 2. This table shows that the mean LN circumference per sample location was not significantly affected by these pathologic abnormalities. On the other hand table 2 shows that there is a strong significant effect (P<0,001) on LN circumference due to sample location.

Effect	DF	DF	F	p
LocationID	2	684	351,1	<.0001
PathScore	2	684	1,3	0,2745

Table 2. This table shows the effect of sample location (25, 50 or 75%) and PathScore on the mean lymph node circumference(cm), where a P value <0,05 represents a significant effect.

A variance component analysis has been done and is shown in table 3. The majority of variance in LN size (57%) is due to the location of the LN in the mesenteric chain, while 36 per cent is due to error, which are animal 'normal' differences. Only 7 per cent is due to differences between farms. This shows that LN sample location has the biggest influence on the circumference of the LN.

Source	Component	Circumference proportion
Var(Sample location)	1,87938	57%
Var(Farm differences)	0,24048	7%
Var(Error)	1,18265	36%
Total	3,30251	

Table 3. Variance in lymph node circumference (cm) divided into three categories, consisting variation due to sample location, farm differences and error.

As shown in table 4, after adjusting for sample location of the LN, the variance is dominated (90%) by error, which is the normal LN difference between animals. 8 per cent of the variance is due to differences between lines and only 2 per cent is due to lamb to lamb differences.

Variance	Component	circumference proportion
Var(Line difference)	0,10734	8%
Var(Lamb difference)	0,03058	2%
Var(Error)	1,28038	90%
Total	1,4183	

Table 4. Variance in lymph node circumference (cm), after adjusting for lymph node sample location, containing line difference, lamb difference and error.

Conclusion

The first hypothesis for this research was that it is possible to detect evidence of *MAP* infection in sheep lymph nodes histologically. The first hypothesis cannot be proven nor rejected, for not enough sheep of different ages have been sampled. Only lambs under an age of 1 year were sampled. Within this age group no histological evidence of *MAP* infection could be found. This might be due to the fact that the sheep were healthy, but it might also be possible that the sampled sheep were too young for histological lesions. The culture and/or PCR results may provide information on the infection status of the sampled sheep. Another reason may be that the estimated prevalence (16% animal level prevalence) was an overestimate; the actual prevalence of Johne's may be much lower.

The second hypothesis was not tested in this research, since no animals were found positive for Johne's disease histologically. Again, the culture and/or PCR results will provide more information on the infection status. Until culture and/or PCR results are available no more conclusions can be made about these hypotheses.

A conclusion that can be made is that LN circumference depends highly on the sample location in the mesenteric LN chain. The smallest circumferences can be found at the 75% sample location, where the largest can be found at the 50% sample site. Coloration, like red, green and yellow, has no effect on the LN size.

More sampling needs to be done to get more LN for histology, preferably from animals of different ages. In this way the histological findings in younger and older animals can be compared. Preferably, these samples should be taken from older animals of the same farms where the lambs for this study were sampled, so these data can be compared.

Discussion

This study was the first stage of an investigation of *MAP* infection rate in sheep and relationship with lymph node (LN) characteristics and circumference. In this study no LN were found positive for *MAP* after histological examination. This could mean that none of the sheep were infected with *MAP*, which would lead to the conclusion that the prevalence of *MAP* is less high than expected. The flock prevalence is estimated around 70% (Thompson, *et al.,* 2002, West, 2002). In deer was found that the prevalence on the Southern Island was higher than on the Northern Island (Glossop, *et al.,* 2008, Hunnam *et al.,* 2010 submitted). This might be the same in sheep.

Another explanation for the absence of histological findings in these LN may be the age of the sampled sheep. The disease has a long incubation time and it may take a few months or years before lesions appear (West, 2002). Clinical disease often does not occur in animals under an age of 2 years (Armstrong, 1956). There is not much known about the age when subclinical infected animals develop lesions. Since all sampled animals were lambs (less than one year old), it might be possible that the sampled animals were too young for showing histological lesions in their LN. Previous studies have shown different results. Begara-McGorum et al. (1998) experimentally infected two week old lambs and found that at 8 weeks post infection small-to-medium sized focal granuloma could be observed in jejunal and ileal Peyer's patches in four of the eight lambs. No other tissues were damaged and no AFO were found. Juste et al. (1994) found that experimentally infected lambs showed granulomatous lesions in the Peyer's patches and in the mesenteric and ileocaecal lymph nodes at 45 days after infection. These findings suggest that lesions can be found in lambs of young age. However, Reddacliff et al. (2003) experimentally infected recently weaned lambs (12 – 16 week old) and found no specific histological lesions in Peyer's patches or MLN in any of the confirmed infected animals in the first months post infection. In these studies all animals were experimentally infected with high doses of *MAP* in a short period of time, which is likely to lead to more extreme infections than natural infections with *MAP*. The fact that no lesions were found in a study with young experimentally infected animals may lead to the conclusion that it is even more unlikely to find lesions in naturally infected young animals. Unfortunately the exact age of the sampled lambs in this study was not recorded. This would have provided some more information about the appearance of lesions at different ages in subclinical infected animals.

The presence of histological lesions also depends on the histo-pathological form of infection. It would be more likely to find granuloma of macrophages filled with AFO in the 'multibacillary' form, than it would be in the 'paucibacillary' form (Turner *et al.,* 2005). It is a possibility that the lambs in this study were infected with a 'paucibaccilary' form, which may explain why so few AFO and granuloma were found.

Samples taken from animals of different ages would provide a lot of information about the sensitivity of histology. These additional samples should preferentially be taken from older animals on the same farms where the lamb LN for this research originated. If the finding of histological lesions for *MAP* is indeed age dependent, the sensitivity of this test is not very high. In that case a culture, PCR or ELISA test would be a better way to detect *MAP* in young animals (Worthington, 2004).

Lesions in the intestines are mostly located at the distal part of the ileum, the caecum, the proximal colon and the draining LN (Armstrong, 1956, Carrigan *et al.,* 1990). This suggests that the LN most likely to be affected are the ileocaecal LN (75% sample location). However, in this study these LN were found to be the ones with the smallest circumferences. It may be that the distal ileum and the caecum are not the area's that are primary affected by the disease. It could also be that the area that is drained by these LN is smaller compared to the other MLN.

In deer was found that 94% of the enlarged LN were *MAP* positive (Hunnam *et al.,* 2010 submitted). Some of the sampled sheep LN had a very large circumference although no histological lesions were found. Most of the LN showed some follicular hyperplasia, which suggests that the immune system was challenged by something. Apart from Johne's disease, there are more diseases and conditions that can cause enlarged LN. Follicular hyperplasia is caused by an antigenic stimulation from the intestines. This may be due to dietary antigens and commensal or pathogenic microorganisms (Hunnam *et al.,* 2010 submitted). This includes protozoan infections such as Eimeria spp. and parasitic infections such as Ostertagia, Trichostrongylus, or Fasciola hepatica (Glossop *et al.,* 2007). The first time the lambs are exposed to foreign material or parasites may cause a reaction of the immune system, presenting as LN being enlarged. It could be that the relationship between LN circumference and infection with Johne's is not as indicative in sheep as in deer. Culture and/or PCR will show if these LN are *MAP* infected or not. Until then no more can be concluded about the relationship between LN size and *MAP* infection in sheep.

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Appendix 1 – review of literature on lymph nodes

Lymph nodes are oval-shaped lymphatic organs that filter lymph before it returns to the blood stream, removing invading microorganisms and various other foreign materials. (Brown 1981, Saunders 2007). They usually have a slight indentation (Figure 7), the hilus, where blood vessels and efferent lymph vessels are located (Brown 1981). The afferent lymph vessels penetrate the rest of the encapsulated surface (Saunders 2007). Both afferent and efferent vessels have valves, ensuring that the filtered lymph will not be mixed with the lymph that still needs to be processed in the lymph node (Saunders 2007). The lymph node is organized into an outer cortex and an inner medulla (Brown 1981). The parenchyma of lymph nodes consists of B and T lymphocytes, antigen-presenting cells and macrophages (Saunders 2007).

Lymph nodes are surrounded by a capsule composed of collagenous connective tissue, collagen and reticular fibers. Trabeculae extend from the capsule into the parenchyma to form open-ended compartments that connect with the medulla (Saunders 2007). These trabeculae provide support for the lymph node and carry blood vessels and nerves (Brown 1981). The afferent vessels that have penetrated the capsule empty into the subcapsular sinus. From the subcapsular sinus several trabecular sinuses are formed that flow along the trabeculae toward the sinuses within the medulla (Saunders 2007). The medullary sinuses form anastomoses creating a network of branches that converge toward the hilus to empty into the efferent lymph vessels (Brown 1981).

The structure of the cortex is uniform, where it consists of spherically shaped lymphatic nodules and diffuse lymphoid tissue. Primary lymphatic nodules contain B lymphocytes entering or leaving the lymph node. Secondary nodules appear to be more lightly stained than primary nodules due to the presence of plasma cells and B lymphocytes. These nodules are also referred to as germinal centers and develop when a lymph node has been challenged by antigens (Saunders 2007).

Between the cortex and the medulla lies the paracortex which contains mostly T lymphocytes (Saunders 2007).

The inner part of the lymph node is the medulla, which is much less organized that the cortex. It contains the medullary cords that are composed of lymphocytes, plasma cells and macrophages. The medullary cords are separated by a network of sinuses and trabeculae. In the sinuses a meshwork of luminal stellate cells are present to slow the movement of new material (Saunders 2007). This facilitates antigen-cell interactions and enables phagocytic activities of the macrophages (Brown 1981). After antigenic stimulation the number of macrophages and plasma cells in the medulla increases. At the same time the germinal centers will develop in the cortex (Brown 1981).The various

organisms that enter the lymph node will be absorbed and digested by the macrophages. These macrophages can be found in the cortex, paracortex and medulla of the lymph node. Macrophages present antgens on their cell surface to the lymphocytes. Immunological islands contain a macrophage surrounded by several B- or T lymphocytes and facilitate this presentation of antigens. After this interaction both B- and T lymphocytes will be activated and enter the blood stream (Brown 1981).
Lymph nodes can vary in size and shape, influenced by what they have been exposed to. Lymph nodes associated with the digestive system might be more active than other lymph nodes, because of the frequency of exposure to foreign material (Saunders 2007).

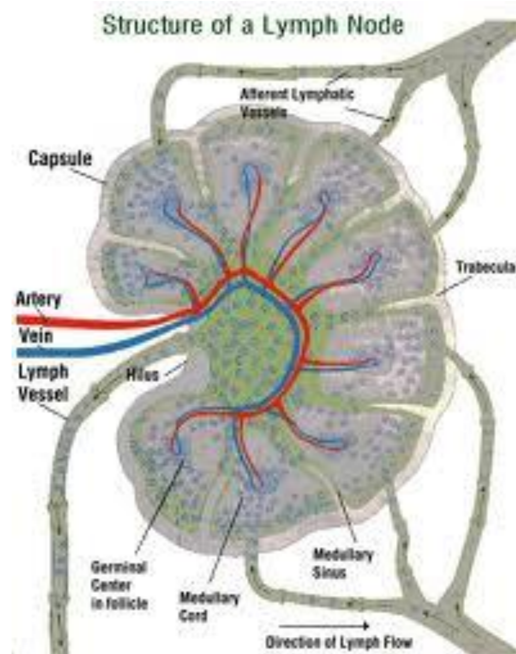

Figure 7: Schematic structure of a lymph node

Appendix 2 – Research protocol

Research Protocol

Title: JOHNE'S DISEASE IN SHEEP
Association between lymph node size and infection rate

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Preamble

Johne's disease in New Zealand sheep was first described by Williamson and Salosbury in 1952. Since then the disease has been diagnosed and confirmed on a lot more farms in both South and North Islands(Armstrong *et al.* 1956., Davindson,1970.).
Johne's Disease (JD, paratuberculosis) is a chronic progressive enteritis due to infection from *Mycobacterium Avium* subspecies *paratuberculosis (MAP).*(Turner *et al.*2005*)*
In ruminants it has become a worldwide problem and it has been recognized in many species such as sheep, cattle, deer, goats and more.(Turner *et al.* 2005).

Sheep infected with *MAP* may eventually develop some clinical signs. The sheep will lose condition, are weak and look emaciated. The wool pulls out easily and after the clinical symptoms appear, death can occur within a few weeks. The feces vary from diarrhea to firm pallets, but the diarrhea is less often seen compared to cattle. (Armstrong,.1956).
The majority of sheep with clinical signs are 3 to 4-year old and clinical signs in sheep under an age of 2 years are rare (Armstrong, 1956). Cattle develop an age-dependent resistance to infection with MAP (Chiodine, *et al.*1984), but little is known about the age that sheep are mostly infected. Not all animals exposed to *MAP* become infected, this depends on immune status and the quantity of bacilli ingested. (Wakelin, 1995). After ingesting an infective dose of the bacterium a long incubation period starts in cattle and sheep, which may explain why the clinical signs usually do not occur under 2 years of age (West, 2002). Some animals may never develop clinical signs and stay subclinical infected. These sheep appear to be normal, but are *MAP* infected and post mortal lesions may be present. These subclinical cases of JD constitute the majority of infections and are a very important aspect of the disease (de Lisle, 1970).
Post mortem examination of sheep with JD showed macroscopic lesions in the distal ileum, the caecum, proximal colon and the associated lymph nodes. The lymph nodes are oedematous and may be up to four times the normal size.(Armstrong,1956).

Tthis project will look at infection rate of *MAP* in sheep and the relationship with lymph node size. Hunnam *et al.* (2010 submitted) found that in deer over 94% of 'abnormal' lymph nodes were infected with *MAP*. 45% of the grossly 'normal' looking MLN in deer was found *MAP* positive.(Stringer *et al.*2010). This suggests that most animals that become infected will be subclinically infected and may not develop any clinical signs at all. Subclinical infection can be measured by fecal, gut and LN sampling. Tests used for detection include Ziehl Neelsen staining, culture, PCR and histology.(Worthington, 2004).

This pilot study will determine if there is a relationship between lymph node circumference and *MAP* infection rate. Mesenteric lymph nodes (MLN) will be collected from slaughter sheep and the lymph node circumference will be measured. Histology will be performed on the MLN and samples will be archived fresh frozen for future culture and or PCR tests, based on the outcome of the histopathology test.

The hypotheses for this research are

1) that it is possible to detect evidence of MAP infection in sheep lymph nodes histologically

2) that there is a relationship between lymph node size and histological evidence of *MAP* infection.

Materials and methods

Sheep will be sampled at a commercial sheep slaughterhouse at Feilding. A flock is defined as sheep farmed at a single location. A line is defined as a shipment of sheep sent for slaughter from the same flock to the same slaughterhouse on the same day. Female sheep are referred to as ewes and male sheep as rams.

The sample size has been determined according to standard population sampling procedures, based on an estimate 16% prevalence of *MAP* infection. A minimum of 172 LN should be sampled, but to compensate the intra-chain correlation, this minimum was multiplied with 2,8 (VIF), which makes the minimum of samples 480 LN (10/line). With 10 samples per line, there will be an 80% chance to detect a positive line.

All tissues will be collected by sterile technique using new equipment for each sample. Fresh samples will be kept chilled in an insulated container.

Herd, age, sex and LN location and colour (normal, red, brown, green, yellow, white, other) will be recorded at the time of sampling. Age will be aggregated into young(lamb) and adult and combined with sex into categories of young ewes, young rams, adult ewes and adult rams.
The circumference of each lymph node will be measured using a flexible measuring tape.

For the histology a 5 to 8 mm wide section will be used at 25% 50% en 75% of the mesenteric chain length. The sections will be stored individually in 10% buffered formalin. Fixed samples will be dehydrated, cleared and embedded in paraffin. Sections will be stained with Haematoxylin and Eosin(H&E) and Ziehl-Neelsen (ZN). Slides will be examined under low (25x), medium (100x) and high-dry (400x) magnification using a …microscoop. Sections taken for culture and PCR will be stored at -80.

N/25 is the 25% sample
N/50 is the 50% sample
N/75 is the 75% sample(ileocaecal)

The culture and PCR sample will be taken from the ileocecal LN, because this LN is the most likely to be affected.

Sample ref	**Kill no.**	**Node**	**Diameter**	**Lymphvessel**	**Other**
LN 1/25					
LN 1/50					
LN 1/75					

Node: 0 if the LN is normal, 1 if the LN is abnormal
Lymphvessel: The presence of enlarged lymph vessels on the serosa of the distal ileum

Other: space for comments. Color, shape, lesions etc. With a scale of +(a bit affected),

++(affected) and +++(very affected)

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