Prevalence of hepatic epithelioid macrophage microgranulomas due to *Mycobacterium avium* subspecies *paratuberculosis* in Lambs on the North Island of New Zealand.

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Abstract

Paratuberculosis causes an infectious wasting condition of domestic cattle and other ruminants with heavy economic losses to domestic livestock industries worldwide. Paratuberculosis is caused by an infection with Mycobacterium avium subspecies paratuberculosis (MAP). Hepatic epithelioid macrophage (HEM) microgranulomas are found in some animals that are infected with MAP. This study, as part of a larger study, was conducted to give insight regarding the prevalence of HEM microgranulomas in lambs on the Manawatu region on the North Island of New Zealand.
In total 400 liver samples of mixed breed lambs aged between 4 and 8 months were biopsied, stained and checked for HEM microgranulomas. No HEM microgranulomas were found in any of the 400 samples taken. Based on the current sample, with 95% certainty, the prevalence of HEM microgranuloma's and MAP type 3c or 3b type lesions in lambs between 4 and 8 months old in the Manawatu region on the North Island of New Zealand is at least less than 0,75%. Further study is needed to give a better insight regarding the prevalence and etiology of MAP in lambs.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, hepatic epithelioid macrophage microgranulomas, prevalence

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a gram-positive, acid-fast organism that causes an infectious wasting condition, also known as Johne's disease (JD) , of domestic ruminant species including deer, sheep, and cattle (Harris & Barletta, 2001). MAP causes a chronic gastrointestinal infection characterized by protein-losing enteropathy. It leads to chronic diarrhea, wasting and could ultimately be fatal in the later stages of the disease mostly on adult ruminant species. However, a large proportion of infected animals remain subclinical infected, with no measurable effect on production (Nielsen & Toft, 2008).

MAP is responsible for heavy economic losses to domestic livestock industries worldwide due to loss of condition, infertility and lower milk production (Benedictus, Dijkhuizen, & Stelwagen, 1987; Stabel, 2015). Estimated losses because of Johne's disease are from $22 to more than $200 per cow inventoried per year, based on the percentage of culled cows with clinical signs (Ott, Wells, & Wagner, 1999).

The primary mechanism for the transfer of *Mycobacterium avium* subspecies *paratuberculosis* from one animal to another is fecal oral transmission (Windsor, PA, 2015). After infection, the animal's cellular immune response leads to granulomatous enteritis. This results in a thick and corrugated intestinal wall mainly in the ileum, but also in the jejunum. As a result of MAP bacteremia and host cellular immune response, sheep with clinical MAP infection will develop epithelioid macrophage microgranulomas in the terminal ileum, mesenteric lymph nodes and other organs (Bower, Begg, & Whittington, 2011; Smith, 2014).

Research by Smith (2014) investigated whether sheep with clinical Johne's disease also show hepatic epithelioid macrophage (HEM) microgranulomas in the liver. If so, could liver biopsy histopathology provide an opportunity to be a potential diagnostic marker. They concluded that sheep with type 3b and 3c type lesions typical of MAP infection, as shown below, could be identified using biopsy core samples for histopathology with a sensitivity and specificity of 96% (95% confidence interval [CI], 0.87-0.99) and 100% (95% CI, 0.95-1)(Smith, 2014) .

Tabel 1. Criteria for Classification of Lesion Types Associated with Natural Paratuberculosis Infection in Sheep (Perez, Marin, & Badiola, 1996; Smith, 2014).

Classification	Definition and Histopathological Description
Type 1	Microscopic lesions only with few granulomas in ileum Peyer's patch, none extending into the mucosa of intestine or jejunum at Peyer's patch or between lymphoid tissue of the ileum Peyer's patches. Few granulomas found in regional mesenteric lymph nodes. No acid-fast organism (AFO) found in either ileum Peyer's patch or mesenteric lymph node.
Type 2	Microscopic lesions only. Multiple granulomas extending into lamina propria mucosae and villus of ileum at the Peyer's patch. Nondiffuse with ileum villus integrity maintained. No lesions in mucosa of ileum outside Peyer's patch lymphoid tissue areas. Similar mesenteric lymph node lesions as Type 1. AFO detected in very few granulomas of ileum Peyer's patch.
Type 3 lesions	Granulomas present in large numbers within the lymphoid tissue of the Peyer's patch, also extending into the mucosa between Peyer's patches including villi. Macroscopic lesions with 3 different subtypes:
Type 3a	Multiple granulomas extending into mucosa between Peyer's patches and causing the villi to enlarge. Always found in ileum and less frequently in jejunum. Lymphocytic and macrophage infiltration around lymphatic and blood vessels of submucosa and serosa. Granulomas always present in mesenteric lymph nodes. Lymphangitis visible in ileum serosal lymphatics. AFO present in ileum mucosal sections and Peyer's patches.
Type 3b	Diffuse granulomatous enteritis with gross mucosal thickening and corrugation with lymphangitis of serosal lymphatics. Oedematous and swollen mesenteric lymph nodes with lymphatic cording over ileum and jejunum. Peyer's patches lose structure due to macrophage dominant granulomas. The lamina propria and mucosa between Peyer's patches has diffuse enteritis with swelling, blunting, and coalescing of villi in ileum through to jejunum. Submucosa lymphocyte and plasma cell infiltrates are common, lymphadenitis with macrophage thrombi present with infiltration of blood vessels and smooth muscle layers of the ileum and jejunum. Mesenteric lymph node and ileocaecal lymph node lost structure with multifocal granuloma formation. Numerous AFO throughout mucosal tissues and mesenteric lymph nodes.
Type 3c	Diffuse granulomatous enteritis. Similar lesions to Type 3b although the predominant inflammatory cell is lymphocytic infiltration of the lamina propria of Peyer's patches and areas between the Peyer's patches including the villi with less macrophage infiltration. Formation of mesenteric lymph node Langhans multinucleate giant cells. AFO absent in most tissues or present in small numbers. Macroscopically similar to Type 3b.

One of the main reasons further research about MAP is important is the possible association between MAP and Crohn's disease, a chronic incurable bowel disease in humans with pathological similarity to Johne's disease of animals (Mishina, Katsel, Brown, Gilberts, & Greenstein, 1996).
MAP has been isolated in different percentages of patients with Crohn's disease, depending on the research. Sanderson et al. (1992) identified MAP in 26 of 40 (65%) Crohn's disease patients and 15 out of 20 (75%) patients with irritable bowel syndrome (Sanderson, Moss, Tizard, & Hermon-Taylor, 1992)
Scanu et al. (2007) isolated MAP in 15 of 20 (75%) patients with irritable bowel syndrome and in 20 of 23 (87%) people with Crohn's disease (Scanu et al., 2007). A review article by McNees, Adrienne L., et al (2015) found a variable frequency (46%-100%) of detected MAP DNA in blood and biopsies of patients with Crohn's disease (McNees, Markesich, Zayyani, & Graham, 2015) .

If a causative link was found between *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease, it would have serious consequences for many countries, including New Zealand, whose economy strongly depends on cattle and sheep industry. New Zealand's red meat exports for 2017-18 exceeded NZ$6.7 billion, where lamb meat export alone was over NZ$3.1 billion (B+LNZ, 2018). Given the fact that in New Zealand, the estimate prevalence of MAP infection for sheep flocks is 76% (posterior probability interval (PPI) 70-81%) and 42% (PPI 35-50%) for beef herds (Verdugo et al., 2014). Johne's disease would change from a disease that affect animal welfare and economic losses, to a disease with potential public health hazard and even bigger economic losses due to possible export stop.

Aim of the study

The objective of this study was to determine the prevalence of hepatic epithelioid macrophage microgranulomas in young lambs by taking liver biopsies from livers in a New Zealand abattoir. At the same time, for other research, a non-invasive light emitting spectroscope was used to see whether spectral imaging can identify these pathological lesions in real time analysis. Both studies are part of a larger ongoing study about hepatic epithelioid macrophage (HEM) microgranulomas and Johne's disease.

Materials and methods

Method of sampling
Sampling took place in the Ovation slaughter premises in Feilding New Zealand. 400 lamb liver samples from 9 random North Island New Zealand farms were randomly sourced on that day. Liver samples were taken were from outward healthy mixed breed lambs between 4 and 8 months that were booked into the kill sheet at the slaughterhouse at that day. No Johne's vaccination was undertaken on these farms or these lambs. These farms have an unknown Johne's status, since they would have required a special tag during the slaughter process if vaccination took place or clinical Johne's was established.
Samples were collected from lamb livers in the offal processing room after they were inspected and washed, immediately prior to being placed in the 15°C chill water baths.

At least 20 livers from each farm and every second liver were collected as it arrived in the processing room until 400 samples were taken.
Each liver was biopsied with a 3mm inner diameter liver biopsy probe (made by Shoof International NZ) from the dorsal serosal surface. The 20mm x 3mm core was then placed into a chilled sterile plastic vacutainer and fixed in 10% formalin in phosphate buffered saline (PBS) awaiting histological preparation away from the Ovation premises.

Histopathology
Samples were taken to Massey university laboratory and processed using sterile instruments. Whole samples were fixated in a tissue processing cassette and placed in 10% PBS buffered formalin. Formalin-fixed tissue samples were dehydrated with graded ethanol and embedded in paraffin blocks. Each core was then sectioned with alternate sections discarded using a 4 µm microtome. Serial longitudinal sections at 4 mm subserosal depth intervals were taken and serially mounted onto 2 glass slides (3 sections per slide) with all 6 sections stained using hematoxylin and eosin stain (HE), giving approximately 360mm^2 liver surface area to examine.
Histological examination was carried out at 10x magnification looking for HEM micro-granulomas (which are present in all sheep with clinical Johne's disease specific type 3b or 3c type lesion in the small intestine (Smith, 2014)). Histopathology was performed blinded to the identity of the lambs.

Statistics

To be able to find the largest prevalence (p) with the probability of no successes out of n trials, $(1-p)^n = 0.05$ for p needs to be solved. Taking logs of both sides, $n \log (1-p) = \log(0.05) \approx -3$. Therefore, since $\log(1-p)$ is approximately $-p$ it could be said that $p \approx 3/n$. This only counts if $n>30$.
If there are no results in n trials, with a 95% confidence interval it could be said that the prevalence have to be lower than 3/n (if n>30) (Van Belle, 2011 ; Eypasch, Lefering, Kum, & Troidl, 1995).

Results

When present, HEM microgranulomas would present themselves, as shown in figure 1, as a lesion pattern uniform throughout the liver (Smith, 2014).
Of the 400 liver samples taken randomly, none of the samples were found to be positive for Hepatic epithelioid macrophage micro-granulomas (Figure 2 and 3)

	HEM +	HEM -	Total
Samples	0	400	400

With 0 results in 400 trials it could be said that the prevalence of HEM microgranulomas in Lambs in the Manawatu region on the North Island of New Zealand is at least lower than 3/n = 3/400 = 0,0075 = 0,75%.

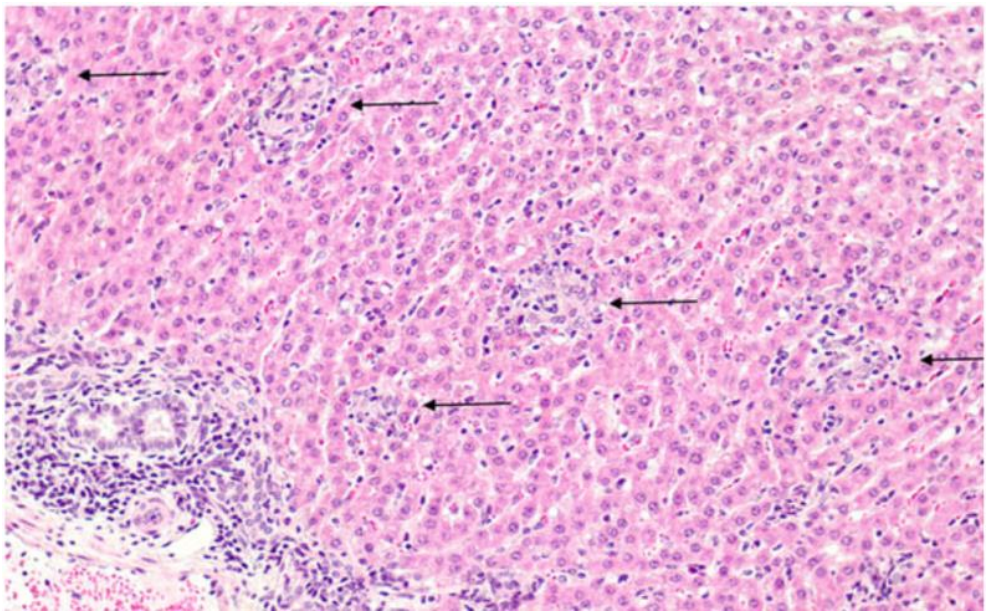

Figure 1. "Liver, sheep: cholangitis and granulomas containing epithelioid macrophages. Ewe had type 3b ileal lesions and clinical Johne's disease. HE " (Smith, 2014).

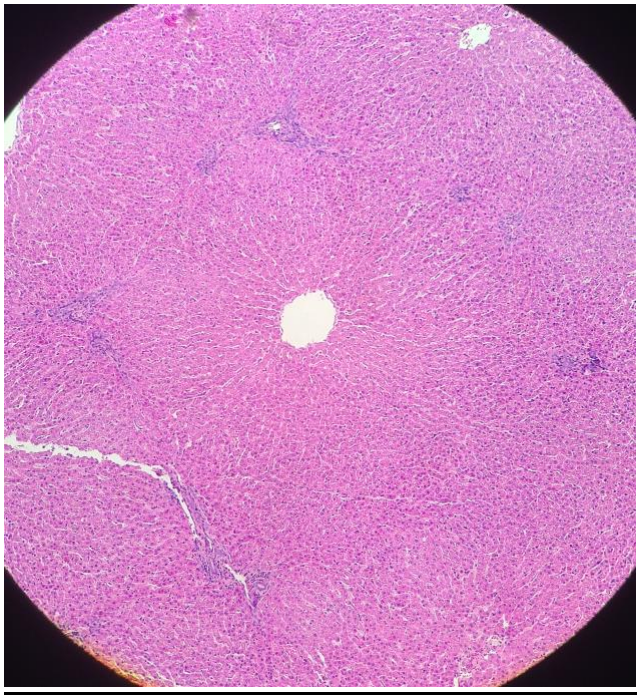

Figure 2. representative view of random taken sample. Normal, healthy liver tissue can be seen

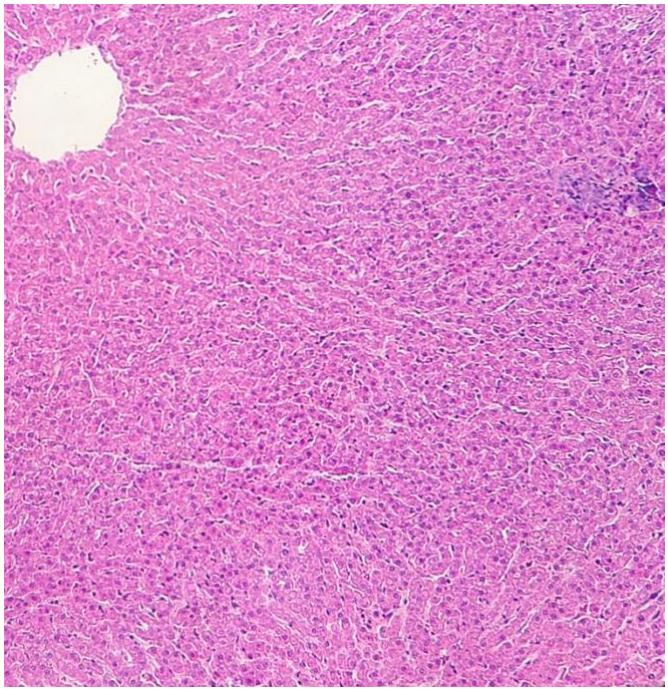

Figure 3. Close up of same picture.

Discussion

As mentioned before, if a causative link was found between *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease, export of meat from New Zealand would be stopped. Therefore lambs were chosen to be tested for HEM microgranulomas, for reason that lamb meat accounts for almost half of the exported red meat production (NZ$ 3,1B vs NZ$ 6.7B total (B+LNZ, 2018)). In case of an export stop, the industry would be hit the hardest in this branch.
The focus of this research and the research parallel to this one was to get an insight in possible *HEM microgranuloma's* in Lambs and if so, could this be detected by a non-invasive light emitting spectroscope.
The results of the study were that no HEM microgranulomas were found in any of the 400 samples taken. Therefore, as shown above, the prevalence of HEM microgranulomas could be calculated to be at least lower than 0,75%.

Multiple errors were made making assumptions and calculating sample size which might be the explanation for not finding any HEM microgranulomas.
The prevalence (p0=0,13%) used in the calculation for sample size before the research, is the prevalence of MAP in ewes and not the prevalence of HEM microgranulomas in ewes. As stated below the prevalence of HEM microgranulomas in ewes would be many times lower compared to the prevalence of MAP.
The assumed prevalence of MAP is calculated by Verdugo et al., 2014 to be 0.13 (13%) and not 0.13% as was used to calculate sample size (Verdugo et al., 2014). With this finding (0,13%), prevalence of HEM microgranulomas, in our research, was estimated to be at least 1% in random taken lambs (p1). It was assumed that the prevalence of HEM microgranulomas in lambs will be higher because they need less MAP intake, compared with ewes, to overwhelm their cell mediated immunity. Thus with same MAP intake lambs will show more extended lesions than ewes. Delgado et al, 2013 showed that, with high dose experimentally infected ewes and lambs, all lambs had type 3a or 3b type lesions while only 1 ewe showed 3a type lesions (Delgado et al., 2013).
One percent prevalence was taken as incidence of the study group, to calculate the sample size needed. A standard power of 80% was needed for the research. To reach this level of power, with the estimated prevalence, at least 315 samples of liver biopsies needed to be taken and investigated, as shown in figure 4 (Hajian-Tilaki, 2011; clincalc.com, 2019). This calculation used to find the sample size needed for this research, as shown in figure 4, compares the incidence of the population to the incidence of the study group. With this calculation it can only be stated that with at least 315 samples of lambs taken, there would be a difference visible in prevalence between the 2 groups. This would mean a comparison is made between the research group and the population which is different from the prevalence of a disease in a population.

$$N = \frac{p_0 q_0 \left\{ z_{1-\alpha/2} + z_{1-\beta} \sqrt{\frac{p_1 q_1}{p_0 q_0}} \right\}^2}{(p_1 - p_0)^2}$$

$$q_0 = 1 - p_0$$

$$q_1 = 1 - p_1$$

$$N = \frac{0.0013 * 0.9987 \left\{ 1.96 + 0.84 \sqrt{\frac{0.01*0.99}{0.0013*0.9987}} \right\}^2}{(0.01 - 0.0013)^2}$$

$$N = 315$$

p_0 = proportion (incidence) of population
p_1 = proportion (incidence) of study group
N = sample size for study group
α = probability of type I error (usually 0.05)
β = probability of type II error (usually 0.2)
z = critical Z value for a given α or β

Figure 4. Calculation of sample size (clincalc.com, 2019)
Standard Z value of 1.96 was taken (confidence of 95%). Standard values for α and β were taken. Respectively 0,05 and 0,2.

Using a different calculation by Naing, Winn & Rusli, 2006, figure 5, only using the expected outcome of 1% prevalence of HEM microgranulomas, the outcome would be in lambs a different sample size.
With an expected prevalence (*P*) of 1%, a confidence (*Z*) of 99% and a precision (*d*) of 0,005 the sample size would be 2636. A precision of 0,005 was chosen because the prevalence was expected to be lower than 10% therefore a precision of half the expected prevalence is taken (Naing, Winn, & Rusli, 2006). A higher confidence of 99% (Z value is 2.58) instead of 95% (Z value is 1.96) was taken, also because of a low expected prevalence. The difference of 6,5 times the taken sample size of 400 could explain not finding any HEM microgranuloma's in the lamb samples taken (Naing, Winn & Rusli, 2006).

$$n = \frac{Z^2 P(1-P)}{d^2}$$

where n = sample size,
Z = Z statistic for a level of confidence,
P = expected prevalence or proportion
(in proportion of one; if 20%, $P = 0.2$), and
d = precision
(in proportion of one; if 5%, $d = 0.05$).

Figure 5. Calculation sample size by (Naing, Winn, & Rusli, 2006).

The prevalence of *Mycobacterium avium* subspecies *paratuberculosis* at an ovine herd level on the North Island of New-Zealand in previous studies was found to be 80% (PPI 71–87) (Verdugo et al., 2014) and 74% (PPI 64% – 76%)(Heuer, Wilson, & Larking, 2011).
On individual level, poor sensitivity and specificity of diagnostic tests for MAP on live animals is a limitation, especially for early or subclinical infections (Nielsen & Toft, 2008; Bower et al.,

2011; Smith, S. et al., 2013). Therefore only 1 study could be found, which calculated the true prevalence of MAP in sheep, finding a prevalence of 0.13 (13%) in sheep (Verdugo et al., 2014).

HEM microgranulomas are only found in systemic infected sheep with type 3b or 3c type lesions (Smith, 2014). Research on herd infected sheep flock showed that type 3b and 3c type lesions were found in 13,9% (type 3b) and 2,4% (type 3c) in 166 adult sheep with proven herd level faecal MAP shedding (Perez et al., 1996). All 4 herds used in this study, at least 2 sheep of each herd had already been diagnosed with Johne's disease. Therefore the prevalence of type 3b and type 3c lesions would be lower on random chosen sheep which might be an explanation for not finding any lesions.

Smith (2014), found a prevalence for type 3b and 3c type lesions to be 31,7% (type 3b) and 8,7% (type 3c) in 126 sheep from 7 North Island New Zealand farms, with a history of clinical JD. An explanation for this high prevalence could be that they were actively looking for sheep with JD, they only selected sheep older than 3 years of age with a body condition score <1.5 (scale 1–5)(Smith, 2014) . Therefore this would not be a representative prevalence in randomly taken sheep.

The prevalence of HEM microgranulomas (out of the studies mentioned above, combined) could be stated to be between 2.2% (16,9% of 13%) and 5,3% (40,4% of 13%). Therefore it would be higher than the 0,75% in our study. An explanation could be that in the study of Verdugo et al., 2014, the prevalence of 13% was found in flocks that were positive for paratuberculosis (78%). In the current study, the Johnee status of tested Lamb flocks was unknown.

Age could be another explanation for not finding any HEM microgranulomas. Many studies agree that younger animals are more susceptible for infection with MAP than adult animals (Payne & Rankin, 1961; Larsen, Merkal, & Cutlip, 1975; Windsor, Peter A. & Whittington, 2010; Delgado et al., 2013) but few describe pathogenesis of MAP related to age. Payne and Rankin (1961), experimentally infected 8, less then 3-month-old calves with MAP. 2,3,4 and 6 months after infection they killed 2 calves each month and described the lesions. None of the calves showed macroscopic lesions in the intestine. Therefore, we can conclude that none had type 3 like lesions and none would have had HEM microgranuloma's in the liver. A difference could be seen between adult cows and calves. Adult cows had more widespread and severe lesions in an earlier stage but recovered from them. This suggests there may be an age resistance to Johne's disease (Payne & Rankin, 1961).

Another study showed similar results to age resistance. Among 13 ewes infected only 1 showed multiple granulomatous lesions, classified as type 3a lesions.

Among 8 lambs, infected at 1 month of age and euthanized at 120- and 220-days post infection, 2 lambs showed type 1 or 2 type lesions, 2 lambs showed 3a type lesions and 4 showed type 3b lesions (Delgado et al., 2013).

The same study also found a relationship between the dose of colony- forming units (CFU) needed to infect a sheep. Of all infected sheep and lamb, only those infected with high-dose suspension (4x106 CFU Map/ml) showed histologically granulomatous lesions typical of paratuberculosis. No lesions were found in low dose infection or control group (Delgado et al., 2013). Suggesting not only age but also the dose needed for infection plays a role in the pathogenesis of *Mycobacterium avium* subspecies *paratuberculosis.*

Most studies to determine lesions related to age used experimentally infected animals. Unfortunately, this is not equal to naturally infected animals. In experimentally infected animals, in early stages, small granulomas were only located in the intrafollicular

areas of the Peyer's patches. The lesions were mainly seen in the ileum and it was difficult to determine bacilli in these lesions. In naturally infected animals these lesions have not been seen (Perez et al., 1996)

Missing HEM microgranulomas by microscopic searching is a small possibility. Research findings by smith 2014 suggested a uniform lesion pattern throughout the right cranio-dorsal lobe of the liver. They found equal numbers of HEM microgranulomas per 200 mm^2 of liver (Smith, 2014) . This suggests if HEM microgranulomas would be present in the livers that were biopsied, HEM microgranulomas would be clearly visible, in big numbers and multifocal, on examination by microscope of HE stained liver biopsy slides.

Conclusion

Because of multiple errors were made, more research is needed to specify the prevalence of HEM microgranuloma's in Lambs. Although no HEM microgranuloma's were found with 400 samples taken, it could be said, with 95% certainty, that the prevalence of HEM microgranuloma's in lambs in the Manawatu region on the North Island of New Zealand is at least lower than 0,75%.

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