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Systemic *Mycobacterium avium* subspecies *paratuberculosis* infection in sheep.

A thesis presented in the fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Science at Massey University, Palmerston North, New Zealand

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Abstract

The systemic infection of organs and skeletal muscle outside the alimentary tract with *Mycobacterium avium* subspecies *paratuberculosis* (Map) has sparingly been mentioned in the many scientific studies undertaken in sheep, yet within the past decade a zoonotic association has been proposed. The occurrence of systemic Map infection at the time of slaughter might enable this organism to be present in food products, such as meat, destined for human consumption, creating a potential link to public health and may therefore attract some attention by the meat industry. There have been very few studies investigating whether meat has potential to expose humans to Map. With this lack of information, it is difficult for the meat industry to make informed decisions in the event that public perception establishes a link with Crohn's disease. Chapter one provides a brief history of Map infection in ruminants and suggests there may be a need to identify steps that could be implemented to mitigate human exposure to Map. The aims for this thesis therefore were to i) determine whether skeletal muscle from naturally infected animals provides a source of Map for humans, ii) provide information on systemic Map infection in sheep, identifying classes of stock that may pose a risk for exposure iii) develop a histological diagnostic test for quantifying the cost of systemic Map infection in sheep with potential use in therapeutic efficacy studies, and iv) provide a potential means to mass screen sheep at time of slaughter using real time spectroscopy to identify systemically infected animals.

Chapter two reviews the source of Map, transmission pathways and subsequent availability of modern diagnostic tests for identifying sheep infected with this organism. There is a lack of published information on systemic Map infection, with little known about how this event develops, how the immune system reacts when Map bacteraemia occurs, whether systemic

Map infection has a cost to production and whether quantification of this cost can be assessed with currently available diagnostic tests.

The aim of Chapter three was to determine whether skeletal muscle from ewes with clinical Johne's disease contained Map and therefore provided a potential source of Map for humans. Fifty one mixed-age, low body condition score ewes (1.5/5), from a farm where clinical Johne's disease had been diagnosed, were necropsied. This included 48 ewes with Map infection confirmed by ileal BACTEC radiometric culture and 21 with clinical Johne's disease confirmed by ileal histopathology. In 18 ewes with clinical Johne's disease, Map was found in the culture of blood (n=13), blood and muscle (n=10) and muscle (n=5). In ewes without clinical Johne's disease, Map was found in 5/30 animals including muscle (n=4) and blood (n=1). It was concluded that meat from ewes with clinical Johne's disease is likely to contain Map and suggested that systemic Map infection may also occur in sheep without clinical disease when managed in direct contact with clinically affected ewes shortly before slaughter.

The presence of Map within skeletal muscle was further investigated in Chapter four with 24 healthy mixed age ewes selected from one farm, which were not in contact with clinically affected ewes. Ileal and mesenteric lymph node cultures identified Map infection in 12/24 ewes. All other tissues and faeces were culture negative, and only 1/24 animals sero-converted. In flocks where Map is present, it appears that up to 50% of animals may be latently infected. Lack of positive culture from blood and muscle samples in latently infected sheep suggests that meat from healthy sheep may not be a source of human exposure to Map.

In New Zealand, the current measure to mitigate human exposure to Map from meat products is the identification of clinically affected sheep prior to slaughter through ante-mortem inspection with emaciated animals rejected at time of slaughter and processed as pet food.

However, this screening process is non-specific with many different causes of emaciation. Currently there are no legal requirements or recommendations from the meat industry for the downgrading of meat from carcases with macroscopic signs of clinical Johne's disease and, as such, meat from these sheep enters the human food chain. Identifying sheep with systemic Map infection is problematic, with diagnosis requiring solid or liquid media culture of Map or polymerase chain reaction (PCR) to identify Map specific DNA. These diagnostic tests are expensive, time consuming and require a high level of expertise. They are therefore unlikely to be adopted by the meat industry as a screening tool for systemic Map infection in sheep. With the aim to develop a diagnostic tool that is relatively quick, simple and cheap, 126 mixed age ewes in poor body condition were euthanised as described in Chapter five and their Johne's disease status determined through histopathology and Ziehl Neelsen stain of the ileum and mesenteric lymph nodes. Sixty ewes were differentiated histopathologically with 51 clinically affected including Type 3b (n=40) and 3c (n=11) and nine not clinically affected with Type 1 (n=5), Type 2 (n=3) and Type 3a (n=1) ileal lesions. Hepatic epithelioid macrophage micro-granulomas (HEM) were observed only in ewes with Type 3b or 3c ileal lesions, all of which were ELISA positive. When present, HEM were in equal densities in liver section and biopsy samples. The sensitivity and specificity for liver histopathology (section or biopsy) for predicting clinical OJD was 96% (95% CI, 87-99%) and 100% (95% CI, 95-100%), respectively, and Cohen's Kappa had an almost perfect level of agreement between HEM formation, ileal pathology and ELISA sero-positivity. This study determined that the presence of HEM provided a surrogate measure of ileal pathology, identified ewes with clinical Johne's disease, and that biopsy samples and post mortem sections were equally suitable for the diagnosis of HEM.

Encouraged by the predictive quality of HEM in Chapter five, it was hypothesised that the identification of HEM from biopsies may provide a method to follow the progression of Map

infection through serial sampling and to quantify the production cost of systemic Map infection. The longitudinal challenge study in Chapter six utilised the identification of HEM as an indicator of systemic Map infection in naïve lambs orally challenged with 1 x 10^9 organisms on ten occasions over 30 days. The presence of HEM was related to live weight gain, body condition score, development of clinical disease or occurrence of self-cure (recovery), and ELISA serology All challenged lambs developed HEM, a higher density of HEM was associated with increased ELISA S/P ratios with a Cohen's kappa substantial level of agreement, and mean weight loss (-2.03kg) from 51 to 154 days post challenge with an almost perfect level of agreement. Thereafter, lower weight gain led to a mean body weight difference of -8kg at 195days compared to non-challenged lambs. Four challenged lambs had to be euthanised due to clinical OJD. After this period, the HEM density and ELISA S/P ratios declined, growth rates increased in the challenged lambs up to 482 days after which no HEM were detected and growth rates were equal between challenged and unchallenged groups. The challenged lambs failed to regain equivalent weights over the 820 days being 11kg lighter at the end of the study despite having equal body condition scores. The challenged lambs were smaller than the unchallenged lambs both in body height and length with multivariate ANOVA analysis determining the post mortem mean skeletal measurements of the poll to rump length and metacarpal/meta-tarsal bones being 4% and 5% shorter, respectively. There were no positive ELISA blood samples or histopathological lesions in any tissues sampled at necropsy from both groups of lambs at the end of the study, suggesting complete cure of the surviving challenged lambs. The findings demonstrated i) that artificial challenge can cause systemic Map infection, ii) systemic infection results in negative growth rates and a loss of body condition, iii) and in addition to the period of retarded growth losses occur from death of some lambs (4/18), iv) that the temporary poor weight gain impacted on the final weight, and v) that recovery to systemic Map infection

appears to occur in survivors of acute disease. Moreover, it was postulated that the identification of HEM from serial liver biopsies may have the potential to determine the therapeutic efficacy of new anti-mycobacterial drugs (such as thalidomide, Appendix one) or vaccines for preventing systemic Map infection.

Chapter seven revisits the histopathological findings described in Chapter five, expanding from the microscopic visual identification of HEM to utilising spectroscopy and hyperspectral image analysis. The aims of this final study included identifying whether a spectral signature for skeletal muscle or liver exists in sheep with Johne's disease and developing an algorithm that can identify the presence of systemic Map infection in sheep. Ninety five mixed aged ewes, of low body condition score from nine farms were euthanised and OJD was confirmed by histopathology in 10 animals. The liver and transected longissimus dorsi muscle were scanned using a visible light to near infrared (Vis-NIR) detector as well as 200 lamb livers from a slaughter house. The histological identification of HEM was used as a surrogate measure of systemic Map infection with HEM recorded in the 10 ewes with Johne's disease and none of the 85 ewes without or the 200 lamb livers. There was no histopathological or hyperspectral differences identified for the transected longissimus dorsi muscle in the 95 ewes. However a a computer generated algorithm identified a hyperspectral signature for liver tissue that when applied, blind to the Johne's disease status of the ewes was able to differentiate all 10 animals with Johne's disease from the 85 ewes and 200 lambs without. This pilot study suggests that spectroscopy may have potential to be a useful real time tool for the identification of sheep with systemic Map infection at the time of slaughter.

In conclusion, disseminated Map infection does occur in sheep with clinical OJD, and meat from these animals can be a source of Map for humans. Meat from healthy sheep or sheep without clinical OJD does not appear to expose consumers to Map. The identification of

HEM in liver biopsies has diagnostic value for identifying sheep with clinical OJD. In naturally infected sheep, HEM only appear when Map infection has progressed to clinical OJD. As opposed to high–dose artificial challenge, systemic Map infection under natural challenge conditions appears to require ileal pathology, suggesting different mechanisms for the occurrence of systemic infection in these two challenge types. Examination of serial liver biopsies and the identification of HEM has enabled the investigation of the production loss due to temporary progression and subsequent resolution of Map infection. The final study in this thesis has tested proof of concept for a new real time diagnostic test that has potential to mass screen sheep within abattoirs at point of slaughter using spectroscopy and hyperspectral analysis. However further research is required to validate this spectroscopic test.

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