Final Report

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Project title: Epidemiological investigation of Spotty Liver Disease in chickens to inform disease control

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In submitting this report, the researcher has agreed to Poultry Hub Australia publishing this material in an edited form.

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Project Summary

Project Title	**Epidemiological investigation of Spotty Liver Disease in chickens to inform disease control**
Project No.	**2018-432**
Date	Start: August 2018 End: June 2019
Project Leader(s)	Dr Thi Thu Hao Van
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Project Aim	This project's aim was to determine the dynamics of *Campylobacter hepaticus* spread within flocks, and identify possible transmission routes in layer farms by investigating the presence of the bacterium in the birds and environmental samples.
Background	Spotty Liver Disease (SLD) is an emerging disease in the Australian poultry industry. It causes significant egg production losses and mortality, especially around peak lay, but can also occur at any stage throughout the production period. The cause of the disease, *C. hepaticus*, was confirmed around 3 years ago but there are no publications on the epidemiology of SLD. This project aimed to investigate the spread of *C. hepaticus* within flocks and identify potential environmental sources of *C. hepaticus.* The goal was to provide information that would help in flock and environmental management to reduce the impact of SLD.
Research Outcome	We found that birds as young as 12 weeks could be infected with *C. hepaticus*, and SLD outbreaks occurred mostly at peak laying period (at 26 weeks) Birds should be regularly monitored for the presence of *C. hepaticus* so that action can be taken to minimise SLD outbreaks. In addition, environmental sources may play an important role in *C. hepaticus* dissemination, especially wild birds, soil, dust and water. The bacteria causing SLD in various farms across the country share similar genome sequences, suggesting that there has been a horizontal transmission among flocks.
Impacts and Outcomes	The results will help the poultry industry in the monitoring and management of SLD

Publications	A paper entitled "*Campylobacter hepaticus* causing Spotty Liver Disease in Chickens: Transmission and routes of infection" has been prepared and submitted for publication.

Executive Summary

This project's aim was to determine the dynamics of *Campylobacter hepaticus* spread within flocks and identify possible transmission routes of *C. hepaticus* in layer farms by investigating the presence of the bacterium in birds and environmental samples. The identification of its transmission routes will assist in developing appropriate biosecurity measures and will help in flock and environmental management to reduce the impact of SLD.

In three farms in Victoria, a total 1007 samples were collected from birds and 69 environmental samples were collected. Birds from each flock were sampled at 2-4 weeks intervals, commencing before the birds were placed into laying farms (16 weeks old), until peak laying period (26-30 weeks). Samples from these main surveyed farms were collected by Scolexia veterinarians. In addition, a total of 1,106 samples from chickens and 53 environmental samples were collected from 56 collection points from different farms around Australia to assist the understanding of SLD epidemiology. Sixteen *C. hepaticus* isolates were subjected to whole genome sequencing to examine the conservation or divergence of *C. hepaticus* genomes in different outbreaks.

We found that birds could be infected with *C. hepaticus* long before laying starts, as young as 12 weeks old, but the peak period for SLD outbreaks was when the birds were 26-27 weeks old. As previously suggested, physiological changes associated with the commencement of laying appears to be a predisposing factor in the emergence of SLD in *C. hepaticus* infected birds.

In one farm birds were infected with *C. hepaticus* at least 8 weeks before clinical SLD became obvious, and SLD occurred when the birds were at peak lay, together with unfavourable conditions on the farm (water supply was not stable during that period). It would be worthwhile to collect samples from birds from several weeks of age till the laying period, to investigate when the birds first become infected with *C. hepaticus*. Subsequently, preventative measures can be put in place if the birds are infected with *C. hepaticus* to prevent SLD outbreaks.

In SLD affected farms, only 10-50% of birds carried *C. hepaticus*. This might be explained by differences in birds' immune status and/or may indicate that *C. hepaticus* has low infectivity, with large inoculums required to establish colonisation.

Understanding where *C. hepaticus* may come from would help to prevent the spread of *C. hepaticus*. We found wild bird droppings from farms in SA and VIC, and flies and rat/mouse droppings from SLD affected farms carried detectable quantities of *C. hepaticus* DNA, detectable by PCR. These organisms, especially wild birds, might be a vector for *C. hepaticus* dissemination.

Moreover, water, soil, mite, and dust samples from SLD affected farms also had detectable levels of *C. hepaticus* DNA. These could be intermediate sources from which organisms may pick up *C. hepaticus* and transfer to chickens, and for transfer between chickens.

Whole genome sequencing and bioinformatic analysis of 16 *C. hepaticus* isolates recovered from SLD-infected farms from 5 states showed that all 16 strains were highly similar.. The results suggested that there has been horizontal transmission among flocks, and environmental reservoirs of the organism might play an important role in spreading *C. hepaticus*. These results emphasise the need for the poultry industry to carefully evaluate flock and environmental management procedures to reduce the impact of SLD.

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Introduction

Spotty Liver Disease (SLD) is an emerging disease in the Australian poultry industry, most notably affecting free range layer flocks but also seen in other chickens. It causes significant egg production losses and mortality, especially around peak lay, but can also occur at any stage throughout the production period.

Our group characterised and formally named the bacterium that causes SLD, *Campylobacter hepaticus* (1). We have developed a reproducible disease induction model, allowing the assessment of various treatment and control options and have developed molecular tools to qualitatively and quantitatively detect the organism in clinical samples (2, 3).

The control of the disease will be assisted by an understanding of the epidemiology of the disease. This may allow elimination of the vectors or causal associations and will assist in defining appropriate biosecurity measures to help in flock and environmental management to reduce the impact of SLD. Therefore, the aim of this study is to investigate the epidemiology of SLD.

Objectives

This project's aim was to investigate the spread of infection within flocks and determine possible transmission routes of *C. hepaticus* in layer farms by investigating the presence of the bacterium in the birds and environmental samples. The objectives of the project were met.

Methodology

a. Sample collection

Cloacal and faecal swabs, bile, and caecum samples were collected at 2-4 weeks intervals in three farms, commencing before the birds were placed into laying farms (16 weeks old), until peak laying period (26-30 weeks). A total of 1007 samples were collected from birds from these three farms and 69 environmental samples, including wild bird species, soil, dust, beetles, flies, rat caecum, and mouse caecum, were also collected (Table 1). Samples from these main surveyed farms were collected by Scolexia veterinarians.

In addition to the temporal study of *C. hepaticus* occurrence on three farms, samples were also collected on an *ad hoc* basic from different farms around Australia, including SA, WA, NSW, QLD, and VIC to enhance the understanding of SLD epidemiology. A total of 1,106 samples from chickens and 53 environmental samples were collected. Samples were collected from chickens across all ages, with or without clinical signs of SLD.

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b. DNA extraction

DNA was extracted from all samples using the DNeasy PowerSoil Kit (Qiagen) or crude DNA was prepared by a boiling method.

c. Polymerase Chain Reaction (PCR)

PCR was carried out, using our established assays (3), to detect the presence of *C. hepaticus*. PCR was performed in 96 well plates using an Eppendorf Mastercycler nexus GSX1 thermal cycler.

d. C. hepaticus isolation

C. hepaticus was isolated from bile samples of SLD affected birds and 16 isolates from diverse locations (6 from Queensland, 6 from Victoria, 2 from Western Australia, 1 from South Australia and 1 from New South Wales) isolated during this study were subjected to whole genome sequencing using the Illumina MiSeq system at RMIT. The aim was to examine the conservation or divergence of *C. hepaticus* genomes in different outbreaks. Sequences were assembled using the A5-miseq pipeline (4). The Average Nucleotide Identity *(*ANI*)* (http://enve-omics.ce.gatech.edu/ani/) was used to compare the genome sequences between the sequenced genomes and the *C. hepaticus* HV10 reference genome of the type strain. An ANI value of more than 95% indicates the compared samples are from the same species. In addition, the genome sequences of the 16 isolates were submitted to RAST (Rapid Annotation using Subsystem Technology) for annotation to predict gene functions. The annotated genes were compared using the Compare Sequence feature of the SEED viewer (5).

Results and Discussions

The survey of *C. hepaticus* infection resulted in the collection of a large number of samples: 2,113 samples from birds and 122 environmental samples. Notable findings from the *ad hoc* sampling of birds included; birds as young as 12 weeks old were found to be infected with *C. hepaticus,* long before laying starts and months before the peak lay period when clinical SLD is most likely to be seen; at 26-27 weeks old. One SLD outbreak was observed in chickens at 60-62 weeks old, and *C. hepaticus* was isolated from these birds. These represent the age extremes at which *C. hepaticus* has been found in birds.

In one farm that the birds were infected with *C. hepaticus* at least 8 weeks before clinical SLD was manifested, and SLD occurred when the birds were at peak lay, together with unfavourable conditions in the farm (water supply was not stable during that period).

Farm 1 was monitored over a 10-week period from 18-28 weeks of age. *C. hepaticus* could not be detected in any of the faecal samples and there were no clinical signs of SLD throughout the monitoring period. Farm 2 was monitored over 13 weeks, from 17-30 weeks of age. *C. hepaticus* was first detected when the birds were 26 weeks old; coinciding with when clinical signs of SLD were seen an confirmed at autopsy. On Farm 3, *C. hepaticus* was detected when chickens were at 24 weeks old, but clinical signs of SLD occurred when the chickens were 26 weeks old.

Both the careful monitoring of farms over an extended time period, and the *ad hoc* sampling of farms, particularly ones in which clinical signs of SLD were just becoming apparent, support earlier finding that SLD is likely to coincide with peak lay. This has led to the hypothesis that physiological changes that occur as birds enter the laying period are predisposing for the emergence of SLD in *C. hepaticus* infected birds. Clearly birds can sometimes be infected weeks before clinical signs become obvious and clinical signs can first appear when birds are older.

An important question is where *C. hepaticus* comes from? We found wild bird droppings from farms in SA and VIC, and flies and rat/mouse droppings from SLD infected farms had detectable levels of *C. hepaticus* DNA, as shown by specific PCR. These organisms, especially wild birds, might be vectors for *C. hepaticus* dissemination. Moreover, we found water, soil, mite, and dust samples from SLD infected farms also had detectable level of *C. hepaticus* DNA (Table 1). These could be intermediate sources from which organisms can pick up *C. hepaticus* and transfer to chickens, and for transfer between chickens.

For environmental samples, *C. hepaticus* DNA was detected by PCR but no viable *C. hepaticus* were recovered. *C. hepaticus* might not be alive at the time of sample collection, or they may enter a viable but non-culturable stage, making isolation impossible.

In SLD affected farms, only 10-50% of birds had detectable levels of *C. hepaticus* in fresh bird droppings and cloacal swab samples. This result demonstrated that some birds might be immune to *C. hepaticus* to be infected, as given the apparent faecal-oral route of infection, it would be expected that all birds within an SLD affected flock would be exposed to *C. hepaticus*. Alternatively, the slow and incomplete spread of *C. hepaticus* throughout an infected flock may indicate that very high inoculums are required to establish colonisation.

Sixteen *C. hepaticus* isolates recovered from bile samples of SLD-affected birds from 5 states were subjected to whole genome sequencing. Average Nucleotide Identity *(*ANI*)* was used to compare the genome sequences between the sequenced genomes and the *C. hepaticus* HV10 reference genome of the type strain. The ANI values, all more than 95%, indicated a high degree of similarity amongst all isolates. In addition, the protein analysis obtained from SEEDViewer coincided with the genome analysis in which the isolates were shown to be closely related. The results suggested that there has been a horizontal transmission among flocks as the genomes of *C. hepaticus* from different regions were highly similar.

Table 1: The presence of *C. hepaticus* in three farms monitored over time

Sample type	*No. samples*	*C. hepaticus positive*							
		PCR (positive/total)						*Culture*	
Farm 1									
Age (weeks)		18	20	22	24	26	28		
Chicken samples	432	0/104	0/61	0/75	0/64	0/69	0/59	Nd	*SLD status*
Beetles, Flies, Rat Faeces, Mouse caecum	14	0/9	0/5					Nd	
Soil	34	1/1	0/18			0/15		Nd	
Farm 2									
Age (weeks)		17	19	22	26	27	30		
Chicken samples	335	0/60	0/50	0/50	7/56	24/68	15/51	3	
Litter, flies, butterflies, earwig, spider	5		0/1	0/1		0/3		Neg	SLD outbreaks started to occur when chickens were 26 weeks old on Farm 2
Wild bird faeces	1						1/1	Neg	
Rat faeces	1						1/1	Neg	
Dust	3				2/2		1/1	Neg	
Water	1						1/1	Neg	
Farm 3									
Age (weeks)		18	21	24	26	28			*C. hepaticus* was detected when chickens were at 24 weeks old, no SLD clinical signs. SLD outbreak occurred when chickens were 26 weeks old on Farm 3
Chicken samples	240	0/45	0/50	4/53	9/40	25/52		1	
Litter, beetles, flies	6	0/2			0/2	0/2		Neg	
Rat faeces	1					1/1		Neg	
Dust	3			1/2		1/1		Neg	
Total	1076								

Implications

The project helps to further understanding of the epidemiology of SLD.Recommendations in the below section will help in flock and environmental management to reduce the impact of SLD.

Recommendations

This study found that birds can be infected with *C. hepaticus* during rear and prior to the onset of lay, without any clinical SLD. SLD outbreaks occurred mainly at peak lay, but could also occur earlier or later in the production cycle. Therefore, it is of epidemiological significance to collect samples from birds from several weeks of age until the peak lay period to investigate when the birds become infected with *C. hepaticus*. As environmental sources are a likely transmission source of *C. hepaticus*, biosecurity methods need to be strictly followed to prevent the spread of this bacteria, such as avoiding standing water on the range. The control of rodents and birds should also be emphasised as an important biosecurity measure to help reduce the spread of SLD.

Acknowledgments

We acknowledge the financial support from Poultry Hub Australia enable us to carry out this research. We thank our industry partner Scolexia and veterinarians from various farms around Australia who provided samples for the study.

Media and Publications

The draft of a paper entitled "*Campylobacter hepaticus* causing Spotty Liver Disease in Chickens: transmission and routes of infection" has been prepared and submitted to Frontiers in Veterinary Science.

We anticipate that the paper will be reviewed within and made available online within 8 weeks, therefore we would highly appreciate if this final report could be withheld from release until then (31/12/2019).

Intellectual Property Arising

No

References

1. Van, T. T. H., Elshagmani, E., Gor, M. C., Scott, P. C., and Moore, R. J. (2016). *Campylobacter hepaticus* sp. nov., isolated from chickens with spotty liver disease. *International Journal of Systematic and Evolutionary Microbiology* 66, 4518–4524.
2. Van, T. T. H., Elshagmani, E., Gor, M.-C., Anwar, A., Scott, P. C., and Moore, R. J. (2017a). Induction of spotty liver disease in layer hens by infection with *Campylobacter hepaticus*. *Veterinary Microbiology* 199, 85–90.
3. Van, T. T. H., Gor, M.-C., Anwar, A., Scott, P. C., and Moore, R. J. (2017b). *Campylobacter hepaticus*, the cause of spotty liver disease in chickens, is present throughout the small intestine and caeca of infected birds. *Veterinary Microbiology* 207, 226–230.
4. Coil D, Jospin G, Darling AE (2015) A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. *Bioinformatics* 15:587-9.
5. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA., … Zagnitko O. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics 9*, 75.