AUSTRALIAN EGG
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Determining the cause and methods of control for 'Spotty Liver Disease'

Final Project Report

A report for the Australian Egg Corporation Limited

by Dr Peter C. Scott

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Foreword

This project was conducted to attempt to identify the causative agent of Spotty Liver Disease by both molecular and *in vitro* techniques, and to assess treatment and control options in the field. The study involved the investigation of field cases and sampling from both affected birds and those in another shed on the same farm, which were not affected. These samples were used to examine the histopathology of affected birds, to undertake genetic analysis of the gut flora of affected and control birds, to examine possible cell-toxicity of sera from affected birds, and to undertake challenge studies to attempt to reproduce the disease.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government.

This report is an addition to AECL's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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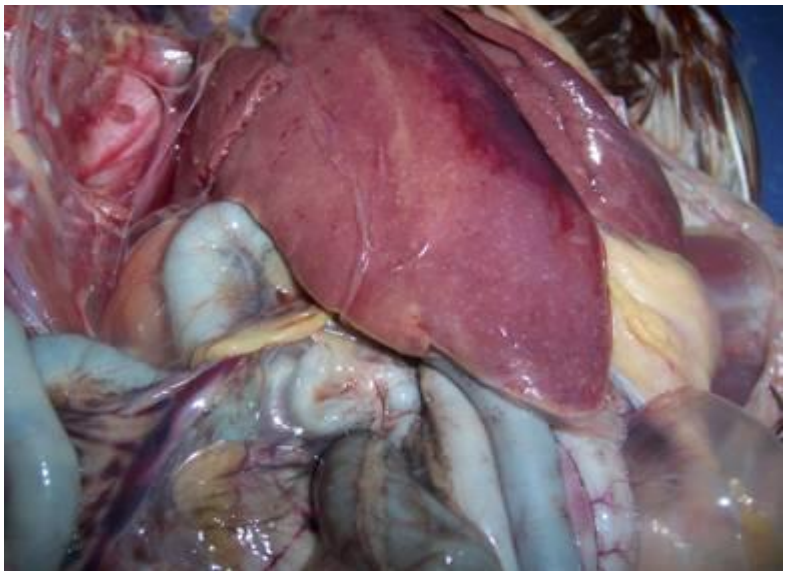
About the Author

Peter Scott is Managing Director of Scolexia and has more than 30 years experience in the Australian poultry industry as a veterinary pathologist, veterinarian, researcher and member of egg industry R&D committees. He has successfully completed many projects for AECL and other industry organisations.



Obvious spots in the liver of an affected bird

A commercial free range farm

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Abbreviations

CEK	Chicken embryo kidney
CDT	Cytolethal Distending Toxin
CTC	Chlortetracycline
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
H.	Helicobacter
H & E	Haematoxylin & eosin
LMH	Hepatocellular carcinoma epithelial
MCFA	Medium Chain Fatty Acids
OTU	Operational Taxonomic Unit
NCTC	National Collection of Type Cultures (Public Health England)
PAS	Periodic acid-Schiff
PC2	Physical Containment Level 2
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
RIRDC	Rural Industries Research and Development Corporation
SLD	Spotty Liver Disease
SPF	Specific pathogen free
UK	United Kingdom
USA	United States of America
V1 or V3	Variable region 1 or 3 of ribosomal RNA gene

Executive Summary

Spotty Liver Disease (SLD) has increased in importance in laying birds in Australia, as a greater proportion of laying birds are housed in free range conditions. Despite many attempts to identify the aetiological agent, presumed to be bacterial because of the conditions rapid curative response to antibiotics, there has been little progression in understanding aetiology and pathogenesis of the disease.

This project was commenced to examine the cause of the disease and possible control options. The project has included a literature review, field investigations including gross and histopathological examinations, challenge studies and *in vitro* toxicity and metagenomic techniques to elucidate the cause and examine suitable control options.

The condition known as Vibrionic Hepatitis which was described in the US in the 1950s appears to be different to the SLD reported in the United Kingdom (UK) and Australia, which involves sudden death in layers, more often around the time of peak production in birds in good condition and in-lay. A decrease in egg production is sometimes associated with the condition. Affected birds have small focal lesions visible on the liver surface, which on histopathological examination consist of multifocal coagulative necrosis.

Two challenge studies using intestinal and caecal contents from affected birds were undertaken without inducing the condition in challenged birds. Cell toxicity studies did not indicate any toxic effects of serum from affected birds. Metagenomic studies were undertaken comparing intestinal and caecal contents from affected and control birds and a *Helicobacter pullorum*-like organism was shown to be more prevalent in the gut flora of the affected birds than that of control birds. *H. pullorum* belongs to a class of organisms that produces a cytolethal distending toxin, and some of the pathology associated with cytolethal distending toxins is consistent with the pathology seen in Spotty Liver Disease.

With respect to management, prevention and control it has been observed that many outbreaks are related to disruptions in bird husbandry particularly as it relates to feed intake, formulation and availability. It has also been associated with free range birds accessing water in the range area. While historically SLD occurred in early lay and predominantly in summer, it now occurs at any time in lay and throughout the year. It is still more common in early lay. The faecal oral cycle still appears to play an essential part in SLD with the disease being almost exclusively seen in birds farmed extensively and, where it does occur in cage or fully slatted systems, there are invariably spatial associations with manure. The use of medium chain organic acids in the diet from the time of transfer to the production sheds, combined with stable management routines and feeding programs, results in a lower incidence of outbreaks and an amelioration of the severity of outbreaks when they occur. On the other hand, the use of probiotics has not at this stage been shown to be helpful in disease control. With respect to treatment, the use of registered antibiotics by water medication is usually effective in the first instance, however, repeated use can quickly lead to a reduction in efficacy through the development of antibacterial resistance.

Further elucidation of the associated organism has occurred in work carried out by Scolexia post the AECL funding, which has confirmed the presence of a *Campylobacter* spp. in field cases in Australia with homology to the organism isolated in the UK. We will soon be assessing a challenge model to enable development of more effective control options and further studies on the use of medium chain fatty acids may also be of benefit in further defining the optimal preventative program for producers in the immediate future.

Overall Conclusions

Most literature regarding SLD has been deficient with respect to a case definition, particularly with respect to pathology. We have developed a clear case definition. SLD is a condition of laying birds in good condition, most likely at the peak of lay and manifesting clinically primarily as sudden death. A higher proportion of free range farms is affected. Outbreaks often occur following an interruption to normal management such as changes to ranging times, nest box issues and feed interruptions. On initial treatment with antibiotics there is a good response but this can diminish with repeat treatments. Gross pathology is chiefly characterised by the presence of multiple focal lesions 1-2 mm diameter, which are usually greyish to white but occasionally red. In general the liver is slightly swollen. Other gross pathology is variable. The hens are observed to be reproductively active and in good body condition. Histology reveals multifocal coagulative necrosis throughout the liver with hepatocellular degeneration and disassociation progressing to loss of hepatocytes with fibrin laking, heterophils in the affected areas as well as hyperaemia/congestion.

We have observed that medium chain fatty acid inclusion in the diet is useful in reducing the incidence and severity of outbreaks, and that the use of some probiotic products in place of the medium chain fatty acids does not reduce the incidence or severity of outbreaks and has in fact been associated with outbreaks.

Metagenomic studies are a very useful tool to further investigate the cause of SLD and other conditions. We demonstrated consistent changes in the flora of affected and unaffected birds. Some bacteria were less prevalent in affected birds including *Lactobacillus coleohominis*, *L. helveticus*, some Clostridiales, some unidentified OTUs and *Bacteroides plebius.* However, other *Bacteroides* spp. were more abundant overall. Whilst differences in gut flora between affected and unaffected birds are suggestive of the role of gut-associated bacteria producing toxins that affect the liver, it is not totally conclusive given the possibility of the condition itself altering gut flora. However, when combined with the frequent absence of bacterial cells in the liver and the response to antibiotic treatment, it is reasonable to suggest that the confirmation of differences in gut flora between affected and unaffected birds in the metagenomic study adds further weight to that hypothesis. Frozen field intestinal and caecal samples stored in either thioglycolate or tryptic soy broths with glycerine did not induce disease in chickens that were exposed orally.

An *H. pullorum* like organism was found to be more prevalent in affected birds than in control birds. However, the subsequent isolation of a *Campylobacter* organism from field cases in Australia by Scolexia and Professor Moore (RMIT University), which has sequence homology to the organism isolated by Crawshaw and Irvine[18] from UK cases suggests that it is the principle pathogen. Its isolation will enable the development of an exposure model that will allow increased progress in assessments of treatment and control methods.

1 Introduction

Spotty Liver is a disease of laying birds, which is associated with increased mortality, particularly around the time of peak production and in some instances associated with a decrease in production. The cause of the condition is not known but suspected to be a bacterium, which produces a toxin that affects the liver. The clinical signs include a brief period of depression in laying birds (usually in good body condition and “in-lay”), and increased mortality. Often birds are found dead without any prior evidence of disease noticed. The notable post-mortem finding is the occurrence of small 1-1.5 mm diameter white, grey or red spots on the surface of the liver. In some cases these are raised. Histologically these appear as a multifocal coagulative necrosis.

The history of the condition is described more fully in the literature review. Briefly, in the 1950s conditions similar to or including pathology, which is now associated with Spotty Liver Disease, were reported in the USA. A similar condition was reported in the UK in the early 2000s. There has been no consistent isolation of bacteria from the lesions, which are often sterile, however Spotty Liver responds to treatment with antibiotics. Spotty Liver was first noted in Australia in the 1980s, affecting both meat and egg laying breed hens in at least some operations of the major commercial producers. It affected primarily poultry housed on the floor and particularly those approaching the peak of lay (60% or greater). It was initially more commonly associated with occurrence in summer.

This study was undertaken to identify the causative agent by both molecular and *in vitro* techniques, and to assess treatment and control options in the field. The study involved the investigation of field cases and sampling from both affected birds and those in another shed on the same farm, which were not affected. These samples were used to examine the histopathology of affected and associated organs, to undertake genetic analysis of the gut flora of affected and control birds, to examine possible cell-toxicity of sera from affected birds, and to undertake challenge studies to attempt to reproduce the disease.

This final report contains some additional information on further work on affected farms in Australia by Scolexia and Professor Moore of RMIT University, which was carried out since the funding for this project ceased. In it we report the isolation of a *Campylobacter* organism with the same sequence identity as an organism reported to have been used to reproduce microscopic lesions similar to Spotty Liver Disease in poultry in the UK.

Figure 1 Dead birds, the most common manifestation of Spotty Liver Disease

2 Literature review

2.1 Definition of the disease

Spotty Liver Disease is a sporadic disease of laying chickens, of unknown cause. The disease is characterised clinically by increased mortality of laying hens that are in good condition, often by decreased production, and pathologically by multiple small foci of necrosis and inflammation throughout the liver in which routine microbiological methods do not identify a consistent causative organism. Most commonly no organisms are observed in the liver lesions.

2.2 Historical background

In the early 1950s there were multiple publications regarding outbreaks of avian infectious hepatitis in multiple states of the USA. An insidious and lingering increase in mortality of 10-15% and lowered egg production by up to 35% were reported in affected flocks.[1,2,3,4,5] In one case the outbreak occurred over several months.[3]

Gross post-mortem changes included normal sized to enlarged mottled livers with irregular white areas of necrosis.[1,5] Another description includes degenerative and haemorrhagic changes in the liver,[3,4] which varied in severity and extent from bird to bird but also lobe to lobe,[1] and could affect only one lobe.[5] Most commonly seen were pin-head sized grey-white foci[2] but lesions could range up to 1 cm in diameter in a round to cauliflower-like shape.[3,5] Other findings reported included ascites, hydropericardium, enlarged and pale heart, enlarged and pale kidneys, and catarrhal enteritis in a small percentage of affected birds.[3]

Microscopic lesions consisted of randomly distributed multifocal to diffuse hepatocellular degeneration and necrosis variably associated lymphocytic and granulocytic inflammation[3] and haemorrhage.[4] Granulomatous foci with fibrinoid necrosis were seen less frequently.[3]

The spleen occasionally contained granulomatous foci. Kidneys exhibited mixed (lymphocytes and heterophils) inflammation of the interstitium with multifocal tubular degeneration associated with heterophilic inflammation. Dilated tubules and inflammatory tubular casts were also 'not uncommon' findings.[3] The bone marrow of affected chickens had increased numbers of immature myelocytes. Mononuclear myocarditis was more commonly seen and more pronounced in younger birds in comparison to mature birds.

Initially routine bacteriological cultures (anaerobic and aerobic) failed to isolate an organism, and diagnosis was made presumptively from histological sections. Inoculation of liver suspension into chicken embryos repeatedly caused the death of the embryos[3,4,5] with variable lesions, mostly consisting of liver and splenic lesions depending on the age of the embryo. Gram negative, motile, cocci and vibrio organisms[4,5] were repeatedly identified from yolk/allantoic fluid from dead embryos. When inoculated into adult chickens there was no mortality but liver lesions comparable to those described above were induced.[5] The organism was cultivated on artificial media from the bile of affected chickens.[4] A few drops of bile were added to blood agar plates, which were incubated at 37.5°C in a Brewer anaerobic jar containing 10% CO_2, 63% methane and 27% air. Isolations were also obtained by using 10% CO_2 in a Brewer jar. A stained smear of the agar at 18-24 hours revealed large numbers of vibrios, despite there being no visible colonies. If incubation continued for 3 days a mucoid growth became visible. Cocci forms appeared to be an older variant of the cultured organism.

Whilst the spectrum of clinical and laboratory signs described in the early US literature certainly included similarities to the disease we see in Australia, they also include differences such as ongoing clinical signs[3] (whereas in Australia it is relatively difficult to find sick birds, as affected birds are generally found dead). These early workers also reported affected birds had shrunken shrivelled, with dry and scaly combs, and reported weight loss. Birds as young

as 10 weeks old were affected with the hepatic conditions they observed. This clinical picture is not the same as that seen in Australian Spotty Liver cases. Many of the pathological changes reported were closer in nature to those related to a typical bacterial hepatitis. The ascites, hydropericardium and heart muscle changes described were also significantly different to the disease we have described in Australia.

Reports of a similar entity sporadically re-emerged in free-range laying flocks in the UK from the early 2000s.[6,7] The reports name the condition avian vibrionic hepatitis or infectious avian hepatitis, despite no consistent bacterial isolations from submitted cases.[7,8] The disease presented as a sudden increase in mortalities with dramatic decrease in egg production in flocks at peak lay and most often over summer.[6] Affected birds were in good condition and most were found dead. Those that were alive were pyrexic and depressed, with palpably enlarged livers. Affected birds had full crops and often had eggs in the oviduct. There was excessive peritoneal and pericardial fluid and occasional peri-hepatitis, but the most striking and consistent finding was a swollen liver with multiple 1-2 mm whitish-grey focal lesions.[6] The histopathology involved acute multifocal necrotic hepatitis.[6] Special stains failed to reveal any organisms.

The condition as seen in the UK behaves like an infectious disease with spread to further sheds and on one occasion to a separate property with limited vehicular contact with the originally affected property[6]. It responded to early administration of antibiotics (chlortetracycline, tiamulin),[6,7] albeit with persistent decreases in egg production. The condition recurred in an affected farm despite depopulation and overwintering, and affected each subsequent flock despite high hygiene standards.[6] The disease continues to appear in the UK and one group has found focal hepatitis lesions in broilers at slaughter.[9] It would appear that the UK literature relates more closely to the disease seen in Australia as described below than those described in the early United States of America (USA) literature.

2.3 Hepatitis in Australia

Spotty Liver syndrome, Spotty Liver Disease (SLD), summer hepatitis and acute focal hepatitis are all terms that refer to a disease entity identified in Australia. There are no peer-reviewed publications pertaining to this disease in Australia, however, several presentations from scientific meetings in addition to two industry research reports for RIRDC are presented as Australian material.

SLD was first noticed in Australia in the late 1980s[10] and is reported in broilers and broiler breeders in addition to caged, barn and free range layer hens[10-13,]. The disease occurs in the eastern states (SA, Victoria, NSW, Queensland) and affects multiple strains of birds from multiple companies.[10]

The disease primarily affects poultry raised on or housed on litter[10-13] and approaching (>60%) peak lay.[10,12,13] There is a higher incidence of the disease in flocks approaching peak lay in summer. [10,12,13] Stressor events, such as husbandry mishaps, ambient temperature rises, dietary changes, water ingress into deep litter sheds or ranging areas, and increased access to the faecal oral cycle are commonly associated with precipitation of outbreaks of SLD. Parasitic worm burdens have been speculated to allow populations of bacteria to proliferate within tissues as a possible trigger to the condition.[10]

Affected flocks experience increased mortalities, reports range from 0.5% per day[10], a doubling of standard daily mortality, to an average of 5-10% per outbreak[11], up to 10% over several weeks[13,] and 27% in one outbreak.[11] Egg production decreases by 5-20% which may persist over several weeks.[11] Flocks affected approaching peak lay will have suboptimal production for the duration of lay.[12]

Only a short period of illness precedes a rapid death.[14] In some cases clinically affected birds may not be noted because the course of illness is peracute.[9] Affected birds exhibit profound

clinical depression and may be pyrexic. Jaundice is noted in a small proportion of affected cases.[9,12] Interestingly one report notes that where flocks are mixed the males were not affected.[9]

On post-mortem examination affected birds are in good condition and often have full crops (suggesting sudden death). They have milliary small (pin head/millet seed sized) pale foci through the liver which range in colour from white-cream to yellow.[12,13,14] Occasional enlarged spleens are noted as is jaundice.[12] Petechial haemorrhages in other organs (pericardial adipose tissue and epicardium) are also reported.[14] Lesions can be found in apparently normal birds.[9]

Histopathological changes in the liver consist of randomly distributed foci of peracute to acute focal hepatocellular degeneration and necrosis with little inflammation.[11,12,13,14] Inflammation when present initially comprises small numbers of heterophils and, as the lesion ages, variable numbers of macrophages and lymphocytes ingress. Histopathological searches for bacterial, fungal and protozoan organisms in affected livers have been unrewarding. Special stains including silver stains, PAS and gram stains fail to reveal organisms.[9,11,13] Although *Campylobacter* like organisms have been reported in a small number of affected livers, these were unable to be isolated for further investigation.[12]

In the words of Dr R. Reece, a recognised expert in poultry pathology, “the gross and histological lesions are dissimilar to other recognised hepatopathies of poultry”. The lesions are distinct from viral hepatitis (e.g. adenoviral inclusion body hepatitis), bacterial abscessation, disseminated intravascular coagulopathy associated with peracute bacterial endotoxaemia, *Chlamydophilia psittaci* infection, fowl cholera, clostridial cholangiohepatitis and migrating larval ascarids. Histologically some foci resemble an acute bacterial effect; others could easily be enterotoxic.[12]

The common observation that the disease responds very quickly to antibiotic therapy[9,12] with rapid cessation of mortalities supports a bacterial component. However, recurrence after cessation of antibiotic therapy is noted.[9] The commonly held hypothesis is for a bacterial toxin[9,13] (possibly clostridial), circulating through the portal veins from the gastrointestinal tract.

Challenge studies have failed to propagate the disease. The only published challenge study in layers[13] used commercial laying inbred white leghorn x bantam birds, 126 weeks of age, which had undergone a moult 12 weeks prior and were at their peak of lay (60-66% eggs/day). These birds were raised and housed on wire and slats, and at the commencement of the trial moved to floor housing with wood chip litter. Housing was in a climatically controlled PC2 facility. Birds were fed commercial layer ration. Two groups were inoculated with intestinal or liver samples from affected birds in a prior field outbreak. The liver samples had been frozen for 20 weeks at -80^{o}C, thawed and homogenised prior to oral dosing. Two groups were also orally inoculated with broths of *Campylobacter coli* and *Clostridium sordellii*, which had been isolated from prior field cases, freeze dried and reconstituted. At the conclusion of the 10-day trial period, no birds had become ill or died. There were no liver lesions consistent with SLD detected on post mortem examination. The researchers[13] acknowledged that there were no additional environmental stressors involved in the trial that may have limited its ability to mimic field conditions and the establishment of disease.

2.4 Overview of SLD like conditions

In addition to the challenge study in layers in Australia, Ceelen’s group inoculated broilers in Belgium with *Helicobacter pullorum* and failed to produce any liver lesions.[15] The only pathology observed were mild lesions in the caeca.

One problem with the literature is the lack of description of pathology in many papers, with some reference to the reports from the 1950s but no actual description of the cases being discussed. This has resulted in a continued uncertainty as to what actual clinical and pathological syndrome is being discussed. The gross and histological features described in cases of "Vibrionic Hepatitis" in the USA are generally different to those described in birds affected by SLD in Australia. These changes may reflect the age of the lesions, with more inflammatory cells ingressing over time, or differences in virulence of a causative organism, or differing causative agents. *Campylobacter coli* has been isolated from SLD affected chickens in Australia[13] but on inoculation into adult chickens failed to reproduce disease. The significance of this organism as a causative agent is currently indeterminate in regards to SLD. *Campylobacter* species are commonly isolated from chicken liver, bile and intestines in laying birds without evidence of disease.[16,17] The disease mysteriously disappeared from the USA in the 1960s and has not been reported since. The cause for the apparent disappearance is not the subject of any published speculation.

While sharing similarities, and named after the "vibrionic hepatitis" in the USA, the UK condition appears more consistent with (and could be argued to be identical to) SLD cases seen in Australia. Gross and histological lesions from cases in the UK resemble those seen in Australian birds.[9,18]

Nevertheless the similarity of lesions in reports of milliary hepatitis is a reminder of the need to continue to fully investigate cases and even in the Australian context, where the bulk of outbreaks seem to yield no significant bacterial growth from liver samples, it is important to undertake microbiological investigations. In 2008, similar lesions were reported in broilers in Grenada with microscopic lesions characterised by multifocal necrotising hepatitis.[19] However, in this case there were obvious intra-lesional bacterial colonies and Aerococcus viridians was isolated from the liver lesions.

2.5 *Helicobacter* like organisms and Cytolethal Distending Toxins

Burnens et al.[20] described the possible association of the *H. pullorum* with lesions of vibrionic hepatitis in poultry and noted that *H. pullorum* was isolated from 50% of chickens examined with liver disease but from only 4% of healthy chickens. *H. pullorum* was first identified and named by Stanley et al. in 1994[21] and was isolated from both poultry and from human patients with gastroenteritis.

H. pullorum and other *Campylobacter* like organisms re-assigned to the genus *Helicobacter* are unlike the early *Helicobacter* spp., which were gastric organisms (e.g. *H. pylori*) and are described as intestinal or enterohepatic organisms[20]. Two of these *Helicobacter* spp. (*H. hepaticus* and *H. bilis*) were implicated in inflammatory liver disease and liver cancer in mice.[22,23,24] This led Burnen's group[20] to examine the relationship between the presence of *H. pullorum* and "Vibrionic Hepatitis". Whilst they found a significant relationship between disease and prevalence of the organism, other surveys have found the organism quite widespread in poultry. Basaran et al.[25] examined the prevalence of the organism in poultry in Turkey and found that 60% of the birds cultured were positive for *H. pullorum*. Ceelen et al.[26] found seven out of eleven flocks examined in Belgium were infected with *H. pullorum*. In positive flocks 33.6% of caeca, 31.8% of colons, 10.9% of jejunums and 4.6% of livers were positive.

Some avian and human derived *Helicobacter pullorum* contain genes that code for a Cytolethal Distending Toxin (CDT).[27] CDT activity is characterised by the appearance of cellular distension, cytoskeletal abnormalities and cytolethality in cultured cell lines. The toxin is coded by *cdtA*, *cdtB* and *cdtC* genes, which are all necessary for cytolethality in *E. coli*. However, not all enterohepatic *Helicobacter* spp. possess *cdt* genes. Intestinal disease in humans caused by *Helicobacter* spp. is associated with the presence of CDT.[28] However, of the isolates examined by these workers only the human derived strains had functional CDT activity, even though the poultry strains examined had CDT coding genes. This situation

could be part of the explanation of the sometimes common occurrence of *H. pullorum* in poultry and poultry products but not necessarily in association with disease.[25,26,28,29,30,31] The factors that cause the *cdt* genes to be active in causing production of cytolethal distending toxins are not well understood, and if the cause of SLD is an *H. pullorum* like organism it may well be the activation of the cdt genes that is a key step in the process of disease initiation. The factors involved in the activation of the *cdt* genes may also help explain some of the differences seen in epidemiology of the disease within a shed and on farm, as well as the difficulty in reproducing the disease experimentally.

3 Field investigations

A wide range of management systems and ages of hens were found to be affected by Spotty Liver Disease in four Australian states during the years 2009 to 2012. These included free range, barn, cage and aviary systems, and birds from 22 up to 80 weeks of age. The average age was 33.8 weeks (or 28.8 weeks if the 80 week old flock is excluded). Outbreaks most commonly occurred within three weeks of peak production. Egg production was variably affected, with some flocks experiencing a drop in egg production in remaining birds and others experiencing mortality but little noticeable effect on hen day production.

On those farms where bird density was measured, there was little noticeable difference in stocking density between the non-affected and the affected sheds. Humid conditions, water lying around the sheds and heavy rain were associated with some of the outbreaks. Disruption to normal management including nest box function and in particular feed deprivation occurred immediately prior to outbreaks in several cases. The presence of helminths occurred at or around the time of outbreaks in some farms.

The use of probiotics was associated with disease on two occasions, whereas the use of organic acids appeared protective on those two farms. On the other hand intermittent use of organic acids was associated with disease on another farm. Scolexia clients using certain organic acids from the time of placement to a month or so past the peak of lay experienced fewer outbreaks of Spotty Liver and those that did have outbreaks experienced lower morbidity than they had in previous outbreaks.

Table 1 lists the outbreaks for which some information has been received. Table 2 lists the mortality rate in some affected flocks.

Table 1 Properties affected by Spotty Liver and reported to Scolexia

Property No	Outbreak/ sample date	Breed	Age (weeks)
1	Jan 2009		
2	Nov 2009	Layer	27
3	Jan 2010	Layer	
4	April 2010	Layer	36
5	April 2010	Layer	28
6	May 2010	Layer	26
5	Aug 2010	Layer	23
7	Aug 2010	Broiler breeder	31
8	Sept 2010		
9	Oct 2010		
9	Nov 2010	Layer	27
9	Nov 2010	Layer	31 & 35 wks
9	Nov 2010	Layer	34
9	Jan 2011		
9	Feb 2011	Layer	25
10	Feb 2011	Layer	25
5	Jun 2011	Layer	22
11	July-11		
9	Sept 2011	Layer	29
5	April 2012		
13	July 2012	Layer	33
14	July 2012		80
15	Jan 2013	Layer	23
16	Jan 2013		
17	Jan 2013	Layer	24 & 46 wks

Table 2 Mortality rates* reported in affected flocks

Birds/shed	No Affected (wk)	% mortality (wk)
14,603	163	1.12
38,000	166	0.44
7,000	150	2.14
33,300	83	0.25
15,100	52	0.34
21,000	40	0.19
30,000	140	0.47
60,000	84	0.14
13,560	460	3.39
15,000	180	1.20
22,500	315	1.4
Average		**1.01**

***** Flocks were treated shortly after diagnosis so this is not a reflection of mortality in untreated flocks.

There are no indications of disease prevalence being influenced by breed, and these results represent a sample of diseased farms over the period involved in the study.

3.1 Clinical signs and gross post mortem findings

One of the difficulties of the project was finding affected birds alive. In most sheds the majority of the birds appeared in good health and the affected birds were found dead, noticed more often in the morning. Sick birds appeared to be most likely discovered early in the morning and simply appeared in varying stages of depression with ruffled feathers and comparative reluctance to move.

In most cases the birds were in good condition and in active lay with developing yolk and often an egg developing in the oviduct. The liver was usually slightly enlarged and swollen with mildly rounded edges and covered in a variable number of discrete small white to yellowish or even red foci 1-2 mm in diameter seen over the liver surface. In many birds the spleen appeared slightly swollen. Whilst other features have been noted, the above are the most consistent findings in the field outbreaks investigated during this project.

In some instances concurrent disease with peri-hepatitis, air-sacculitis, egg peritonitis and or pericarditis have been noted, the most common being egg peritonitis. Unusually the clinicians have found typical “Spotty Liver” foci on livers of birds selected and culled from “control” sheds. More usually the birds from control (unaffected) sheds have shown no morphological signs of Spotty Liver. Given the difficulty in finding live but diseased birds the significance of finding apparently healthy birds with liver lesions is suggestive that subclinical Spotty Liver may be another manifestation of the disease.

Figure 2 Spotty Liver in a layer hen. Note both red and white lesions present on the liver surface

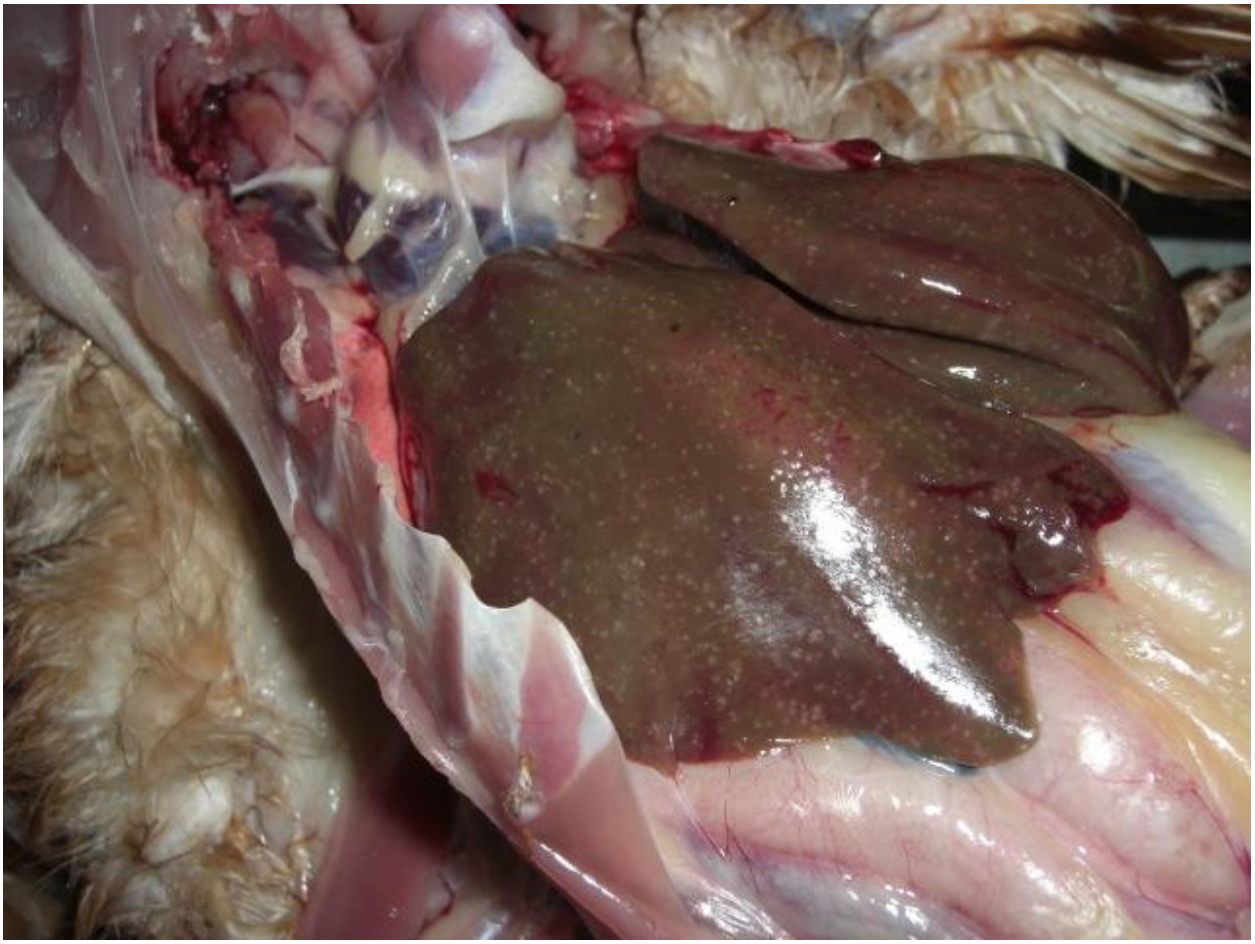

Figure 3 Typical Spotty Liver lesions in a layer hen

Note the consistency in the size of the lesions.

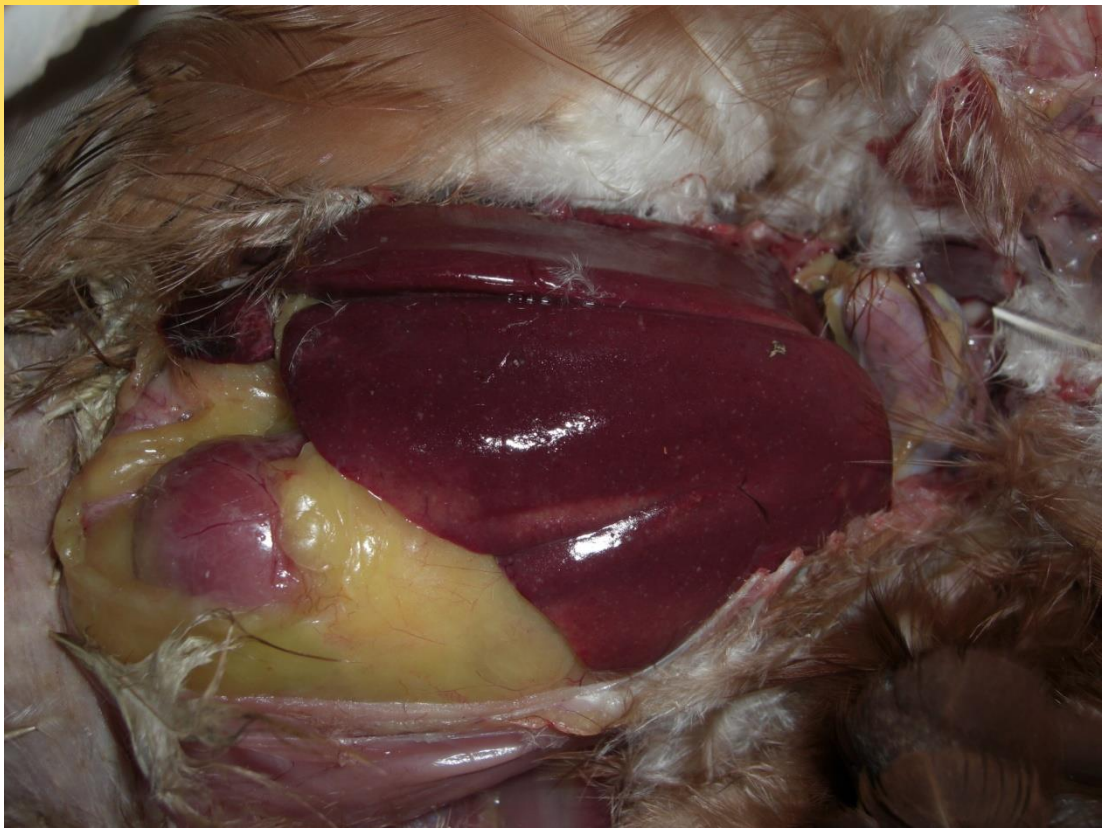

Figure 4 Further Spotty Liver lesions in a bird in good condition

4 Histopathology

4.1 Histopathology

Cases from various states were examined, in varying detail, in a number of laboratories by different pathologists. Some pathologists would report simply a “multifocal coagulative necrosis” in the liver, where those pathologists more closely associated with this project would describe more detail as listed below in the descriptions for each organ regularly examined. Overall changes in the liver were more consistent than in other organs. Where a particular pathology was only reported for one case it is not listed below, (for further detail on field investigations please contact Scolexia).

4.1.1 Liver

- the “spots” of this condition can be broadly described as a multifocal coagulative necrosis
- over 80% of cases thoroughly examined had hepatocellular degeneration and disassociation progressing to loss of hepatocytes with fibrin “laking” and heterophils in the affected areas as well as hyperaemia / congestion (with increased erythrocytes) in affected and surrounding areas
- over 50% of cases had Kupffer cells exhibiting phagocytosis and lipid vacuolation of hepatocytes (which was queried as possible evidence of fatty change)
- over 40% of cases described had evidence of fibrin thrombi in sinusoids and/or some in central veins
- approximately 26% of cases examined showed “pavementing” of leucocytes along the endothelium of larger veins
- in 30% of cases there was evidence of periportal lymphoplasmacytic infiltration.

Less frequently observed were the following:

- foci of heterophilic vasculitis with degeneration of intima and fibrin exudation (18.5%)
- occasional gout tofi scattered through parenchyma (18.5%)
- aggregations of heterophils, lymphocytes and plasma cells around portal areas and adjacent to central veins adjacent to vascular walls and extending through the wall (14.8%)
- pale staining hepatocyte cytoplasm but normal nuclear detail, disarray of hepatocellular chord architecture with the space of Disse accentuated (7.4%)
- increased individual (or 2/3 clusters) cell(s) necrosis throughout the parenchyma (not in foci of necrosis) affecting both hepatocytes and Kupffer cells (7.4%)
- "typical" foci of necrosis contain viable hepatocyte nuclei with esosinophilic debris degenerate and effete heterophils (7.4%)
- small mononuclear (mostly lymphocytic) aggregates in the parenchyma (7.4%).

4.1.2 Spleen

- the most common finding reported in the spleens of affected cases was reticular cell hyperplasia (in approximately half of the cases examined)
- in 28% of cases, small fibrin thrombi were seen throughout the vessels and parenchyma
- approximately a quarter of the cases had increased numbers of plasma cells throughout the parenchyma.

Less frequently observed were the following:

- fibrin lakes and necrotic cell destruction (16%)
- hyperplasia of dendritic cells/histocytic cells (16%)

- an increased number of phagocytic macrophages including the occurrence of erythrophagocytosis (16%)
- moderate diffuse congestion (12%)
- depleted of follicular units, increased prominence of central antigen presenting cells (8%)
- scattered foci of cell debris (necrosis) (8%).

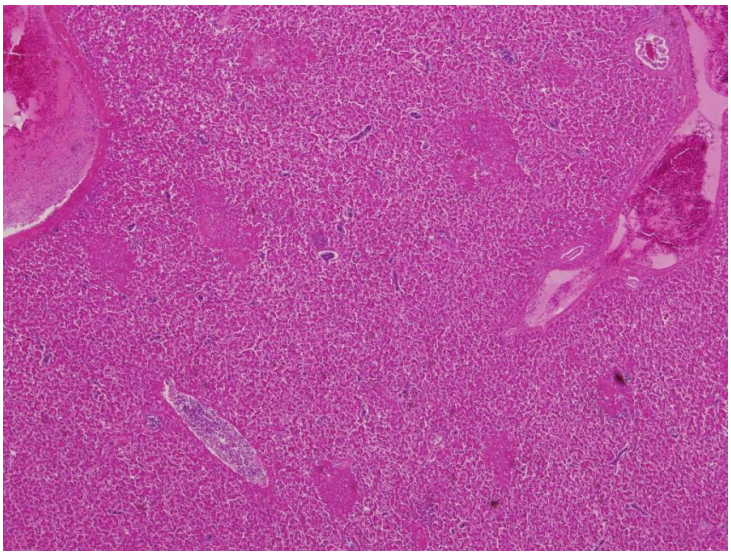

Figure 5 Multiple necrotic lesions in a bird affected by Spotty Liver

H & E stain.
Magnification 4x40

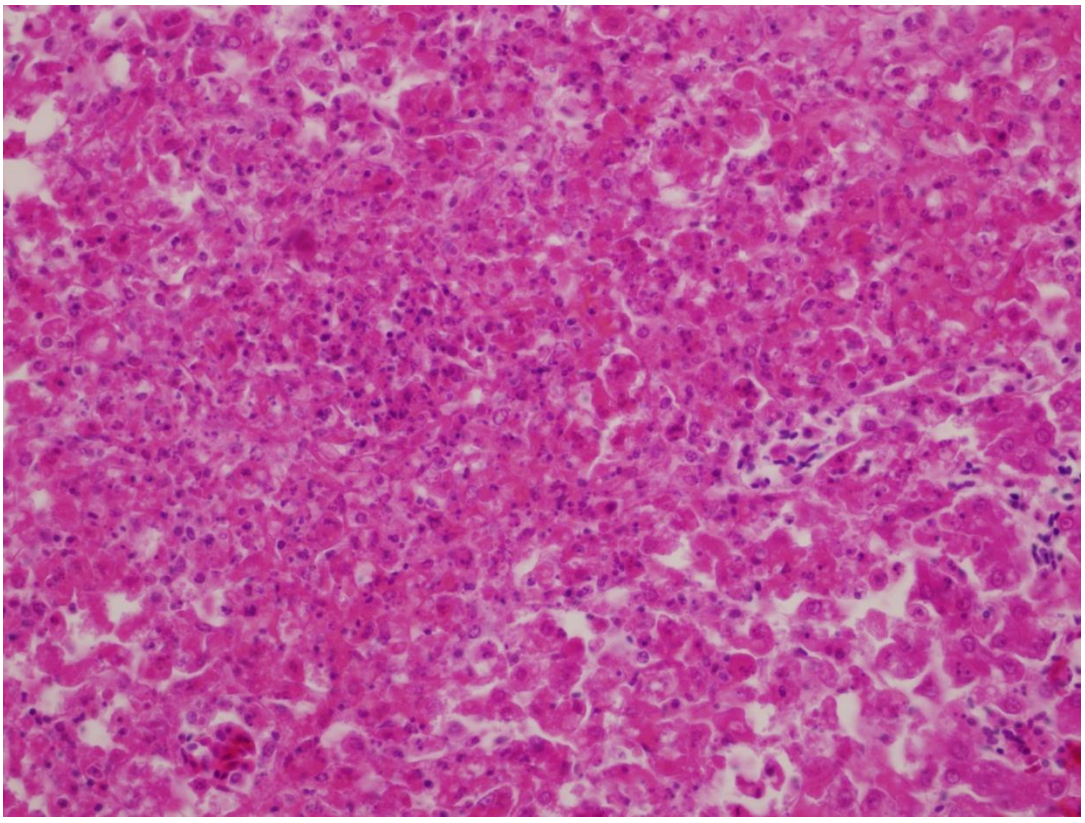

Figure 6 Hepatocellular dissolution necrosis

H & E stain.
Magnification 4x400

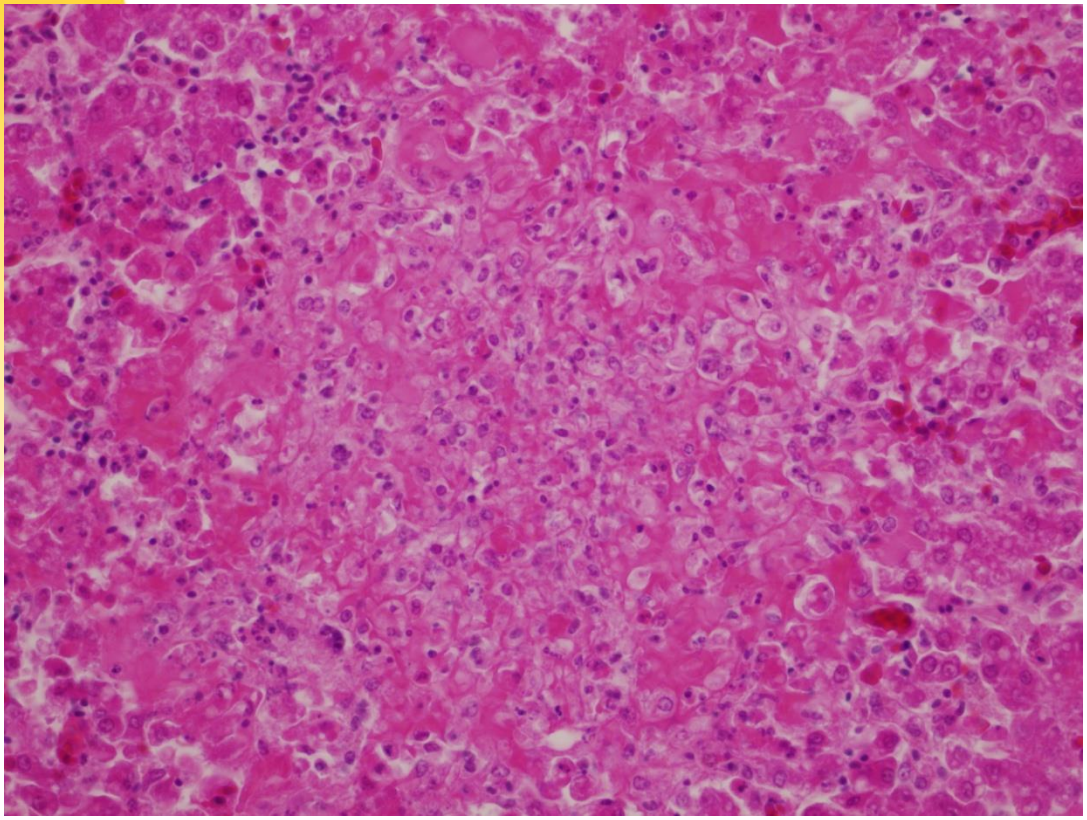

Figure 7 Liver affected by Spotty Liver with typical fibrin laking
H & E stain.
Magnification 4x1,000

4.1.3 Small Intestine

- All small intestines from SLD cases demonstrated an increased number of plasma cells and lymphocytes in both crypt and villous lamina propria.
- Over half of the cases also having a focal infiltration of heterophils in the lamina propria.
- Just over a third of cases also had a histiocytic / heterophilic inflammation and fibrosis or fibroplasia of the serosa and/or mesentery.
- Two cases (17%) had hyperaemic vessels of the villous tips.

4.1.4 Large Intestine

- The pathology of the large intestine was similar to the small intestine with the majority of cases (67%) having lymphocyte and plasma cell infiltration of the lamina propria.
- 28% had small foci of heterophils inflammation in the lamina propria.
- Some cases (17%) showed dilation of the Peyers patches with herniation of the crypt epithelium.
- Two cases (11%) had a histiocytic / heterophilic inflammation and fibrosis or fibroplasia of the serosa and/or mesentery.

Table 3 Cases of Spotty Liver investigated with histopathology

Property Number	Sample date	Age (weeks)	Lab submission no	Shed No	Total birds	Cases (week)	Epidemiological features	House type	Gross pathology
4	21/04/2010	36	Micro Uni Melb: CM10-0167	6	14,603	163	Worms, range bare, water on range, sparrows, feed supply interruptions, non-continuous inclusion of medium chain organic acids	Free range	Spotty liver, some with oedema in the peritoneum. SI empty, caecae full, bile dark
6	7/05/2010	26	2010-1699-MH		38,000	166	Mortalities spiked a week after peak production. IB vaccinated 2 wks ago, mortalities a week prior	Cage	"hepatitis" - spotty liver, livers enlarged. Spleens enlarged
5	27/04/2010	28							
1	21/01/2009		09-4422157	9					Spotty liver
8	7/09/2010		P10-71264						Spotty liver - swollen, Mild fibrogelatinous perihepatic effusion. Some spleens slightly enlarged, mottled.
9	6/10/2010		ACE-5003-11						Histo slides
9	9/11/2010	27	Uni Melb 966-10 Patient No 267521	4				Cage	
10	18/02/2011	As above							Well feathered, reasonable body condition, active ovaries, spotty liver, slightly enlarged spleens

Table 3 (continued) Cases of Spotty Liver investigated with histopathology

Property No	Sample date	Age (weeks)	Lab submission no	Shed No	Total birds	Cases (week)	Epidemiological features	House type	Gross path
3	7th + 20/01/2010		Ace 5439/09 & 5438/09	15 & 16			Stagnant water lying (mostly outside free range area. Not ranging at time. Bottom shed not affected		
2	24/11/2009	27	PIRSA 5439/09		21,000	40	Increased water consumption prior to outbreak		
11	Jul-11		Summary from Tom Grimes						Enlarged livers with spotty liver
5	5/08/2010	23		1	30,000	140	Cold and wet, birds not yet ranging. Medium chain organic acids in the diet. Treated with CTC and began again 5 d post tx	Free range aviary	
15	4/1/2013	23	0147/13	42 & 43	22,500	315	Followed hot weather. Foggers in shed.	Free range but not at time	Enlarged lives with spots, Petechial haemorrhages in abdominal, serosal & coronary fat in 3 ex 8 birds

5 Metagenomics

5.1 Introduction

Diseased livers have often been completely negative for bacterial isolation, and microscopic analysis also sometimes fails to detect any sign of bacterial involvement within the liver. For this reason, we hypothesised that the disease pathology within the liver may result from the action of a systemic toxin mobilised from a bacteria in the gut via the circulation to the liver. Therefore, we have investigated the bacteria within the gut as a potential source of toxin acting on the liver. The approach that has been taken was to characterise the structure of the gut microbiota in an attempt to identify bacterial species that are more abundant in diseased birds than in healthy birds. As culture methods have been unsuccessful in identifying the causative agent, alternative methods were required. Recent advances in DNA sequencing technology have opened up new ways of analysing bacterial populations and identifying specific tags for particular bacteria. These high throughput sequencing technologies (Roche/454 pyrosequencing) have been used in this study to investigating the diversity of bacteria present in the gut of SLD affected birds.

Samples from eight independent disease outbreaks have been analysed. Over the timeframe of this project, four separate reports on examinations of gut microbiota from diseased (SLD) and healthy birds were written. These separate reports can be found in the appendices.

The candidate bacterium that has been identified, initially as OTU 47, had 16S rRNA genes that had 98.8% sequence identity to the 16S rRNA gene of *Helicobacter pullorum* NCTC 12824.

In the latest work undertaken by Drs Dana Stanley and Robert Moore, we extended the analysis of the candidate bacterium by carrying out whole metagenome analysis of the sample in which the candidate organism was most prevalent. We have shown that the candidate is related to but distinct from *Helicobacter pullorum*. There is no close match to the genome in the publicly available sequence databases and so we conclude that the candidate bacterium represents a new species. The derivation of genome sequence data has allowed the design of a series of PCR assays for the organism. These PCR assays should allow a more detailed survey of samples from disease outbreaks and will allow us to test the hypothesis that this new organism is the causative agent of Spotty Liver Disease.

5.2 Material and methods

5.2.1 Samples

Samples from eight outbreaks were collected. At each site samples were taken from both diseased birds and healthy birds. The number of samples from each outbreak included in the final analysis is shown in Table 4.

Samples were also collected from an infection trial in which material from affected birds was used to infect healthy birds. Control material from healthy birds was also used. One batch of the material used to infect birds was prepared using gut samples stored in Tryptic Soy broth with glycerine and a second batch was prepared from gut samples stored in Thioglycolate broth with glycerine. The number of birds sampled and sequenced from each group is shown in Table 5.

5.2.2 DNA preparation from gut samples

Material from the intestinal content and caecal samples were resuspended in 250 µl of phosphate buffered saline. Total DNA was isolated using the method of Yu and Morrison[36]. Briefly, a sample was transferred to a 2 ml screw cap tube with lysis buffer (500 mm NaCl, 50 mM Tris-HCl pH 8.0, 50 mM EDTA and 4% sodium dodecyl sulfate) and sterile zirconium beads and then homogenised using a Precellys 24 tissue homogeniser (Bertin Technologies) at

maximum speed of 6500 rpm, twice, 3 x 10 seconds each time. Following centrifugation the supernatant was collected, ammonium acetate was added and nucleic acid was precipitated with isopropanol, followed by ethanol wash. After centrifugation the pellet was resuspended in Tris-EDTA buffer and digested with DNase-free RNase and proteinase K to remove RNA and protein. The DNA was finally purified on a QIAamp column (Qiagen) according to the manufacturer's instructions. DNA quantity and quality was measured on a NanoDrop ND-1000 spectrophotometer.

5.2.3 PCR amplification of 16S ribosomal RNA gene sequences

DNA derived from the bacteria of the birds was processed to amplify the 5' end (V1-V3 region) of the eubacterial 16S ribosomal RNA genes. DNA was amplified using Bio-Rad iProof DNA polymerase. Each PCR reaction contained 25 µl of iProof 2X master mix (containing buffer, nucleotides and iProof enzyme), 2 µl of each primer (final concentration 0.5 µM), 1.5 µl DMSO, 0.5 µl 50 mM $MgCl_2$ and template DNA made up to 19 µl in water. The primers used were designed to amplify the V1-V3 region of the 16S rRNA gene (forward primer, 5' AGAGTTTGATCCTGG 3'; reverse primer, 5' TTACCGCGGCTGCT 3'). Each primer also included sequences to facilitate the sequencing of products in the Roche/454 system and the reverse primers consisted of a related set of primers that differed in "barcode" sequences; specific sequences introduced into the primers to allow tagging of individual samples in a multiplex sequencing system. The PCR reactions were performed in an Eppendorf Mastercycler using the following conditions: 98°C for 60 seconds then 25 cycles of 98°C for 5 seconds, 40°C for 30 sec, 72°C for 30 sec; elongation at 72°C for 10 min then hold at 5°C. The efficiency of PCR amplification of each sample was assessed by running 10 µl of the PCR mix on a 1.5% agarose gel.

Table 4 Number of samples from each disease outbreak that were sequenced

Outbreak No.	Healthy Bird Samples		Diseased Bird Samples	
	Caecum	Intestine	Caecum	Intestine
1	6	5	6	5
2	6	6	6	6
3	6	0	6	0
4	4	0	4	0
5	5	3	8	7
6	4	3	3	2
7	3	3	3	0
8	2	3	4	0

Table 5 Number of samples sequenced from infection trial bird gut samples

Sampling Point	Treated*		Controls*	
	Thioglycolate	Tryptic Soy	Thioglycolate	Tryptic Soy
Day 3	9	6	0	3
Day 7	8	7	2	3
Day 11	11	11	2	3

* Treated birds were dosed with gut contents from field cases of SLD, controls with gut contents of birds without signs of SLD.

5.2.4 High throughput sequencing and analysis of 16S amplicons

The amplified products from each animal were pooled using approximately equal amounts of each PCR product. The pooled samples were sequenced using the Roche/454 FLX+ Genome Sequencer and Titanium chemistry. The output sequence file was analysed using a number of publicly available software packages and databases. The Sff files were burst into fasta and qual files using PyroBayes[37] and chimeric sequences removed using pintail[38]. Sequence quality trimming settings were: sequence length 300-600 bases, no ambiguous sequences, minimum average quality score of 25 and maximum homopolymer run of 6 nucleotides, using QIIME[39]. OTUpipe[40], combining USEARCH and UCLUST scripts[41,42] was used to perform denoising error-correction, abundance and amplicon estimation and OTU (Operational Taxonomic Unit) picking. After OTUs were assigned, using 97% sequence similarity, all of the remaining analysis used QIIME software, using QIIME defaults unless stated otherwise. Genus and/or species names were assigned using a Blast method against the GreenGenes database[43] and further confirmed using the EzTaxon database[44]. All samples represented by less than 1000 sequences were removed from the analysis.

5.2.5 Metagenomics

Whole metagenome analysis involves the sequencing of all the genomes in a complex microbial population, such as that recovered from the gut of a spotty liver affected bird. Our initial 16S rRNA gene sequencing targeted the characterisation of a single gene across the many different bacteria present but whole metagenome analysis attempts to characterise all genes across all the genomes. Because metagenome analysis generates datum that is thousands of times more complex than 16S rRNA gene analysis, it is only possible to do one or a few samples in sufficient detail to give useful results.

The 16S rRNA gene analysis demonstrated that the candidate organism, OTU 47, makes up only a small proportion of the total microbial population in any sample. To maximise the chances of recovering useful information from a metagenomic analysis we used the specific DNA sample isolated from SL outbreak material, which the 16S rRNA gene analysis had demonstrated to carry the highest level of OTU 47. We anticipated that even when using this sample the amount of OTU 47 sequence identified would be low, with very sparse coverage across its genome. To increase the value of recovered DNA sequence information we chose to use a specialised sequencing method on our Roche/454 sequencing machine. Long paired-end sequencing allows sequences that are several kilobases apart to be linked to each other and hence assists in assembling sequence data. This information was supported by a 2 x 250 bp sequencing run on an Illumina MiSeq instrument.

5.3 Results

5.3.1 16S ribosomal RNA gene amplification

Samples amplified satisfactorily and gave clean products with very low amounts of non-target bands. An example of the gel analysis of the PCR amplification is shown in Figure 8. Most samples amplified satisfactorily and progressed to sequencing. For a small proportion of the samples (8 of 127) PCR amplicons could not be obtained and so they could not be included in the analysis.

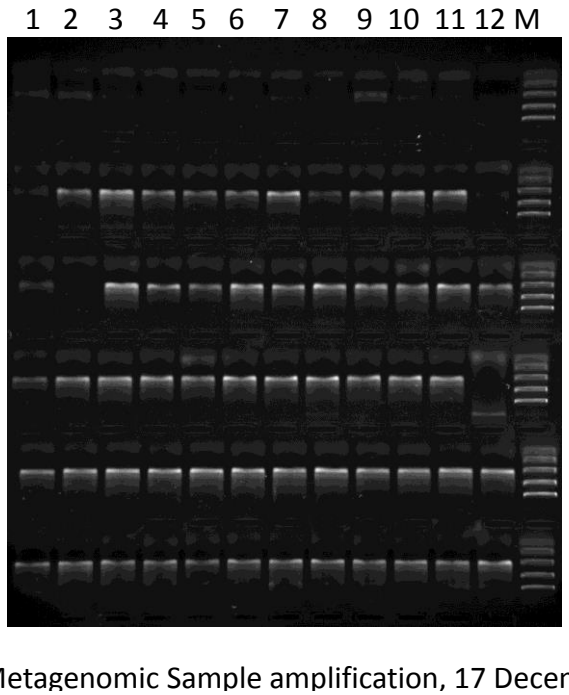

Figure 8 Example of gel analysis of amplification of 16S PCR products

5.3.2 Sequence output

Sequence data was recovered from 119 field samples, which gave a total of 773,921 raw sequence reads. After quality trimming, 513,895 reads were retained; an average of 4,318 reads per sample.

For the experimental infection trial 157,032 raw sequence reads were obtained from the 65 samples. After quality trimming 133,716 sequence reads remained for analysis; an average of 2057 reads per sample.

5.3.3 Experimental infection trial

The experimental infection trial did not provide any indication of the bacterium that might be responsible for SLD. The only obvious sample clustering that could be seen was based only on the day samples were taken (Figure 9). No clustering of samples was seen based on the source of bacteria used to inoculate birds.

5.3.4 Intestinal samples are dominated by *Lactobacillus* species

To explore the data principal component analysis (PCoA) plots were inspected (Figure 10). As expected the most obvious clustering of samples was based on the tissue origin of samples;

that is whether derived from caecal content or intestinal content. Component 1 accounted for a large percentage (64.84%) of the variation seen between samples. In general the microbiota samples from intestinal content were more tightly clustered than the caecal derived samples.

The ileal samples were quite distinct from the caecal samples because they were strongly dominated by *Lactobacillus* species. A phylogenetic analysis (Figure 11) shows that most ileal samples consisted of greater than 90% *Lactobacillus* species whereas the caecal samples rarely had more than 20% *Lactobacillus*. The domination of the intestinal samples by *Lactobacillus* species meant that very little depth of data was seen for other bacterial species. Therefore, the rest of the analysis concentrated on the caecal derived samples.

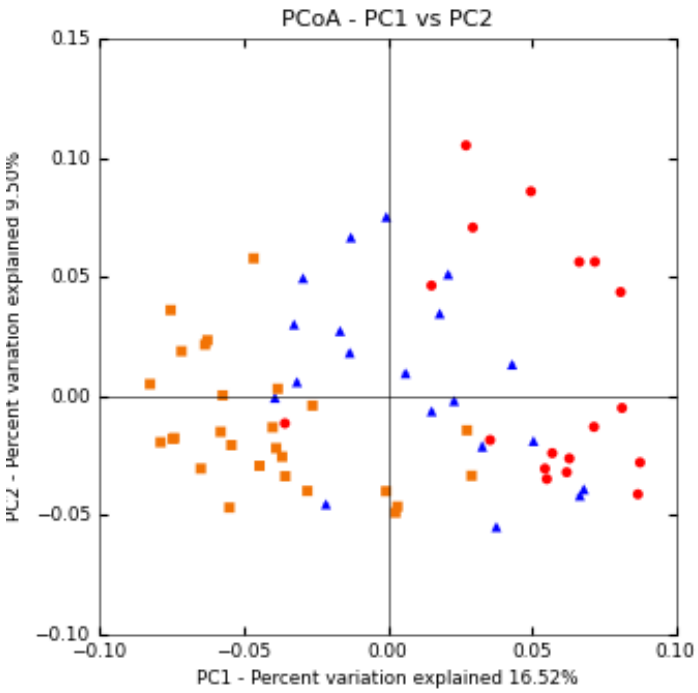

Figure 9 PCoA plot of Bray-Curtis results from analysis of experimental infection samples

Red circles indicate samples from day 3.
Blue triangles indicate samples from day 7.
Orange squares indicate samples from day 11.

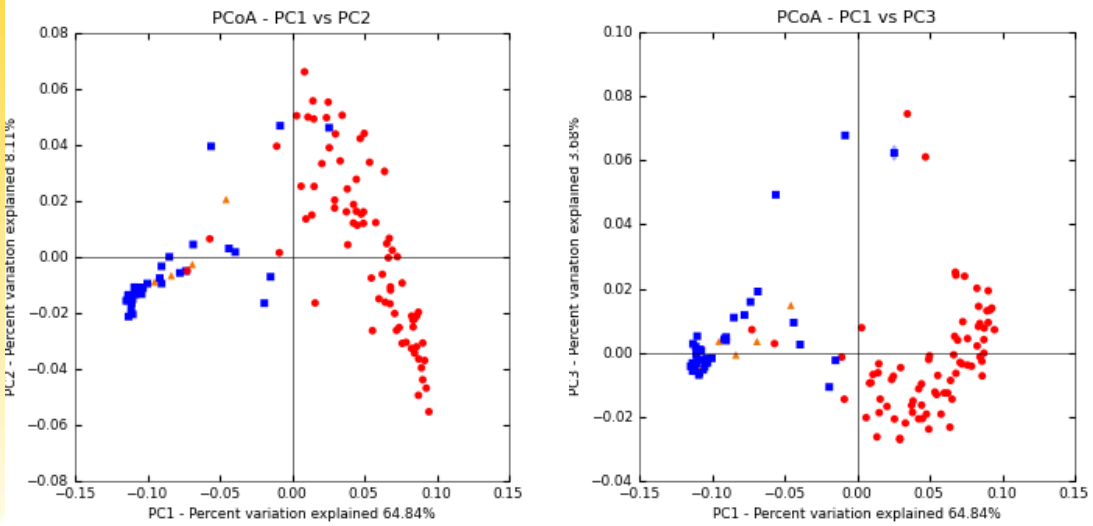

Figure 10 PCoA plots using weighted Unifrac results

Red circles represent 16S samples derived from caecal content.
Blue squares represent samples derived from intestinal content.
Orange triangles represent samples derived from ileal content.

Background: PCoA plots are designed to show the overall relatedness of samples, the closer samples are plotted to each other the more similar they are. There are a range of algorithms that can be used to define the similarity (or dissimilarity) of samples that are used in PCoA plots. Within the software packages the plots are interactive such that mousing over a symbol reveals which sample it is derived from. 3D plots can also be produced.

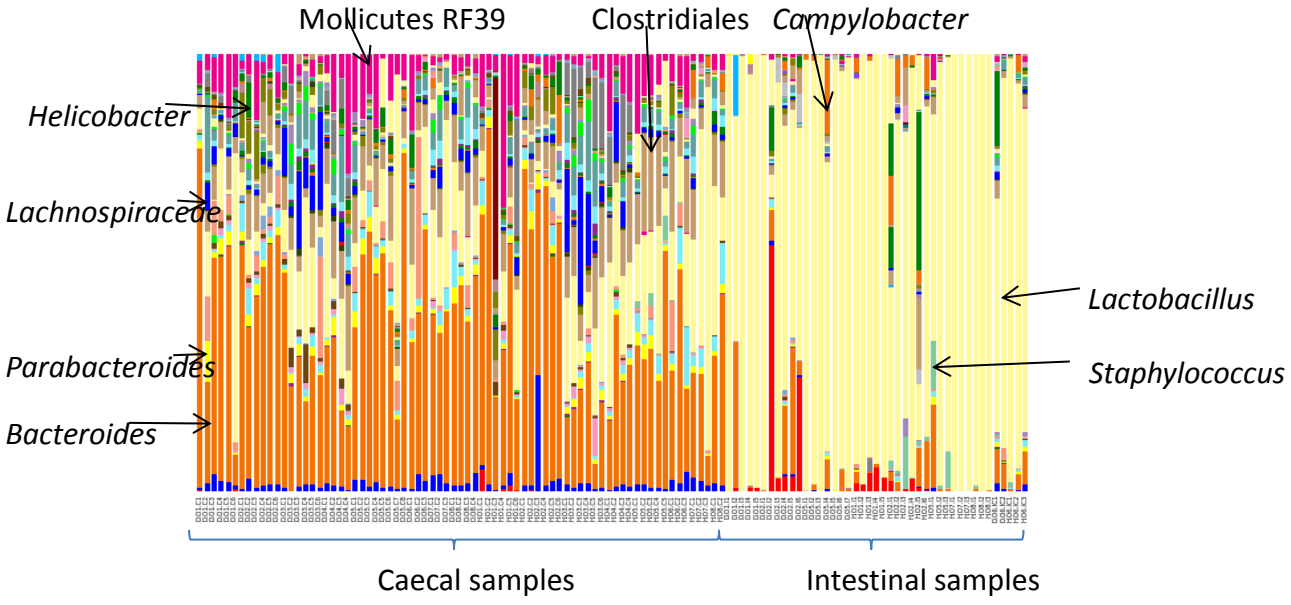

Figure 11 Phylogenetic analysis of all samples at the genus level

The caecal samples are on the left and the intestinal samples are on the right.
Each column represents the total microbiota for a particular sample, coloured and divided proportionally to the type of bacteria present.
The colours representing a number of the different bacterial groups have been identified by the labels and arrows.

5.3.5 Samples cluster according to the disease outbreak from which they are derived

The PCoA analysis also clearly showed that samples clustered according to the disease outbreak from which they were derived (Figure 12). Also, outbreaks 1 to 4 were somewhat clustered together as were outbreaks 5 to 8. Outbreaks from the same properties, but sampled at different times were clustered together. For example: outbreaks 1 and 2 were both from Farm 3, sampled 12 days apart; outbreaks 3 and 4 were both from Farm 5, sampled one week apart; and outbreaks 5 and 6 were both from Farm 5, sampled 7 weeks apart. It is to be expected that such clustering would be seen as the caecal microbiota of birds from one property is likely to have been influenced by such factors as common origin and in some cases a common batch of birds, similar environments and husbandry practices, as well as similar feed and water.

5.3.6 Samples do not cluster according to health status

We further examined the clustering analysis for any evidence of clustering based on health status. No clustering could be discerned (Figure 13). Because of the differences in microbiota composition between outbreaks, the global analysis to compare healthy and diseased birds may be confounded by the wide spread of results. Therefore clustering by health status was further examined by considering the clustering analysis of the caecal samples from individual outbreaks (Figure 14). Clearly there are no gross systematic differences in the overall structures of microbiota from healthy and diseased birds. This is consistent with the hypothesis that a single pathogenic bacterial species, perhaps of fairly low abundance, is responsible for disease pathogenesis. To identify such a hypothesised bacterium it was necessary to look in more detail at the microbiota analysis, down to the level of each bacterial species identified.

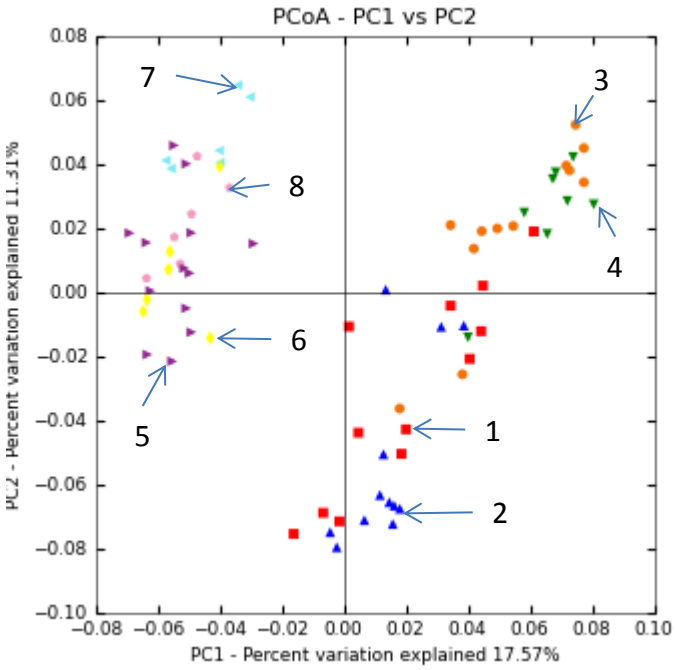

Figure 12 PCoA plot using Bray-Curtis results from the caecal samples

The coloured symbols corresponding to samples from each outbreak are indicated by the numbers and arrows.

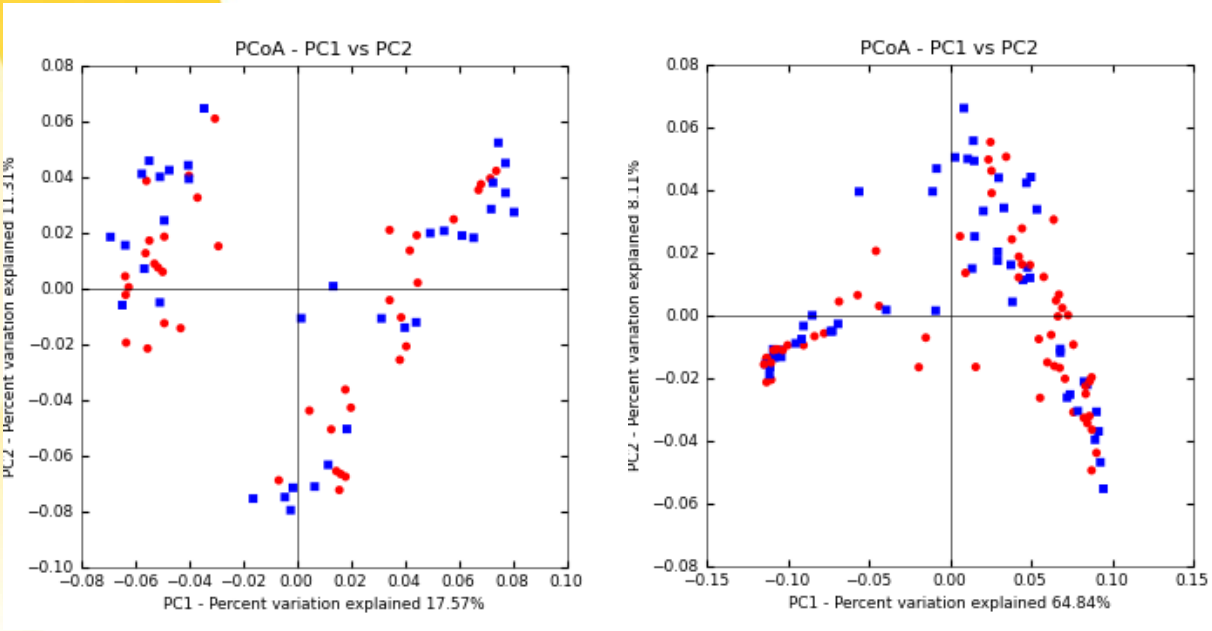

Figure 13 PCoA plots colour coded for health status

The left panel plots the Bray-Curtis results for the caecal samples (related to Figure 12).
The right panel plots the weighted Unifrac results for all samples (related to Figure 10).
Blue squares indicate samples from healthy birds.
Red circles indicate samples from SLD birds.

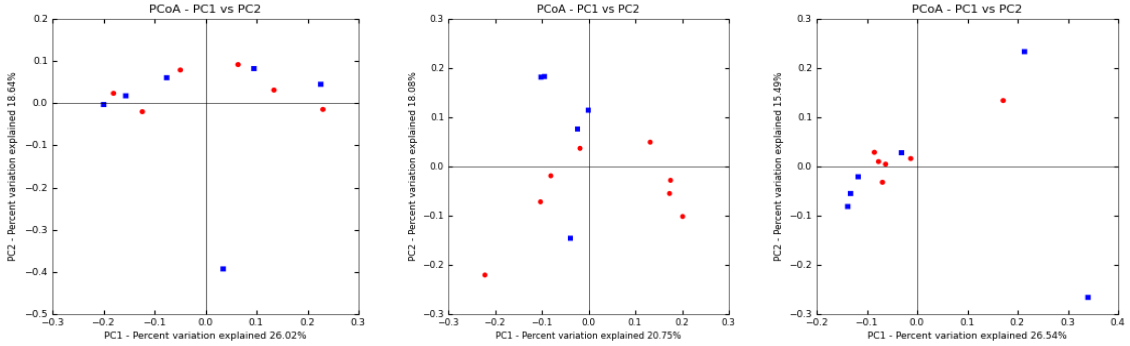

Figure 14 PCoA plots of Bray-Curtis results from caecal samples of outbreaks 1, 2 and 5

Blue squares indicate samples from healthy birds.
Red circles indicate samples from SLD birds.

Background: In this type of high throughput 16S rRNA based analysis of microbiota the analysis is based on operational taxonomic units (OTU). For the purposes of discussion, the OTU can be regarded as roughly equivalent to a bacterial species when the sequence clustering to produce the OTUs is set at a similarity level of 97%. This means that all the sequences that are classified within a particular OTU have a similarity of at least 97%. If the OTUs are based on a lower level of similarity then, depending on the percentage similarity, the OTU would be more equivalent to a genus, family or order. Once sequences are clustered into OTUs a representative sequence can then be compared with phylogenetic databases to determine if the OTU is related to a known bacterial species.

5.3.7 Statistical analysis of OTU abundance identifies a potential pathogen

The analysis of the sequence data results in a table in which the abundance of each OTU is mapped against each sample. Statistical tools can then be used to identify OTUs in which variations in abundance across samples correlate with variations in some other sample characteristic, for example which outbreak the sample is from or the health status of the bird.

When ANOVA was used to interrogate the data from each outbreak in isolation, it was found that for each outbreak there were a number of OTUs that correlated with health status (healthy or SLD) at a statistically significant level ($p<0.05$), however, no single OTU correlated at statistically significant levels across more than 3 of the 8 outbreaks. When all the data was pooled to create a single data set, and therefore give greater statistical power, ANOVA identified 12 OTUs that were differentially abundant between healthy and diseased birds at $p<0.05$; these OTUs are shown in Table 6. If the working hypothesis is correct then the pathogen causing SLD would be expected to be in higher abundance in the diseased birds. Therefore, a relevant OTU should be more abundant in diseased birds than in healthy birds. Only 3 of the 12 differential OTUs had a ratio of greater than one. OTUs 1229 and 267 were classified by reference to the GreenGenes database[43] down to genus level and identified as *Bacteriodes*. OTU 47 was classified to the genus *Helicobacter*. When the data set was investigated in more detail it was seen that OTU 1229 was only identified in 3 of the outbreaks (2, 5, and 6) and OTU 267 was seen in 5 of the outbreaks (1, 2, 3, 4, and 7) (data not shown). OTU 47 was identified in all 8 outbreaks (Table 7) and on this basis is a candidate as the potential pathogen causing SLD.

Although OTU 47 was detected in all outbreaks it was only more abundant in the diseased birds in 6 of the 8 outbreaks and the difference between abundance in diseased and healthy birds was only statistically significant in a single outbreak, although the overall pooled results for OTU 47 also reached statistical significance. In some outbreaks (e.g. 1 and 4) substantial differences were seen in the abundance of OTU 47 but did not reach statistical significance because of the high bird-to-bird variation within a group and the low number of samples that were available from some outbreaks – note that the outbreak that did show statistical significance was also the outbreak with the greatest number of samples.

Table 6 OTUs identified as differing in abundance between healthy and diseased birds

OTU	Probabil - ity	H_mean[a]	D_mean[a]	D/H[b]	Consensus Lineage[c]
90	0.0078	0.0034	0.0014	0.4136	p__Firmicutes; c__Clostridia; o__Clostridiales
222	0.0094	0.0051	0.0026	0.5113	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides
19	0.0150	0.0235	0.0064	0.2730	p__TM7; c__TM7-3; o__I025; f__Rs-045
657	0.0203	0.0014	0.0005	0.3500	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides; s__Bacteroides plebeius
1229	0.0215	0.0002	0.0006	4.0250	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides
94	0.0216	0.0018	0.0005	0.2625	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__Lactobacillus coleohominis
316	0.0224	0.0010	0.0003	0.3387	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales
1059	0.0237	0.0090	0.0022	0.2487	p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__Lactobacillus helveticus
32	0.0240	0.0085	0.0013	0.1490	p__Firmicutes; c__Clostridia; o__Clostridiales
97	0.0271	0.0084	0.0038	0.4600	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides
267	0.0352	0.0003	0.0012	4.2778	p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Bacteroidaceae; g__Bacteroides
47	0.0408	0.0026	0.0073	2.7897	p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Helicobacteraceae; g__Helicobacter

[a] percentage of OTU in data set, [b] ratio of proportion in diseased birds compared to healthy birds, [c] taxonomic assignment of OTU by reference to GreenGenes database.

Table 7 Prevalence of OTU 47 across the outbreaks

Outbreak	Probability[a]	H_mean	D_mean	D/H ratio
CO1	0.3693	0.0004	0.0030	8.5000
CO2	0.7547	0.0097	0.0072	0.7407
CO3	0.1646	0.0005	0.0025	4.6667
CO4	0.0841	0.0013	0.0167	12.4000
CO5	0.0493	0.0000	0.0164	Inf
CO6	0.6612	0.0022	0.0039	1.8333
CO7	0.3027	0.0054	0.0007	0.1333
CO8	0.3552	0.0000	0.0008	Inf
All	0.0408	0.0026	0.0073	2.7897

[a] Probability that the difference between the abundance in healthy and diseased birds is by chance.

5.3.8 OTU 47 is closely related to *Helicobacter pullorum*

A sequence representative of OTU 47 was used to interrogate the EzTaxon database[44] to identify the most closely related cultured bacteria. OTU 47 had 98.8% identity to *Helicobacter pullorum* NCTC 12824.

5.3.9 Metagenome sequencing

The Roche/454 metagenomic sequencing run produced 1.17 million sequence reads, and the Illumina MiSeq run produced about 15 million reads. Database searches indicated that no more than 0.7% of this sequence information had homology to *Helicobacter* sequences. Figure 15 indicates the phylogenetic composition of the sample as indicated by MG-RAST analysis of a random subset of 2.4 million sequences of MiSeq data.

The low level of the candidate organism has meant that only a small amount of fragmentary genomic DNA sequence information has so far been mined from the metagenomic data. This has not allowed the construction of any form of whole genome assembly but has given sufficient information to allow the design of new PCR assays to assist in ongoing characterisation of clinical material derived from Spotty Liver cases.

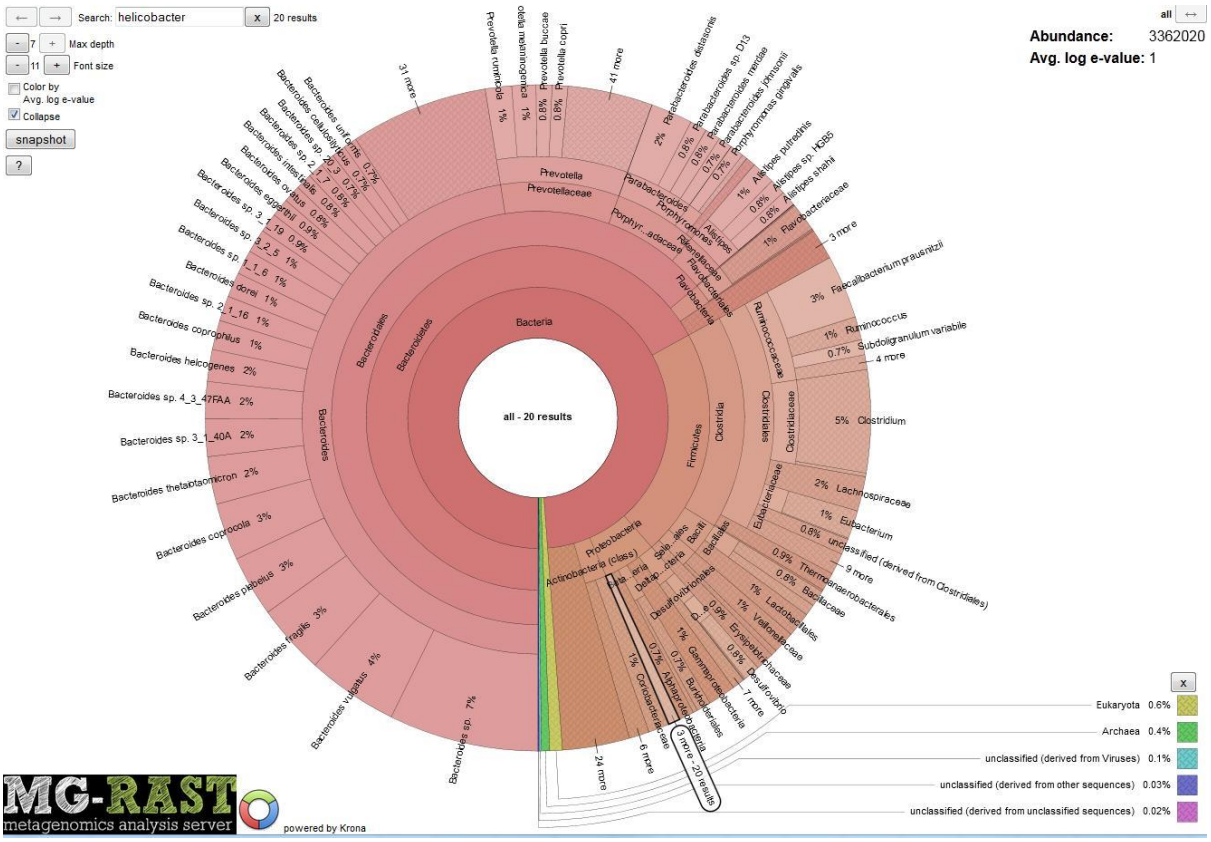

Figure 15 Phylogenetic composition of the sequenced DNA sample from SLD affected hen caecum, as determined by MG-RAST analysis[45]

The small helicobacter related proportion is contained within the highlighted segment at about 5 o'clock on the diagram.

Using paired-end sequence reads from the Roche/454 run it was possible to select out all those sequence reads that corresponded to the OTU 47 16S rRNA gene sequence. By using the other, non-16S, segment of each paired-end read it was possible to "walk out" into the genome to find other genomic regions that were linked to 16S in the target genome. When the new sequences were investigated by searching against the public sequence databases it was

commonly found that the sequence had no good match to anything previously characterised. Other regions showed significant homology to *Helicobacter* genomes but generally not to *H. pullorum* but rather *H. hepaticus*, *H. cinaedi*, and other helicobacters. This indicates that the candidate organism is unlikely to be a strain of *H. pullorum* but rather some other previously uncharacterised *Helicobacter*.

5.3.10 PCR development

Some of the newly identified genomic regions of the candidate organism were used to design PCR primer pairs. The aim was to develop a specific and reliable PCR assay to monitor for the presence of the organism in DNA extracts prepared from clinical material. Five primer pairs were designed. All pairs produced PCR products when used on the DNA sample that was used for metagenomic analysis (Figure 16).

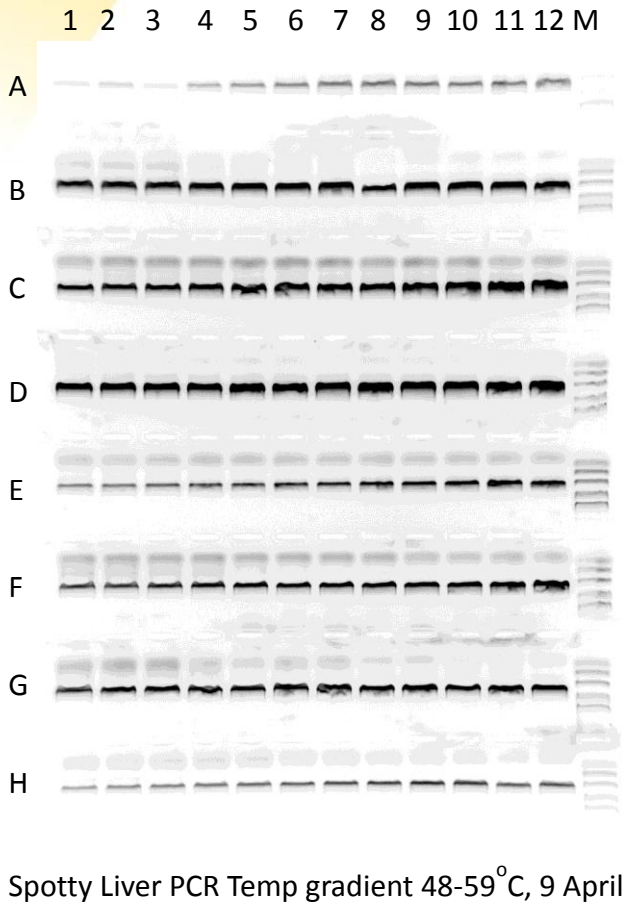

Figure 16 Gradient PCR to determine temperature optimum for PCR reactions

PCRs were carried out in a 96-well plate and samples run on a 1.5% agarose gel for visualisation.
Temperature increased in 0.5°C increments across the plate from wells 1 to 12.
Five primer pairs were used: T1-T2 in row A, T3-T4 in row B, T5-T6 in rows C and F,
T7-T8 in rows D and G, and T15-T16 in rows E and H.
Bioline Taq polymerase was used in rows A to E and New England Biolabs Q5 polymerase was used in rows F to H.
The template DNA in all tracks was from the DNA sample that had been used for metagenome analysis.
Molecular weight markers are loaded in track M.

Each of the PCR primer pairs was used on a series of samples, including known positive and negative controls. An example of the results is shown in Figure 17. The primers appear to provide specificity for the candidate organism and under the PCR cycling conditions used, the assay is semi-quantitative in that samples which from deep 16S sequencing were expected to

have the highest levels of the candidate gave the strongest PCR bands, while samples with lower levels gave less intense PCR bands. Negative controls did not produce a band. Interestingly, the candidate organism was readily detected in caecal samples from a series of birds (Figure 17, positions C6 to C10) but was not detected in the DNAs prepared from small intestine content samples from the same birds (Figure 17, positions C1 to C5). This indicates that culturing efforts should be concentrated on caecal samples rather than small intestine samples.

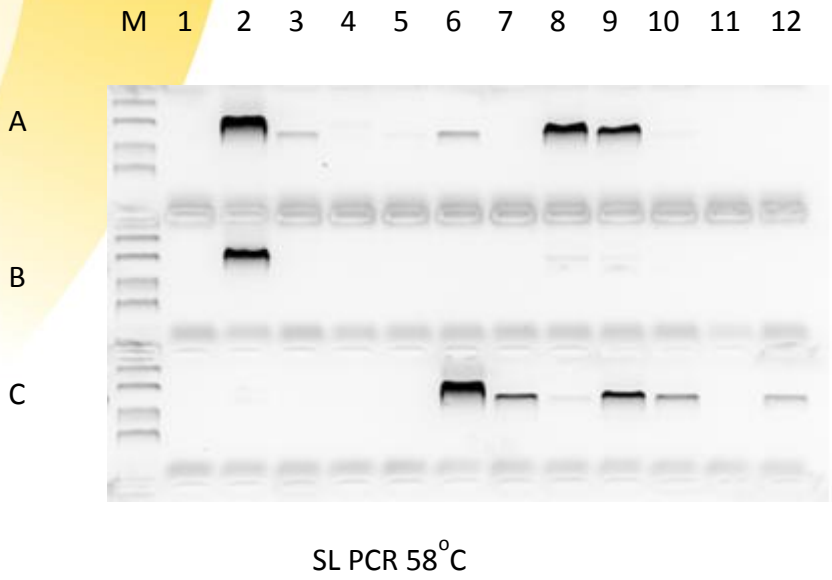

Figure 17 PCR using primer pairs T7-T8 (rows A and C) and T15-T16 (row B)

Positions 2A and 2B use the same template as used in Figure 16.
Positions 7A and 7B are no template controls, and positions 11A and 11B are a mouse caecal DNA negative control.
Note that the T7-T8 primer pair amplifies a band in a series of clinical sample DNAs derived from caecal samples (C6 to C10), but not in the intestinal sample DNAs from the same birds (C1 to C5).

5.4 Discussion

OTU 47, initially identified as *H. pullorum* related, has been identified as the potential pathogen responsible for the development of Spotty Liver Disease. *H. pullorum* was first recognised and named in 1994[21]. It has been commonly found in healthy chickens[25,26,30,31,32,33] and turkeys[34]. The organism has been reported in faeces and intestinal contents of meat chickens in Australia[29]. There have been tentative suggestions of a link between *H. pullorum* and SLD but no convincing evidence has been reported[20,21] and no signs of liver pathology have been reproduced in an infection model[15,35]. *H. pullorum* is difficult to differentiate from other Helicobacters and Campylobacters, and so this is consistent with early suggestions that *Campylobacter* and a “vibrio” like organism may be involved with SLD. *H. pullorum* may be a zoonotic agent as infections in humans have been implicated in gastrointestinal diseases and in liver disease[21,27]. *Helicobacter* spp. have been associated with liver disease in mice[22,23]. A toxin, cytolethal distending toxin, has been identified in avian and human isolates of *H. pullorum*[27,28].

The evidence produced in this study, which has lead to the conclusion that OTU 47 may be involved in SLD, is certainly not overwhelming but it seems a remarkable coincidence that the single candidate identified is closely related to an organism that has previously been suggested by other workers to be involved in disease. OTU 47 was seen in healthy birds as well as diseased birds. This suggests that OTU 47 can be non-pathogenic; this could be because a critical population level is required to induce disease, other predisposing factors are required, or strain differences determine pathogenicity. There is certainly good evidence that there are significant levels of strain diversity, both in human and chicken isolates[46]. In six out of eight sets

of outbreak samples OTU 47 was detected at higher levels in the SLD birds than in healthy control birds, as our hypothesis would predict. The finding that in two sets of outbreak samples OTU 47 was more abundant in healthy birds than in SLD birds does not necessarily argue strongly against the hypothesis, as it is not clear which part of the gastrointestinal tract (GIT) should ideally be assayed for the pathogen. This study has concentrated on samples from the caecum but other areas of the gut may be of equal or greater importance as the place of residence of the pathogen.

Culturing of the candidate organism has proven difficult, so alternative approaches to advance our knowledge of the organism have been sought. Metagenomic analysis has been carried out to generate genomic sequence information beyond the 16S rRNA gene sequence originally used to identify the candidate organism. Because there is only low-level coverage of the candidate organism, OTU 47, in the metagenomic data, only fragmentary genomic information has so far been defined. It is clear from the fragmentary genomic data that the candidate organism, although probably a helicobacter, is not currently represented in the publically available sequence databases and is likely to be distinct from *H. pullorum*. Because of this clear separation from *H. pullorum* it was inadvisable to use the currently published PCR assay[47] for further analysis of clinical samples. The fragmentary genomic sequence data was sufficient to allow the design of new PCR assays which, in initial work, appear to be specific for the target bacterium.

5.5 Future work

There is a clear path forward for future work with two key areas of activity – the first directed towards more confidently establishing the identity of the pathogen via survey work of clinical material from disease outbreaks, and the second aimed at culturing the candidate organism and then going on to experimentally reproduce the disease.

The new PCR assays can now be used to interrogate clinical material from cases of Spotty Liver Disease, with several goals in mind:

- Firstly, to test how strong the correlation is between disease and presence of the candidate organism.
- Secondly, to identify new clinical samples that carry a higher load of OTU 47, which could be subjected to metagenomic sequencing in order to achieve a more complete characterisation of the genome.
- Thirdly, the PCR could be used to monitor OTU 47 growth in enrichment cultures – it has proven difficult to isolate potential colonies of OTU 47 in a single step primary plating procedure but it might be possible to enrich in liquid culture with the assistance of the PCR assays.

From the current analysis, only fragmentary pieces of the OTU 47 genome have been recovered from the metagenomic sequencing. It would be valuable to analyse the existing data in more detail and also generate more complete information from new sequencing efforts. A good draft genome sequence of the organism would allow an analysis of its biochemical potential and may reveal ways in which it could be cultured. Successful culturing of the organism is likely to give a much higher probability of experimentally reproducing the disease. A draft genome would also facilitate a search to discover key virulence factors, including extracellular toxins, which might be responsible for disease pathogenesis. Even without successfully culturing the organism it may be possible to design vaccines based on such virulence factors but it would be difficult to test experimental vaccines without having also developed an *in vivo* disease induction model to reproduce the disease.

6 Cell toxicity study

As discussed, the current theory best explaining the pathogenesis and development of SLD in laying birds is the production of a bacterial toxin in the gut of the affected birds, which then has its effect in the liver. In an attempt to further define the role of toxins in the disease pathogenesis we obtained serum from diseased birds. This was frozen and later the University of Melbourne thawed the serum and inoculated it into kidney and liver related cell lines, as reported on the following page.

The lack of cytopathic changes in the cell lines examined does not preclude the role of a toxin in SLD. Issues such as the impact of freezing on the toxin, concentration in general circulating serum and time of collection from the affected birds may all have an adverse impact on the likelihood of demonstrating toxicity in the cell lines.

APCAH
Asia Pacific Centre for Animal Health

THE UNIVERSITY OF MELBOURNE

1st March 2012

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Accessions SCX 17, SCX 18 & SCX 19

Spotty Liver serum toxicity in cells

Primary chicken embryo kidney and LMH (a chicken hepatocellular carcinoma cell line) cells were seeded into 24 well plates (1mL per well) and incubated at 37°C/5% CO_2 for 48 hours until monolayers were 80 % confluent.

Media was removed and 0.05mL of serum, taken from chickens affected by spotty liver and unaffected chickens, were applied using one well per sample. Serum from SPF chickens was used as a negative control. Uninoculated cells were treated with newborn calf serum. The plates were then incubated at 37°C for 30 minutes.

Maintenance media was added to all wells (M199 for CEK cells, DMEM for LMH cells). The media was not supplemented with extra calf serum.

The plates were incubated at 37°C/5% CO_2 for 6 days. The cells were examined and compared with union

There were no differences observed between cells in wells inoculated with SPF serum, serum from unaffected birds or affected birds. The only difference observed was that in wells with chicken serum, the cell monolayers became confluent more rapidly than those supplemented with calf serum. This was expected as chicken cells are known to show improved growth in the presence of chicken serum.

There was no evidence of cytopathic effect or changes due to toxicity in any cells.

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7 Challenge studies

Figure 18 The author Dr P Scott examining birds from the challenge study

7.1 Purpose

The purpose of the study was to recreate the clinical condition that occurs in the field or at least the subclinical condition that is recognised on post mortem examination of affected birds.

We also wished to identify if the causal organism is an anaerobe or an aerobe. This would allow a more focussed approach to identifying the causal organism(s). We also aimed to use tissues and intestinal material from the birds that may develop clinical signs and lesions for controlled genetic characterisation.

7.2 Materials and methods

Two studies were undertaken with Hy-Line Brown layers sourced from a commercial farm. The birds had received no antibiotic or organic acid treatment prior to the study and the in-study feed was also free of such additives. In each study 110 birds were orally dosed with caecal and intestinal contents from either healthy birds or birds affected by SLD. These samples had been stored in either Thioglycolate or Tryptic Soy broths with added glycerine to allow freezing of the samples. The birds were wing tagged with individually numbered tags and bodyweights were recorded. Treatment groups are described below in Table 8.

Table 8 Experimental design

Group	Treatment	Animals per group
A	Intestinal content from healthy birds (aerobic)	15
B	Intestinal content from healthy birds (anaerobic)	15
C	Intestinal content from affected birds stored in Tryptic Soy broth	40
D	Intestinal content from affected birds stored in Thioglycolate broth	40

The hypotheses tested were:
H_0: Group (C or D) Spotty Liver Rate = Group (A or B) Spotty Liver Rate.
H_1: Group (C or D) Spotty Liver Rate > Group (A or B) Spotty Liver Rate.

7.2.1 Dose and route of administration

Two (first study) or four (second study) mL of intestinal and caecal contents combined was administered by mouth using a 5 mL syringe inserted into the corner of the mouth and the birds allowed to naturally swallow the contents.

7.2.2 Monitoring

Birds prior to and after challenge were monitored for normal behavioural activity including drinking, feeding and egg laying. Specifically birds were monitored for depression, inappetence and any other abnormal signs. Birds were monitored a minimum of 2 times daily prior to challenge.

7.2.3 Autopsy

The autopsy included examination of all major organ systems with sampling for histopathology from the liver, kidney, spleen, small intestine and caecum. In addition samples from the mid small intestine and caecum were removed including mucosa, and stored in both anaerobic and aerobic media as well as samples frozen for metagenomic studies.

On days 3, 5, 7, 9 and 11, three birds from each of groups A and B, and 8 birds from each of groups C and D were autopsied and samples collected labelled and stored.

7.3 Results

The average weight of the birds at the start of Study 1 was 1.901 kg and the birds were 33 weeks old. The average weight of the 23 week old birds used at the start of Study 2 was 1.5919 kg. No birds showed symptoms of disease or required removal from the study. On autopsy examination some birds were not in lay but otherwise no significant changes to any organ system were noticed. In Study 1, 17.3% were not in lay, and in Study 2, 70% of the birds were not in lay at the time of autopsy. No Spotty Liver like spots were observed in any group except in one bird, and these were not related to pathology associated with Spotty Liver when examined histologically.

Metagenomic studies from the birds did not demonstrate any difference in the intestinal or caecal 16S rDNA genomic profiles.

7.4 Discussion

In this study we were unable to reproduce Spotty Liver Disease or even the associated liver changes that have been observed in otherwise healthy birds. With respect to hypothesis testing, the null hypothesis is accepted (that there was no difference in Spotty Liver Disease between treated and control birds). In addition the lack of any difference in 16S rDNA metagenomic profiles from birds treated with ingesta from affected and control birds suggests that our challenge material did not influence gut flora.

Earlier metagenomics studies and the evidence provided by response to treatment with antibiotics are clear evidence of the role of gut bacteria in the disease. Possible causes of failure to reproduce the disease in this case include a need to include both aerobic and anaerobic flora in the inoculum, the failure of the relevant organisms to survive freezing even with glycerol added to the broths, failure of the broths to enable survival of the relevant organisms regardless of freezing and an inadequate dose of the organism. It is also possible that the site of collection of the organism (mid intestine and caecum) was not the correct site or that the organism was not present in high numbers at the time of collection.

In the second study a significant number of birds were not in lay at the time of autopsy and most field cases are seen to have been in good condition and in production at the time of death. It may be necessary in future studies to allow a longer pre-challenge interval in the experimental pens so that birds begin to lay again after transport to the site. It is not possible to speculate whether any adverse response to transport and a new environment would cause more of a physiological demand than that which birds experience when they are near and at the peak of production. In addition, the birds used in Study 2 were lighter than those in the previous study and lighter than the breed standard for this age (1.6 kg actual compared with 1.8 kg).

8 Treatment, control, nutrition and husbandry

Figure 19 Free range layer chickens

In the face of the uncertainly about the causal organism(s) and physiological and husbandry issues involved in the occurrence of SLD, controlled studies of treatment and control options have not been undertaken although management changes from recommended preventive measures have allowed some assessment of the comparative benefits of some synbiotics in the control of SLD. The conclusions drawn here are based on empirical field evidence.

Treatment with antibiotics has been useful in the face of outbreaks. In Australia treatment of hens laying eggs for human consumption is restricted to either chlortetracycline or Linco-Spectin® (Zoetis Australia Pty Ltd), which is a combination of lincomycin (222 mg/g) and spectinomycin (445 mg/g). Chlortetracycline products are generally cheaper and tend to be used more frequently, however, multiple treatments of the same flock result in a reduction in the response to antibiotic treatment. Both the response to treatment and the apparent onset of antibiotic resistance suggest a bacterial cause of the condition.

The prophylactic use of antibiotics should be viewed with caution because, while preventing outbreaks of SLD in early lay, repeated use can lead to subsequent outbreaks of SLD being unresponsive to Chlortetracycline, presumably due to the development of resistance by the causative bacteria.

It is preferable to treat outbreaks of SLD with water-soluble chlortetracycline for short periods of 3 to 5 days while concurrently reviewing the management stressors. On occasions repeat treatment and 2 weeks of in-feed treatment will be required.

With respect to the management of rearing, there appears to be an advantage rearing birds on a floor system because there are significantly less adaptation pressures on the birds after transfer, around water and feed intake.

In regard to the various prebiotics and probiotics that have been utilised, it is the medium chain fatty acids (MCFA) that have demonstrated most promise in reducing the occurrence and severity of outbreaks. However, in general, they appear to have no significant therapeutic value in altering the course of an outbreak and equally limited value in preventing recurrent outbreaks, if introduced after the outbreak has commenced. To be advantageous their use requires incorporation well before the onset of lay. They will not compensate for poor husbandry.

Other combinations of feed additives (synbiotics) or in water prebiotics have not resulted in any universal observation of the ability to prevent SLD. In two instances where producers have switched some sheds from recommended programs of MCFA inclusion in the diet to the inclusion of probiotics, the birds in the sheds receiving probiotics have become affected by SLD, while the birds in sheds still receiving MCFA have not been affected by SLD.

The formulation of the feed should be consistent and vary little between the various ration stages. The quality of the raw materials needs to be consistent and the incorporation of new season cereal grains needs to be carried out with caution, and with the necessary enzymes.

Any disruptive feeding patterns need to be avoided, and the husbandry practices of birds maintained consistently through the production period. Outbreaks of SLD have been observed following feeding equipment issues, or changes to times of pop-hole opening and subsequent disruption of the normal feeding patterns.

Variations in water source and patterns of water intake need to be avoided; something difficult to do with free range birds.

On some occasions flocks that have MCFA in the feed and are under good husbandry conditions may have low background mortalities that are within standard expectations, but SLD can be identified in some of these mortalities. In such cases it is recommended that Chlortetracycline (CTC) treatment not be undertaken. It should always be noted that the use of CTC has the disadvantage of creating a microflora imbalance that can aid the colonisation of *Enterobacteriaceae*, which can result in other bird health and food safety issues.

In flocks with repeatedly relapsing SLD, it may not be apparent whether this is a recurrent whole-of-flock condition or is a new outbreak within a sub-population of previously unaffected birds. If the former, this raises questions about the ability of a flock to establish a protective immunity and whether the disease could be prevented by vaccination or controlled exposure. In comparable avian diseases, where the organism can chronically colonise the host (including Necrotic Enteritis and *Salmonella*), a robust solution by vaccination alone has not been identified.

SLD continues to be a condition that frustrates the Australian poultry industry and those veterinarians who service it.

9 Isolation of putative causal organism of Spotty Liver Disease in Australia

Further to the completion of the initial draft of this report, additional work, funded by the Poultry CRC, by Scolexia and Professor Moore (RMIT University) on Spotty Liver cases on commercial farms has led to the isolation of a *Campylobacter* species from field cases of Spotty Liver Disease in Australia as described below. A paper describing the new bacterial species and formally naming it *Campylobacter* hepaticus has been published. It is clear that this new organism for the Campylobacter/Helicobacter group is the cause of current Spotty Liver Disease outbreaks.

Details of the characteristics of the new bacterium can be found in:

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. doi: 10.1099/ijsem.0.001383

10 Discussion

Considerable progress has been made in our understanding of SLD with recognition of the distinction between the syndrome first reported in the USA and known as Vibrionic Hepatitis and SLD, as reported in the UK and Australia. We have a better indication of the causal organism and the possible mechanism of pathogenesis with the production of Cytolethal Distending Toxin (CDT) by Helicobacter type organisms explaining lesions in the liver, and the variable expression of the CDT genes explaining the difficulty in reproducing the disease, and the inconsistent findings with respect to epidemiology of the condition. In the process we have also learned more about the “normal” flora of the poultry intestine and caeca and have demonstrated consistent changes in the flora of affected and unaffected birds. We have shown that metagenomic studies are a very useful tool to further investigate the cause of SLD and other conditions. We have observed that the use of medium chain fatty acids is useful in reducing the incidence and severity of outbreaks, and that the use of some probiotic products in place of the medium chain fatty acids does not appear to reduce the incidence or severity of outbreaks.

We can clearly state the case definition as involving laying birds in good condition most likely at the peak of lay (at least in the first instance) and manifesting clinically primarily as sudden death. The average age of birds during outbreaks was 33.8 weeks with a range of 22 to 80 weeks and a median age of 28 weeks. All types of management systems were affected, with a higher proportion of free range farms affected. Outbreaks often occur following an interruption to normal management such as changes to ranging times, nest box issues and feed interruptions. On initial treatment with antibiotics there is a good response but this can diminish with repeat treatments. Gross pathology is chiefly characterised by the presence of multiple focal lesions 1-2 mm diameter, which are usually greyish to white but occasionally red. In general the liver is slightly swollen. Other gross pathology is variable. The hens are observed to be reproductively active and in good body condition. Histology reveals multifocal coagulative necrosis throughout the liver with hepatocellular degeneration and disassociation progressing to loss of hepatocytes with fibrin laking, heterophils in the affected areas as well as hyperaemia/congestion. Given the previously mentioned issues with investigators not describing pathology of the cases they discuss, and the mixed pathology and clinical signs observed in the 1950s, the establishment of a case definition is an important contribution.

Attempts to reproduce SLD by various methods (dosing birds with gut contents of liver or intestinal and caecal contents from affected birds or with particular organisms) described in the literature review did not succeed in reproducing the disease. The two challenge studies undertaken in this project did not lead to the creation of a viable challenge model. In all instances, the intestinal and caecal samples used in the challenge studies were frozen prior to inoculation, and whilst in our studies we immediately stored samples in both tryptic soy and thioglycolate broths, no attempt was made in any study to collect samples in a microaerophilic atmosphere. It is possible that either or both issues affected the ability to reproduce SLD. Whilst difficult, the collection of samples in a microaerophilic atmosphere and/or the use of unfrozen intestinal and caecal contents may increase the likelihood of establishing a challenge model in hens. The lack of cytopathic effects of serum from affected birds on chicken kidney and hepatocellular carcinoma cell lines does not rule out the hypothesis that the disease involves a toxin produced by gut bacteria. However, further testing of other cell lines and repeat testing of those cell lines used but with fresh unfrozen serum from affected birds should be undertaken.

The metagenomic evidence produced in this study suggests that an *H. pullorum* like organism may be involved in the disease; this has previously been suggested by other workers. OTU (Operational Taxonomic Unit) 47 was seen in healthy birds as well as diseased birds. This suggests that OTU 47 can be non-pathogenic. This could be because a critical population level is required to induce disease, other predisposing factors are required, or strain differences determine pathogenicity. There is certainly good evidence that there are

significant levels of strain diversity, both in human and chicken isolates. However, the subsequent isolation of a *Campylobacter* organism from field cases in Australia by Scolexia and Professor Moore (RMIT University), which has sequence homology to the organism isolated by Crawshaw and Irvine[18] from UK cases, will enable the development of an exposure model that will allow increased progress in assessments of treatment and control methods.

In addition, some bacteria were less prevalent in affected birds including *Lactobacillus coleohominis*, *L. helveticus*, some Clostridiales, some unidentified OTUs and *Bacteroides plebius.* However, other *Bacteroides* spp. were more abundant overall. Whilst differences in gut flora between affected and unaffected birds are suggestive of the role of gut associated bacteria producing toxins that affect the liver, it is not totally conclusive given the possibility of the condition itself altering gut flora. However, when combined with the frequent absence of bacterial cells in the liver and the response to antibiotic treatment, it is reasonable to suggest that the confirmation of differences in gut flora between affected and unaffected birds in the metagenomic study adds further weight to that hypothesis.

This AECL project, involving examination of the literature, field and laboratory studies, case definition, histopathology and challenge studies has been the basis for the further work conducted by Scolexia and Professor Moore (RMIT University), which has allowed us to isolate what appears to be the causative organism, with the proposed name of *Campylobacter hepaticus* sp. nov., from field cases in Australia. Crawshaw and Irvine[18] isolated this organism from field cases in the UK and, whilst not reproducing the disease, were able to reisolate the organism from birds challenged by intraperitoneal infection and observe microscopic lesions similar to those seen in SLD cases, although without reproducing visible spots.

The priority of future work by Scolexia and Professor Moore should be to reproduce Koch's Postulates using *Campylobacter hepaticus* since isolated from field cases and develop a satisfactory disease model to both extend our understanding of the disease and pathogenesis, and to allow for the evaluation of various control and treatment options. This should involve the use of oral challenge and the reproduction of visible liver spots as seen in normal disease outbreaks, using laying hens close to the peak of lay.

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12 Plain English Summary

Project Title:	Determining the cause and methods of control for 'Spotty Liver Disease'
AECL Project No	1SX091
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Objective	This project aimed to identify the causative agent of Spotty Liver Disease (SLD) by both molecular and *in vitro* techniques, and to assess treatment and control options in the field.
Background	Spotty Liver is a disease of laying birds, which is associated with increased mortality, particularly around the time of peak production and in some instances associated with a decrease in production. The cause of the condition is not known but suspected to be a bacterium, which produces a toxin that affects the liver and endothelial tissue. The clinical signs include a brief period of depression in laying birds (usually in good body condition and "in-lay"), and increased mortality. Often birds are found dead without any prior evidence of disease noticed. SLD has increased in importance in laying birds in Australia, as a greater proportion of laying birds are housed in free range conditions. Despite many attempts to identify the aetiological agent, presumed to be bacterial because of the condition's rapid curative response to antibiotics, there has been little progression in understanding aetiology and pathogenesis of the disease. It has been observed that many outbreaks are related to disruptions in bird husbandry particularly as it relates to feed intake, formulation and availability. It has also been associated with free range birds accessing water in the range area. While historically SLD occurred in early lay and predominantly in summer, it now occurs at any time in lay and throughout the year. It is still more common in early lay.
Research	The project included a literature review, field investigations including gross and histopathological examinations, challenge studies, and *in vitro* toxicity and metagenomic techniques to elucidate the cause and examine suitable control options. The study involved the investigation of field cases and sampling from both affected birds and those in another shed on the same farm, which were not affected. These samples were used to examine the histopathology of affected birds, to undertake genetic analysis of the gut flora of affected and control birds, to examine possible cell-toxicity of sera from affected birds, and to undertake challenge studies to attempt to reproduce the disease.

Outcomes	Two challenge studies using intestinal and caecal contents from affected birds were undertaken without inducing the condition in challenged birds. Cell toxicity studies did not indicate any toxic effects of serum from affected birds. Metagenomic studies were undertaken comparing intestinal and caecal contents from affected and control birds. An *H. pullorum* like organism (in the Campylobacterales order) was found to be more prevalent in affected birds than in control birds. However, the subsequent isolation of a *Campylobacter* organism from field cases in Australia by Scolexia and Professor Moore (RMIT University), which has sequence homology to the organism isolated by Crawshaw and Irvine from UK cases, suggests that it is the principle pathogen. Its isolation will enable the development of an exposure model that will allow increased progress in assessments of treatment and control methods.
Implications	With respect to management, prevention and control it has been observed that many outbreaks are related to disruptions in bird husbandry particularly as it relates to feed intake, formulation and availability. It has also been associated with free range birds accessing water in the range area. The faecal oral cycle still appears to play an essential part in SLD with the disease being almost exclusively seen in birds farmed extensively and, where it does occur in cage or fully slatted systems, there are invariably spatial associations with manure. The use of medium chain organic acids in the diet from the time of transfer to the production sheds, combined with stable management routines and feeding programs, in some cases results in a lower incidence of outbreaks and an amelioration of the severity of outbreaks when they occur. On the other hand, the use of some probiotic has not at this stage been shown to be helpful in disease control. Overall the attempted control of SLD with the various prebiotics, probiotics, phytogenic and similar products available has been with minimal success. With respect to treatment, the use of registered antibiotics by water medication is usually effective in the first instance; however, repeated use can quickly lead to a reduction in efficacy through the development of antibacterial resistance.
Key Words	eggs; spotty liver disease; Helicobacter; Campylobacter; metagenomics study
Publications	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. doi: 10.1099/ijsem.0.001383

13 Appendices

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