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Researcher Contact Details
Professor Robert Moore
RMIT University
Email: rob.moore@rmit.edu,au

Dr Peter Scott
Scolexia Pty Ltd
Email: pscott@scolexia.com,au

Dr ThiThuHao Van
RMIT University
Email: thithuhao.van@rmit.edu,au

Dr Arif Anwar
Scolexia Pty Ltd
Email: aanwar@scolexia.com,au

In submitting this report, the researcher has agreed to the Poultry CRC publishing this material in its edited form.

Poultry CRC Ltd Contact Details
PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: admin@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

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Executive Summary

Spotty Liver Disease (SLD) occurs mainly in layer chickens, particularly free-ranging birds but also less commonly in barn and cage birds and parent stock. It is characterized by multiple grey/white spots in the liver, loss of egg production and in an increase in mortality. Although the disease has been recognised for over 60 years the cause of the disease had not been identified. It is of course difficult to identify, develop, and test control measures for the disease if the cause of the disease is not known and an experimental disease induction system isn't available.

We undertook this project to address these issues and attempt to isolate the pathogen responsible for SLD. Because diseased birds respond to some antibiotic treatments it has long been assumed that the pathogen is a bacteria. Over the years various bacteria have been prosed as the cause however no definitive proof supporting any particular bacterium has been forthcoming.

We have successfully isolated a bacterium from the liver and bile of commercial layer birds suffering from SLD. We have biochemically characterised the bacteria and undertaken molecular analysis to show that it represents a previously unrecognised species of *Campylobacter*. We have called this new pathogen *Campylobacter hepaticus* and have formally published this new name.

We then went on to show that layer birds inoculated with *C. hepaticus* developed SLD. At both the microscopic and macroscopic levels the liver lesions produced appeared to be identical to typical field cases of the disease. We reisolated *C. hepaticus* from the diseased birds. Our experiments fulfil Koch's postulates for the identification of a pathogen causing a specific disease and hence we can take this as conclusive evidence that *C. hepaticus* is the cause of SLD.

With the pathogen identified and isolated and an experimental disease induction process in place this research provides the basic tools that will now allow this disease to be addressed in a rational way. Ways of managing and treating the disease can now be developed and tested and diagnostic assays can be developed to investigate disease epidemiology.

Introduction

Spotty liver disease (SLD) in chickens can cause severe reduction in egg output and increased mortality in layer flocks (Crawshaw and Young, 2003; Grimes and Reece, 2011). The disease is characterised by multiple, grey/white miliary spots in the liver, usually 1-2 mm in diameter. Descriptions of gross pathology suggest that the conditions variously referred to as avian vibrionic hepatitis, avian infectious hepatitis, summer hepatitis and miliary hepatitis probably represent the same disease (Forsyth et al., 2005; Peckham, 1958; Sevoian et al., 1958). The disease was first reported in the United States in 1954 and there have since been reports from a number of countries including Canada, the United Kingdom and Germany (Crawshaw and Irvine, 2012; Truscott and Stockdale, 1966; Tudor, 1954). In Australia SLD has been intermittently seen in layers, and on occasions in the broiler breeder industry, for several decades but in recent years, with the increase of extensively farmed layers, it has become more common and is now a major concern, causing significant problems in regard to both mortalities and production (Scott, 2016; Grimes and Reece, 2011). Therefore, there is an increased need to understand the aetiology of disease so that rational approaches can be taken to the development of effective control measures.

Despite efforts from a number of research groups throughout the world the cause of the disease had not been established. It has long been suspected that a bacterium may be the cause of SLD because affected birds recover when treated with some antibiotics. Various bacteria have been speculated to be the cause of the disease, including a “vibrio”, *Camplyobacter jejuni*, *Campylobacter coli*, *Helicobacter pullorum* and clostridia, but there has been no conclusive evidence for any of these (Boukraa et al., 1991; Burnens et al., 1996; Grimes and Reece, 2011; Jennings et al., 2011; Peckham, 1958). It has been reported that often no bacteria could be isolated from the livers of affected birds and microscopically no bacteria were apparent within the liver lesions. This led to speculation that the causative organism may colonise a site remote to the liver (e.g. the gastrointestinal tract) and produce a toxin which can traffic to the liver and cause the typical lesions seen in SLD.

Because of these observations reported in the literature our earlier work on SLD, funded by AECL, focused on the microbiota present in the gastrointestinal tract (GIT) of birds. We compared the microbiota of diseased and healthy birds from the same flocks in an attempt to determine if the pathogen could be identified by its differential abundance between sick and healthy birds. We did identify some candidates, one of which was related to but not identical to *Helicobacter pullorum,* based on 16S ribosomal RNA gene sequence and a small amount

of other genomic sequence that we linked to the organism by whole metagenome analysis of one affected bird's microbiota. Our initial goal in this new CRC project was to attempt to culture the candidate *H. pullorum*-like organism. As we were about to commence the project a study from England indicated that the causative organism may be a *Campylobacter* (Crawshaw et al., 2015). We therefore used bacterial isolation methods designed to culture members of both *Campylobacter* and the related *Helicobacter* genus.

Objectives

Our goal was to attempt to determine the pathogen responsible for SLD and to establish basic tools, such as cultured isolates and a reliable disease model, to facilitate research on treatment options for the disease. Such enabling tools would provide the immediate ability to evaluate products such as prebiotics and short chain fatty acid formulations for SLD control and, in the longer term, the development of vaccines.

The specific goals were:

(i) determine the pathogen responsible for SLD,
(ii) define conditions to culture the causative organism, and
(iii) establish an experimental disease induction model.

Methodology

Bacterial Isolation

Initially, bacterial isolation was attempted from the livers of SLD affected birds from five layer flocks. Briefly, livers were collected in Stuart's transport medium (Oxoid) and transported from the point of collection to the laboratory on ice. We arranged to have the samples in the laboratory within 24 hours of collection. Portions of the livers were aseptically macerated in 5 mL tubes containing modified Preston broth (Crawshaw *et al*., 2015). The tubes were incubated at 37 °C in microaerobic conditions for two days. Following pre-enrichment, samples were plated onto Brucella agar with 5% horse blood (HBA) and incubated under the same conditions as above. The plates were examined after three and seven days of incubation. The suspected *Campylobacter/Helicobacter* colonies (Gram negative and oxidase positive) were subcultured onto HBA to obtain pure cultures for storage.

Bacterial Species Identification

For more detailed identification, we selected 10 isolates, 2 from each SLD affected flock, (HV10, DisRed, D4, 4L, 12L, 17L, 19L, 22L, 27L and 29L) representing different farms and

different regions in Australia (Victoria and Queensland states), thereby representing a geographically and epidemiologically independent set of isolates. A wide range of biochemical tests were performed to characterise the isolates, using previously described methods (Nakari et al., 2008; On and Holmes, 1992, 1991, 1995; Ursing et al., 1994). All tests were performed at least twice with *C. jejuni* strain 81116 (NCTC11828), *C. coli* strain NCTC 11366 and *Enterococcus cecorum* 20L (RMIT collection) used as controls. The biochemical characteristics tested included a Gram-stain reaction, motility test using hanging drop method, catalase, oxidase, and urease production tests, hydrolysis of hippurate and indoxyl acetate, reduction of nitrate, H_2S production in triple-sugar iron agar, requirement for H_2, growth tests included temperature tolerance at 25 °C, 37 °C and 42 °C under microaerobic conditions, growth under aerobic and anaerobic conditions on 5% blood agar at 37 °C as well as NaCl, glycine, bile, triphenyltetrazolium chloride, metronidazole, and cefoperazone tolerance, growth on Nutrient and MacConkey agar; and antibiotic susceptibility tests (nalidixic acid and cephalotin) by disk diffusion method.

Partial 16S rRNA gene sequences of the isolates were generated to determine the phylogenetic position of the isolates. Primers with the sequences (5'-3'): AGTTTGATCCTGGCTCAG and ACGGTACCTTGTTACGACTT were used to amplify 16S rRNA gene sequences (Hunt et al., 2013). The 16S rRNA gene sequences obtained were compared to those in the National Center for Biotechnology Information (NCBI) GenBank database using the megaBLAST algorithm. The 16S rRNA gene sequences obtained from NCBI for *Campylobacter* reference sequences and the 16S rRNA gene sequences of the novel species were aligned using Clone Manager 9 (Scientific & Educational Software, Denver, Colaroda, USA). A phylogenetic tree was constructed in MEGA6 (Tamura et al., 2013) using the neighbour-joining method. Bootstrap analysis was performed with 1000 resampled datasets. Partial *hsp60* gene sequencing was performed as described by Debruyne et al. (2009); further analysis was performed as for the 16S rRNA gene which was described above.

Electron Microscopy

Morphology characteristics of all four strains were determined using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM cells were grown on Brucella horse blood agar for 60 hrs and resuspended in 0.1M PBS. Droplets (20 µL) of bacterial suspensions were placed on a sheet of Parafilm and Formvar carbon coated grids were floated on them for ten minutes. Grids were then blotted dry and placed onto a drop of 2% (v/v) phosphotungstic acid (pH 6.6) negative stain for 1 minute. Grids were placed onto a drop of water to wash and blotted dry and viewed using a JEOL1010 TEM microscope.

Scanning electron microscopy (SEM) was carried out using an agar thin layer method modified from Matsuguchi et al. (1977). This method does not involve any mechanical process such as centrifugation or pipetting that can damage the flagella. Briefly, *C. hepaticus* NCTC 13823T (=CIT) strain was grown on HBA for 3 days and the cells were harvested in Brucella broth. Twenty microliter of the culture were dropped on the soft HBA surface (0.8% agar containing two agar layers and a coverslip was placed between the two layers) and the plates were incubated for two days. After incubation, the coverslip was taken together with the top agar layer containing the bacterial cells on the surface. The cells were then fixed with 1% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.2). The samples were dehydrated in increasing concentrations of ethanol (50 %, 70 %, 90%, 95% and 100%). Hexamethyldisilizane was used for drying the specimens. Dried samples were sputter coated and imaged using the FEI Verios 460L**.**

Whole Genome Sequencing

Genomic DNA was extracted using an Isolate II genomic DNA kit (Bioline). The genomic library preparation was performed using the Nextera XT DNA Library Preparation Kit and sequenced using Illumina MiSeq with 2x300 bp paired-end reads. The A5-miseq pipeline was used to assemble the genome (Coil et al., 2015).

DNA-DNA hybridization (DDH) has traditionally been used to compar whole genomes of organisms to determine relatedness. However, with the ready availability of whole genome sequence data, facilitated by the revolution bought about by Next Generation Sequencing (NGS) technologies, more precise whole genome comparisons can be made. The average nucleotide identity (ANI) can now be used as a superior alternative to DDH (Konstantinidis and Tiedje, 2005). It has been suggested that an ANI value of less than 95% indicates the compared samples are from different species. The ANI calculations (Goris et al., 2007) was carried out using the ANI calculator (http://enve-omics.ce.gatech.edu/ani/).

Invasion Assay

The invasiveness of the *C. hepaticus* NCTC 13823^T strain to immortalized chicken liver cells, LMH (ATCC CRL-2117) (Kawaguchi et al., 1987), was compared to that of other *Campylobacter* species (*C. jejuni* 81116, *C. lari* 54/6 and *C. upsaliensis* 54/2) using a cellular invasion assay. The LMH cells were grown in Waymouth's medium (Gibco) supplemented with 10% fetal bovine serum at 37C in a 5% CO_2 humidified atmosphere. For the invasion assay, LMH cells were grown in 24-well plastic plates seeded at 10^5 cells per well and incubated for 48 hours before infection. The culture of each *Campylobacter* strain was

prepared by taking several colonies from 48 h bacterial growth on HBA and suspending in Brucella broth. The OD_{600} value for the bacterial suspensions was adjusted to 0.1 which is equivalent to approximately 10^8 CFU/mL and 100 µl from this bacterial suspension were inoculated into each well containing a confluent monolayer of LMH cells. The viable counts of the bacterial suspensions used in the assay were directly measured by plating out dilutions on HBA plates. The 24-well tray was incubated at 37°C in microaerobic conditions for 5 h to allow the bacterial strains to invade the LMH cells. The cells were then washed with PBS twice, then 1 ml of Waymouth's media/FBS containing 400 µg/ml gentamycin was added to each well and the plates were incubated in 5% CO_2 at 37°C for 90 min to allow killing of bacterial cells that had not invaded. Gentamycin kills the extracellular bacterial cells but cannot penetrate the eukaryotic cell membrane and hence the internalised bacterial cells are protected (Zeitouni et al., 2013). After gentamycin treatment, the plates were washed 3 times with PBS to remove gentamycin and the cells were lysed by adding 200 µl of 0.3% triton-X-100 to each well. The cell lysates were diluted by the addition of 800 µl of PBS and the plates were incubated in 5% CO_2 at 37°C for 15 min. The lysate was diluted further and plated out on HBA plates to evaluate the number of viable bacteria that had invaded the LMH cells. The invasive ability was expressed as the percentage of the inoculum surviving the gentamycin treatment relative to the initial inoculum. For each strain, the invasion assay was performed at least twice, in triplicate at each time point.

For statistical analysis of the data, the mean values were compared using the Mann-Whitney test. Mean values were considered to be significantly different if the *p* value was less than 0.01.

Chicken Challenge Experiments

Trial 1

A bacterial challenge trial was carried out to demonstrate the pathogenicity of this organism in chickens. The animal experimentation was approved by the Wildlife and Small Institutions Animal Ethics Committee of the Victorian Department of Economic Development, Jobs, Transport and Resources (approval number 14.16). Eight groups of three chickens (26-week-old Hy-Line layer hens) were housed in separate cages next to each other, in which four groups were used as control and four groups were challenged by direct oral gavage with 1×10^9 CFU of *C. hepaticus* NCTC 13823^T strain in 1 mL of Brucella broth. The birds were feed *ad libitum* with a standard, antibiotic-free layer diet. The control chickens were given 1 mL of Brucella broth. Half of the birds from each group were sacrificed after 7 days and the other half at 13 days post-challenge. The livers were examined for lesions and segments were collected for isolation of *C. hepaticus* and histopathological examination. Bile

samples from all chickens were taken aseptically from the gall bladder and cloacal swabs were collected for bacteriology. Samples were kept on ice, transported to the laboratory and processed as soon as possible. *C. hepaticus* was isolated from liver and bile as described above.

Trial 2

A second challenge experiment was undertaken in which the challenge dose of *C. hepaticus* was increased to 1 x 10^{10} CFU and the times when the health of the birds' livers were assessed was at days 5 and 7 post-challenge. The other details of the trial and sampling were the same as for the first trial.

Histology

Liver was fixed in 10% neutral phosphate-buffered formalin. The tissues were trimmed into a 5 mm cubes and embedded in paraffin wax blocks. Sections were cut and then stained with haematoxylin and eosin. The tissues were processed by Ace Laboratory Services, Bendigo East, Australia.

Confirmation of Identity of *C. hepaticus* Reisolated from Challenged Birds

Isolation and identification of *C. hepaticus* from the tissue samples of experimentally infected birds was carried out as described by Van et al. (2016) for liver samples. Isolation from bile samples was done without the enrichment step; 20 µL of bile was spread directly onto Horse blood agar plates and incubated at 37ºC in microaerobic conditions for 3 days. Suspected *Campylobacter* colonies (Gram negative and oxidase positive) were subcultured onto HBA to obtain pure cultures for further characterization and storage. 16S ribosomal RNA gene amplicon sequencing were performed to characterise the isolates (Van et al 2016).

The primers 5'AGAGTTTGATCNTGGCTCAG3' and 5'GGACTACHVGGGTWTCTAAT3' were used to amplify the 16S rRNA gene. The expected size is approximately 800 bp. The PCR conditions were 98°C for 1 min, 35 cycles of 98°C for 10 s; 49°C for 30 s and 72°C for 30 s, final extension at 72°C for 10 min. The amplified products were Sanger sequenced at Micromon, Monash University, Victoria, Australia.

Results

Bacterial Isolation, Identification and Characterisation

It quickly became apparent that the culturing from most liver samples resulted in isolation plates with an apparent monoculture of bacterial colonies with a pale, watery appearance. Eighteen bacterial isolates were recovered from 27 liver samples from 5 different flocks. Of the 9 liver samples from which isolates were not recovered 7 failed because of gross contamination of the primary isolation plate and on 2 culture attempts nothing was recovered.

Sequencing of 16S rRNA genes revealed that all strains shared identical 16S rRNA sequences and this sequence was identical to the NCBI database entry for *Campylobacter* sp. FARM4 2011/1, isolated from and SLD case in England (Crawshaw *et al*., 2015). The 16S rRNA gene sequence shares ≤98.54 % sequence similarity to other *Campylobacter* species with validly published names; the closest species being *C. coli*. A 16S rRNA gene sequence similarity of 98.65 % can be used as the threshold for differentiating two species (Kim et al., 2014), therefore this bacterium is qualified as a new species. Furthermore, the neighbour-joining dendogram of representative 16S rRNA gene sequences (Fig. 1) indicated that the strains formed a robust clade (100 % bootstrap support) that was clearly distinct from other *Campylobacter* species.

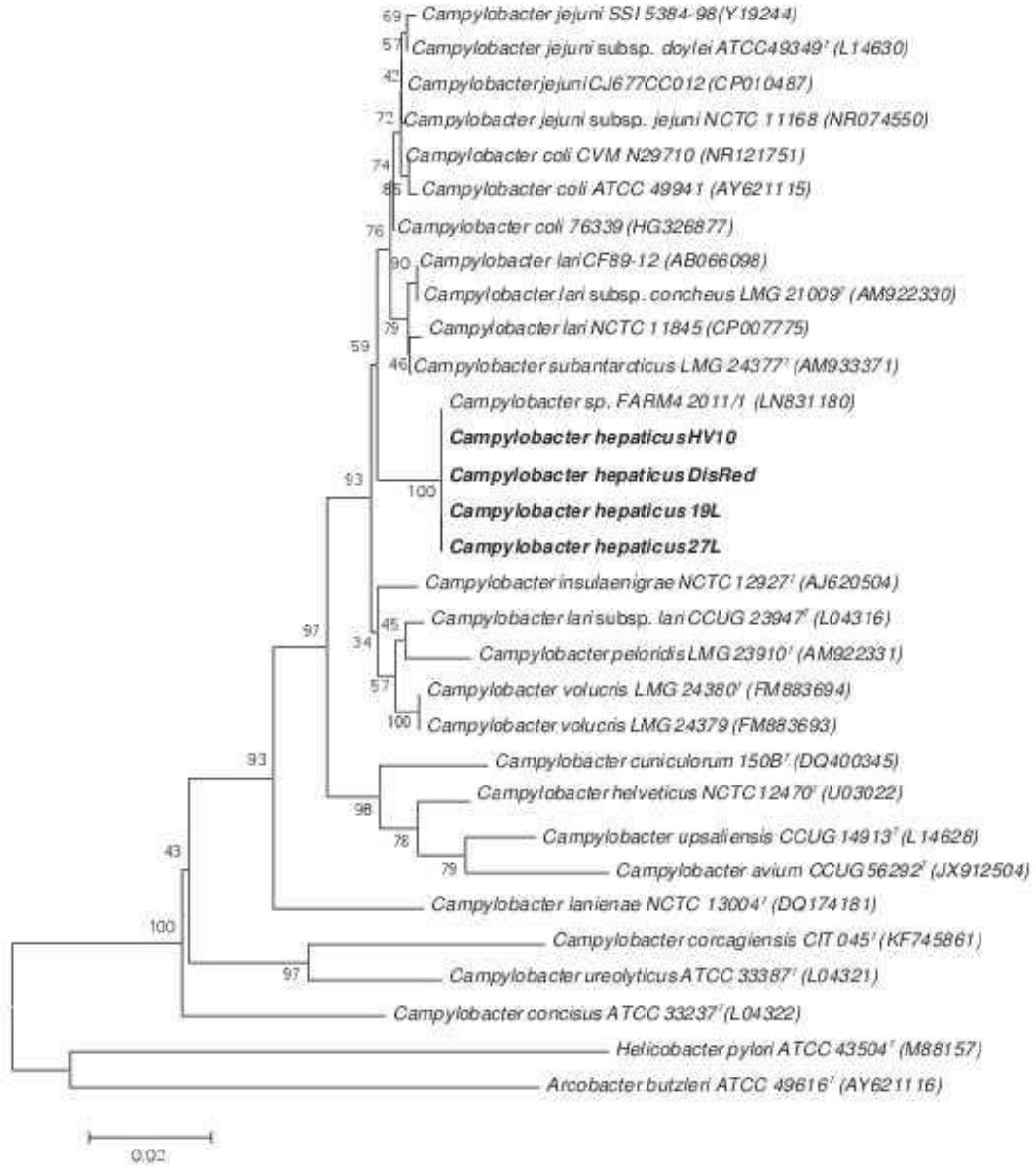

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences constructed by the neighbour-joining method. Bootstrap values (%), calculated from 1,000 repetitions, are indicated at the nodes. Bar, 0.02 substitutions per nucleotide position. ^{T} indicates type strain. Four *C. hepaticus* sp. nov. strains are shown but the other 6 isolates characterised all have identical sequences.

To further investigate the phylogenetic relationship of the new *Campylobacter* isolates another phylogenetic tree was built based on the heat shock protein 60 (*hsp60*) gene sequences, as the level of interspecies sequence variation of this gene is greater than that of the 16S rRNA gene, and therefore can provide better resolution for species classification (Kärenlampi et al., 2004). The partial *hsp60* sequences (555 bp) from four strains were used to generate a phylogenetic tree. The strains tested had identical *hsp60* gene sequences,

and interspecific sequence similarities were no higher than 90 %. As seen in the 16S rRNA gene phylogenetic tree, the strains formed a robust clade that was distinct from recognised *Campylobacter* species (Fig. 2). The 16S rRNA and *hsp60* gene sequences of the type strain (HV10) have been deposited in GenBank under the accession numbers KU886019 and KU886020 respectively.

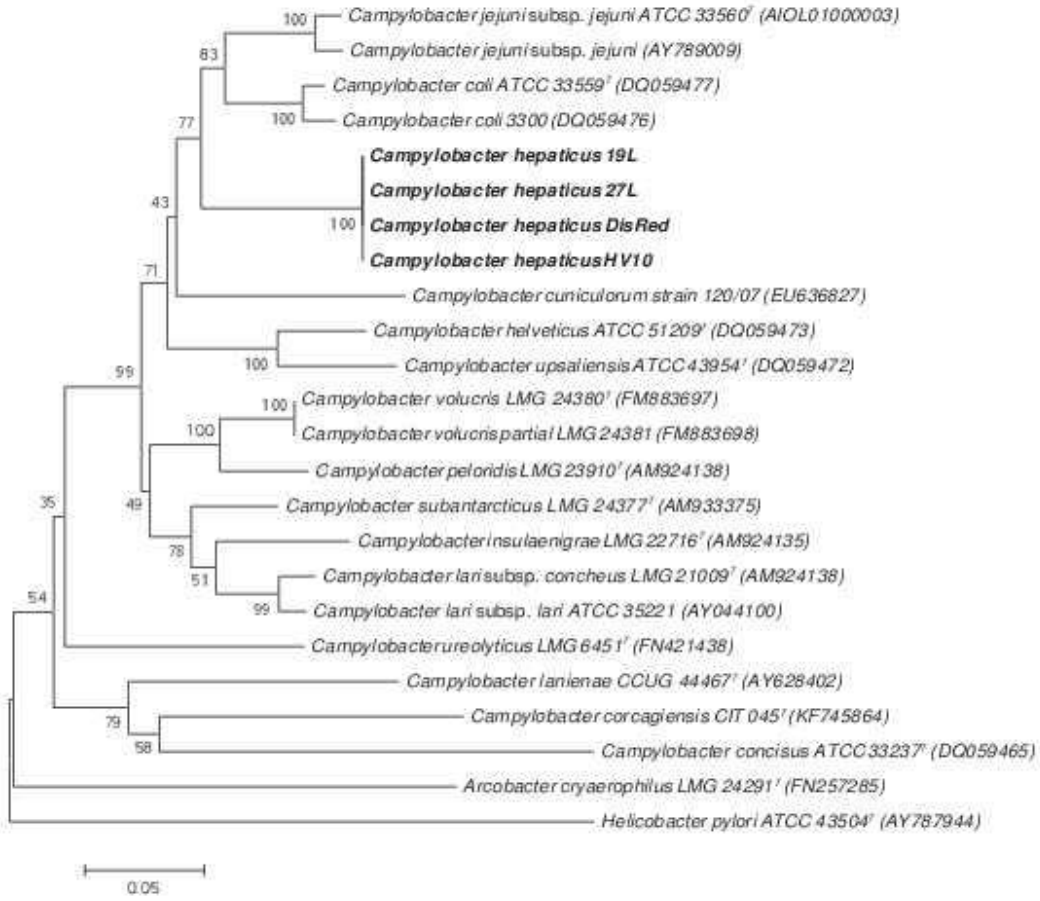

Figure 2. Phylogenetic tree based on *hsp60* gene sequences constructed by the neighbour-joining method. Bootstrap values (%), calculated from 1,000 repetitions, are indicated at the nodes. Bar, 0.05 substitutions per nucleotide position. ^{T} indicates type strain.

Whole genome sequence data was obtained from the proposed type strain, HV10, and three other strains. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession LUKK00000000. The version described in this report is LUKK01000000. The draft genome sequence of strain HV10 indicated a genome size of 1,482,384 bp with a G+C content of 27.9 mol%; this is clearly lower than the DNA base composition range of 29-47 mol% previously reported for the genus *Campylobacter* (Debruyne et al., 2008). The chromosome is predicted to contain 1,520 open reading frames of which 1,471 are protein coding sequences and 49 are predicted rRNA genes. The results

indicate that the 4 novel strains are highly similar, with ANIs of greater than 99%, and are distinct from other *Campylobacter* species, with ANIs of less than 84% (Table 1).

Table 1. Average nucleotide identity (ANI) values obtained from the comparison of *C. hepaticus* HV10 genome against three other *C. hepaticus* isolates from this study and other related *Campylobacter* species.

Species	**NCBI/Genebank Accession**	**ANI two-way (%)**
Campylobacter hepaticus strain 27L	This study	99.99%
Campylobacter hepaticus strain 19L	This study	99.53%
Campylobacter hepaticus strain DisRed	This study	99.99%
Campylobacter jejuni subsp. *jejuni* NCTC 11168 = ATCC 700819	NC_002163.1	83.21%
Campylobacter jejuni RM1221	NC_003912.7	83.15%
Campylobacter coli 76339	HG326877.1	80.52%
Campylobacter coli RM1875	NZ_CP007183.1	80.65%
Campylobacter lari NCTC 11845	NZ_CP007775.1	77.63%
Campylobacter subantarcticus LMG 24377	NZ_CP007773.1	76.95%
Campylobacter insulaenigrae NCTC 12927^T	NZ_CP007770.1	77.33%
Campylobacter volucris LMG 24379	NZ_CP007774.1	77.13%
Campylobacter peloridis LMG 23910^T	CP007766.1	77.13%
Campylobacter cuniculorum DSM 23162	NZ_KK211203.1	77.39%
Campylobacter ureolyticus RIGS 9880	NZ_CP012195.1	74.64%
Campylobacter concisus strain ATCC 33237^T	NZ_CP012541.1	76.18%

The comparison of the biochemical characteristics of the SLD isolates with other closely related *Campylobacter* species of the genus *Campylobacter* is presented in Table 2. Unlike most other species of the genus *Campylobacter*, most of the 10 strains of this novel species hydrolyse hippurate and half of the strains could not reduce nitrate.

Table 2. Phenotypic characteristics differentiating the novel *C. hepaticus* sp. nov. strains from other species of the genus *Campylobacter.*

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
Oxidase	+	+	+	+	V	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	V	+	+	+	+	+
Catalase	+	V	V	+	-	+	+	_	+	+	(+)	+	V	-	-	+	+	+	V	+	+	+	+	-	+	(-)	+	V*	+	-	V	+
Urease	-	-	V	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	V*	NA	-	-	NA
Hydrolysis of:																																
Hippurate	(+)	+	-	-	-	-	-	(-)	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Indoxyl acetate	+	+	-	+	-	V	+	V	-	-	-	-	V	+	-	-	-	-	+	+	-	NA	-	-	-	+	V	-	-	+	V	-
Reduction of:																																
Nitrate	V	+	V	+	(-)	(+)	+	+	+	+	(+)	+	(+)	+	-	+	+	+	-	+	+	+	+	(-)	NA	+	+	(+)	+	+	+	+
H_2S Production (TSI)	-	-	V	-	-	+	-	(-)	-	-	-	-	-	-	-	+	+	-	-	-	-	NA	-	+	NA	-	V	+	-	-	-	-
α-Haemolysis	-	-	-	(-)	(-)	-	+	(-)	-	NA	V	-	-	+	-	V	V	NA	+	+	+	NA	+	-	NA	+	+	+	+	+	V	NA
H_2 requirement	-	V	-	-	+	-	-	+	-	-	-	-	+	-	+	V	V	NA	-	-	-	NA	-	+	NA	+	+	-	NA	-	+	NA
Growth at/in/on:																																
25 °C (microaerobic)	-	-	-	-	-	NA	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37 °C (microaerobic)	+	+	+	+	+	+	+	V	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	V	+	+	+	+	+
42 °C (microaerobic)	+	+	+	+	(+)	+	(+)	V	(+)	V	-	-	V	+	(-)	+	+	-	-	+	+	+	+	+	+	(-)	V	+	+	+	V	+
37 °C (anaerobic)	-	-	+	-	+	+	-	+	(-)	+	V	+	+	-	+	-	+	-	-	-	+	NA	-	+	NA	+	+	+	+	-	V	+
37 °C (aerobic)	-	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MacConkey agar	-	-	+	V	-	-	-	(+)	(+)	(+)	V	-	(+)	-	-	V	V	NA	-	-	+	-	-	(+)	NA	-	+	V	(-)	-	V	-
Glycine (1%)	+	-	V	(+)	(-)	+	-	+	+	+	(-)	+	+	V	+	+	V	+	(-)	+	-	(+)	+	V	+	+	V	+	(+)	+	+	-
NaCl (2%)	-	-	-	-	(-)	+	-	V	-	NA	-	+	+	-	NA	-	-	-	-	-	-	+	+	-	(+)	V	+	+	+	-	+	-
NaCl (4%)	-	NA	-	-	-	NA	NA	-	-	-	-	-	NA	-	NA	-	-	-	-	-	-	-	-	-	NA	-	-	NA	NA	-	+	NA
Bile (1%)	+	V	NA	(+)	-	+	NA	-	+	NA	+	NA	-	+	NA	+	NA	NA	+	+	NA	NA	NA	(+)	NA	-	-	V	+	+	V	w
2,3,5-triphenyltetrazolium chloride, TTC (0.04%)	+	-	NA	+	-	-	V	V	_	+	-	-	-	-	-	-	-	NA	V	+	NA	+	+	-	NA	-	-	-	NA	V	-	-
Metronidazole (4 mg/L)	+	NA	NA	(+)	(-)	NA	NA	-	(+)	NA	V	NA	-	-	-	(-)	V	+	(-)	+	+	+	+	(+)	+	-	+	(-)	(-)	(+)	-	+
Cefoperazone (64 mg/L)	-	NA	NA	(+)	-	-	NA	(+)	NA	NA	NA	NA	-	V	(-)	NA	NA	+	NA	+	NA	NA	NA	NA	NA	-	-	-	NA	(-)	-	-
Resistance to:																																
Cephalotin (30 µg)	+	+	-	+	-	NA	(+)	-	-	NA	-	-	-	-	-	(-)	-	+	-	+	+	+	+	-	(-)	-	-	-	-	(-)	NA	+
Nalidixic acid (30 µg)	V	-	V	-	(+)	+	V	+	+	NA	V	+	V	-	V	+	+	+	-	-	+	-	(+)	(+)	(+)	(+)	-	(+)	+	-	-	+
DNA G+C content (mol %)	27.9	35	NA	31	37-41	31.9	32.4	45-46	33-35	NA	33-34	33.6	44-46	34	32.5	35-36	31-33	NA	31	30-31	36	30	29-30	36-38	29	45-46	44-46	29-33	30	32-36	28-30	29

Taxa: 1 = *C. hepaticus* HV10 (n = 10); 2, *C. avium*; 3, *C. canadensis*; 4, *C. c*oli; 5, *C. concisus*; 6, *C. corcagiensis*; 7, *C. cuniculorum*; 8, *C. curvus*; 9, *C. fetus* subsp. *fetus*; 10, *C. fetus* subsp. *testudinum*; 11, *C. fetus* subsp. *venerealis*; 12, *C. geochelonis*; 13, *C. gracilis*; 14, *C. helveticus*; 15, *C. hominis*; 16, *C. hyointestinalis* subsp. *hyointestinalis*; 17, *C. hyointestinalis* subsp. *lawsonii*; 18, *C. insulaenigrae*; 19, *C. jejuni* subsp. *doylei*; 20, *C. jejuni* subsp. *jejuni*; 21, *C. lanienae*; 22, *C. lari* subsp. *concheus*; 23, *C. lari* subsp. *lari*; 24, *C. mucosalis*; 25, *C. peloridis*; 26, *C. rectus*; 27, *C. showae*; 28, *C. sputorum*; 29, *C. subantarcticus*; 30, *C. upsaliensis*; 31, *C. ureolyticus*; 32, *C. volucris*. +, 90-100%; (+) 75-89%; V, 26-74%, (-), 11-25; -, 0-10%; NA, not available;* test results differ between *C. sputorum* biovar *sputorum* (catalase and urease negative), *paraureolyticus* (catalase negative, urease positive) and *fecalis* (catalase positive, urease negative). Data for reference taxa were taken from On et al. 1995; Logan et al. 2000; Piccirillo *et al.* 2016. TSI: triple sugar-iron agar.

Electron microscope studies of the SLD isolates showed, via TEM, that the cells were typically S-shaped, with bipolar unsheathed flagella (Fig. 3). SEM of whole colonies showed that as well as the s-shaped and longer spiral cells there were also a few of coccoid morphology (Fig 4). Such coccoid cells have been reported previously in aging cultures of other campylobacters.

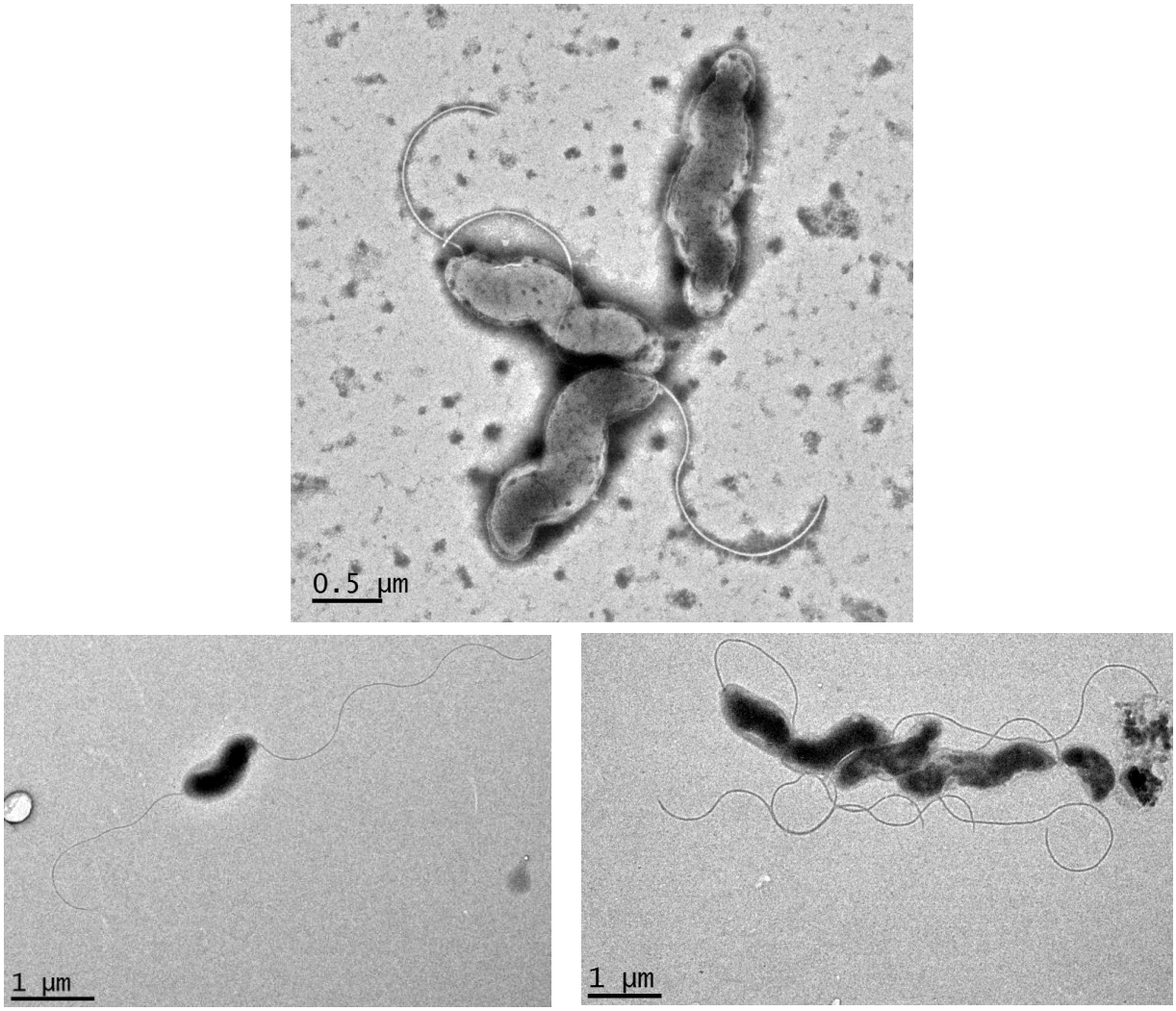

Figure 3. Transmission electron micrographs of *Campylobacter hepaticus* showing their long bipolar flagella and S- shaped. Scale bar is indicated in each photomicrograph.

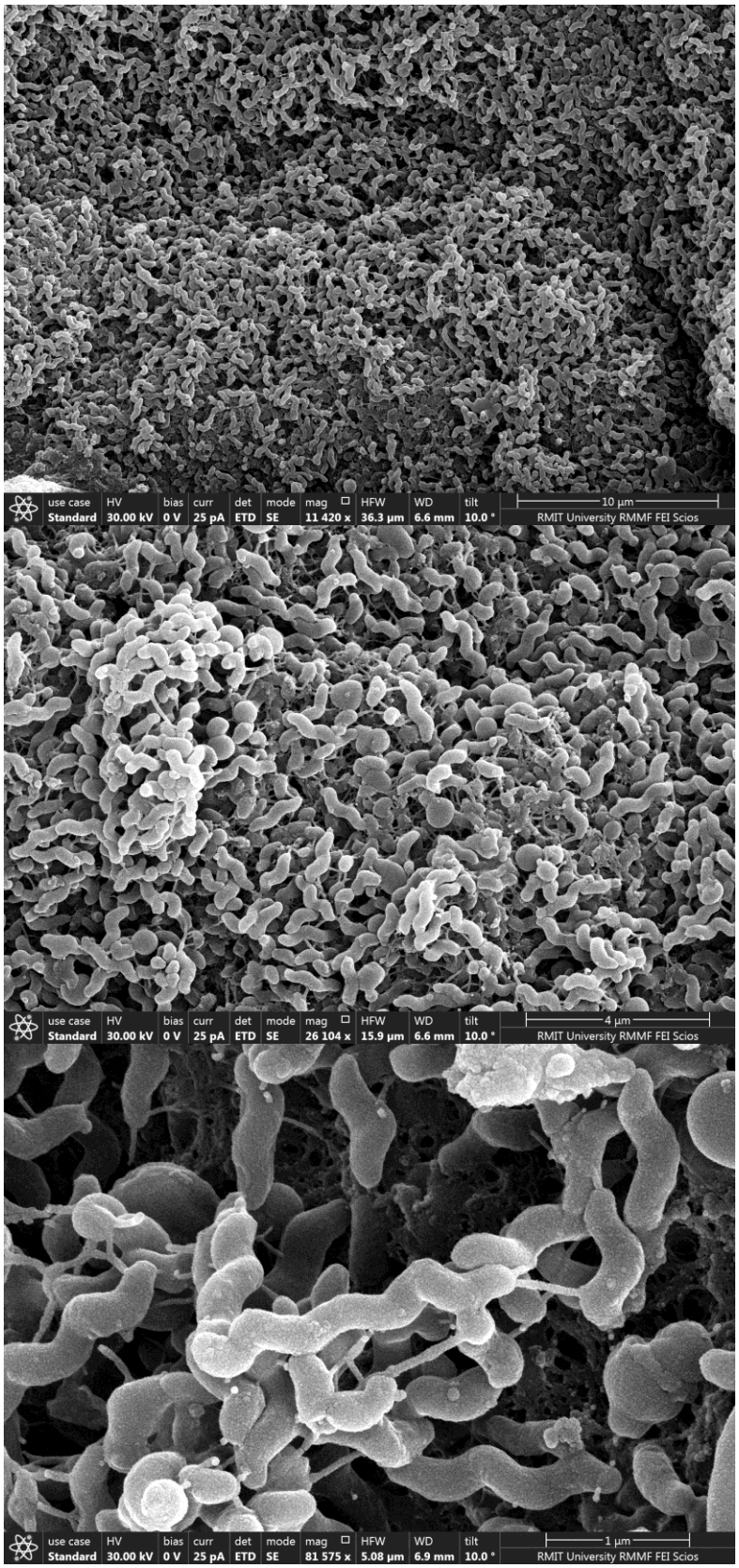

Figure 4. Scanning electron micrographs of *Campylobacter hepaticus*. The surface of an intact colony grown on an agar surface was imaged. Different magnifications are shown; see scale bars.

Description of *Campylobacter hepaticus* sp. nov.

Based on the above results we were able to propose that the SLD isolates represented a new species of *Campylobacter*. We proposed the name *Campylobacter hepaticus* for this new species and that name has now been officially published (Van et al., 2016). The official description of this new species is as follows.

Campylobacter hepaticus (he.pa'ti.cus. N.L. masc. adj. hepaticus (from Gr. adj. hepatikos) of the liver, from which the bacterium was first isolated).

Cells are Gram negative, S-shaped, 0.3-0.4 µm wide and 1.0-1.2 µm long after 3 day incubation on Brucella horse blood agar in a microaerophilic atmosphere at 37 °C. Colonies are wet, creamy coloured, some convex, some are flat and spreading. They could vary in size and morphology after long incubation times. Cells appeared coccoid after 12 days of incubation. The isolates are non-haemolytic. They are motile and possess a single flagellum at both poles. They are catalase and oxidase positive and urease negative. Strains may differ in their ability to reduce nitrate or hydrolyse hippurate. The type strain hydrolyses hippurate but could not reduce nitrate. Hydrogen sulphide is not produced in triple-sugar iron medium. All strains hydrolyse indoxyl acetate. No growth under aerobic conditions at 37 °C, anaerobic at 37 °C or microaerobic at 25 °C. All are able to grow at 37 °C and 42 °C microaerobically. All strains do not require hydrogen to grow. All strains can grow on nutrient agar without blood, most of them cannot grow on MacConkey agar. Strains grow on blood agar medium supplemented with 1 % glycine, 1 % bile, metronidazole (4 mg/L) and grow weakly on 0.04 % 2,3,5-triphenyltetrazolium chloride. All strains are resistant or show intermediate resistance to nalidixic acid (30 µg per disc) and most of them are resistant to cephalothin (30 µg per disc) by disc diffusion tests. The G+C content of the type strain is 27.9 mol%. This novel species has been isolated form birds with spotty liver disease and is likely to be pathogenic to chickens, but pathogenicity to human is unknown. The type strain, HV10, has been deposited as NCTC13823^T (=CIP 111092^T), and was isolated from the liver of a chicken with SLD in 2015.

Invasion assay

The invasiveness of *C. hepaticus* NCTC 13823^T strain into LMH cells was found to be high compared to other species of *Campylobacter* tested. The invasion index for *C. hepaticus* was within the range of 0.27-0.80 of the starting bacterial inocula while that of *C. jejuni, C. lari and C. upsaliensis* strains were 0.06-0.16, 0.007-0.020 and 0.002-0.005 respectively. The *C. hepaticus* strain had a statistically significantly higher LMH cell invasion mean than

C. jejuni (*p*= 0.0002), *C. lari* (*p*=0.0014) and *C. upsaliensis* (*p*= 0.00138) strains. There was also significant difference in the invasiveness of *C. jejuni* compared to *C. lari* or *C. upsaliensis* but there was no significant difference in the invasiveness of *C. lari* and *C. upsaliensis* strains (p=0.013) (Fig. 5).

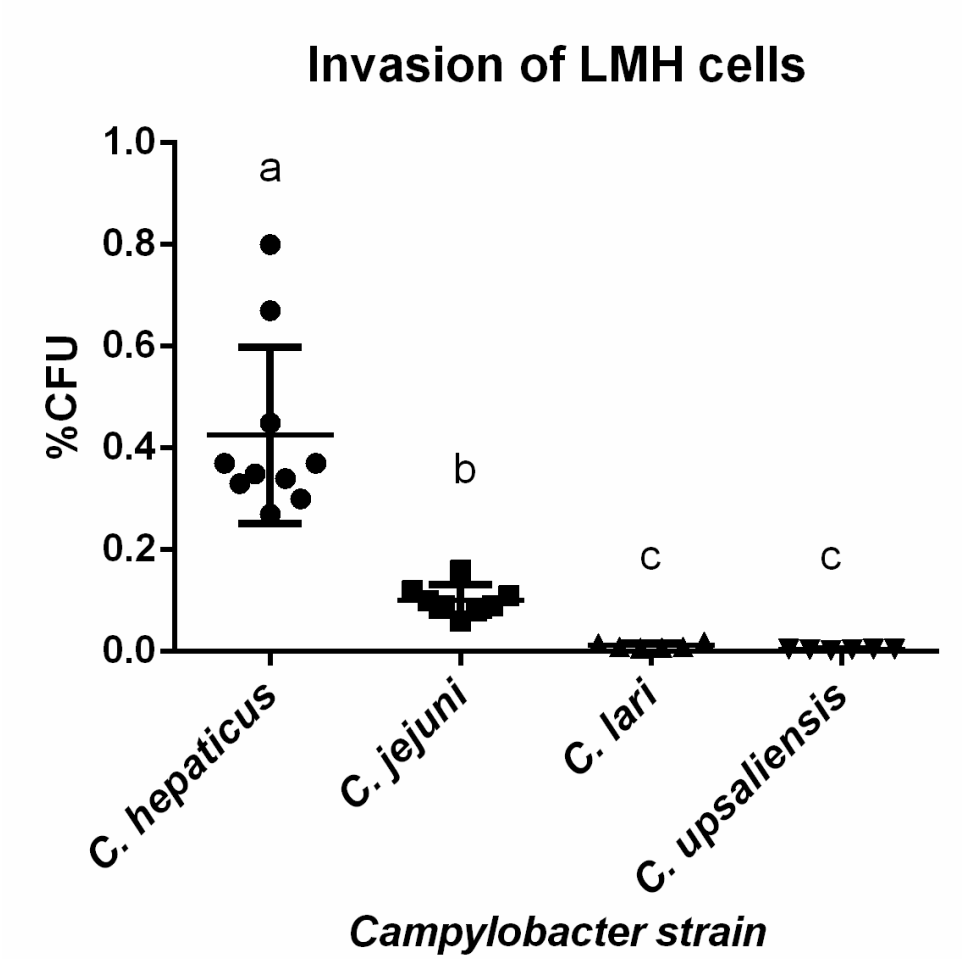

Figure 5. Invasion of *C. hepaticus* and other *Campylobacter* species. The results are presented as percentages of internalized bacterial cells compared to the starting inocula. n ≥ 6. The means and standard deviations are shown by bars. Means with the different letter represents statistically significant values at p≤0.01.

Chicken Challenge Experiments

Trial 1

In the first trial one bird from the orally challenged group died at 6 days post-challenge (dpc). The rest of the birds showed no external signs of illness during the course of the experiment. Examination of the liver of the bird that died showed it to have severe multifocal hepatitis (Fig. 6). After 7 dpc all the necropsied birds in the orally challenged group showed moderate

multifocal hepatitis (>30 spots on the liver). After 13 dpc the birds in the orally challenged group showed little to mild multifocal hepatitis (<30 spots on the liver). At post mortem examination of the livers of the control birds no gross lesions were visible at either of the sampling points in the experiment. *C. hepaticus* was reisolated from the livers of all the challenged birds but could not be isolated from any of the control birds. One putative *C. hepaticus* reisolate from each bird was confirmed by sequencing of their 16S rRNA gene.

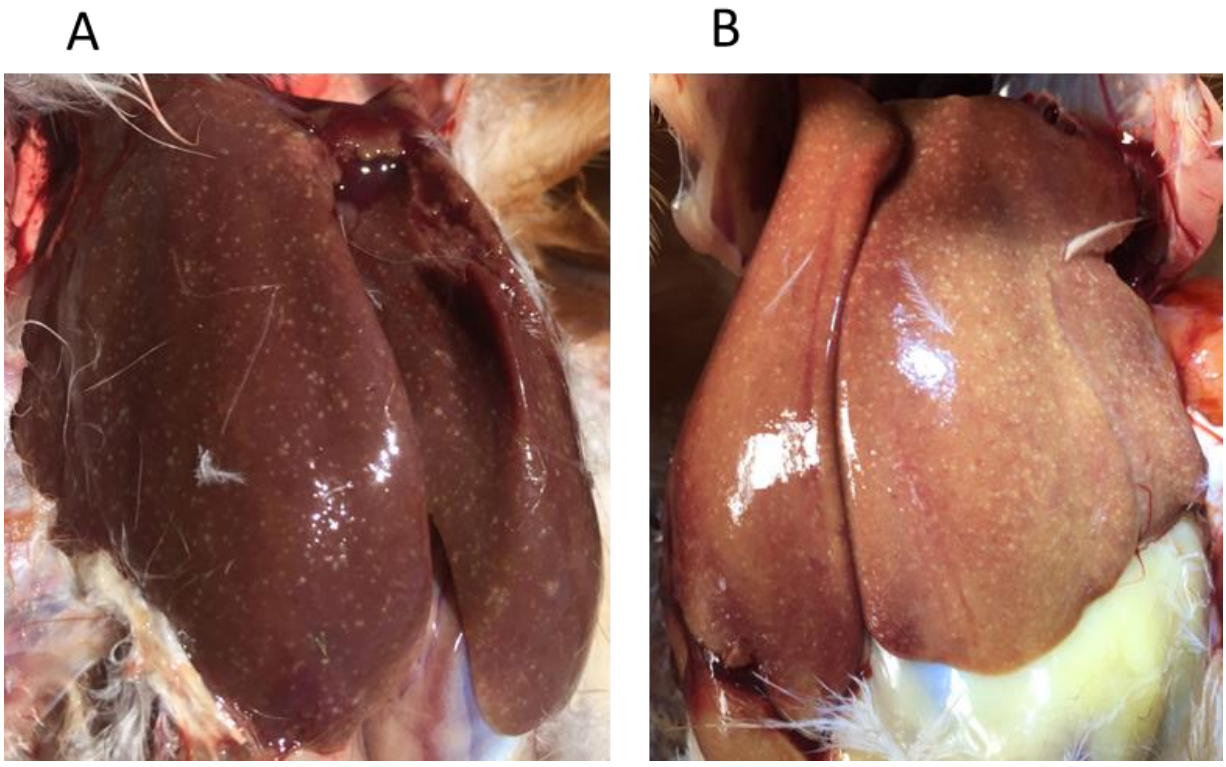

Figure 6. A: Severe multifocal hepatitis in the liver of the bird that died. B: The liver of a bird from Trial 2 necropsied at 5 dpc.

The histopathology of the livers of all birds (7 dpc) showed that the controls did not have lesions. Livers of the birds that were challenged with *C. hepaticus* NCTC 13823^{T} all exhibited lesions similar to those found in clinical cases of SLD. The liver of the dead bird showed scattered, often extensive, areas of coagulative hepatocellular necrosis throughout the section. Figure 7 shows the histopathology of the livers of two challenged birds.

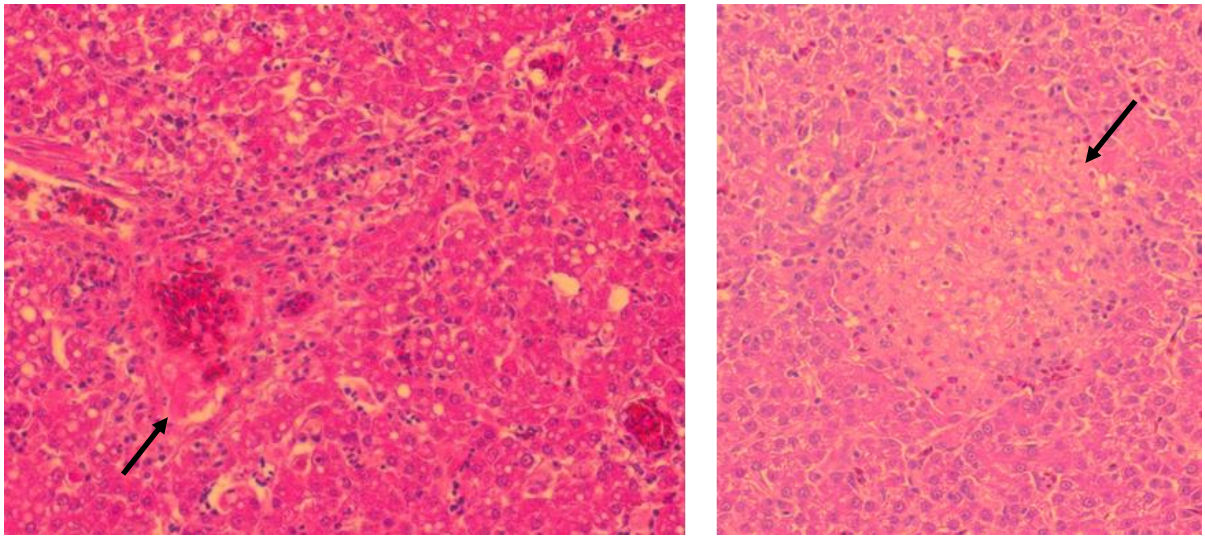

Figure 7. Multifocal necrotising hepatitis with fibrinogranulocytic foci (indicated by arrows) in the livers of two challenged chickens (haematoxylin and eosin stain).

Trial 2

At 5 days post-challenge no spots were seen on the livers of the birds in the control group and no bacteria were isolated from the liver and bile samples of these birds. All the challenged birds had lesions typical of clinical cases of SLD with the extent of disease ranging from little to severe multifocal hepatitis; some with just a few spots on the liver to other birds with hundreds or thousands of white spots on the liver. *C. hepaticus* was isolated from the bile and liver samples of all the challenged birds. At 7 dpc, again, no gross lesions were visible and no *C. hepaticus* were isolated from the liver and bile samples of the birds in the control group. Five out of six challenged birds showed little to moderate multifocal hepatitis but no bird showed the hundreds to thousands of spots as seen in some birds at 5 dpc and *C. hepaticus* was recovered from their bile and liver samples. Table 3 shows the detailed results of bacteriological and liver examination of the challenged birds from Trial 2. The level of disease, as indicated by the number of spots visible on the surface of the liver, varied across the challenged birds and we could see that now and in the future there was a need to have a standardised classification of disease severity. We therefore suggest a disease scoring system in the legend of Table 3.

Table 3: SLD lesion scoring and the presence of *C. hepaticus* in challenged birds from the second trial

Bird ID	Necropsy		Disease severity in	Isolation of *C. hepaticus**	
	5 dpc	7 dpc	liver^	from liver	from bile
4	✓		1	++	++
5		✓	2	+	++
6		✓	2	+	+
10	✓		3	+	+++++
11		✓	3	++	+++
12		✓	1	++	+
16	✓		2	+	+
17	✓		2	+	+++
18	✓		5	++++	+++++
19	✓		4	++	+++
20		✓	3	++	++++
21		✓	0	-	-

^ Disease severity measured by the number of miliary spots on liver: score of 0 indicates no spots; 1 indicates 1-5 spots; 2 indicates 6-20 spots; 3 indicates 21-100 spots; 4 indicates 101-1000 spots; 5 indicates more than 1000 spots.

* Growth of *C. hepaticus* was recorded according to the number of bacterial colonies recovered on HBA plates after incubation for 3 days following the standard methodology. +, 1-5 colonies; ++, 6-50 colonies; +++, 51-150 colonies, ++++, 151-300 colonies; +++++, too numerous to count.

Discussion

We successfully isolated bacteria from the livers of birds affected by SLD. These bacterial isolates were shown to be of a previously unrecognised species which we have named *Campylobacter hepaticus*. We then went on to first show that *C. hepaticus* could invade cultured live cells and then subsequently showed these isolates could induce lesions in the livers of infected birds that were indistinguishable from field clinical cases of SLD.

The accepted method of demonstrating that a particular pathogen is the cause of a disease is to address the 4 elements of Koch's postulates (Grimes, 2006). We have done this: (1) *C. hepaticus* was isolated from each of the outbreaks of SLD that we investigated; (2) *C. hepaticus* was recovered from diseased birds and grown in pure culture; (3) challenge of chickens of the appropriate type and age with *C. hepaticus* caused disease; and (4) *C. hepaticus* was reisolated from the experimentally challenged diseased birds. Therefore, each of the steps in Koch's postulates has been fulfilled and so it can be confidently concluded that *C. hepaticus* is the cause of SLD.

Based on the appearance of the cultured bacteria and some biochemical properties, but more strongly on the shared 16S rRNA gene sequences, it appears that the organism that we have isolated, characterised, and named, is similar to that reported by Crawshaw *et al.* (2015). It therefore appears likely that the SLD seen in Australia and England is the same disease. It will be interesting to see if related organisms can be isolated from other geographical locations.

Previous investigations that have attempted to identify and isolate bacteria that may cause SLD have variously identified "vibrios", based on microscopic morphology of apparently associated bacteria, and more recently Campylobacters. The Campylobacters have generally been assumed to be *C. jejuni*, although the basis for this assignment is not clear. Bacteria from the related genus, *Helicobacter*, have also been suggested as having an association with the disease. All these bacteria are related to the *C. hepaticus* that we have now identified as the cause. It would be interesting to know if previous researchers have seen this organism but were unable to recognise it as a new species. Perhaps it is more likely that previous isolation attempts were unsuccessful because *C. hepaticus* either does not grow or struggles to grow on some of the *Campylobacter* selective media that has previously been used.

Crawshaw and colleagues challenged 4 week old specific pathogen free (SPF) chickens, by intraperitoneal injection, with the *Campylobacter* that they isolated from SLD affected birds (Crawshaw et al., 2015). Microscopic lesions were apparent in the livers of some challenged birds, however, no gross lesions were visible on post mortem examination and the challenge did not cause mortality. In contrast, in the challenge trials reported here, one bird died 6 dpc and macroscopic lesions, typical of field clinical cases of disease, were seen in all but one of the challenged birds. Histological examination of the livers showed that the bird that died and most of the birds that survived had severe multifocal hepatitis with extensive areas of coagulative hepatocellular necrosis throughout the sections evaluated, indistinguishable

from the histopathology of clinical cases of disease. In the first challenge trial there was obvious and significant disease in the livers of birds necropsied at 7 dpc. However, when the remaining birds were necropsied at 13 dpc less disease was seen. From this we concluded that the peak of disease, as indicated by macroscopically visible liver lesions, must be within the first week post-challenge. Therefore, in the second trial we investigated liver lesions at 5 and 7 dpc and again found that birds at the earlier time-point generally had more severe disease. From this we conclude that the peak of disease is likely to be seen at or before 5 dpc and the birds can recover, at least in terms of the obvious liver lesions disappearing, over the course of a couple of weeks. The difference in the outcomes of the current bird challenge experiments and that of Crawshaw *et al.* (2015) may be due to the age of birds, the type of birds, the challenge strains used and/or the method of bacteria growth and the mode of inoculation used in the experiments. Chickens in egg production are undergoing some physiological changes that, from field observations, may represent a predisposing factor in the development of SLD (Grimes and Reece, 2011). Disease outbreaks are often associated with this transition to peak egg-laying. Earlier work to experimentally induce the disease mainly challenged chicken embryos or day old chicks and recorded deaths but usually not liver lesions (Delaplane et al., 1955; Moore, 1958). The work presented here is the first clear and reproducible example of an SLD induction model that fully recapitulates the gross liver lesions typical of clinical cases of disease.

The invasion assay showed that *C. hepaticus* was significantly more invasive to chicken liver cells (LMH) than the *C. jejuni*, *C. lari* and *C. upsaliensis* strains tested in parallel. The *C. jejuni* 81116 strain has previously been shown to be invasive and this property has been a useful tool in the study of the role of various gene products in virulence (Fearnley et al., 2008; Wassenaar et al., 1991). The high invasive capabilities of *C. hepaticus* may explain why this organism can cause lesions in the livers of infected chickens and the invasion assay may be a useful *in vitro* tool to investigate aspects of the genetic basis of pathogenicity.

Implications

With the causative agent of SLD now definitively identified and with the availability of a robust experimental disease induction model the way is open to develop and test new methods to control or limit the impacts of the disease. To date antibiotic treatment has been one of the more effective ways to ameliorate disease but with the occurrence of some antibiotic resistance in some treatment regimens and the need to move to more sustainable

non-antibiotic control methods within the poultry industry there is a need for other treatment options such as vaccination and symbiotic feed additives. The *in vitro* invasion assay will assist in the investigation of *C. hepaticus* virulence factors which may be suitable antigenic targets for vaccine design.

Recommendations

This foundation research has identified the organism responsible for SLD and hence the target for methods to treat the disease. In addition it provides a disease model in which to properly test such treatments. Further research is now enabled to investigate ways of ameliorating SLD. There are two clear tracks that should be pursued; (i) evaluation of feed supplements such as short chain fatty acids, yeast extracts, essential oils, prebiotics and probiotics, as alternatives to antibiotics, and (ii) vaccines against *C. hepaticus* – there are a number of formats worth investigating – killed, subunit, live attenuated, and live vectored antigen delivery. The development of diagnostic assays to detect the pathogen within flocks and in the environment should be a priority. Such assay could be PCR based assays to detect pathogen DNA or immune based assays to directly detect pathogen proteins or chicken immune responses to exposure to the pathogen. More basic research is also needed to try to understand the origins and epidemiology of *C. hepaticus* infections and the natural chicken immune response to infection.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	**Confirmation of a putative Spotty Liver pathogen**
Poultry CRC Sub-Project No.:	1.2.6
Researcher:	Prof Robert Moore
Organisation:	RMIT University
Phone:	+61 3 9925 7580
Fax:	+61 3 9925 7110
Email:	rob.moore@rmit.edu.au
Sub-Project Overview	The goal of this Sub-Project was to identify the pathogen responsible for Spotty Liver Disease (SLD). This has been achieved.
Background	SLD can cause mortality and loss of egg production in layer birds and is also seen in other sectors of the poultry industry. It was first identified in the US over 60 years ago but identifying the cause of the disease has proven elusive. The disease has been present in the Australian poultry industry for several decades but recently it has been seen more frequently and is becoming a major concern for poultry producers, particular in the layer industry. It has long been suspected that the disease is caused by a bacterial infection and various different bacterial species have been suggested as the cause. However, despite the efforts of multiple laboratories around the world, no causal bacterium had been defined.
Research	We were able to isolate bacteria from the liver of diseased birds by refining the culturing conditions used. Molecular and biochemical analysis showed that a *Campylobacter* was isolated from most diseased livers. The *Camplyobacter* was shown to be a new species that we named *C. hepaticus*. *C. hepaticus* does not grow on some of the media that is commonly used for *Camplyobacter* isolation, perhaps explaining why it has not been isolated previously. We then went on to show that *C. hepaticus* could induce SLD in layer birds and we could reisolate the bacterium from the experimentally infected birds. This definitively demonstrated that *C. hepaticus* is the cause of SLD.
Sub-Project Progress	The bacterium *Campylobacter hepaticus*, which causes SLD, has been identified, isolated, characterised and formally named as a newly identified species. The pathogen's causal role in SLD has been demonstrated and an effective experimental method for disease induction in layer birds has been developed.
Implications	With the cause of the disease now definitively identified and a disease model available the way is now open to develop and evaluate new methods of disease control such as vaccines and symbiotic products.
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 Van, T.T.H., Elshagmani, E., Gor, M.C., Anwar, A., Scott, P.C., and Moore, R.J. (2016) Fulfillment of Koch's postulates shows that spotty liver disease in layer hens is caused by *Campylobacter hepaticus*. Submitted to Veterinary Microbiology.