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Development of immunological assays to assist the control of Spotty Liver Disease in chickens

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Project Summary

Project Title	Development of immunological assays to assist the control of Spotty Liver Disease in chickens
Project No.	2019-114
Date	Start: November 2019 End: October 2022
Project Leader(s)	Dr Thi Thu Hao Van
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Project Aim	The project aim was to develop innovative immunological assays to detect *C. hepaticus* antibodies and antigens in birds, by using three approaches: (i) a whole cells antigen ELISA; (ii) an ELISA based on recombinant immunodominant antigens; and (iii) a monoclonal-based ELISA to detect circulating antigen.
Background	Spotty Liver Disease (SLD) is an emerging disease of serious concern in the egg production industry, as it causes significant egg loss and mortality in layer hens. *Campylobacter hepaticus* has been confirmed to be the causative agent. Current detection methods for SLD, such as PCR and culturing, only detect an active infection and will not give any indication of a past infection from which the bacteria have been cleared. An immunological assay, on the other hand, can provide information on previous infections and therefore is crucial for epidemiological studies and vaccine development against SLD. This project aimed to develop immunological assays to allow monitoring of infection in flocks and therefore assist in the control of SLD in chickens.
Research Outcome	Two immunoassays capable of detecting *C. hepaticus* specific antibodies present in the sera of infected birds were successfully developed, with high sensitivity (93–97.6%) and specificity (95–95.5%). The first assay, SLD-ELISA1, uses *C. hepaticus* total protein extract (TPE) as the antigen coating on ELISA plates. The second assay, SLD-ELISA2, uses filamentous hemagglutinin adhesin (FHA) as a capture ligand to identify *C. hepaticus* specific antibodies. SLD-ELISA2 is more user-friendly and standardisable than SLD-ELISA1 for screening antibody responses to *C. hepaticus* exposure in hens as it is based on a purified recombinant protein. A sandwich ELISA, SLD-ELISA3, using polyclonal antibodies generated against recombinant FHA protein fragments, $FHA_{623-953}$ (F2) and $FHA_{1628-1899}$ (F4) was also developed. The assay is specific to *C. hepaticus* but has low sensitivity for chicken tissue samples and therefore may not be useful for field samples. However, the polyclonal antibody generated for SLD-ELISA3 may be useful for the development of an immunohistochemistry assay.
Impacts and Outcomes	The methods developed in this study are useful for detecting the exposure of layer chickens to *C. hepaticus* to help in flock and environmental monitoring and management to reduce the impact of SLD. It can also be used to test the efficacy of experimental vaccines in any vaccine development program against *C. hepaticus*.

Publications	Muralidharan C, Anwar A, Wilson TB, Scott PC, Moore RJ & Van TTH. (2020). Development of an enzyme-linked immunosorbent assay for detecting *Campylobacter hepaticus* specific antibodies in chicken sera – a key tool in Spotty Liver Disease screening and vaccine development. *Avian Pathology*, 49:658-665. Muralidharan C, Anwar A, Quinteros JA, Wilson TB, Scott PC, Moore RJ & Van TTH. (2022). The use of filamentous hemagglutinin adhesin to detect immune responses to *Campylobacter hepaticus* infections in layer hens. *Frontiers in Veterinary Science* (under peer review). Muralidharan C, Huang J, Anwar A, Scott PC, Moore RJ & Van TTH. (2022). Prevalence of *Campylobacter hepaticus* specific antibodies among commercial free-range layers in Australia. *Frontiers in Veterinary Science* (under peer review).

Executive Summary

This project's aim was to develop innovative immunological assays to detect *C. hepaticus* antibodies and antigens in birds, by testing three approaches: (i) a whole cells antigen ELISA; (ii) an ELISA based on recombinant immunodominant antigens; and (iii) monoclonal-based ELISA to detect circulating antigen. The immunoassays provide useful tools for monitoring the exposure of poultry flocks to *C. hepaticus* infection and can be used to direct and support vaccine development.

For the first milestone, an ELISA immunological assay to detect *C. hepaticus* antibodies in chicken serum using *C. hepaticus* total proteins, named as SLD-ELISA1, was developed. The assay uses *C. hepaticus* total protein extract (TPE) as the antigen coating on ELISA plates. The cross-reactivity of *C. hepaticus* antibodies with closely related *C. jejuni* and *C. coli* antigens was successfully overcome by pre-absorbing the sera using *C. jejuni* cell extracts. The assay was validated using sera samples from both naturally and experimentally infected birds, birds vaccinated with formalin killed bacteria, and sera samples from SLD negative birds (control group). The optimised SLD-ELISA1 assay had 95.5% specificity and 97.6% sensitivity. A research paper titled "Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for detecting *Campylobacter hepaticus* specific antibodies in chicken sera – a key tool in Spotty Liver Disease (SLD) screening and vaccine development" has been published in Avian Pathology.

For the second milestone, an ELISA based on recombinant immunodominant antigens, SLD-ELISA2, was developed. An immunoproteomic approach that combined bioinformatics analysis, western blotting, and liquid chromatography mass spectrometry (LC MS/MS) protein profiling was used. A Fragment of filamentous hemagglutinin adhesin (FHA), $FHA_{1628-1899}$, with *C. hepaticus* specific antigenicity was identified. Recombinant $FHA_{1628-1899}$ was used as antigen coating on ELISA plates to capture $FHA_{1628-1899}$ specific *antibodies* in SLD-ELISA2. This assay is more user-friendly and standardisable than SLD-ELISA1 as it based on a purified recombinant FHA fragment, which is easier to prepare than TPE and can be fully quality controlled. This study is the first report of the use of FHA from a *Campylobacter* species in immunoassays, and it also opens future research directions to investigate the role of FHA in *C. hepaticus* pathogenesis and its effectiveness as a vaccine candidate. A research paper titled "The use of filamentous hemagglutinin adhesin to detect immune responses to *Campylobacter hepaticus* infections in layer hens" has been submitted for publication to Frontiers in Veterinary Science. This assay has been successfully used in a large scale investigation of *C. hepaticus* specific antibodies among commercial free range layers in Australia (PHA 2020-226). A paper entitled: "Prevalence of *Campylobacter hepaticus* specific antibodies among commercial free-range layers in Australia" has been submitted to Frontiers in Veterinary Science and it is in revision stage.

For the third milestone, a sandwich ELISA, SLD-ELISA3, was developed using polyclonal antibodies generated against recombinant FHA protein fragments, $FHA_{623-953}$ (F2) and $FHA_{1628-1899}$ (F4). The recombinant FHA-F2 was recognised on western blots probed using pooled SLD positive sera. FHA-F4, that was previously determined as specific for *C. hepaticus* (and used as coating antigen in SLD-ELISA2). Therefore, polyclonal rabbit antibodies were generated against a mixture of recombinant FHA-F2 and FHA-F4 and was used as capture and detection antibodies for SLD-ELISA3. The limit of detection (LOD) of *C. hepaticus* whole cells was determined to be 0.6×10^7 cfu/ml and the LOD of total protein extracts from *C. hepaticus* was determined to be 1 µg/ml. The assay is specific for *C. hepaticus*; however, the sensitivity is only 20% for bile samples and did not distinguish liver extracts from *C. hepaticus* positive and negative birds. This is likely because the level of *C. hepaticus* in the tissue is

lower than the detection limit. Therefore, the assay may not be suitable to detect *C. hepaticus* in chicken tissue samples. However, the polyclonal antibody produced for SLD-ELISA3 can be used to develop an immunohistochemistry assay for use on tissue sections to detect the binding between antibody and antigen to localise specific antigens and bacteria in tissues via fluorescent microscopy.

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1. Introduction

Spotty Liver Disease (SLD) causes serious issues for the Australian Poultry Industry, most notably affecting free range layer flocks but also seen in other chickens. The cause of the disease had been a mystery for over 60 years, until several years ago when a group in the UK reported the isolation of a new bacterium in chickens infected with SLD (Crawshaw et al. 2021) and our group characterised and formally named the bacterium that causes SLD, *Campylobacter hepaticus* (Van et al. 2017). The disease was first reported in the United States in 1950s, and there have since been reports from various countries including Canada, the United Kingdom and Germany, and recently in the United States, New Zealand and Jordan (Courtice et al. 2018; Crawshaw & Irvine 2012; Gregory et al. 2018; Hananeh & Ababneh 2021; Tudor 1954).

Currently, antibiotic therapy is providing the most successful means of SLD control. However, antibiotic resistant field isolates of *C. hepaticus* have been identified and it would be preferable to use methods other than antibiotic therapy to control the disease. Feed additives have been investigated, but only a few have shown any signs of helping in SLD control. Vaccination against *Campylobacter* disease had, historically, proved difficult, and the ability of the disease to reoccur in flocks indicates that vaccine development will be challenging. Thus, the control of the disease may be dependent on understanding the epidemiology of the disease and eliminating the vectors, predisposing factors, or causal associations.

Thanks to Poultry Hub Australia (PHA) support of our SLD epidemiology project, we have investigated a large number of birds for active *C. hepaticus* infection, using PCR. This work helped to identify possible sources and routes of infection and will help in the control of SLD by highlighting biosecurity and monitoring measures (Phung et al. 2019). To further understand the epidemiology of SLD and provide additional tools for the monitoring of flocks, we proposed to use bird serology to detect antibodies against *C. hepaticus.* Unlike PCR, which can only detect active infections in which the organism and hence its DNA is present, an enzyme-linked immunosorbent assay (ELISA) should be able to detect previous infection. The development of a serological diagnostic method is also the key to understanding the nature of *C. hepaticus* infection, i.e. whether the first infection can provide immunity to SLD or if the birds remain susceptible to a second infection during another disease outbreak (recurring). Hens can recover from SLD, and it is assumed that immune responses are important.

The aim of this project was to develop innovative immunological assays to detect *C. hepaticus* antibodies and antigens in birds, by testing three approaches: (i) a whole cells antigen ELISA; (ii) an ELISA based on recombinant immunodominant antigens; and (iii) monoclonal-based ELISA to detect circulating antigen. The methods developed in this study have been used to examine the exposure of layer chickens to *C. hepaticus* to assist in SLD management. It can also be used in vaccine development studies to test the efficacy of experimental vaccines.

2. Objectives

The project objectives were:

- Development of ELISA1 immunological assay to detect *C. hepaticus* antibody using whole cell lysates (SLD-ELISA1).
- Development of an ELISA based on recombinant immunodominant antigens (SLD-ELISA2).
- Development of monoclonal-based ELISA to detect circulating antigen (SLD-ELISA3).

3. Methodology

3. 1. Development of SLD-ELISA1 immunological assay to detect *C. hepaticus* antibody using whole cell lysate

3.1.1 Preparation of total protein extracts (TPE)

C. hepaticus HV10 (NCBI Accession number CP031611), *C. jejuni* 81116 (NCTC 11828), and *C. coli* NCTC 11366 were grown on horse blood agar (HBA) plates (Brucella broth (Oxoid) supplemented with 1.5% agar (Oxoid) and 5% horse blood (Equicell)) under microaerophilic conditions using Campygen packs (Oxoid). The bacterial cells were harvested in 500 µl phosphate buffered saline (PBS) and stored at -20°C for 2 hours or overnight to enhance the cell disruption efficiency. The cell suspension was thawed on ice and sonicated to obtain the whole cell lysate (WCL). WCL was centrifuged at 8000 x g for 10 minutes to collect the supernatant, which was named the total protein extract (TPE). The standardised TPE solution (OD_{280}= 1) was used as the antigen to coat wells for the ELISA assay.

3.1.2 Collection of chicken sera

Forty-one SLD positive samples (nine from formalin killed vaccine inoculated, 10 naturally infected, and 22 experimentally challenged birds) and 22 SLD negative samples (5 field sampled birds with no history of SLD and 17 negative controls used in an infection/challenge trial) included in the study. 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).

3.1.3 Enzyme linked immunosorbent assay (ELISA)

Fifty microlitres of antigen solution (*C. hepaticus* TPE in PBS, pH 7.2) was coated on each well of 96 well plates. Primary antibody solution (chicken sera) was used either with or without pre-absorption with *C. jejuni* proteins, as described in the reduction in cross-reactivity by pre-absorption section. Goat anti-chicken Ig-Y-HRP (Thermo Fisher Scientific) was used as secondary antibody and Novex 3,3',5,5'- tetramethylbenzidine (TMB) chromogenic substrate (Invitrogen) was added to each well as the chromogenic substrate. The absorbance was measured at 652 nm using a microplate reader (POLARstar Omega Plate Reader Spectrophotometer, BMG LABTECH).

3.1.4 Investigation of *C. jejuni*, *C. coli* and *C. hepaticus* cross-reactivity

Sera samples from nine birds inoculated with formalin killed *C. hepaticus* $HV10^T$ to induce immune responses, five negative controls, and ten diseased birds naturally infected with SLD, were tested for *C. jejuni* and *C. coli* cross-reactivity. Adjacent wells in ELISA plates were coated with *C. hepaticus,*

C. jejuni, and *C. coli* TPE. ELISA was performed as described above with 0.01 OD TPE antigen coating, 1 in 1000 dilution of chicken sera, and 1 in 2000 dilution of secondary antibody.

3.1.5 Reduction in cross-reactivity by pre-absorption

The primary antibody/sera solutions were pre-absorbed with *C. jejuni* TPE (OD_{280} 1) to reduce the non-specific binding of *C. jejuni* antibodies that may be present in the sera samples. An experimentally determined volume of *C. jejuni* TPE (OD_{280} 1) was added to the primary antibody/sera solution and incubated for 45 minutes at room temperature. The solution was then centrifuged at 3700 x g for 10 minutes and the supernatant was used as the pre-absorbed primary antibody solution to probe the antigen coated wells.

3.1.6 Assessment of the ELISA in the presence of *C. jejuni* and *C. coli* antibodies

To determine if the assay was affected by presence of *C. jejuni* or *C. coli* antibodies, caecal samples of six *C. hepaticus* negative control birds from the challenge trial were tested for the presence of *C. jejuni*, *C. coli* or both DNA, using the previously reported multiplex PCR (Van et al. 2018). The PCR result was then compared with the ELISA response.

3.1.7 Optimisation of the ELISA parameters

The following ELISA parameters were optimised. (i) Volume of *C. jejuni* TPE for pre-absorption. The optimal volume of *C. jejuni* TPE to be used for pre-absorption was determined by testing the addition of different volumes of OD1 *C. jejuni* TPE (1, 2, 3, 4 and 5 µl) per 100 µl of the primary antibody solution. (ii) Antigen coating. Various dilutions of *C. hepaticus* TPE were tested for optimal ELISA performance, absorbance (OD_{280}) 1, 1 in 10, 100, 200, 400, 800, 1600 and 3200 dilution of OD_{280} 1 TPE. (iii) Antibody dilutions. Different dilutions of chicken sera, 1 in 100, 250, 500, 750, 1000, 2000, 4000 and 5000 were used in the primary antibody titre. Secondary antibody (goat anti-chicken Ig-Y-HRP) was tested at dilutions 1 in 1000, 2000, 4000, 6000 and 8000. All samples were assayed in triplicates and standard deviations were calculated.

3.1.8 Evaluation of ELISA performance

The assay was validated using 63 samples (22 negative and 41 positive samples, including sera from naturally and experimentally infected birds). Each sample was tested at least twice, with triplicate wells for each assay. The assay sensitivity and specificity were calculated from the absorbance values of the 63 sera samples, as described by Chénard et al. (1998). One-way analysis of variance (ANOVA) tests was performed to determine the statistical significance of the ELISA results.

3.2. Development of an ELISA based on recombinant immunodominant antigens (SLD-ELISA2)

3.2.1 Prediction of *C. hepaticus* specific immunogenic proteins using bioinformatics analysis

The whole genome sequences of 14 Australian *C. hepaticus* strains were annotated using the Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al. 2008; Overbeek et al. 2014) to identify predicted proteins encoded by the *C. hepaticus* core genome. Annotated protein sequences from *C. hepaticus* type strain HV10T were used in all bioinformatics analysis (Van et al. 2016). The bioinformatics workflow used to predict *C. hepaticus* specific immunogenic proteins is outlined in Figure 1. Proteins with high antigenicity scores and significant amino acid differences of the *C. hepaticus* sequence compared to *C. jejuni* and *C. coli* sequences within the epitope regions were

shortlisted for cloning and expression studies. The conservation of shortlisted protein encoding genes in all 14 *C. hepaticus* strains was also checked.

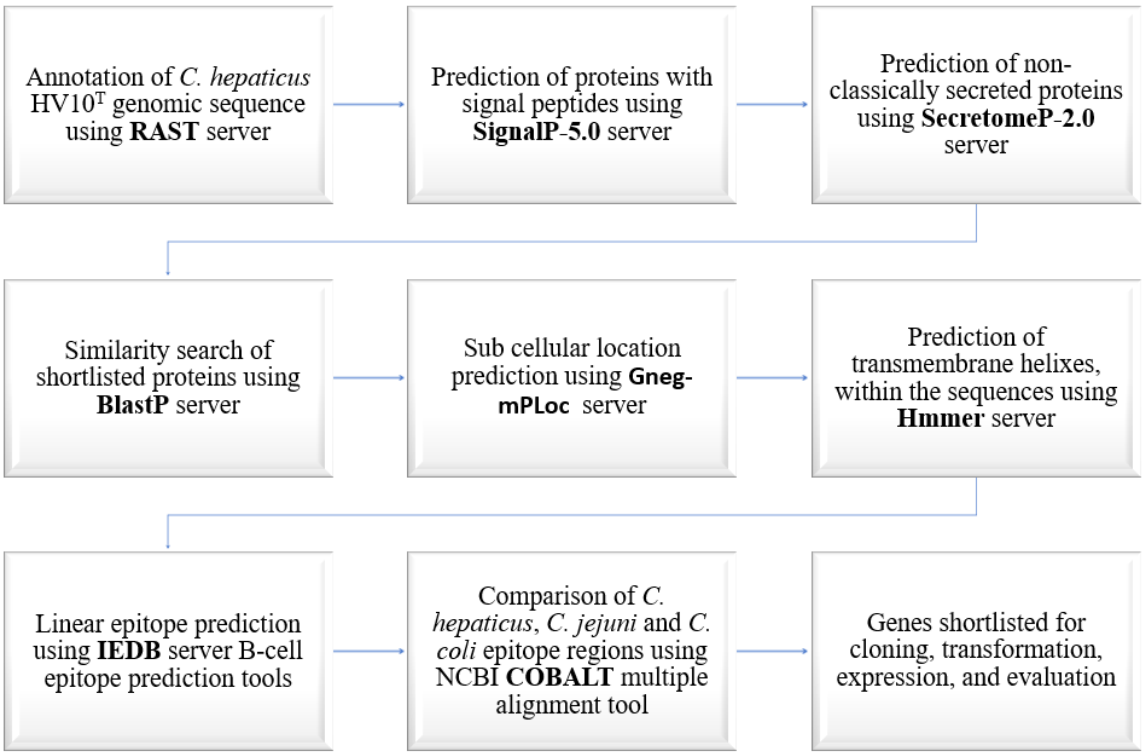

Figure 1 Bioinformatics workflow used in the prediction of *C. hepaticus* specific immunogenic proteins

3.2.2. Identification of *C. hepaticus* specific immunogenic proteins by western blotting

C. hepaticus HV10^T, *C. jejuni* 81116, and *C. coli* NCTC 11366 total protein extracts (TPE) were prepared as described previously (Muralidharan et al. 2020). Pre-absorbed primary antibody solution was obtained after spinning down the normal primary antibody solution incubated for one hour with 50 µL each of OD 4 *C. jejuni* and *C. coli* TPEs. Protein bands from *C. hepaticus*, *C. jejuni* and *C. coli* TPE lanes on PVDF membranes probed using normal and pre-absorbed primary antibody solutions were compared, and the unique bands that were present only in *C. hepaticus* were noted. Two-dimensional (2D) gel electrophoresis was also performed using Zoom IPG runner system and reagents to resolve the bands further as per manufacturers protocol (Thermo Fisher Scientific).

3.2.3 Protein identification using liquid chromatography electrospray ionisation tandem mass spectrometry (LC ESI MS/MS)

Proteins bands in Coomassie stained gels that corresponded to *C. hepaticus* specific bands identified by western blotting were excised and trypsin digested. Proteins present within the bands were identified by LC ESI MS/MS (Monash Proteomics and Metabolomics facility, Melbourne, Australia). Data analysis was performed using the Byonic search engine (ProteinMetrics) by comparing experimentally obtained peptide spectra with theoretical peptide spectra generated from a RAST annotated *C. hepaticus* HV10^T protein database and SwissProt database separately. The ten top ranked proteins obtained from the *C. hepaticus* HV10^T proteins database comparison were inspected for potential immunogenic proteins.

3.2.4 Determination of immunogenicity of shortlisted proteins by cloning, expression, and western blotting

Protein encoding genes that were shortlisted though the bioinformatics and western blotting/ LC ESI MS/MS analysis were synthesised and cloned in the pET-28a (+) -TEV vector (GenScript gene synthesis service). For smaller proteins, the whole coding sequence was used but for larger proteins, a fragment of the coding sequence containing IEDB predicted immunogenic regions was used. The genes were codon optimised for expression in *Escherichia coli.* The immunogenicity of expressed proteins was determined by western blotting using SLD positive and SLD negative bird sera. The recombinant protein recognised by the majority of SLD positive sera and fewest SLD negative sera was purified on immobilised metal affinity chromatography (IMAC) polypropylene columns (Sigma) packed with nickel-nitriloacetic acid (Ni-NTA) agarose (Thermo Fisher Scientific).

3.2.5. Development of ELISA using purified recombinant protein, SLD-ELISA2

A total of 115 sera samples were used for the development and validation of SLD-ELISA2, including sera samples from 48 experimentally infected birds, 10 naturally infected birds, and 57 negative control birds. The experimental infection trials at the Scolexia facility were approved by Agriculture Victoria, Wildlife and Small Institutions Animal Ethics Committee (Approval number 33.21).

All sera samples were initially assayed using SLD-ELISA1 to confirm their SLD immune response status. Sera from two naturally infected birds, two experimentally infected birds and three negative control birds were used for the optimisation of SLD-ELISA2. Purified recombinant protein at 0.25, 0.5, 1.0 and 2.0 µg/ml of PBS was tested to determine the optimal protein concentration for coating ELISA plates. Bird sera at one in 500, 1000, 2000 and 4000 dilutions were tested to identify the optimal sera dilution that could differentiate SLD positive and SLD negative sera samples. Nunc Maxisorp 96 well plates (Thermo Fisher Scientific) were coated overnight at 4°C with the recombinant protein (50 µl/well). TMB substrate (Invitrogen) was used for chromogenic detection (50 µl/well). The absorbance was read at 450 nm on a POLARstar Omega Plate Reader Spectrophotometer (BMG LABTECH) following the addition of 50 µl of stopping solution (2 M sulphuric acid).

The cut-off value for the assay was calculated as the absorbance mean plus two standard deviations (SD) of the negative control samples (Barajas-Rojas et al. 1993). Assay sensitivity and specificity was calculated as described previously (Chénard et al. 1998). Statistical analysis was carried out using GraphPad Prism (9.0.2) software (San Diego, CA, USA). A one-way ANOVA test was performed assuming equal Gaussian distribution of residuals and equal standard deviations with 95% confidence interval. Multiple comparison was performed by comparing the mean of each group with the mean of every other group.

3.3. Development of monoclonal-based ELISA to detect circulating antigen (SLD-ELISA3)

3.3.1. Selection of antigens for monoclonal and polyclonal antibody preparation

The FHA protein is 1899 aa long, therefore it was divided into 4 fragments of 272–674 aa to make it easier to express in *E. coli*. The amino acid sequence of FHA and the coverage of recombinant protein fragments FHA-F1 (594 aa), FHA-F2 (331 aa), FHA-F3 (674 aa), and FHA-F4 (272 aa) are shown in Figure 2.

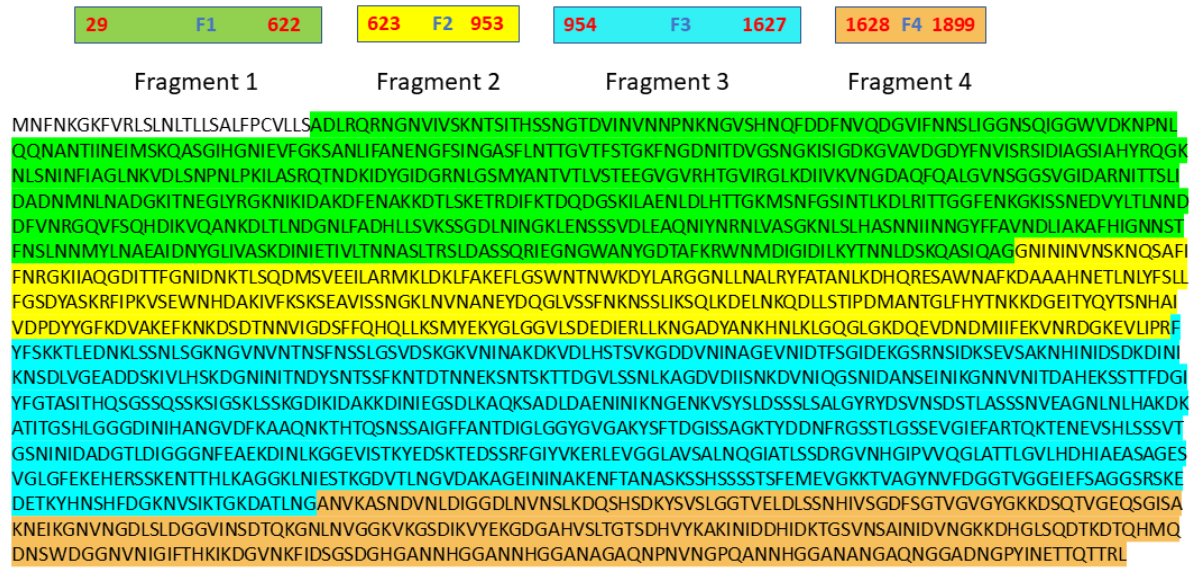

Figure 2 The pictorial representation and amino acid sequence of FHA, divided into four regions, F1, F2, F3 and F4, highlighted in green, yellow, blue, and orange respectively

All four fragments of the FHA protein had multiple epitope regions confirmed by B-cell epitope prediction using the Immune Epitope Database (IEDB) server (Jespersen et al. 2017). The immunogenicity of FHA-F4 (denoted as $FHA_{1628\text{-}1899}$) was demonstrated and used in SLD-ELISA2. The remaining three gene fragments were synthesised and cloned in pET-28a (+) -TEV vector by GenScript, USA, transformed, expressed in *E. coli* BL21 (DE3) (New England Biolabs) and tested for immunogenicity. *E. coli* cell lysates from each clone were tested for immunogenicity by performing western blotting. Recombinant proteins recognised by the majority of SLD positive sera and least SLD negative sera were purified on immobilised metal affinity chromatography (IMAC) polypropylene columns (Sigma) packed with nickel-nitriloacetic acid (Ni-NTA) agarose (Thermo Fisher Scientific, USA). The elutes containing target proteins were pooled and buffer exchanged using PBS to remove imidazole or urea using Amicon 10 kDa ultracentrifuge filter unit (Merck).

3.3.2. Polyclonal and monoclonal antibody production

FHA-F4 was used to produce monoclonal antibodies using the GenScript monoclonal antibody synthesis service. Five BALB/c mice were immunised three times with purified recombinant protein to generate the antibody pool. GenScript polyclonal antibody service was also used to generate rabbit polyclonal antibodies against a mixture of recombinant FHA-F2 and FHA-F4. The sera from rabbits were pooled and affinity purified with the pure recombinant proteins. A portion of the purified polyclonal antibody was conjugated with horseradish peroxidase (HRP) enzyme to enable its use as a detection antibody.

3.3.3. Validation of the polyclonal antibodies and the sera from immunised mice for monoclonal antibody production

The sera from five BALB/c mice immunised with FHA-F4 was validated using western blot and ELISA. The mice sera were used as the primary antibody to check if the antibody could recognise the recombinant FHA-F4 and native FHA protein in *C. hepaticus* total protein extract (TPE). HRP conjugated goat anti-mouse IgG polyclonal antibody (Sigma) was used as the secondary/detection antibody.

The unlabelled anti-FHA-F2F4 polyclonal antibody was validated by using the polyclonal antibody as the primary antibody and mouse anti-rabbit IgG-HRP as the detection antibody on PVDF membranes blotted with recombinant proteins FHA-F2, FHA-F4 and *C. hepaticus* total proteins. HRP conjugated polyclonal antibody was validated by using them as detection antibody on the blotted membrane. The cross-reactivity of polyclonal antibodies against *C. jejuni, C. coli*, *E. coli*, and *C. bilis* total proteins was also checked.

3.3.4. Development and optimisation of ELISA using polyclonal antibodies, SLD-ELISA3

Polyclonal anti-FHA-F2F4 unlabelled antibody was used as the capture antibody and polyclonal anti FHA-F2F4-HRP conjugated antibody was used as the detection antibody in ELISA3. Various concentrations of capture antibody, such as 2, 4, 8, 16, and 32 µg/ml polyclonal anti-FHA-F2F4 antibody, in PBS was tested. *C. hepaticus* whole cells at ten-fold dilutions of OD_{600} 1 cell suspension in PBS (corresponding to OD_{600} 0.6 x 10^8 cfu/ml) from 10^4–10^7 cfu/ml were tested. *C. hepaticus* TPE at concentrations 0.1 µg/ml, 1 µg/ml and 10 µg/ml were also tested.

3.3.5. Determination of the cross-reactivity of polyclonal antibodies to *C. hepaticus* FHA against *C. jejuni* and *C. coli* proteins

The highest concentrations of *C. jejuni*, and *C. coli* cell suspensions and TPE in comparison with the optimised *C. hepaticus* cell suspensions and TPE were assayed to determine the cross-reactivity of polyclonal antibodies against *C. jejuni*, and *C. coli*. Whole cell suspensions at 10^7 and 10^8 cfu/ml and TPEs at 10 µg/ml and 100 µg/ml concentrations were used.

3.3.6. Validation of SLD-ELISA3 using bile and liver samples

Five bile samples that had culture confirmed presence of *C. hepaticus* and two bile samples from negative control birds were used to validate the assay. The liver samples from three positive control birds that showed liver lesions and three negative control birds were also tested to determine the assay validity for tissue samples.

4. Results and discussions

4.1. Development of SLD-ELISA1 immunological assay to detect *C. hepaticus* antibody using whole cell lysate

4.1.1. Reducing cross-reactivity

The most challenging issue in developing the *C. hepaticus* ELISAs was to provide differentiation from immune responses to the closely antigenically related species *C. jejuni* and *C. coli*. The cross-reactivity issue of *C. hepaticus* antibodies was successfully overcome by pre-absorbing the sera using *C. jejuni* proteins (Figure 3A; Figure 4, Columns 5 and 9 (normal sera) vs Columns 7 and 11 (preabsorbed sera with *C. jejuni*) when SLD-negative sera were used; Figure 4, Columns 4, 8 and 12 where the signals from *C. jejuni* coated wells were absent in the wells using preabsorbed sera). Figure 4 also illustrates a clear differentiation between SLD positive and negative samples as the signals were obtained only on *C. hepaticus* coated wells for SLD positive samples (Column 3) and not on SLD negative samples (Columns 7 and 11) when pre-absorbed sera were used. In addition, the positive samples were unaffected by preabsorption (Figure 4, Column 3).

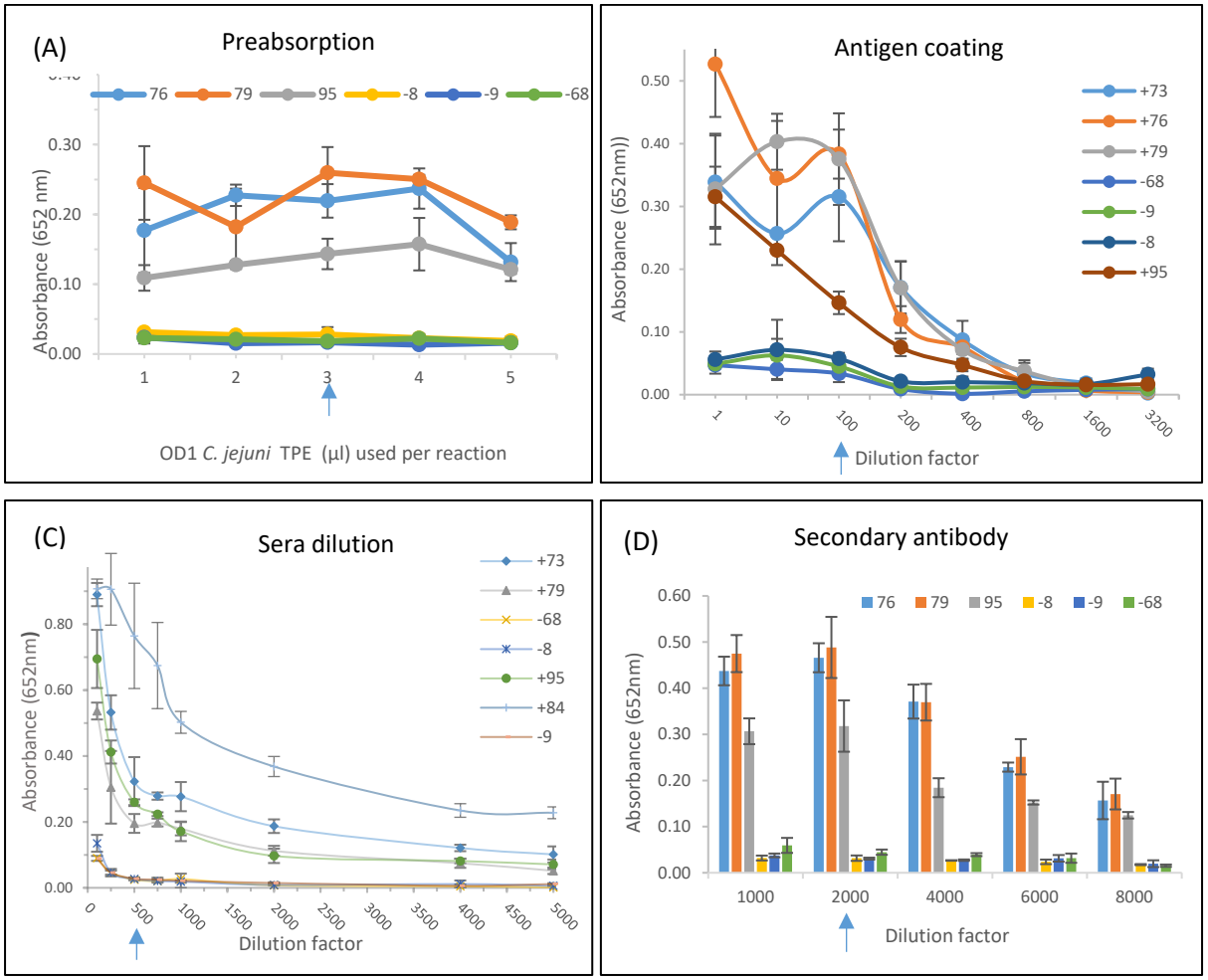

Figure 3 Optimisation of ELISA parameters

(A) Assay of volume of TPE to use for sera pre-absorption.
(B) Assay of effects of antigen coating dilution on ELISA performance.
(C) Assay of effects of primary antibody (chicken sera) dilution.
(D) Assay of secondary antibody dilution.
The conditions selected for ongoing use in the standardised ELISA are indicated by the blue arrows.
Serum used: +73, +76, +79, +95 are SLD positive sera; -8, -9, -68 are SLD negative sera.

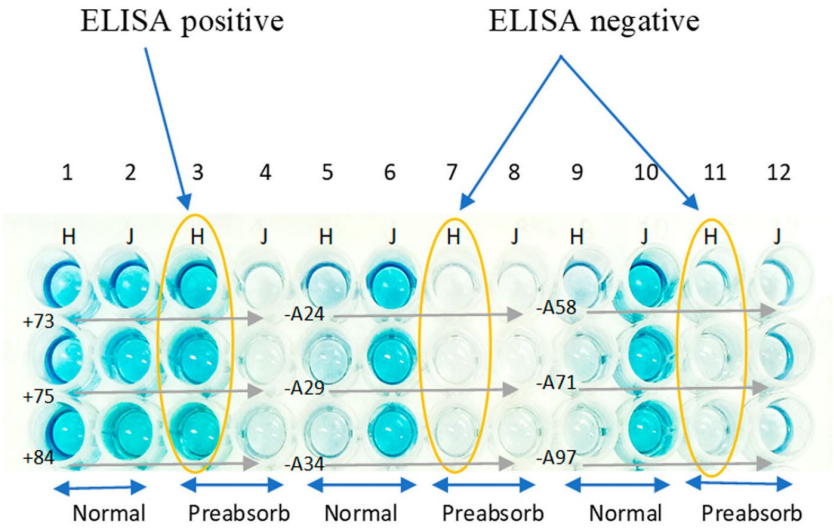

Figure 4 ELISA plate showing the specificity of the ELISA assay developed in this study

Adjacent wells in the plate were coated with *C. hepaticus* and *C. jejuni* TPE to demonstrate cross-reactivity of chicken sera.
H = *C. hepaticus* coating on wells in Columns 1, 3, 5, 7, 9 an 11.
J = *C. jejuni* coating on wells in Columns 2, 4, 6, 8, 10 and 12.
The results showed that the cross-reactivity issue of *C. hepaticus* antibodies with their closely related *C. jejuni* and *C. coli* antigens was successfully overcome by pre-absorbing the sera using *C. jejuni* proteins and *C. jejuni* or *C. coli* infection did not compromise the identification of *C. hepaticus* specific antibodies.
Negligibly low signals were obtained for negative samples on both *C. jejuni* and *C. hepaticus* coated wells.

4.1.2. Optimisation of ELISA parameters

A series of experiments was performed to define the ELISA assay conditions that gave the best resolution between positive and negative samples. The final conditions selected for standardisation of the assay were: volume of OD_{280} 1 *C. jejuni* TPE to be used for pre-absorption per reaction = 3 µL (Figure 3A); antigen coating dilution = 1 in 100 (Figure 3B), primary antibody (chicken sera) dilution = 1 in 500 (Figure 3C), secondary antibody dilution = 1 in 2000 (Figure 3D).

4.1.3. ELISA is not affected by the presence of *C. jejuni* or *C. coli* antibodies

Assay specificity and reliability was further demonstrated by testing the sera of the six *C. hepaticus* negative control birds (-A24, -A28, -A34, -A58, -A71 and -A97, Figure 4) which were shown to carry *C. jejuni* or *C. coli* in the birds' fecal samples (by multiplex PCR (Van et al. 2018)). Also, ELISA using *C. jejuni* as an antigen showed positive signals, demonstrating that these birds raised antibody responses against *C. jejuni* (Figure 4, Columns 6 and 10). When ELISA were carried out using *C. hepaticus* as antigen, these sera showed negative signals, demonstrating that *C. jejuni* or *C. coli* infection did not compromise the identification of *C. hepaticus* specific antibodies (Figure 4, Columns 7 and 11).

4.1.4. Validation of ELISA

Forty-one SLD-positive (9 from formalin-killed inoculated *C. hepaticus*, 10 were naturally infected, and 22 were inoculated with live *C. hepaticus*) and 21 SLD-negative samples were tested in the ELISA validation assay.

Therefore, ELISA based on whole cell lysate was successfully developed. The cross-reactivity between antibodies to other campylobacters and the *C. hepaticus* antigen preparation was reduced by

pre-absorbing the sera against *C. jejuni* TPE, prior to use in the ELISA. This successfully removed most of the cross-reacting antibodies from sera. The pre-absorption of anti-*Campylobacter* sera has been previously reported in which *in vitro* grown cultures of *C. jejuni* were used to pre-absorb patient sera and *in vivo* induced antigens of *C. jejuni* were identified by a 2D-gel immunoblotting method (Hu et al. 2013). The ELISA assay developed using whole cell lysate has high sensitivity (97.6%) and specificity (95.5%), and it was successfully used to detect anti-*C. hepaticus* antibodies in the sera from birds inoculated with the HV10 strain and sera from one field infection.

4.2. Development of an ELISA based on recombinant immunodominant antigens (SLD-ELISA2)

4.2.1. Identification of prospective immunogenic proteins by bioinformatics analysis

The RAST server predicted 1521 protein encoding genes (PEGs) in the *C. hepaticus* HV10^T genome, of which 167 were predicted to contain signal peptides, and 85 were predicted as non-classically secreted proteins. Among those 252 proteins, 142 proteins that shared less than 85% amino acid identity with *C. jejuni* and *C. coli* homologs were shortlisted for further analysis. From 142 candidates, 19 proteins that had high IEDB predicted antigenicity scores, predicted extracellular or outer membrane location, and significant amino acid differences of the *C. hepaticus* sequence compared to *C. jejuni* and *C. coli* sequences within the epitope regions predicted using IEDB server were shortlisted for cloning and expression studies. Figure 5 summarises the strategy used for shortlisting 19 prospective candidates for expression, cloning, and immunological studies.

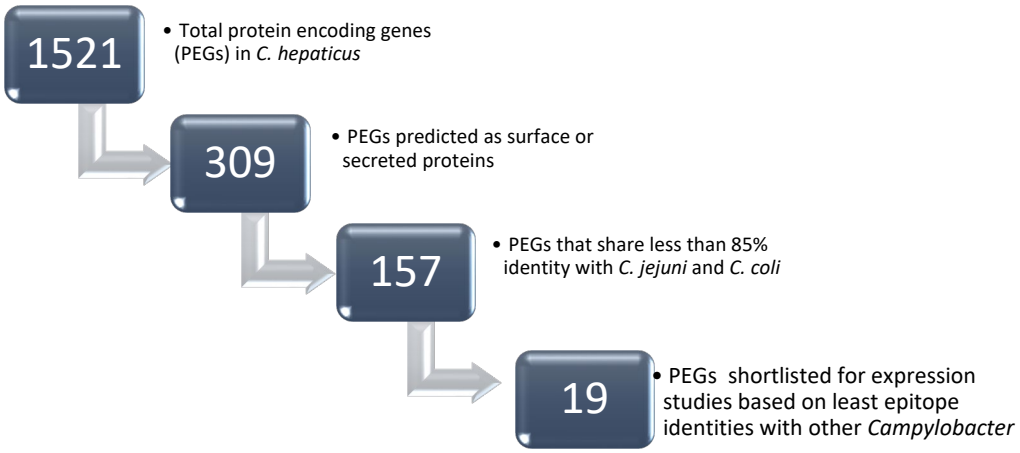

Figure 5 Progression of shortlisting protein encoding genes for expression studies

4.2.2. Identification of *C. hepaticus* specific immunogenic proteins by western blotting and LC-MS/MS protein profiling

Western blot of *C. hepaticus*, *C. jejuni,* and *C. coli* total proteins probed with pooled SLD positive sera showed bands at 220 kDa and 25 kDa that were only present in *C. hepaticus,* as shown in Figure 6. Western blotting using pre-absorbed SLD positive pooled sera also retained the strong band at 220 kDa. Therefore, the 220 kDa and 25 kDa bands were analysed using LC MS/MS to determine the identity of the immunogenic proteins present within the bands.

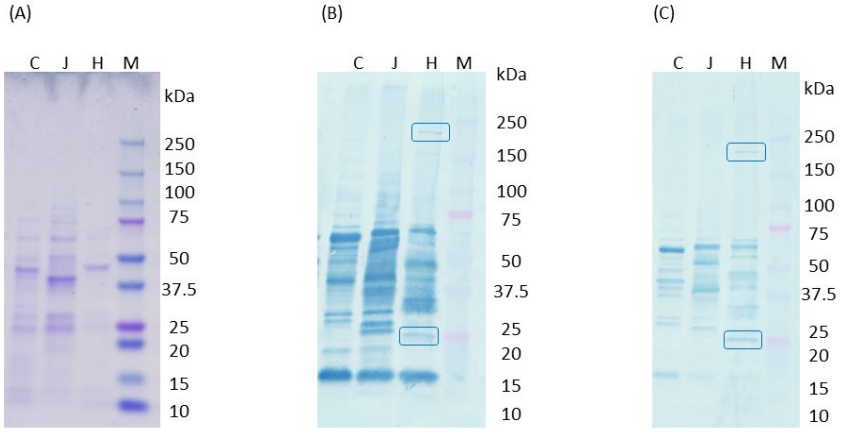

Figure 6 Western blot of *C. hepaticus*, *C. jejuni,* and *C. coli* total proteins probed with pooled SLD positive sera

(A) Coomassie stained gel.
(B) PVDF membrane probed with SLD positive pooled sera that showed specific bands at 220 kDa and 25 kDa regions.
(C) PVDF membrane probed with preabsorbed SLD positive pooled sera that still showed the specific bands at 220 kDa and 25 kDa.

Lane M – Precision plus protein ladder (Bio-Rad).
Total protein profiles of *C. hepaticus* - H, *C. jejuni* - J and *C. coli* - C.

As expected, a mixture of proteins was identified within each band. The top band (220 kDa) matched well with a protein annotated as filamentous hemagglutinin adhesin (FHA, PEG 1316). It is the largest protein encoded in the *C. hepaticus* genome and is composed of 1899 amino acids. The proteins in the successive rankings were RNA polymerase beta subunit proteins and cytoplasmic proteins, which also matched to *C. jejuni* proteins in the SwissProt database search. Hence, they were not considered likely to be immunogenic proteins. Moreover, no bands were observed around the 220 kDa region in *C. jejuni* or *C. coli* protein lanes on western blots. The band at 25 kDa produced major outer membrane protein (MOMP, PEG 140) as the top match. Lower ranked proteins were cytoplasmic and non-immunogenic proteins. The top ranked proteins that matched with the two *C. hepaticus* specific bands at 220 kDa and 25 kDa, encoded by PEGs 1316 and 140 were also identified as potential specific antigen candidates using bioinformatics analysis.

4.2.3. Determination of immunogenicity of shortlisted proteins by cloning, expression, and western blotting

Seventeen out of the 19 cloned genes/gene fragments were successfully expressed in *E. coli*. Pooled SLD positive sera was used in western blotting to determine the immunogenicity of the 17 expressed recombinant proteins. The recombinant proteins encoded by PEGs 407, 433, 486 and 1316 were recognised by the antibodies in pooled sera. The four proteins showed distinct bands of the expected size on western blots probed using pooled SLD positive sera, Coomassie staining and anti-his western blotting, and each was absent in the uninduced *E. coli* cell lysates. However, when probed using individual SLD positive and SLD negative sera, only the recombinant protein encoded by PEG 1316, a fragment of FHA, showed *C. hepaticus* specific immunogenicity (Figure 7). Therefore, the recombinant FHA fragment produced by *E. coli* harbouring the PEG 1316 gene fragment was used in the development of SLD-ELISA2. The recombinant FHA fragment was named as $FHA_{1628\text{-}1899}$, to indicate the length and position of amino acid residues within the native FHA protein that were included in the recombinant protein.

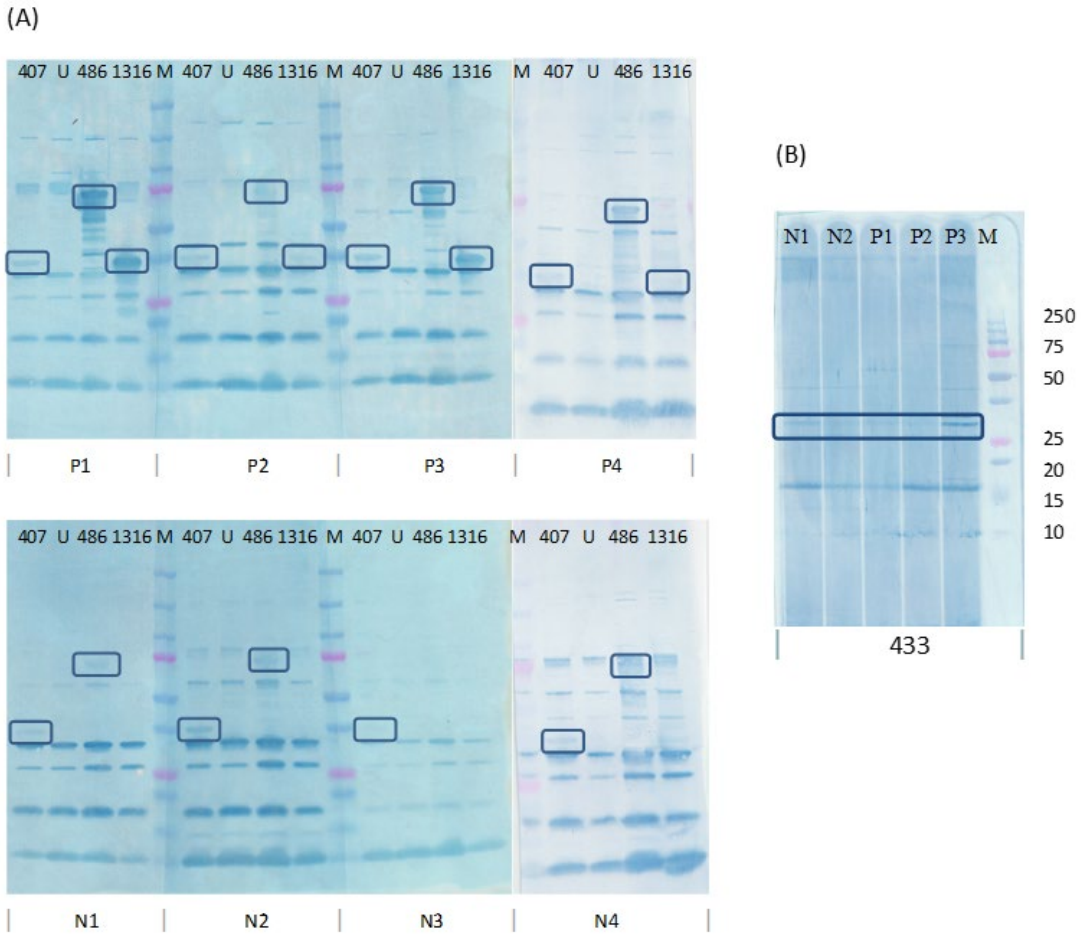

Figure 7 Determination of *C. hepaticus* specific immunogenicity of the recombinant proteins coded by PEGs 407, 433, 486, and 1316 by western blotting using individual SLD positive and negative sera

(A) PVDF membrane blotted with *E. coli* cell lysates (PEGs 407, 486, and 1316) and probed with sera from four SLD positive birds, P1, P2, P3, and P4, and four negative control birds, N1, N2, N3, and N4.

(B) PVDF membrane blotted with *E. coli* cell lysate (PEG 433) and probed with sera from three SLD positive birds, P1, P2, and P3, and two negative control birds, N1, and N2.

Only the recombinant protein encoded by PEG 1316 showed *C. hepaticus* specific immunogenicity as it was recognised by SLD positive sera and not by the negative control sera.

The recombinant proteins encoded by PEGs 433, 407, and 486 cross-reacted with antibodies in several SLD negative sera.

M – Precision plus protein ladder (Bio-Rad).

4.2.4. Development of SLD-ELISA2 using purified recombinant $FHA_{1628-1899}$

The sera samples used to develop and test SLD-ELISA2 from naturally and experimentally infected birds were positive in SLD-ELISA1, and sera samples from all negative control birds were negative in SLD-ELISA1. The optimal antigen coating concentration on ELISA plates was identified as 0.5 µg of purified protein/ml. One in a thousand dilution of sera was determined to be optimal for distinguishing SLD positive and SLD negative samples (Figure 8).

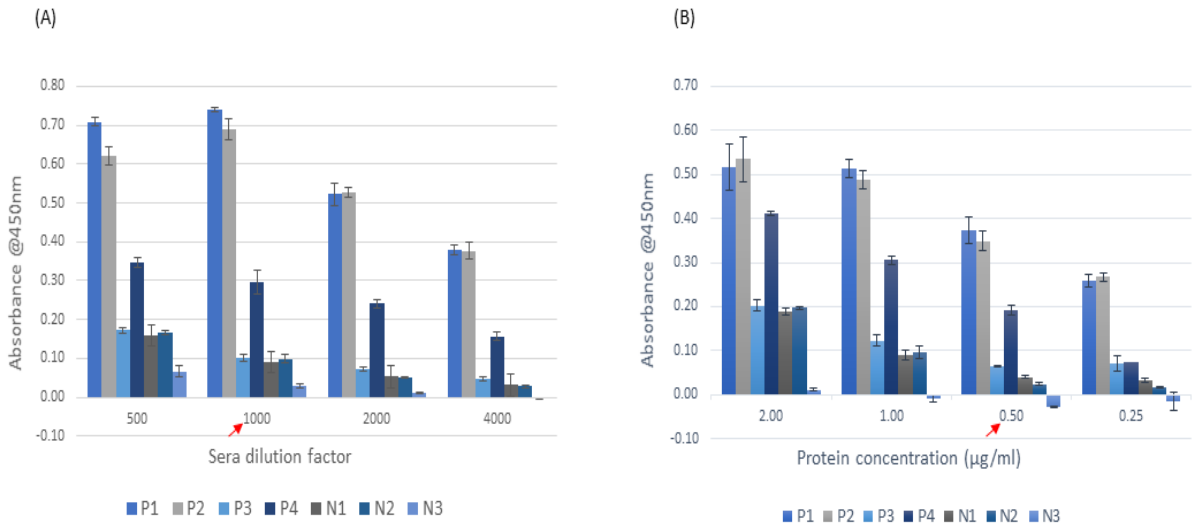

Figure 8 Optimisation of ELISA parameters using four SLD positive (S1, S2, C86, J3) and three SLD negative (A97, B84, B98) sera samples

(A) One in thousand dilution of sera was considered as the optimum to reduce non-specific binding.
(B) 0.5 µg/ml was the desirable concentration to minimise the non-specific binding of SLD negative sera samples.

The cut-off value for the assay was calculated as 0.224 (mean + 2SD of negative control samples). SLD-ELISA2 results were congruent with SLD-ELISA1 in 94% (108/115) of the samples. All 10 sera samples from the naturally infected group, and 44 of 48 samples from the experimentally infected group, had absorbance values well above the cut off value 0.224, resulting in 93.1% assay sensitivity (Figure 9). The assay specificity was 94.73% with the absorbance value of all but three sera samples from the negative control group below the cut-off value. Statistical analysis using one way ANOVA confirmed that the absorbance values of the naturally and experimentally infected groups were significantly different to the negative control group with a p-value less than 0.0001 (Figure 9).

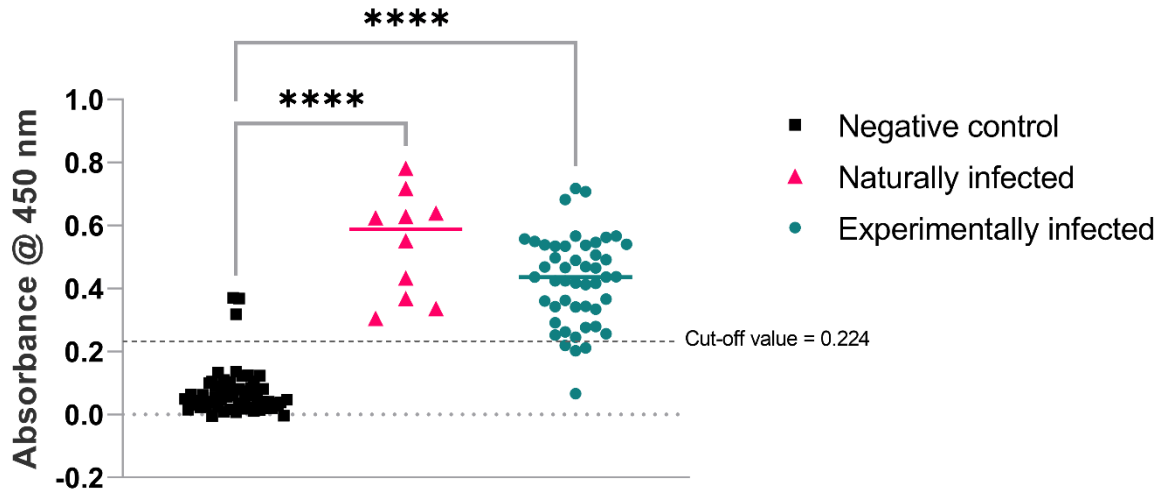

Figure 9 Absorbance produced by anti-FHA antibodies present in the bird sera used in SLD-ELISA2

Ordinary one-way ANOVA pairwise comparison results were highly significant with p-value < 0.0001 for both naturally and experimentally infected groups compared to the negative control group.

In summary, $FHA_{1628-1899}$ was identified as a *C. hepaticus* specific immunogenic protein from a group of 19 proteins that were progressively shortlisted from 1518 *C. hepaticus* PEGs after extensive bioinformatic analysis. The identity of *C. hepaticus* FHA to FHA in other *Campylobacter* species was less than 72%. The identity of the immunogenic fragment identified in this study, $FHA_{1628-1899}$ was even lower (< 64%). This low identity explains the *C. hepaticus* specific immunogenicity of $FHA_{1628-1899}$. The present study is the first report of the use of an FHA from a *Campylobacter* species in immunological assays. Furthermore, $FHA_{1628-1899}$ is 100% conserved among 12 Victorian and South Australian *C. hepaticus* strains and 96% conserved among the two Queensland strains (Van et al. 2019). $FHA_{1628-1899}$ also shared 98.55% identity with a USA *C. hepaticus* strain (Arukha et al. 2021) and 97.80% identity with UK *C. hepaticus* strains (Petrovska et al. 2017). The high similarity of $FHA_{1628-1899}$ among *C. hepaticus* strains isolated from geographically distinct locations suggests the universal application of this assay. The specificity and sensitivity of SLD-ELISA2, 95% and 93% respectively, were very close to that of SLD-ELISA1 for both experimentally and naturally infected samples. SLD-ELISA2, based on the purified recombinant FHA fragment, is more user-friendly and standardisable than SLD-ELISA1 for screening antibody responses to *C. hepaticus* exposure in hens.

4.3 Development of monoclonal-based ELISA to detect circulating antigen (SLD-ELISA3)

4.3.1. Selection of antigens for antibody preparation

FHA-F4 that was previously determined as specific for *C. hepaticus* (and used for coating antigen in ELISA2) was used to immunise the mice to produce monoclonal antibodies that could be used as the detection antibody.

Out of the remaining three FHA fragments, the recombinant FHA-F2 showed immunogenicity on western blots probed using pooled SLD positive sera (Figure 10). PVDF membrane blotted with FHA-F2 expressed *E. coli* cell lysate recognised antibodies in four out of five sera collected from SLD positive birds. However, it also cross-reacted with the antibodies present in two out of five sera collected from negative control birds (Figure 11). Therefore, FHA-F2 and F4 were chosen to immunise the rabbits for polyclonal antibody production.

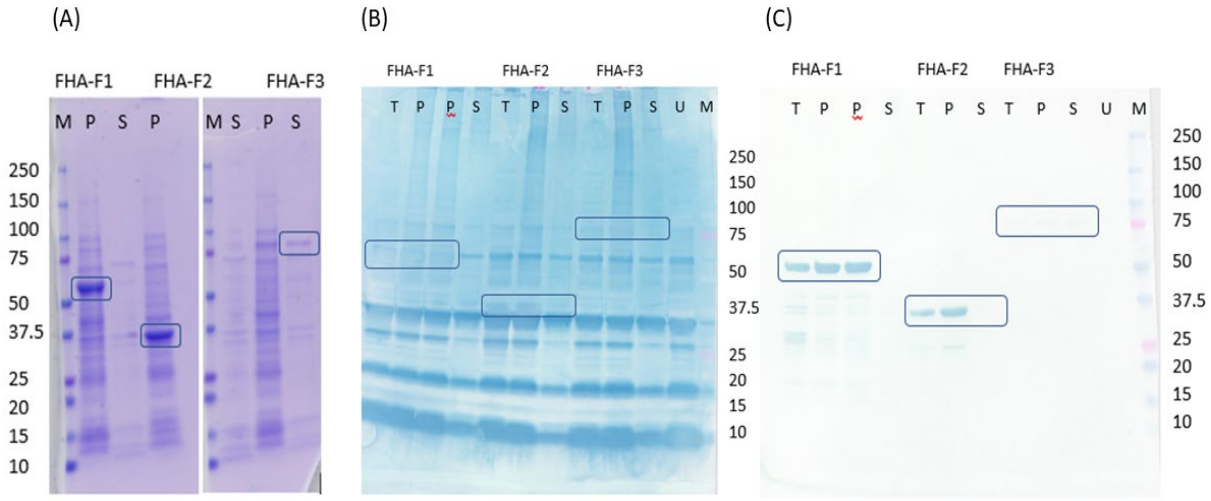

Figure 10 Determination of the expression and immunogenicity of recombinant FHA fragments F1, F2, and F3

(A) Coomassie stained gel of the expressed *E. coli* cell lysates that showed recombinant proteins of expected sizes in blue boxes.
(B) PVDF membrane blotted with expressed *E. coli* cell lysates and probed using pooled SLD positive sera recognised FHA-F2 only.
(C) Anti-his tag western blotting that showed recombinant proteins of expected sizes in blue boxes.
T – Total proteins after sonication of the expressed *E. coli* cells.
P – Pellet obtained after sonication and centrifugation.
S – Supernatant obtained after sonication and centrifugation.
U – Uninduced *E. coli* cell lysates.
M – Precision plus protein marker.

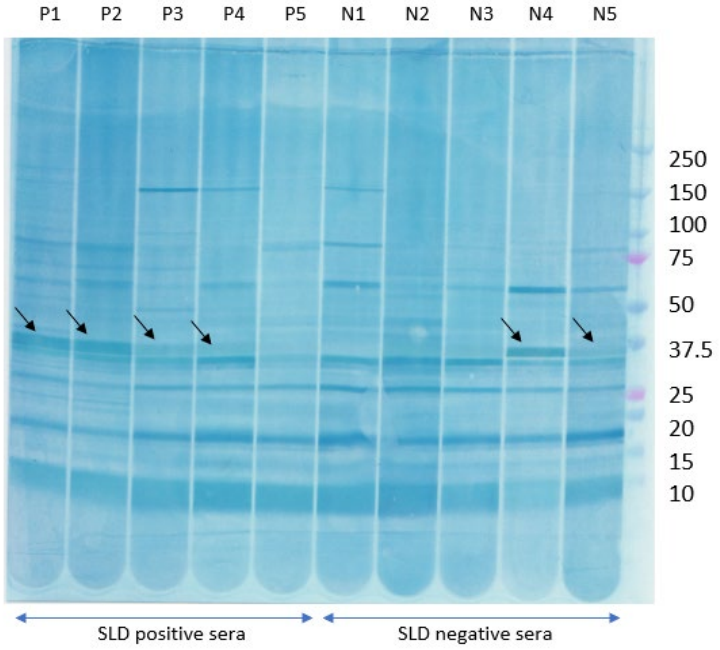

Figure 11 Determination of *C. hepaticus* specific immunogenicity of recombinant FHA-F2

PVDF membrane blotted with FHA-F2 expressed *E. coli* cell lysate and probed with individual SLD positive and negative sera recognised antibodies in four out of five sera from SLD positive birds and two out of five sera from negative control birds.
P1 – P5 are sera collected from positive control birds.
N1 – N5 are sera collected from negative control birds.
The black arrows represent the recombinant FHA-F2 recognised by the antibodies present in bird sera.

4.3.2 Validation of antibodies from mice immunised for monoclonal antibody production

The sera from five BALB/c mice immunised with FHA-F4 only recognised the recombinant FHA-F4 protein. None of them recognised the native FHA protein. Extensive analysis was undertaken to try to understand why none of the sera raised to FHA-F4 could recognise the native FHA, but no clear explanation could be found. Therefore, the monoclonal antibody production was not progressed to hybridoma production stage. Instead, both recombinant proteins (FHA-F2 and FHA-F4) were used as pool for polyclonal antibody production. Anti-FHA-F2F4 polyclonal antibody recognised both FHA-F2 and FHA-F4 recombinant proteins and native FHA from *C. hepaticus* as demonstrated in Figure 12. The antibodies did not cross-react with *C. jejuni*, *C. coli,* or *E. coli* proteins. Anti-FHA-F2F4 polyclonal antibody therefore was used for both capture and detection antibody for ELISA3.

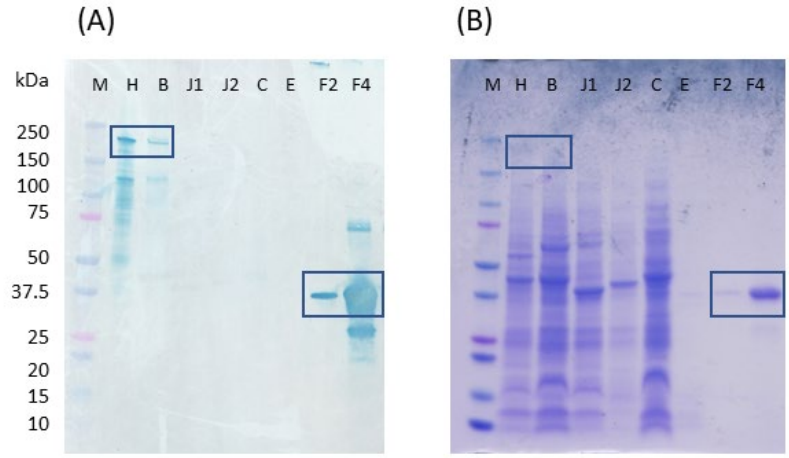

Figure 12 Validation of anti-FHA-F2F4 polyclonal antibodies

(A) PVDF membrane blotted with whole cell lysates and probed using anti-FHA-F2F4 polyclonal antibodies. The antibodies recognised native FHA in *C. hepaticus* and *C. bilis* cell lysates and recombinant FHA-F2 and FHA-F4, highlighted in blue boxes.

(B) Coomassie stained gel indicating bands at expected sizes for native FHA and recombinant FHA fragment.

The blue boxes show the native FHA in *C. hepaticus* and *C. bilis* cell lysates and recombinant FHA-F2 and FHA-F4.

H – *C. hepaticus.*

J1 & J2 – Two different strains of *C. jejuni.*

C – *C. coli.*

E – *E. coli.*

B – *C. bilis.*

F2 – FHA-F2.

F4 – FHA-F4.

M – Precision plus protein ladder (Bio-Rad).

4.3.3. Development and optimisation of ELISA using polyclonal antibodies, SLD-ELISA3

The optimal concentration of unconjugated anti-FHA-F2F4 polyclonal antibody used for coating was determined to be 16 µg/ml. The limit of detection (LOD) of *C. hepaticus* cells was identified as 0.6×10^7 cfu/ml given the OD 1 culture of *C. hepaticus* corresponds to 0.6×10^8 cfu/ml) and the LOD of total protein extracts from *C. hepaticus* was determined to be 1 µg/ml. (Figure 13).

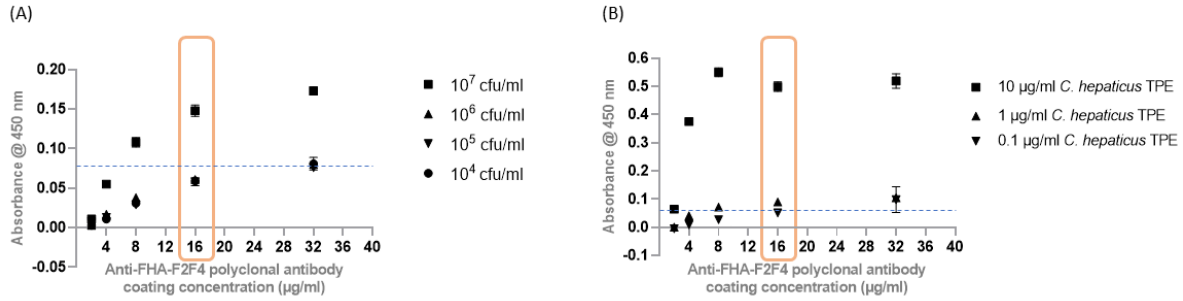

Figure 13 Determination of coating antibody concentration, and limit of detection of native *C. hepaticus* FHA

Determination of coating antibody concentration and LOD of *C. hepaticus* whole cells (A) and *C. hepaticus* TPE (B).

4.3.4. Determination of the cross-reactivity of Ab with *C. jejuni* and *C. coli* proteins

Similar to the observations in western blotting, *C. jejuni* and *C. coli* proteins did not cross-react with the polyclonal antibody pair used in SLD-ELISA3 (Figure 14). The assay cut-off value was determined to be 0.067 calculated as the mean + 2 SD of the absorbance of *C. jejuni* and *C. coli* whole cells (10^7 cfu/ml) and 10 µg/ml *C. jejuni* and *C. coli* TPEs. The absorbance from 10^8 *C. hepaticus* whole cells was 20 times higher than the absorbance from the corresponding *C. jejuni*, and *C. coli* cells. The absorbance of 10 µg/ml *C. hepaticus* TPEs was 10 times higher than the absorbance from the corresponding *C. jejuni*, and *C. coli* TPEs.

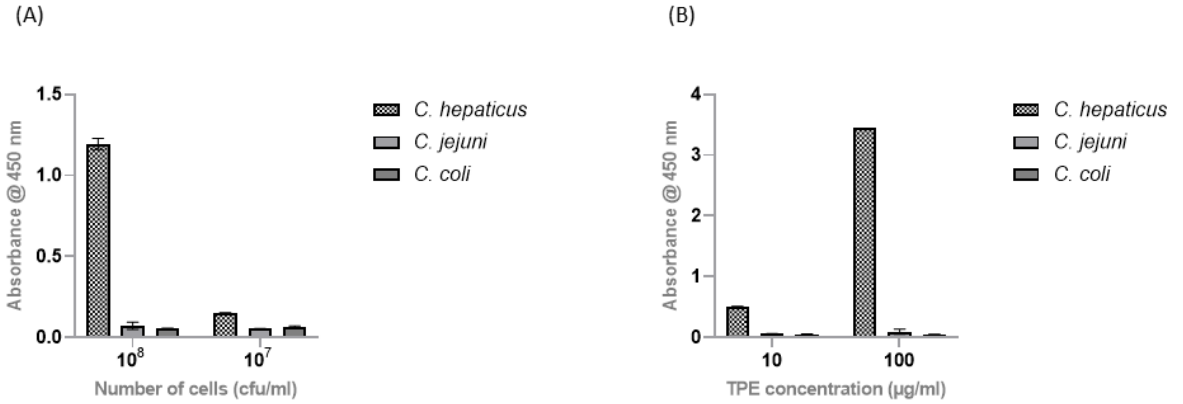

Figure 14 Determination cross-reactivity with *C. jejuni*, and *C. coli* proteins

Whole cells (A) and total protein extracts, TPE (B).

4.3.5. Assay validation using bile and liver samples

One of the five *C. hepaticus* culture positive bile samples tested positive with SLD-ELISA3 (20% assay sensitivity). SLD-ELISA3 did not distinguish liver extracts from *C. hepaticus* positive and negative birds prepared by tissue homogenisation using bead beating or sonication (Figure 15). This indicated the limitation of SLD-ELISA3 to test liver samples. Previous studies using PCR and microscopy have indicated that generally there are only low numbers of *C hepaticus* cells in liver tissues for SLD affected birds.

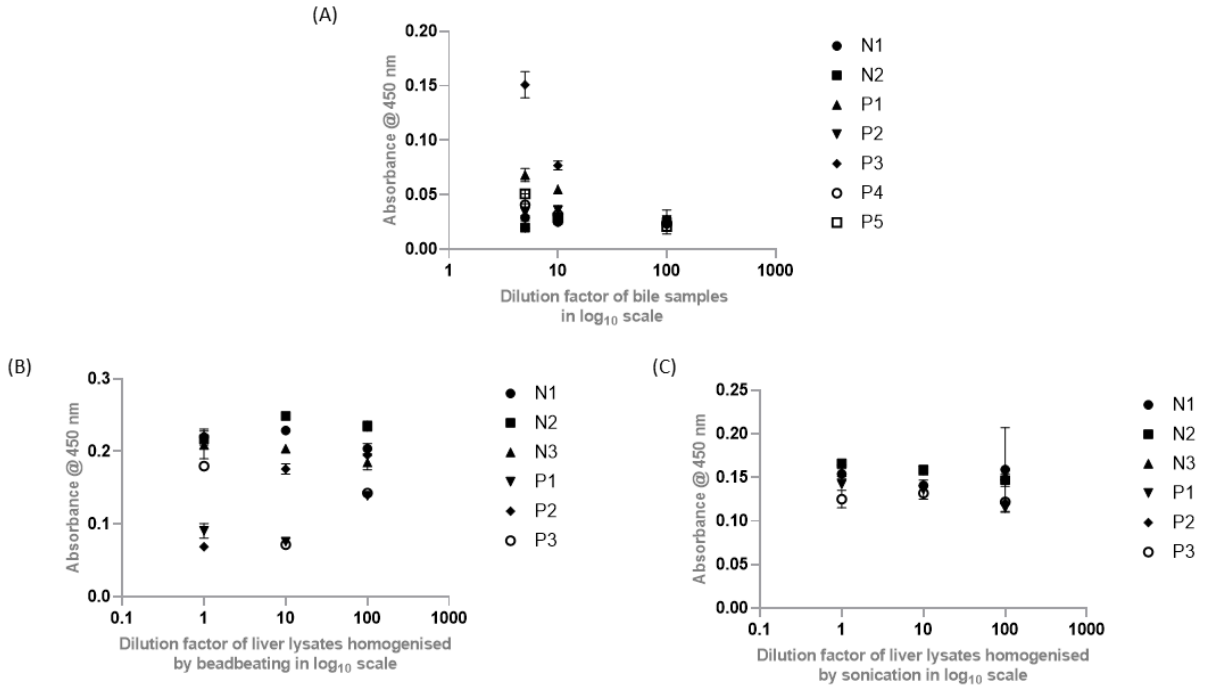

Figure 15 Validation of SLD-ELISA3 using liver and bile samples

(A) Absorbance of bile samples from two negative control birds (N1 and N2) and five experimentally infected birds (P1 – P5). One of the five bile samples from experimentally infected birds, P3 had detectable levels of FHA protein.
Absorbance of liver homogenates of from three negative control birds (N1 – N3) and three experimentally infected birds. (P1 – P3) prepared by bead beating (B) and sonication (C).
All liver samples had absorbance values higher than the cut-off value.

In summary, a sandwich ELISA (SLD-ELISA3) was developed to detect the localisation of *C. hepaticus* inside bird tissues. The limit of detection (LOD) of *C. hepaticus* whole cells was determined to be 0.6×10^7 (6×10^6 cfu/ml) and the LOD of total protein extracts from *C. hepaticus* was determined to be 1 µg/ml. The assay sensitivity is only 20% when *C. hepaticus* culture positive bile samples were used. SLD-ELISA3 did not distinguish liver extracts from *C. hepaticus* positive and negative birds. This is likely because the level of *C. hepaticus* in the tissue is lower than the detection limit. Therefore, this assay may not be useful to detect *C. hepaticus* in tissue samples, but the polyclonal antibody produced in this study can be used to develop an immunohistochemistry assay.

Implications

The novel ELISAs developed in this study will be a valuable diagnostic tool to monitor the immune status of flocks that are susceptible to SLD. It will not only assist in screening birds in the field for *C. hepaticus* exposure but will also equip researchers with a rapid detection method for anti-*C. hepaticus* immune responses during vaccine trials, and be a powerful tool for use in epidemiological studies. SLD-ELISA2, based on *C. hepaticus* protein filamentous hemagglutinin adhesin, is a more convenient option than SLD-ELISA1, especially when working with large sample sizes as in the identification of the seroconverted birds in farms or in assessing the immune response of birds in large scale experimental infection studies.

Recommendations

With immunological tools now available to detect *C. hepaticus* antibodies in chickens (SLD-ELISA1 and SLD-ELISA2), flocks can be investigated for past and present *C. hepaticus* infection for appropriate biosecurity measures to minimise the impact of SLD.

C. bilis has been recently confirmed also to cause SLD, therefore the effectiveness of SLD-ELISA1 and SLD-ELISA2 in detecting *C. bilis* antibodies in *C. bilis* infected birds needs to be determined.

The polyclonal antibody raised against FHA F2 and F4 in this study could be used to develop an immunohistochemistry assay as it specifically recognises *C. hepaticus*. Such an assay is necessary to study the trafficking of *C. hepaticus* in the chicken body during early infection and progression to disease. Understanding the movement of *C. hepaticus* within chickens is likely to be important for identifying and developing appropriate vaccine targets.

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Media and Publications

Muralidharan C, Anwar A, Wilson TB, Scott PC, Moore RJ & Van TTH. (2020). Development of an enzyme-linked immunosorbent assay for detecting *Campylobacter hepaticus* specific antibodies in chicken sera - a key tool in Spotty Liver Disease screening and vaccine development. *Avian Pathology*, 49:658-665.

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Muralidharan C, Huang J, Anwar A, Scott PC, Moore RJ & Van TTH. (2022). Prevalence of *Campylobacter hepaticus* specific antibodies among commercial free-range layers in Australia. *Frontiers in Veterinary Science* (under peer review).

Intellectual Property Arising

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