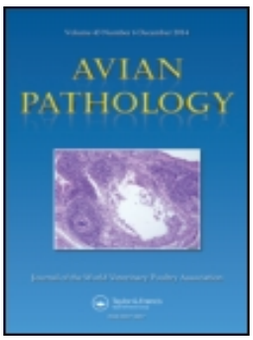

Avian Pathology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/cavp20

# 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

**Chithralekha Muralidharan , Arif Anwar , Timothy B. Wilson , Peter C. Scott , Robert J. Moore & Thi Thu Hao Van**

**To cite this article:** Chithralekha Muralidharan , Arif Anwar , Timothy B. Wilson , Peter C. Scott , Robert J. Moore & Thi Thu Hao Van (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, DOI: 10.1080/03079457.2020.1813252

**To link to this article:** https://doi.org/10.1080/03079457.2020.1813252



Published online: 23 Sep 2020.

AVIAN PATHOLOGY
https://doi.org/10.1080/03079457.2020.1813252

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

OPEN ACCESS Check for updates

# 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

Chithralekha Muralidharan[a], Arif Anwar[b], Timothy B. Wilson[b], Peter C. Scott[b], Robert J. Moore [a] and Thi Thu Hao Van[a]

[a]School of Science, RMIT University, Bundoora, Australia; [b]Scolexia Pty Ltd., Moonee Ponds, Australia

**ABSTRACT**

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. The causative agent is a newly identified Gram-negative bacterium, *Campylobacter hepaticus,* and knowledge about *C. hepaticus* pathogenesis and the potential for vaccine development is still in its infancy. 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 in vaccine development against SLD. In the present study, we have developed the first immunoassay capable of detecting *C. hepaticus*-specific antibodies present in the sera of infected birds. The assay uses *C. hepaticus* total protein extract (TPE) as the antigen coating on enzyme-linked immunosorbent assay (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 serum samples from SLD-negative birds (control group). The optimized ELISA assay had 95.5% specificity and 97.6% sensitivity. The immunoassay provides a useful tool for monitoring the exposure of poultry flocks to *C. hepaticus* infection and can be used to direct and support vaccine development.

**HIGHLIGHTS**

- The first immunoassay developed for Spotty Liver Disease (SLD).
- A useful method for detecting *C. hepaticus*-specific antibodies in birds.
- Highly specific (95.5%) and sensitive (97.6%) assay.
- A key tool for use in epidemiological studies and vaccine development.

**ARTICLE HISTORY**
Received 31 March 2020
Accepted 17 August 2020

**KEYWORDS**
*Campylobacter hepaticus*; Spotty Liver Disease; ELISA; sera; immunoassay; pre-absorption; chicken

## Introduction

Spotty Liver Disease (SLD) is a condition of egg-layers often associated with a decline in egg production, egg quality, and sometimes sudden death. Affected birds typically have numerous white, cream, or grey spots on the surface of the liver, representing gross necrotic lesions (Burch, 2005; Grimes & Reece, 2011; Crawshaw & Irvine, 2012; Scott, 2016). Although the disease was first reported during the 1950s in the USA (Tudor, 1954), the causative organism was only recently identified as a novel campylobacter species, *Campylobacter hepaticus* (Van *et al.*, 2016). SLD outbreaks have occurred frequently in Australia since the mid-1980s, and the incidence of disease has increased in recent years, coinciding with the consumer driven shift towards free-range egg farming (Grimes & Reece, 2011). SLD has recently been reported from the USA and the UK, indicating its potential to become an international problem within the global egg industry (Crawshaw & Irvine, 2012; Crawshaw *et al.*, 2015; Gregory *et al.*, 2018).

The methods available to date to detect *C. hepaticus* from SLD cases have been limited to culture and DNA-based approaches (Van, Gor, *et al.*, 2017). Culturing the bacterium from microbiologically complex samples, such as faeces or environmental samples, is a tedious and time-consuming task as it takes 3–7 days to grow on culture plates and culture can be challenging as a selective medium for *C. hepaticus* isolation is yet to be developed (Phung *et al.*, 2020). When grown on nutrient agar or Brucella agar supplemented with blood, other campylobacters routinely present in

**CONTACT** Thi Thu Hao Van thithuhao.van@rmit.edu.au School of Science, RMIT University, PO Box 71, Bundoora, Victoria 3083, Australia

samples, like *Campylobacter coli*, *Campylobacter jejuni* and many other bacteria, out-grow the plate and make it difficult to identify *C. hepaticus*. Therefore, samples of bile and liver, in which *C. hepaticus* is often the only bacterium present, are the samples of choice for culture confirmation of *C. hepaticus* involvement in SLD. The culturing method is only useful if the pathogen is alive in the sample provided for detection.

A qPCR assay and an end-point PCR assay using primers targeting the glycerol kinase gene, one of the unique genes present in *C. hepaticus,* have been successfully used to detect *C. hepaticus* from liver, gut, bile, faeces and environmental samples (Van, Gor, *et al.*, 2017; Phung *et al.*, 2020). A multiplex PCR assay employing primers for the *C. jejuni*-specific hippuricase (*hipO*) gene and the *C. coli*-specific serine hydroxymethyltransferase (*glyA*) gene, along with *C. hepaticus*-specific glycerol kinase gene primers, has also been developed as a diagnostic tool to simultaneously detect and differentiate these three pathogens in poultry and environmental samples (Van *et al.*, 2018).

Culturing or DNA-based approaches will not give any indication of the exposure to *C. hepaticus* in healthy or recovered birds that have cleared the bacteria. Serological methods of detection are potentially capable of detecting *C. hepaticus*-specific antibodies present in chicken serum produced by a previous SLD infection or vaccination. The cross reactivity of *C. hepaticus* antigens with antibodies to other common campylobacters was anticipated to be one of the major challenges in developing a species-specific assay. Nearly 70% of the genes in *C. hepaticus* are conserved in *C. jejuni* and *C. coli* strains (Van *et al.*, 2019). It is not uncommon to find *C. jejuni* and *C. coli* in flocks since most chickens get exposed to them early in their life and a majority harbour them as commensal bacteria in the intestine and caecum (Lindblom *et al.*, 1986; Van *et al.*, 2018). Hence, the serological assay needs to differentiate between the presence of antibodies against these closely related species and antibodies to *C. hepaticus*.

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. A serological diagnostic method is therefore pivotal in determining the feasibility of developing a vaccine against SLD.

## Materials and methods

### 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 plates (Brucella broth, Oxoid, Altrincham, UK) supplemented with 1.5% agar (Oxoid, UK) and 5% horse blood (Equicell, Bayles, VIC, Australia) under microaerophilic conditions using Campygen packs (Oxoid, UK). The bacterial cells from 2–3 culture plates were harvested in 500 µl phosphate buffer saline (PBS) and stored at −20°C for 2 h or overnight to enhance the cell disruption efficiency. The cell suspension was thawed on ice and sonicated (Branson 250 Digital Sonifier connected with microtip probe, Branson Ultrasonic Corporation, Danbury, CT, USA) for six (15 s on, 30 s off) cycles, to obtain the whole cell lysate (WCL). WCL was centrifuged at 8000× *g* for 10 min to collect the supernatant which was named the TPE. The optical density ($OD_{280}$) of the TPE solution was adjusted to 1 by addition of PBS. The $OD_{280}$ was measured using a Nanodrop one instrument (Thermo Fisher Scientific, Waltham, MA, USA). The standardized TPE solution was used as the antigen to coat wells for the enzyme-linked immunosorbent assays (ELISA).

### Collection of chicken sera

A total of 63 serum samples (nine birds intramuscularly injected with formalin-killed *C. hepaticus* HV10 cells to induce immune responses, five birds with no history of SLD (negative controls), 10 SLD-positive cases from a farm in Victoria in 2018, and 39 birds from an infection/challenge trial using live *C. hepaticus* HV10 (22 challenged and 17 unchallenged negative controls)) were included in the study. Therefore, there were 41 SLD-positive samples (nine from formalin-killed, 10 naturally infected and 22 challenged birds) and 22 SLD-negative samples (five with no history of SLD and 17 negative control used in an infection/challenge trial) included in the study. The disease was induced as described by Van, Elshagmani, *et al.* (2017). SLD-positive and negative control birds were confirmed through PCR and/or visible signs in autopsy. The whole blood samples were collected aseptically using 21-gauge needles and 10 ml syringes and were transferred to the laboratory in 15 ml centrifuge tubes. Serum was obtained as the supernatant by spinning the sample tubes at 2000× *g* for 15 min. The bird 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).

### Enzyme-linked immunosorbent assay (ELISA)

Nunc MaxiSorp™ high protein-binding capacity 96 well ELISA plates (Thermo Fisher Scientific) were used for the assay. Briefly, 50 µl of antigen solution

(*C. hepaticus* TPE in PBS, pH 7.2) was added to each well and incubated for 2 h at room temperature or overnight at 4°C. The antigen solution was poured off and wells were washed using 200 µl PBS with 0.05% tween 20 (PBST). The non-specific binding sites were then blocked by adding 200 µl of blocking solution (5% skim milk powder in PBS) and incubated for 2 h at room temperature or overnight at 4°C. One hundred microlitres of primary antibody solution (chicken sera diluted in blocking solution) were added to each well and incubated for 2 h at room temperature or overnight at 4°C. The sera were used either with or without pre-absorption with *C. jejuni* proteins, as described below in the reduction in cross reactivity by pre-absorption section. The wells were washed four times using 200 µl PBST and probed with 100 µl secondary antibody, goat anti-chicken Ig-Y-HRP (Thermo Fisher Scientific) diluted in blocking solution, for 2 h, followed by four washing steps using 200 µl PBS. Finally, 50 µl of Novex 3,3′,5,5′- tetramethylbenzidine chromogenic substrate (Invitrogen, Waltham, MA, USA) was added to each well. The plates were sealed using clear sealing tape (Thermo Fisher Scientific) during all incubation steps. The absorbance was measured after 20–30 min at 652 nm using a microplate reader (POLARstar Omega Plate Reader Spectrophotometer, BMG LABTECH, Ortenberg, BW, Germany).

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

Serum samples from nine birds inoculated with formalin-killed *C. hepaticus* HV10 to induce immune responses, five negative controls, and 10 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. All 63 samples included in this study were also assayed on 0.01 OD TPE *C. hepaticus* coated plates with dilutions of sera and secondary antibody used above.

### *Reduction in cross reactivity by pre-absorption*

The primary antibody/serum 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 serum samples. An experimentally determined volume of *C. jejuni* TPE ($OD_{280}$ 1) was added to the primary antibody/sera solution and incubated for 45 min at room temperature. The solution was then centrifuged at 3700× *g* for 10 min and the supernatant was used as the pre-absorbed primary antibody solution to probe the antigen coated wells.

### *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.

### *Optimisation of the ELISA parameters*

Serum samples from nine birds inoculated with formalin-killed *C. hepaticus* cells (SLD-positive) and five birds with no history of SLD (SLD-negative) were used for the optimization of the ELISA protocol. (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. Primary and secondary antibody solutions were prepared in blocking solution (5% skim milk powder in PBS). Primary antibody titre was optimized by testing different dilutions of chicken sera, 1 in 100, 250, 500, 750, 1000, 2000, 4000 and 5000. Five dilutions of secondary antibody, goat anti-chicken Ig-Y-HRP, 1 in 1000, 2000, 4000, 6000 and 8000 were assayed for optimal performance in tested serum samples. All samples were assayed in triplicates and standard deviations were calculated.

### *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 serum samples, as described by Chenard *et al.* (1998). One-way analysis of variance test was performed to determine the statistical significance of the ELISA results.

## Results

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

ositive signals were observed in *C. hepaticus, C. jejuni* and *C. coli* protein coated wells for all samples (Figure 1). Although the absorbance values from *C. hepaticus, C. jejuni* and *C. coli* coated wells were different, they fell within a narrow absorbance range

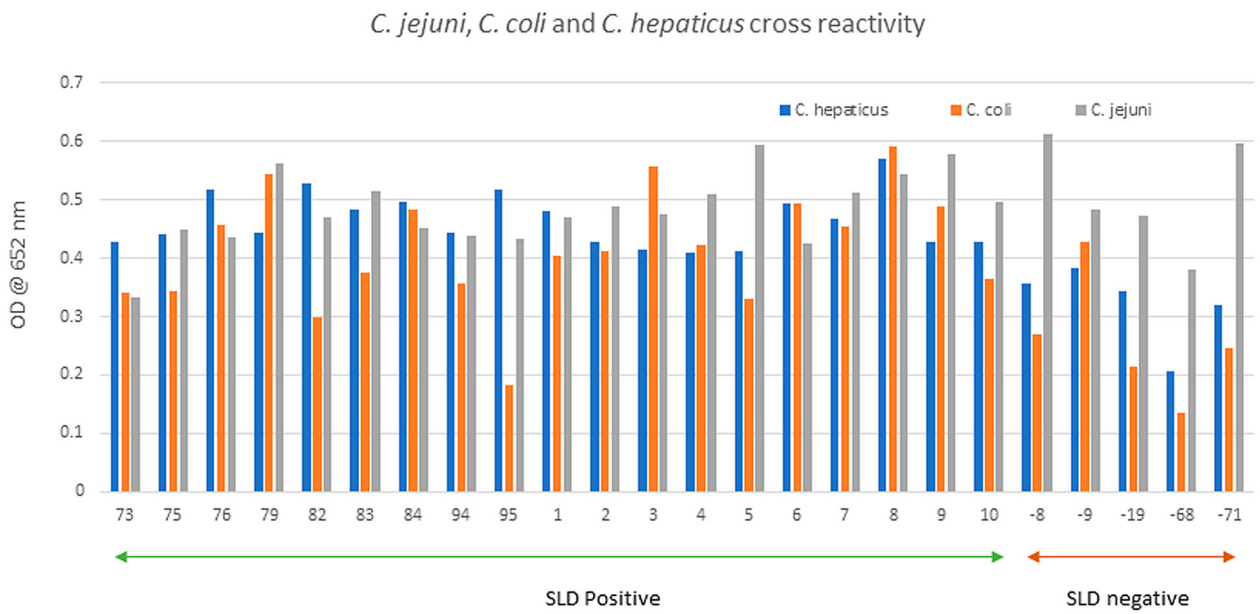


**Figure 1.** *C. jejuni*, *C. coli* and *C. hepaticus* cross reactivity. Samples 73–95 were SLD positive-samples from birds inoculated with formalin-killed *C. hepaticus* cells. Samples 1–10 were sera from naturally-infected birds and −8 to −71 were negative control serum samples.

(0.3–0.5) for most samples. In SLD-negative samples, strong absorbance signals were observed in *C. hepaticus* coated wells and the absorbance readings from *C. jejuni* coated wells were higher than that from *C. hepaticus* coated wells, suggesting cross reactivity of *C. hepaticus* proteins with *C. jejuni* antibodies. Also, when ELISA was performed using all 63 samples included in this study, on *C. hepaticus* coated plates, 39 out of 41 SLD true positives were ELISA positive, but 7 out of 22 negative control samples were also ELISA positive (data not shown). This result further suggested the cross reactivity of *C. jejuni* antibodies with *C. hepaticus* proteins.

### Reduction in cross reactivity by pre-absorption

*C. jejuni* TPE was chosen to pre-absorb the primary antibody solution as the cross reactivity indicated by the ELISA result (Figure 1) showed stronger absorbance signals from *C. jejuni* proteins compared to *C. coli*. Figure 2 illustrates the reduction in cross reactivity of six SLD-negative serum samples (-A24, -A29, -A34, -A58, -A71 and -A97) using pre-absorption. Weak signals were obtained for all negative samples on *C. hepaticus* coated wells (Lanes 5 and 9) using normal sera. The signals were strong on *C. jejuni* coated wells (Lanes 6 and 10). The signals from the same samples were significantly reduced when they were

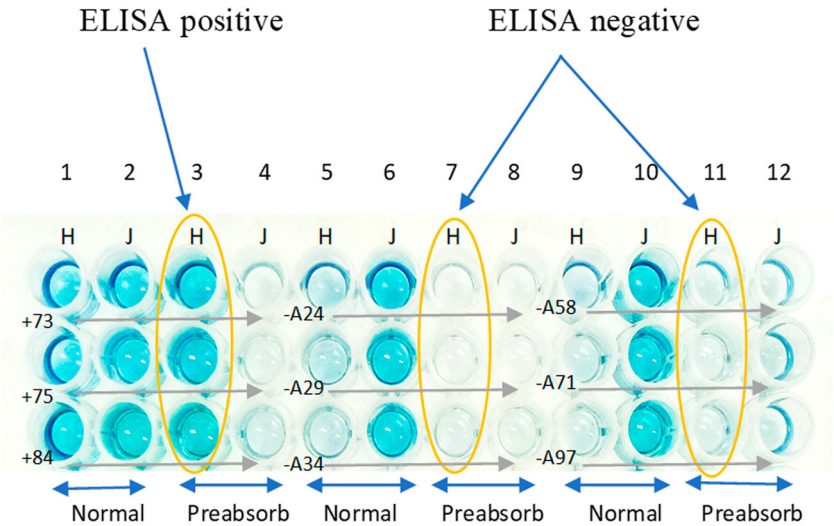


**Figure 2.** Reduction in cross reactivity by pre-absorption. H = *C. hepaticus* coating on wells in columns 1, 3, 5, 7, 9 and 11. J = *C. jejuni* coating on wells in columns 2, 4, 6, 8, 10 and 12. Normal serum was used in columns 1, 2, 5, 6, 9 and 10. Pre-absorbed sera was used in columns 3, 4, 7, 8, 11 and 12.

pre-absorbed with *C. jejuni* TPE and probed on *C. hepaticus* coated wells (Lanes 7 and 11) and *C. jejuni* coated wells (Lanes 8 and 12). The signals from positive samples (+73, +75 and +84) shown in Lane 1 were unaffected by pre-absorbtion (Lane 3). Therefore, the cross reactivity issue of *C. hepaticus* antibodies with their closely related *C. jejuni* antigens was successfully overcome by pre-absorbing the sera using *C. jejuni* proteins.

### *Assessment of the ELISA in the presence of C. jejuni or C. coli antibodies*

The multiplex PCR assay detected *C. jejuni* DNA in five and *C. coli* DNA in three out of six *C. hepaticus*-negative samples tested (SLD-negative samples shown in Figure 2). ELISA using *C. jejuni* TPE as antigen coating showed positive signals, demonstrating that these birds had raised antibody responses against *C. jejuni* (Figure 2, Columns 6 and 10). But when ELISA was carried out using *C. hepaticus* TPE as antigen coating, these sera showed negative signals, demonstrating that *C. jejuni* or *C. coli* infection did not compromise the identification of *C. hepaticus*-specific antibodies (Figure 2, Columns 7 and 11). Negligibly low signals were obtained for pre-absorbed negative samples on both *C. jejuni* and *C. hepaticus* coated wells. Therefore, it can be concluded that the ELISA developed in this study is not affected by the presence of *C. jejuni* or *C. coli* antibodies.

### *Optimisation of ELISA parameters*

(i) Volume of *C. jejuni* TPE for pre-absorption. All tested volumes of *C. jejuni* TPE ($OD_{280}$ 1) significantly reduced the absorbance signals from negative samples (Figure 3(A)). Both 3 µl and 4 µl gave comparable signals for the positive sera. Therefore the lower volume, 3 µl, was chosen as the volume of *C. jejuni* TPE ($OD_{280}$ 1) solution to be used for pre-absorption per reaction or per well; i.e. 3 µl of *C. jejuni* TPE ($OD_{280}$ 1) solution added to 100 µl of chicken serum diluted in blocking

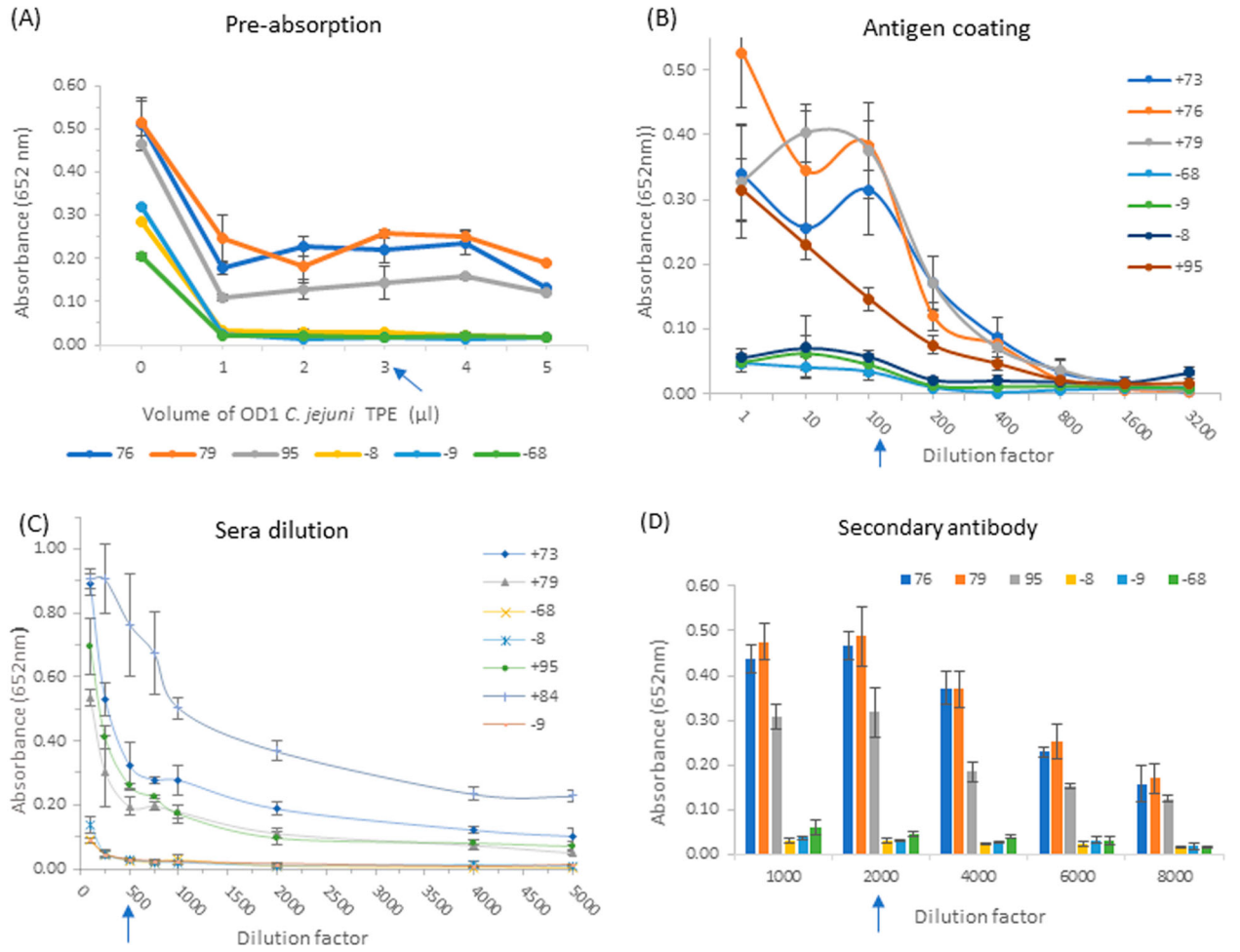


**Figure 3.** Optimisation of ELISA parameters. (A) Assay of volume of TPE to use for serum pre-absorption in three SLD-positive and three SLD-negative serum samples. (B) Assay of effects of antigen coating dilution on ELISA performance in four SLD-positive and three SLD-negative serum samples. (C) Assay of effects of primary antibody (chicken serum) dilution in four SLD-positive and three SLD-negative samples. (D) Assay of secondary antibody dilution in three SLD-positive and three SLD-negative serum samples. The conditions selected for ongoing use in the standardized ELISA are indicated by the blue arrows. Sera used: +73, +76, +79, +95 are SLD positive sera; −8, −9, −68 are SLD negative sera.

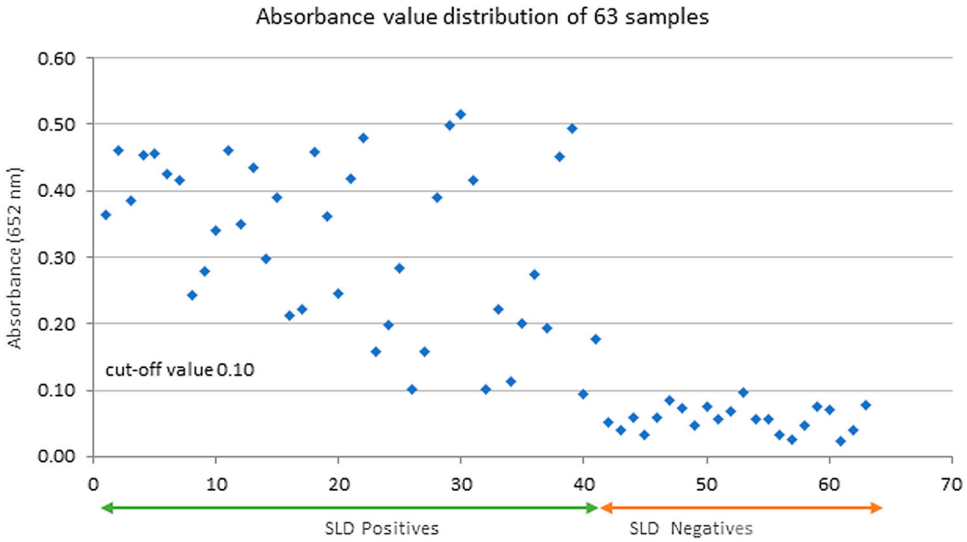


**Figure 4.** Distribution of the absorbance values of 63 samples in ELISA. All 41 positive samples are clustered towards the left, and 22 negative samples on the right.

solution. (ii) Antigen coating. One-hundred-fold dilution of OD1 *C. hepaticus* TPE (i.e. final OD of 0.01) was found to be ideal to effectively differentiate SLD-positive and negative samples (Figure 3(B)). The assay sensitivity was reduced when dilutions above one-hundred-fold were used. (iii) Antibody dilutions. Five-hundred-fold dilution of chicken sera was optimal for obtaining a clear cut-off between SLD-positive and negative serum samples (Figure 3(C)). One in 2000 dilution of secondary antibody was found to be the optimal dilution to obtain reliable absorbance signals without being a limiting factor for SLD-positive samples. The absorbance values of the SLD-positive samples dropped proportionately with further dilutions (Figure 3(D)).

Based on the optimisation of ELISA parameter results, the volume of *C. jejuni* TPE (OD 1) to be used for pre-absorption (3 µl per 100 µl diluted serum solution) and the dilution factors for antigen coating (100), primary antibody (500), and secondary antibody (2000) were applied in the ELISA assay.

### *Evaluation of ELISA performance*

The absorbance value distribution of 63 samples assayed by the ELISA is shown in Figure 4. Out of 22 sera from birds with no known exposure to *C. hepaticus*, and therefore not expected to have anti-*C. hepaticus* antibodies, 21 were ELISA negative, with their absorbance value below the cut-off value of 0.1. Therefore, the assay specificity was calculated to be 95.5%. It should be noted that the one negative serum sample which was scored as a false positive gave high absorbance readings in repeated assays, suggesting the bird may have been exposed to *C. hepaticus* previously.

Of the 41 sera from birds known to have been exposed to *C. hepaticus*, only one was scored as ELISA negative, indicating an assay sensitivity of 97.6%. The assay cut-off value of 0.1 was chosen based on the performance of all the 63 samples with a focus on assay specificity and sensitivity.

The test was proved statistically significant (*P*-value = 0) by performing one-way analysis of variance comparison based on Dunnett method assuming equal variance.

## Discussion

ELISA is a reliable and affordable diagnostic tool for many infectious diseases. There are several ELISA kits available in the market for testing *Campylobacter*-specific IgG/IgA antibodies or antigens in human faecal samples, for example the RIDASCREEN® Campylobacter ELISA kit (Buissonniere *et al.*, 2018). However, these kits are only suitable for detection of antibodies from humans. The assays used for screening *C. jejuni* antibodies from chicken sera, like the one described by Radomska *et al.* (2016), would not be suitable for the specific detection of *C. hepaticus* antibody due to the presence of antibodies to other common campylobacters in the sera, generated against previous or current *C. jejuni* or *C. coli* infections. The PCR results obtained in the present study also support the above statement by confirming the presence of *C. jejuni, C. coli* or both in the caecal samples of some of the *C. hepaticus*-negative birds tested.

Many of the antigenic targets that have been used in serological detection of campylobacters are membrane surface proteins or flagellar proteins, because of their obvious immunogenic nature (Yeh *et al.*, 2013; Yeh *et al.*, 2016). The use of *C. jejuni* outer membrane proteins and formalin-inactivated whole cells as antigens for ELISA and immuno-blotting tests, demonstrated by Islam *et al.* (2014), was successful in detecting *C. jejuni* or *C. coli* infections in humans. However, we initially presumed that the use of WCL will not

work for our assay because of its cross reactivity with these common *Campylobacter* antigens. Other work by Gerstenecker *et al.* (1992) on *H. pylori* detection using an enzyme immunoassay, reported the reduction in assay specificity by using WCL (60.7%) against 92%, obtained using a specific antigenic membrane-associated protein. In the current study, 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. So, one of the major challenges in the assay development was utilized to improve the reliability of the assay.

The pre-absorption of anti-Campylobacter sera has been previously reported by Hu *et al.* (2013), where *in vitro* grown cultures of *C. jejuni* were used for pre-absorbing patient sera to identify *in vivo* induced antigens of *C. jejuni* by a 2D-gel immuno-blotting method.

In the present study, when ELISA was performed using serum without pre-absorption, seven negative samples gave false positive results. This suggested the presence of *C. jejuni* or *C. coli* antibodies in the serum samples, and the presence of *C. jejuni* and/or *C. coli* DNA in the birds from which the sera were derived was confirmed by PCR in a number of cases. When the sera were pre-absorbed with *C. jejuni* proteins, most of the general *Camplylobacter* antibodies were removed leaving primarly *C. hepaticus*-specific antibodies in the primary antibody solution. Thus, all but one of the 22 putative negative samples were below the cut-off value, making the assay specific for *C. hepaticus* when the assay was performed using pre-absorbed sera. Choi *et al.* (2018) also demonstrated an improvement in the specificity of a multiplexed opsonophagocytic killing assay for group B *Streptococcus* by reducing the non-specific killing with the use of pre-absorbed sera. The ELISA assay 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, as described in the results. In another challenge, the *C. hepaticus 44L* strain was used and the assay successfully detected antibodies induced by this strain (data not shown). It has been reported that the genomes of *C. hepaticus* strains are highly similar (Van *et al.* 2019), therefore the expectation is that the immunoassay could reliably test antibody responses produced by other *C. hepaticus* strains.

The novel ELISA developed in this study will be a valuable diagnostic tool to monitor the immune status of flocks that are susceptible to SLD. The ELISA is highly sensitive (97.6%), specific (95.5%) and convenient to use. It will not only assist in screening birds in the field for *C. hepaticus* exposure, but also equips researchers with a rapid detection method for anti-*C. hepaticus* immune responses during vaccine trials and will be a powerful tool for use in epidemiological studies.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

The authors acknowledge the financial support of Poultry Hub Australia that enabled us to carry out this research [PHA Project No. 2019-414].

## ORCID

*Robert J. Moore* http://orcid.org/0000-0002-0776-5861

## References

Buissonniere, A., Benejat, L., Charron, P., Bessede, E., Lehours, P., Valdenaire, G., Richer, O. & Megraud, F. (2018). A new kit to detect *Campylobacter* species in stool specimens: the Orion GenRead Campylobacter®. *European Journal of Clinical Microbiology and Infectious Diseases*, 37, 1585–1587.

Burch, D. (2005). Avian vibrionic hepatitis in laying hens. *The Veterinary Record*, 157, 528.

Chenard, G., Bloemraad, M., Kramps, J.A., Terpstra, C. & Dekker, A. (1998). Validation of a monoclonal antibody-based ELISA to detect antibodies directed against swine vesicular disease virus. *Journal of Virological Methods*, 75, 105–112.

Choi, M.J., Noh, J.Y., Cheong, H.J., Kim, W.J., Lin, S.M., Zhi, Y., Lim, J.H., Lim, S., Seo, H.S. & Song, J.Y. (2018). Development of a multiplexed opsonophagocytic killing assay (MOPA) for group B *Streptococcus*. *Human Vaccines & Immunotherapeutics*, 14, 67–73.

Crawshaw, T.R., Chanter, J.I., Young, S.C., Cawthraw, S., Whatmore, A.M., Koylass, M.S., Vidal, A.B., Salguero, F.J. & Irvine, R.M. (2015). Isolation of a novel thermophilic *Campylobacter* from cases of spotty liver disease in laying hens and experimental reproduction of infection and microscopic pathology. *Veterinary Microbiology*, 179, 315–321.

Crawshaw, T.R. & Irvine, R.M. (2012). Spotty liver syndrome in poultry in Great Britain. *The Veterinary Record*, 170, 317–318.

Gerstenecker, B., Eschweiler, B., Vogele, H., Koch, H.K., Hellerich, U. & Kist, M. (1992). Serodiagnosis of *Helicobacter pylori* infections with an enzyme immunoassay using the chromatographically purified 120 kilodalton protein. *European Journal of Clinical Microbiology & Infectious Diseases*, 11, 595–601.

Gregory, M., Klein, B., Sahin, O. & Girgis, G. (2018). Isolation and characterization of *Campylobacter hepaticus* from layer chickens with Spotty Liver Disease in the United States. *Avian Diseases*, 62, 79–85.

Grimes, T. & Reece, R. (2011). "Spotty Liver disease" – an emerging disease in free range egg layers in Australia. In D. Frame (Ed.), *Proceedings of the sixtieth western poultry disease conference*, Sacramento, CA, 53–56.

Hu, Y., Shang, Y., Huang, J., Wang, Y., Ren, F., Jiao, Y., Pan, Z. & Jiao, X.A. (2013). A novel immunoproteomics method for identifying *in vivo*-induced *Campylobacter*

*jejuni* antigens using pre-adsorbed sera from infected patients. *Biochimica et Biophysica Acta*, 1830, 5229–5235.

Islam, D., Ruamsap, N., Aksomboon, A., Khantapura, P., Srijan, A. & Mason, C.J. (2014). Immune responses to *Campylobacter* (*C. jejuni* or *C. coli*) infections: a two-year study of US forces deployed to Thailand. *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, 122, 1102–1113.

Lindblom, G.B., Sjogren, E. & Kaijser, B. (1986). Natural *Campylobacter* colonization in chickens raised under different environmental conditions. *Journal of Hygiene*, 96, 385–391.

Phung, C., Vezina, B., Anwar, A., Wilson, T., Scott, P.C., Moore, R.J. & Van, T.T.H. (2020). *Campylobacter hepaticus*, the cause of Spotty Liver Disease in chickens: transmission and routes of infection. *Frontiers in Veterinary Science*, 6, 506.

Radomska, K.A., Vaezirad, M.M., Verstappen, K.M., Wosten, M.M.S.M., Wagenaar, J.A. & Putten, V.J.P.M. (2016). Chicken immune response after *in ovo* immunization with chimeric TLR5 activating flagellin of *Campylobacter jejuni*. *PLoS One*, 11, e0164837.

Scott, P.C. (2016). *Determining the cause and methods of control for "Spotty Liver Disease"*. Final Project Report. AECL Publication No. 1SX091A, Australian Egg Corporation Limited, North Sydney, NSW, Australia.

Tudor, D.C. (1954). A liver degeneration of unknown origin in chickens. *Journal of the American Veterinary Medical Association*, 125, 219–220.

Van, T.T.H., Anwar, A., Scott, P.C. & Moore, R.J. (2018). Rapid and specific methods to differentiate foodborne pathogens, *Campylobacter jejuni, Campylobacter coli*, and the new species causing Spotty Liver Disease in chickens, *Campylobacter hepaticus*. *Foodborne Pathogens and Disease*, 15, 526–530.

Van, T.T.H., Elshagmani, E., Gor, M.C., Scott, P.C. & Moore, R.J. (2016). *Campylobacter hepaticus* sp. nov., isolated from chickens with spotty liver disease. *International Journal of Systematic and Evolutionary Microbiology*, 66, 4518–4524.

Van, T.T.H., Elshagmani, E., Gor, M.C., Anwar, A., Scott, P.C. & Moore, R.J. (2017). Induction of spotty liver disease in layer hens by infection with *Campylobacter hepaticus*. *Veterinary Microbiology*, 199, 85–90.

Van, T.T.H., Gor, M.C., Anwar, A., Scott, P.C. & Moore, R.J. (2017). *Campylobacter hepaticus*, the cause of spotty liver disease in chickens, is present throughout the small intestine and caeca of infected birds. *Veterinary Microbiology*, 207, 226–230.

Van, T.T.H., Lacey, J.A., Vezina, B., Phung, C., Anwar, A., Scott, P.C. & Moore, R.J. (2019). Survival mechanisms of *Campylobacter hepaticus* identified by genomic analysis and comparative transcriptomic analysis of *in vivo* and *in vitro* derived bacteria. *Frontiers in Microbiology*, 10, 107.

Yeh, H.Y., Hiett, K.L., Line, J.E., Oakley, B.B. & Seal, B.S. (2013). Construction, expression, purification and antigenicity of recombinant *Campylobacter jejuni* flagellar proteins. *Microbiological Research*, 168, 192–198.

Yeh, H.Y., Telli, A.E., Jagne, J.F., Benson, C.L., 2nd, Hiett, K.L. & Line, J.E. (2016). Epitope mapping of *Campylobacter jejuni* flagellar capping protein (FliD) by chicken (*Gallus gallus domesticus*) sera. *Comparative Immunology, Microbiology and Infectious Diseases*, 49, 76–81.