Evaluation of a *Campylobacter jejuni N*-glycan-ExoA glycoconjugate vaccine to reduce *C. jejuni* colonisation in chickens

Prerna Vohra[1,2#], Cosmin Chintoan-Uta[1], Abi Bremner[1], Marta Mauri[3], Vanessa S. Terra[3], Jon Cuccui[3], Brendan W. Wren[3], Lonneke Vervelde[1], Mark P. Stevens[1], on behalf of the Glycoengineering of Veterinary Vaccines Consortium.

[1]The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Edinburgh, EH25 9RG, United Kingdom.

[2]Institute for Immunology and Infection Research, School of Biological Sciences, Charlotte Auerbach Road, University of Edinburgh, Edinburgh EH9 3FF, United Kingdom.

[3]Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT, United Kingdom.

Running title: Glycoconjugate vaccine against *Campylobacter* in chickens.

Keywords: Glycoconjugate, vaccine, *Campylobacter*, colonisation, chickens

[#]Address correspondence to Dr Prerna Vohra, Institute for Immunology and Infection Research, School of Biological Sciences, Charlotte Auerbach Road, University of Edinburgh, Edinburgh EH9 3FL, United Kingdom. E-mail Prerna.Vohra@ed.ac.uk; Tel. +44 (0) 131 650 6763.

Abstract

Campylobacter jejuni is the leading bacterial cause of human gastroenteritis worldwide and handling or consumption of contaminated poultry meat is the key source of infection. Glycoconjugate vaccines containing the *C. jejuni N*-glycan have been reported to be partially protective in chickens. However, our previous studies with subunit vaccines comprising the *C. jejuni* FlpA or SodB proteins with up to two or three *C. jejuni N*-glycans, respectively, failed to elicit significant protection. In this study, protein glycan coupling technology was used to add up to ten *C. jejuni N*-glycans onto a detoxified form of *Pseudomonas aeruginosa* exotoxin A (ExoA). The glycoprotein, G-ExoA, was evaluated for efficacy against intestinal colonisation of White Leghorn chickens by *C. jejuni* strains M1 and 11168H relative to unglycosylated ExoA. Chickens were challenged with the minimum dose required for reliable colonisation, which was 10^2 colony-forming units (CFU) for strain M1 and and 10^4 CFU for strain 11168H. Vaccine-specific serum IgY was detected in chickens vaccinated with both ExoA and G-ExoA. However, no reduction in caecal colonisation by *C. jejuni* was observed. While the glycan dose achieved with G-ExoA was higher than FlpA- or SodB-based glycoconjugates that were previously evaluated, it was lower than that of glycoconjugates where protection against *C. jejuni* has been reported, indicating that protection may be highly sensitive to the amount of glycan presented and/or study-specific variables.

Introduction

Campylobacter remains the most common bacterial cause of human gastroenteritis globally, having caused an estimated 95 million illnesses and 21,000 deaths worldwide in 2010 [1]. Up to 80% of human campylobacteriosis cases are associated with the avian reservoir [2] and the consumption and handling of contaminated poultry meat are undeniably key risk factors [3]. In the United Kingdom, 63,946 laboratory-confirmed cases of human campylobacteriosis were recorded in 2017 [4], with 9.3 cases predicted to be unreported for every one captured by national surveillance [5] and an estimated annual direct cost to the economy of GBP 50 million [6].

Human campylobacteriosis can range in severity from mild gastroenteritis to acute self-limiting haemorrhagic diarrhoea with severe inflammation and may lead to long-term sequelae including reactive arthritis and inflammatory neuropathies such as Guillain–Barré Syndrome [7–10]. In contrast, poultry are generally unaffected despite carrying large numbers of *C. jejuni* in their gastrointestinal tract [11,12] although decreased growth performance in chickens harbouring *C. jejuni* asymptomatically [13] and gut damage, inflammatory responses and diarrhoea have been reported in some broiler breeds [14]. Controlling *Campylobacter* in poultry is essential to reduce a key foodborne zoonosis and could also enhance poultry productivity and welfare in some instances. Risk assessment models have predicted that a hundred-fold reduction in *C. jejuni* on fresh chicken carcasses could reduce human infections by twelve-fold [15].

Vaccination has been explored as a strategy to control *C. jejuni* in poultry. Apart from conserved and immunodominant protein antigens, the *C. jejuni N*-linked heptasaccharide has also been demonstrated to be partly protective against colonisation [16–18]. This glycan is highly conserved in *Campylobacter* species [19] and can be coupled to acceptor proteins containing the D/E-Y-N-X-S/T glycosylation sequon via the action of the enzyme PglB, which is encoded on the naturally-occurring *N*-glycosylation system (*pgl* locus) of *C. jejuni*. The *C. jejuni pgl* locus when transferred to *E. coli* can mediate the *N*-glycosylation of proteins modified to contain one or many glycosylation sequons to produce recombinant glycoconjugate proteins in a low-cost and efficient process termed protein glycan coupling technology (PGCT) [20]. PGCT has been used to produce glycoconjugates against numerous pathogens including *Francisella tularensis* [21,22], *Staphylococcus aureus* [23] and

Shigella flexneri [24]. In a previous study [25], we used PGCT to couple up to two or three *C. jejuni N*-glycans to immunogenic *C. jejuni* proteins FlpA and SodB, respectively, which had previously been shown to reduce colonisation in chickens. We evaluated the efficacy of these glycoconjugates against experimental *C. jejuni* challenge but did not observe a significant reduction in colonisation despite detecting high levels of protein antigen-specific serum IgY in vaccinated chickens. To refine our approach, in this study we selected detoxified *Pseudomonas aeruginosa* exotoxin A (ExoA) as a heterologous immunogenic carrier protein based on its known ability to improve immunogenicity of polysaccharide- and protein-based vaccines [26,27] and modified it to contain ten glycosylation sequons [22]. A similar heavily glycosylated toxoid, based on a detoxified variant of *Corynebacterium diphtheriae* ToxC modified to contain 9 glycosylation sequons, was previously shown to reduce *C. jejuni* colonisation in chickens by 4 to 6 $\log_{10}$ [16]. Moreover, greater glycosylation has been associated with improved protection as observed in a murine model of tularemia where ExoA glycosylated with polymerised *Francisella tularensis* O-antigen glycans at ten glycosylation sequons was fully protective against a hypervirulent strain whereas ExoA modified at two glycosylation sequons was less than 50% protective [21,22]. The efficacy of the novel glycoconjugate, G-ExoA, produced by PGCT relative to unglycosylated ExoA was evaluated against experimental *C. jejuni* challenge with strains M1 and 11168H using the minimum challenge doses for each.

Materials and methods

Bacterial strains and culture conditions

Campylobacter jejuni M1 [28] and 11168H [29] were routinely cultured on charcoal-cephoperazone-deoxycholate agar (CCDA) at 40°C under microaerophilic conditions (5% O_2, 5% CO_2 and 90% N_2). Liquid cultures were prepared in Mueller-Hinton (MH) broth that was allowed to equilibrate with the microaerophilic atmosphere overnight before inoculation and incubation for 16 h with shaking at 400 rpm. For oral challenge of chickens, cultures were adjusted based on a standard curve of colony-forming units (CFU) ml^{-1} relative to absorbance at 600 nm and viable counts were confirmed by retrospective plating of ten-fold serial dilutions on CCDA.

Animal experiments

White Leghorn chickens from *Campylobacter*-free flocks were obtained on the day of hatch from a Home Office licensed breeding establishment (National Avian Research Facility, University of Edinburgh) and housed in groups of up to twenty in colony cages. Groups were of mixed sex and individuals were wing-tagged for identification. Water and sterile irradiated feed based on vegetable protein (DBM Ltd., UK) were provided *ad libitum*. Animal experiments were conducted at the Moredun Research Institute according to the requirements of the Animals (Scientific Procedures) Act 1986 under project licence PCD70CB48 with the approval of the local Ethical Review Committee. Chickens were monitored twice daily. Post-mortem examinations were conducted following culling by cervical dislocation.

Dose titration and colonisation dynamics of *C. jejuni* 11168H

The minimum challenge dose of *C. jejuni* 11168H required for reliable caecal colonisation of chickens was determined as described previously for *C. jejuni* M1 [25]. An overnight culture containing approximately 10^9 CFU ml^{-1} was diluted in MH broth to prepare four inocula such that a dose of 100 µl would contain approximately 10^2, 10^3, 10^4 and 10^5 CFU of *C. jejuni* 11168H, respectively. At day 15 of age, 6 chickens per group were challenged by oral gavage with 100 µl of these cultures. Post-mortem examinations were performed on day 7 post-challenge. Contents from both caeca of each bird were mixed together in equal quantities and ten-fold serial dilutions were prepared in phosphate-buffered saline (PBS) and plated on CCDA to determine viable *C. jejuni* per gram of caecal contents in individual chickens.

Following determination of the minimum challenge dose of *C. jejuni* 11168H, twelve chickens were challenged with the minimum dose at day 15 of age to determine the dynamics of colonisation. Post-mortem examinations of four chickens were conducted on days 1, 3 and 5 post-challenge and bacteria were enumerated from both caeca of each bird by plating ten-fold serial dilutions on CCDA as above.

Preparation of ExoA glycosylated and unglycosylated variants

Briefly, full length detoxified ExoA from *Pseudomonas aeruginosa* was modified to contain ten sequons to enable *N*-glycosylation by PglB, a DsbA signal peptide to direct it to the periplasm for

glycosylation and a *C*-terminal 6-His tag for affinity purification, and cloned under the control of an L-arabinose-inducible promoter in pEC415 (*amp*^{R}) [22]. The plasmid (pEC415-GT-ExoA) was introduced by electroporation into *E. coli* SDB1, a *wecA*- *waaL*- strain suitable for PGCT. Glycosylated ExoA (G-ExoA) was produced in the presence of pACYC*pgl* (*cat*^{R}), a plasmid that contains all the necessary genes to produce the *C. jejuni N*-glycan and a functional PglB to mediate *N*-glycosylation [20]. Unglycosylated ExoA was produced from the same plasmid but in the presence of pACYC*pglB*::*kan*, where the PglB is non-functional [30].

For vaccine production, transformants were grown overnight at 37°C in lysogeny broth (LB) with the appropriate antibiotics. The following day, the cultures were diluted 1:100 and grown at 37°C under shaking conditions (180 rpm) until an OD_{600} of 0.8 was reached. 0.4% (w/v) L-arabinose was added to the cultures and they were grown for a further 16 h at 28°C. Cell pellets were collected by centrifugation at 5400 *g* for 30 minutes at 4°C, resuspended in ice-cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and subjected to five rounds of mechanical lysis using a pre-chilled Stansted High Pressure Cell Disruptor (Stansted Fluid Power Ltd., UK) under 60,000 psi (410 MPa) in continuous mode. The lysate was centrifuged at 10,000 g for 60 minutes at 4°C and the supernatant was collected for protein purification using Ni-affinity chromatography. The supernatant was combined with Ni-NTA resin (Ni-NTA, Qiagen, Germany) for 1 hour at 4°C. The column was then washed with 200 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) and the proteins were eluted using 2 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Protein fractions were pooled and concentrated using buffer exchange columns Vivaspin 2 (Vivaproducts, UK) into PBS containing 20% glycerol and 5% glucose. Purity of glycoconjugates and antigens was assessed by Coomassie staining of proteins resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using an anti-His antibody (Clone His.H8, Thermo Fisher) at 1:5,000 followed by an anti-mouse IRDye 680 RD antibody (LiCor) at 1:10,000 for protein detection and biotin-conjugated soybean agglutinin (bioSBA, Vector Laboratories) at 1:5,000 followed by IRDye Streptavidin 800 CW (LiCOR) at 1:5,000 dilution for *N*-glycan detection. Protein concentration was determined by NanoDrop (ThermoFisher, UK), using the extinction coefficient determined for the modified proteins calculated using Prot Pram (Expasy): ExoA extinction coefficient 92,820 M^{-1} cm^{-1}, Abs (0.1%) = 1.25, Molecular weight (MW) =

74,127.31Da. Quantification of glycosylated and unglycosylated protein in the G-ExoA preparation was done by densitometry using the LiCOR software.

Animal trials to evaluate the efficacy of ExoA glycosylated and unglycosylated variants as *C. jejuni* vaccines

Vaccines were prepared by mixing purified ExoA or G-ExoA with Montanide™ ISA 70 VG (Seppic, France) at a ratio of 30% protein and 70% adjuvant. Groups of up to fifteen chickens were vaccinated on days 6 and 16 of age intramuscularly with 100 µl of the vaccine preparation containing 95 µg protein divided equally between two pectoral muscles. Mock-vaccinated chickens were injected with a mixture of 30% protein buffer (20% (v/v) glycerol, 5% (w/v) glucose in PBS) and 70% Montanide™ ISA 70 VG. Two independent trials of this design were performed: trial 1 with *C. jejuni* M1 as the challenge strain and trial 2 with *C. jejuni* 11168H. *C. jejuni* M1, a highly virulent strain reported to have caused a rare direct transmission event from poultry to human [28], and *C. jejuni* 11168H, a laboratory-adapted less virulent strain, were selected to study vaccine efficacy across a range of strains. At day 20, chickens were challenged by oral gavage with 100 µl of culture containing the minimum challenge dose of the respective *C. jejuni* strains. Post-mortem examinations were performed on days 27 and 37 of age, 7 and 10 days post-challenge, respectively. This regimen (Figure S1) was chosen to permit comparison with earlier studies [25,31]. Contents from both caeca of each bird were collected as described above and ten-fold serial dilutions were plated on CCDA to determine viable counts per gram in individual chickens.

Analysis of humoral immune responses following vaccination

Blood was collected by cardiac puncture at post-mortem examination and serum was stored at -80°C following centrifugation of clotted blood at 1000 *g* for 10 min at 4°C. Blood was also collected from the brachial veins of six chickens per group at days 16 and 20, prior to 2nd vaccination and challenge, respectively, for longitudinal assessment of responses. Three of these chickens were culled at day 27 while the other three were culled at day 37. Serum IgY levels were quantified by enzyme-linked immunosorbent assays (ELISA). To quantify vaccine-specific responses, 96-well plates were coated with 0.5 µg ml^{-1} of ExoA or G-ExoA in carbonate-bicarbonate buffer and incubated 4°C overnight. Plates were washed with PBS containing 0.05% (v/v) Tween 20 and 100 µl of 1:100 serum diluted in

PBS was added per well. Control wells were mainained to which no serum was added. Plates were incubated at 37°C for 1 h and then washed as above. Rabbit anti-chicken IgY-horseradish peroxidase (HRP) antibody at 1:3000 (Sigma, UK) was used to detect bound serum IgY. Plates were washed twice, tetramethylbenzidine (TMB) substrate (BioLegend, UK) was added and the plates were incubated for 10 min at room temperature in the dark. The reaction was stopped using 2M H_2SO_4 and absorbance at 450 nm adjusted against absorbance at 620 nm ($A_{450/620}$) was measured using a plate reader with background correction using the values of the no serum control wells (Multiskan Ascent, Thermo, UK).

In trial 2 only, five additional chickens from each group were culled at days 16 and 20, prior to 2nd vaccination and challenge, respectively. Blood was collected by cardiac puncture and vaccine-specific responses were quantified as above. To quantify *N*-glycan-specific responses, ELISAs were performed using SodB and G-SodB [25] as coating antigens with the expectation that no anti-SodB IgY would be detected in these birds as they were sampled pre-challenge but anti-*N*-glycan IgY may be detected following vaccination with G-ExoA only.

Statistical analysis

Statistical tests were performed in GraphPad Prism version 8.00 (GraphPad Software). Differences in colonisation levels and humoral responses between groups of chickens in the vaccination trials at each time point were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistical significance ($P \leq 0.05$) is indicated with asterisks (*). Data are represented graphically as median values with 95% confidence intervals.

Results

Determining the minimum dose of *C. jejuni* 11168H required for reliable intestinal colonisation

C. jejuni 11168H is a hypermotile clonal derivative of NCTC 11168 that reliably colonises the gastrointestinal tract of chickens when administered at a dose of 10^6 CFU [32]. Following dose titration of *C. jejuni* 11168H, bacteria were detected in the caeca of chickens challenged with 10^4 and 10^5 CFU at day 7 post-challenge at colonisation levels of 9.09 and 8.62 $\log_{10}$ CFU g^{-1}, respectively (Figure 1A). No bacteria were detected in groups challenged with 10^2 and 10^3 CFU. A dose of 10^4

CFU was therefore selected as the minimum challenge dose for effective caecal colonisation by *C. jejuni* 11168H. Following challenge with this minimum dose, bacteria were only detected in two out of four chickens at a median level of 2.18 $\log_{10}$ CFU g^{-1} at day 1 post-challenge (Figure 1B). However, by day 3, median bacterial levels rose to 3.04 $\log_{10}$ CFU g^{-1} and increased to 8.13 $\log_{10}$ CFU g^{-1} by day 5 (Figure 1B).

Characterisation of vaccines

His-tagged ExoA and G-ExoA were affinity-purified from *E. coli* using the same modified construct (Figure 2A) in the absence or presence, respectively, of a functional PglB to perform *N*-glycosylation. The presence of ExoA in both preparations was verified by SDS-PAGE and glycosylation of only G-ExoA was clearly visible as ten bands above the ExoA protein (Figure 2B). Western blotting using an anti-His antibody to detect ExoA (Figure 2C) and a biotinylated lectin against the *C. jejuni N*-glycan (Figure 2D) confirmed these findings. Semi-quantitative analysis by densitometry indicated that 80% of protein in the G-ExoA preparation was glycosylated. Therefore, each G-ExoA vaccine dose contained approximately 9.85 µg of *N*-glycan.

Effect of vaccination on *C. jejuni* colonisation

The efficacy of ExoA and G-ExoA against *C. jejuni* colonisation was evaluated in two independent trials (Figure S1). In trial 1, chickens were challenged with 10^2 CFU of *C. jejuni* M1 and caecal colonisation was assessed at days 27 and 37 (Figure 3A). At day 27, a statistically significant reduction in colonisation was observed in the G-ExoA-vaccinated group as compared to the ExoA-vaccinated group but this was likely owing to the variation in colonisation levels observed in the G-ExoA-vaccinated group. At day 37, a significant reduction in colonisation was observed in the ExoA-vaccinated group compared to the mock-vaccinated group but not compared to the G-ExoA-vaccinated group.

In trial 2, chickens were challenged with 10^4 CFU of *C. jejuni* 11168H (Figue 3B). No reduction in colonisation was observed at day 27 in either vaccinated group. However, statistically significant

reduction in colonisation was observed at day 37 in both ExoA- and G-ExoA-vaccinated groups compared to the mock-vaccinated group.

Induction of humoral immune responses

Significantly elevated vaccine-specific serum IgY was detected in ExoA and G-ExoA-vaccinated groups in both trial 1 (Figure 4A, B) and trial 2 (Figure 4C, D) at days 27 and 37 compared to mock-vaccinated chickens. However, no significant differences were observed between ExoA and G-ExoA-vaccinated groups. Longitudinal analysis of humoral immune responses in a subset of chickens from each group showed higher vaccine-specific IgY levels following the second vaccination at day 20 as expected. In trial 1, these responses were elevated further in all vaccinated chickens following challenge with *C. jejuni* M1. In trial 2, the magnitude of responses following 2nd vaccination were similar to post-challenge levels in trial 1. However, an increase in responses was not observed consistently after challenge with *C. jejuni* 11168H and antibody levels were lower at day 37. There was no correlation between levels of vaccine-specific IgY and caecal colonisation.

In trial 2, serum IgY levels against the vaccines and *C. jejuni N*-glycan were measured from an additional five chickens (not included in Figure 4) culled after the first and second vaccinations. As observed in the longitudinally sampled chickens in Figure 4, vaccine-specific IgY increased following 2nd vaccination. No responses against SodB, an immunogenic *C. jejuni* protein, were observed as expected as these chickens were *Campylobacter*-free and the samples were collected pre-challenge. However, responses against its glycosylated form, G-SodB, were also lacking, suggesting that *N*-glycan-specific responses in the G-ExoA-vaccinated group were either absent or below the limit of sensitivity of the assay. The increase in bound serum IgY over time and relative to the mock-vaccinated group suggests reponses to contaminating *E.coli* antigens in the vaccine preparations.

Discussion

The control of *C. jejuni* in poultry is essential to reduce human campylobacteriosis. Vaccination has been explored as a strategy to reduce colonisation by *C. jejuni* in chickens and glycoconjugate vaccines containing the *C. jejuni N*-glycan have been reported to be efficacious in doing so. In a previous study, we conjugated *C. jejuni N*-glycans to *C. jejuni* proteins FlpA and SodB, which had

individually been shown to reduce colonisation in chickens [31,33], to test if glycosylation improved their efficacy. However, both the proteins and the glycoconjugates were unable to reduce *C. jejuni* colonisation in chickens [25], albeit the vaccination regimen differed from that previously used for SodB [33]. We speculated that different challenge strains, vaccination schedules, chicken lines and indeed levels of vaccine glycosyaltion could explain the differences in our results and published reports. FlpA and SodB had been modified to contain two and three glycosylation sequons, respectively. However, in a previous study in which chickens were vaccinated with an antigen coupled to up to nine *N*-glycans colonisation was reduced by 4 to 6 $\log_{10}$ [16]. Moreover, the antigen to which the *N*-glycans were coupled was an inactive form of *Corynebacterium diphtheriae* ToxC, a known immunogen. Therefore, in this study we tested whether a highly glycosylated heterologous antigen, a detoxified form of *Pseudomonas aeruginosa* exotoxin A with up to ten *C. jejuni N*-glycans (G-ExoA), could reduce *C. jejuni* colonisation in chickens.

Vaccination trials were performed using a previously used schedule (Fig S1) [25] to evaluate the efficacy of G-ExoA against two strains of *C. jejuni* - M1 and 11168H. As done previously for *C. jejuni* M1, first, the minimum challenge dose for effective colonisation of the caeca was determined for *C. jejuni* 11168H. The rationale for this was partly due to concern that *C. jejuni* M1 may colonise the avian gut so proficiently that it overcomes vaccine-mediated immunity, as was recently suggested by colonisation phenotypes for strain M1 in inbred chicken lines exhibiting heritable differences in resistance [34], relative to findings with other strains [35]. Despite a 100-fold difference in minimum challenge dose for strains M1 and 11168H of 10^2 and 10^4, respectively, both strains showed similar growth dynamics in the caeca, reaching peak colonisation levels by 5 days post-challenge.

Vaccination of chickens with both ExoA and G-ExoA elicited strong vaccine-specific serum IgY responses. ExoA has previously been used as a carrier protein in vaccines to enhance antibody production against poorly immunogenic proteins, for example against the Pfs25 malaria protein [26], and against polysaccharides including the Vi toxin of *Salmonella* Typhi [27] or the O-antigen of *Francisella tularensis* [21,22]. However, in this study, antibody levels were not significantly higher in the G-ExoA group and anti-vaccine responses increased over time in both ExoA- and G-ExoA-vaccinated groups. Further, vaccine-specific IgY continued to increase in trial 1 following challenge

with *C. jejuni* M1 but in trial 2 vaccine-specific IgY reached higher levels before challenge than in trial 1. Also, antibody levels only increased mariginally after challenge with *C. jejuni* 11168H and then reduced by day 37. The same batch of vaccines was used for both trials and the vaccines did not contain *C. jejuni* proteins. Therefore, the differences in IgY levels pre-challenge may be caused by natural variation between hatches. The boosted response detected in ExoA and G-ExoA-vaccinated groups is therefore, not *N*-glycan-specific. Attempts to detect *N*-glycan-specific IgY in chickens post-vaccination but prior to challenge proved difficult owing to the difficulties in producing highly pure *N*-glycans for ELISA. Instead, purified G-SodB [25] was used as a proxy for *C. jejuni N*-glycans and unglycosyalted SodB was used as a negative control but no differences in serum IgY levels were observed between ExoA or G-ExoA vaccinated groups. Together, these observations suggest a lack of relevant anti-*N*-glycan responses.

The results of this study are in contrast with previously published work in which chickens were vaccinated with an inactive form of *Corynebacterium diphtheriae* ToxC modified with nine *N*-glycans in which a reduction in *C. jejuni* colonsation of 4 to 6 $\log_{10}$ was observed [16]. While the same breed of chickens was used here, the vaccination schedules differed: Nothaft *et al.*, 2016 vaccinated chickens at days 7 and 21 and challenged on day 28, and here chickens were vaccinated on days 6 and 16 and challenged on day 20. The challenge strains also differed: Nothaft *et al.*, 2016 used *C. jejuni* 81-176 whereas *C. jejuni* M1 and 11168H were used here, and the variation between phenotypes of *C. jejuni* strains, even within the same lineage, are well documented [36]. In both studies, the vaccine dose was calculated based on the protein content, which was 100 µg for the ToxC glycoconjugate and 95 µg for G-ExoA. Despite comparable amounts of protein, the vaccines contained roughly 15 µg and 10 µg *N*-glycan per dose, respectively, which is considerably different. Moreover, the ToxC glycoconjugate was administered with Freund's complete and incomplete adjuvants at 1st and 2nd vaccination, respectively, at a 1:1 ratio, and in a volume of 300 µl whereas G-ExoA was mixed with a less reactogenic commercially-used adjuvant at a 30:70 ratio (G-ExoA:adjuvant) in a total volume of 100 µl to comply with local Ethical Review Committee requirements. These differences in protocols could account for the contrasting results of the two studies.

Vaccine development against *C. jejuni* is marred by variability in study design, vaccine design and delivery as well as the *C. jejuni* challenge strains used. Significant variability can also exist within and between replicate groups. There is a need to adopt consistent protocols that allow a direct comparison of efficacy across trials and for vaccines to be tested repeatedly and against diverse bacterial strains. For consistency with previous work, the glycosylated inactivated toxin vaccine, G-ExoA, was tested using the same protocols used to test our previous glycoconjuagtes [25] and was also tested against two *C. jejuni* challenge strains simultaneously. The glycan dose for G-ExoA was estimated to be higher than for G-FlpA (3 µg) and G-SodB (9 µg) previously tested, but was still insufficient to reduce colonisation of chickens by either *C. jejuni* strain despite the induction of vaccine-specific humoral responses. While it is known that mucosal secretory IgA (sIgA) plays a greater role in clearance of *C. jejuni* [37] and that serum IgY levels do not always correlate with a reduction in *C. jejuni* colonization in chickens, demonstrating vaccine-specific serum IgY is nonetheless important to assess immunogenicity of the carrier protein as well as the *C. jejuni N*-glycan. It is possible that the route of administration of glycoconjugate vaccines via mucosal surfaces might induce high levels of protective sIgA. However, in this study intra-muscular administration was used to enable comparisons with published research [16,25]. Further optimisation of glycoconjugate vaccines and investigations into alternative routes of administration such as sprays, optimal delivery vehicles such as nanoparticles and mucosal adjuvants is needed to achieve avian responses of an adequate magnitude and nature to elicit protection against *C. jejuni*.

Funding

The authors gratefully acknowledge funding from the Biotechnology & Biological Sciences Research Council (BBSRC; grant reference BB/N001591/1) and BBSRC strategic investment in The Roslin Institute (BBS/E/D/20002174). The funders played no role in study design, data anlysis and interpretation of data, writing the manuscript or the decision to submit the manuscript for publication.

Acknowledgements

We wish to thank staff at the Moredun Research Institute for assistance with animal experiments.

References

[1] Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, et al. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLoS Med 2015;12:e1001923.

[2] EFSA. Scientific Opinion on *Campylobacter* in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. EFSA J 2011;9:2105.

[3] Tam CC, Higgins CD, Neal KR, Rodrigues LC, Millership SE, O'Brien SJ, et al. Chicken consumption and use of acid-suppressing medications as risk factors for *Campylobacter* enteritis, England. Emerg Infect Dis 2009;15:1402–8.

[4] PHE. Zoonoses Report UK 2017. Public Health England, London, United Kingdom. https://www.gov.uk/government/publications/ zoonoses-uk-annual-reports. Public Heal England, London, United Kingdom Https//WwwGovUk/Government/Publications/ Zoonoses-Uk-Annual-Reports 2017:1–99.

[5] Tam CC, Rodrigues LC, Viviani L, Dodds JP, Evans MR, Hunter PR, et al. Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. Gut 2012;61:69–77.

[6] Tam CC, O'Brien SJ. Economic Cost of *Campylobacter*, norovirus and rotavirus disease in the United Kingdom. PLoS One 2016;11:e0138526.

[7] Nachamkin I, Allos BM, Ho T. Campylobacter species and Guillain-Barré syndrome. Clin Microbiol Rev 1998;11:555–67.

[8] McCarthy N, Giesecke J. Incidence of Guillain-Barré syndrome following infection with *Campylobacter jejuni*. Am J Epidemiol 2001;153:610–4.

[9] Nachamkin I. Chronic effects of *Campylobacter* infection. Microbes Infect 2002;4:399–403.

[10] Pope JE, Krizova A, Garg AX, Thiessen-Philbrook H, Ouimet JM. *Campylobacter* reactive arthritis: a systematic review. Semin Arthritis Rheum 2007;37:48–55.

[11] Beery JT, Hugdahl MB, Doyle MP. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. Appl Environ Microbiol 1988;54:2365–70.

[12] Sahin O, Morishita TY, Zhang Q. *Campylobacter* colonization in poultry: sources of infection and modes of transmission. Anim Heal Res Rev 2002;3:95–105.

[13] Awad WA, Molnár A, Aschenbach JR, Ghareeb K, Khayal B, Hess C, et al. *Campylobacter*

infection in chickens modulates the intestinal epithelial barrier function: Innate Immun 2014;21:151–60.

[14] Humphrey S, Chaloner G, Kemmett K, Davidson N, Williams N, Kipar A, et al. *Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affects bird welfare. MBio 2014;5:e01364-14.

[15] Rosenquist H, Nielsen NL, Sommer HM, Nørrung B, Christensen BB. Quantitative risk assessment of human campylobacteriosis associated with thermophilic *Campylobacter* species in chickens. Int J Food Microbiol 2003;83:87–103.

[16] Nothaft H, Davis B, Lock YY, Perez-Munoz ME, Vinogradov E, Walter J, et al. Engineering the *Campylobacter jejuni N*-glycan to create an effective chicken vaccine. Sci Rep 2016;6:26511.

[17] Price NL, Goyette-Desjardins G, Nothaft H, Valguarnera E, Szymanski CM, Segura M, et al. Glycoengineered outer membrane vesicles: a novel platform for bacterial vaccines. Sci Rep 2016;6:24931.

[18] Nothaft H, Perez-Muñoz ME, Gouveia GJ, Duar RM, Wanford JJ, Lango-Scholey L, et al. Co-administration of the *Campylobacter jejuni N*-glycan based vaccine with probiotics improves vaccine performance in broiler chickens. Appl Environ Microbiol 2017;83:e01523-17.

[19] Kowarik M, Young NM, Numao S, Schulz BL, Hug I, Callewaert N, et al. Definition of the bacterial *N*-glycosylation site consensus sequence. EMBO J 2006;25:1957–66.

[20] Wacker M, Linton D, Hitchen PG, Nita-Lazar M, Haslam SM, North SJ, et al. *N*-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. Sci (New York, NY) 2002;298:1790–3.

[21] Cuccui J, Thomas RM, Moule MG, D'Elia R V, Laws TR, Mills DC, et al. Exploitation of bacterial *N*-linked glycosylation to develop a novel recombinant glycoconjugate vaccine against *Francisella tularensis*. Open Biol 2013;3:130002.

[22] Marshall LE, Nelson M, Davies CH, Whelan AO, Jenner DC, Moule MG, et al. An O-Antigen glycoconjugate vaccine produced using protein glycan coupling technology is protective in an inhalational rat model of tularemia. J Immunol Res 2018;2018.

[23] Wacker M, Wang L, Kowarik M, Dowd M, Lipowsky G, Faridmoayer A, et al. Prevention of *Staphylococcus aureus* infections by glycoprotein vaccines synthesized in *Escherichia coli*. J Infect Dis 2014;209:1551–61.

[24] Riddle MS, Kaminski RW, Di Paolo C, Porter CK, Gutierrez RL, Clarkson KA, et al. Safety and immunogenicity of a candidate bioconjugate vaccine against *Shigella flexneri* 2a administered to healthy adults: a Single-Blind, Randomized Phase I Study. Clin Vaccine Immunol 2016;23:908–17.

[25] Vohra P, Chintoan-uta C, Terra VS, Bremner A, Cuccui J, Wren BW, et al. Evaluation of glycosylated FlpA and SodB as subunit vaccines against *Campylobacter jejuni* colonisation in chickens. Vaccines 2020;8..

[26] Qian F, Wu Y, Muratova O, Zhou H, Dobrescu G, Duggan P, et al. Conjugating recombinant proteins to *Pseudomonas aeruginosa* ExoProtein A: a strategy for enhancing immunogenicity of malaria vaccine candidates. Vaccine 2007;25.

[27] Lanh MN, Van Bay P, Ho VA, Thanh TC, Lin FYC, Bryla DA, et al. Persistent efficacy of Vi conjugate vaccine against typhoid fever in young children. N Engl J Med 2003;349.

[28] Friis C, Wassenaar TM, Javed MA, Snipen L, Lagesen K, Hallin PF, et al. Genomic characterization of *Campylobacter jejuni* strain M1. PLoS One 2010;5:e12253.

[29] Macdonald SE, Gundogdu O, Dorrell N, Wren BW, Blake D, Stabler R. Draft genome sequence of *Campylobacter jejuni* 11168H. Genome Announc 2017;5. https://doi.org/10.1128/genomeA.01556-16.

[30] Linton D, Dorrell N, Hitchen PG, Amber S, Karlyshev A V., Morris HR, et al. Functional analysis of the *Campylobacter jejuni N*-linked protein glycosylation pathway. Mol Microbiol 2005;55.

[31] Neal-McKinney JM, Samuelson DR, Eucker TP, Nissen MS, Crespo R, Konkel ME. Reducing *Campylobacter jejuni* colonization of poultry via vaccination. PLoS One 2014;9:e114254.

[32] Abouelhadid S, North SJ, Hitchen P, Vohra P, Chintoan-Uta C, Stevens M, et al. Quantitative analyses reveal novel roles for *N*-Glycosylation in a major enteric bacterial pathogen. MBio 2019;10:3.

[33] Chintoan-Uta C, Cassady-Cain RL, Al-Haideri H, Watson E, Kelly DJ, Smith DGE, et al. Superoxide dismutase SodB is a protective antigen against *Campylobacter jejuni* colonisation in chickens. Vaccine 2015;33:6206–11.

[34] Chintoan-Uta C, Wisedchanwet T, Glendinning L, Bremner A, Psifidi A, Vervelde L, et al. Role of cecal microbiota in the differential resistance of inbred chicken lines to colonization by

Campylobacter jejuni. Appl Environ Microbiol 2020;86.

[35] Psifidi A, Fife M, Howell J, Matika O, van Diemen PM, Kuo R, et al. The genomic architecture of resistance to *Campylobacter jejuni* intestinal colonisation in chickens. BMC Genomics 2016;17.

[36] Pascoe B, Williams LK, Calland JK, Meric G, Hitchings MD, Dyer M, et al. Domestication of *Campylobacter jejuni* NCTC 11168. Microb Genomics 2019;5.

[37] Lacharme-Lora L, Chaloner G, Gilroy R, Humphrey S, Gibbs K, Jopson S, et al. B lymphocytes play a limited role in clearance of *Campylobacter jejuni* from the chicken intestinal tract. Sci Rep 2017;7:45010–90.

Figure legends

Figure 1. Dose titration and colonisation dynamics of *C. jejuni* 11168H.

(A) Following challenge with 10^2 to 10^4 CFU of *C. jejuni* 11168H, the minimum challenge dose for reliable caecal colonisation was determined to be 10^4 CFU. No bacteria were detected at lower doses (N.D.) (▼). (B) The caecal colonisation dynamics following challenge with 10^4 CFU of *C. jejuni* 11168H showed increasing bacterial loads over time as expected.

Figure 2. Vaccine preparations evaluated against *C. jejuni*.

(A) A schematic of the construct from which His-tagged ExoA and G-ExoA were produced in the absence and presence of a functional PglB for *N*-glycosylation, respectively. (B) ExoA and G-ExoA preparations were visualised using SDS-PAGE. ExoA was detected by its size (indicated by the arrow) in both preparations and glycosylation of G-ExoA was evident by the presence of ten bands the protein. (C) Protein profiles of ExoA and G-ExoA were confirmed by western blotting using an anti-His antibody. (D) *N*-glycosylation of G-ExoA only was confirmed by lectin staining.

Figure 3. Caecal colonisation by *C. jejuni* following vaccination and oral challenge.

(A) In trial 1, at day 27, a statistically significant reduction in *C. jejuni* M1 levels was observed in the G-ExoA-vaccinated group (●) compared to the Exo-A-vaccinated group (●). At day 37, only colonisation in the ExoA-vaccinated group was statistically lower than the mock-vaccinated

group (▲). (B) In trial 2, no reduction in colonisation was observed in vaccinated chickens at day 27. However, at day 37, colonisation in both ExoA- and G-ExoA-vaccinated groups was statistically lower than the the mock-vaccinated group. *$P \leq 0.05$

Figure 4. Induction of immune responses following vaccination and oral challenge. (A) In trial 1, ExoA-vaccinated (●) and G-ExoA-vaccinated (●) groups had significantly higher levels of serum IgY against the ExoA preparation compared to mock-vaccinated chickens (▲) at days 27 and 37 but there were no differences between the groups. Longitudinally sampled birds (coloured individually: B1 ●, B2 ●, B3 ●, B4 ●, B5 ● and B6 ●) showed increasing elevated levels of IgY until after challenge with *C. jejuni* M1 (D27). (B) The same pattern of responses was observed against G-ExoA in trial 1. (C) In trial 2, vaccinated groups had significantly higher levels of serum IgY against the ExoA preparation compared to mock-vaccinated chickens at days 27 and 37 but there were no differences between the groups. Longitudinally sampled birds had higher IgY levels following 2nd vaccination (D20) than in trial 1 that increased marginally after challenge with *C. jejuni* 11168H (D27) in most chickens. (D) The same pattern of responses was observed against G-ExoA in trial 2. *$P \leq 0.05$

Figure 5. Induction of humoral immune responses before challenge in trial 2. (A) Increased levels of vaccine-specific IgY were seen in ExoA-vaccinated chickens (■) compared to mock-vaccinated (▲) chickens at days 16 and 20, before 2nd vaccination and challenge, respectively. (B) The same was observed for G-ExoA-vaccinated chickens (■). (C) Negligible amounts of IgY were detected against SodB as expected. (D) Similar IgY levels were observed against G-SodB indicating a lack of *N*-glycan-specific responses or levels below the limit of assay sensitivity.

Figure 1

Click here to access/download;Figures;Figure1.eps

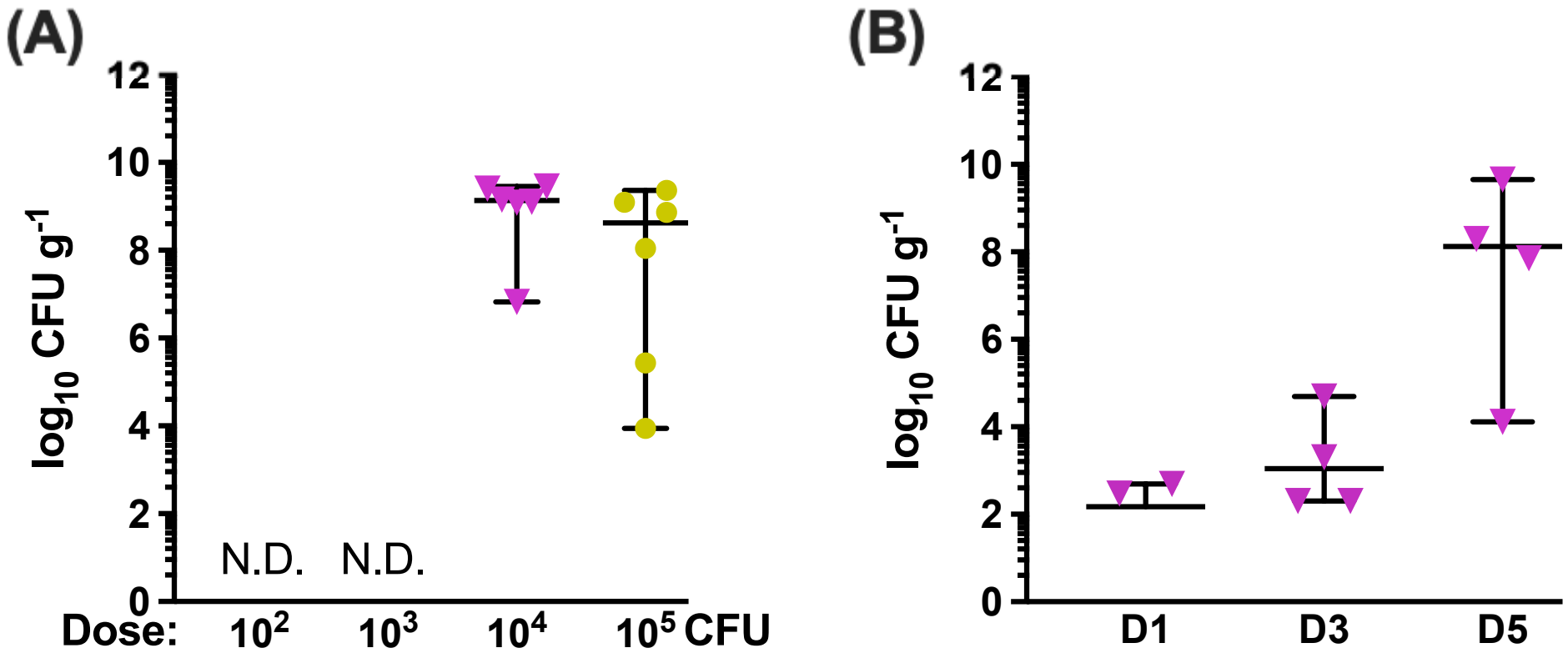

Figure 2

Click here to access/download;Figures;Figure2.pdf

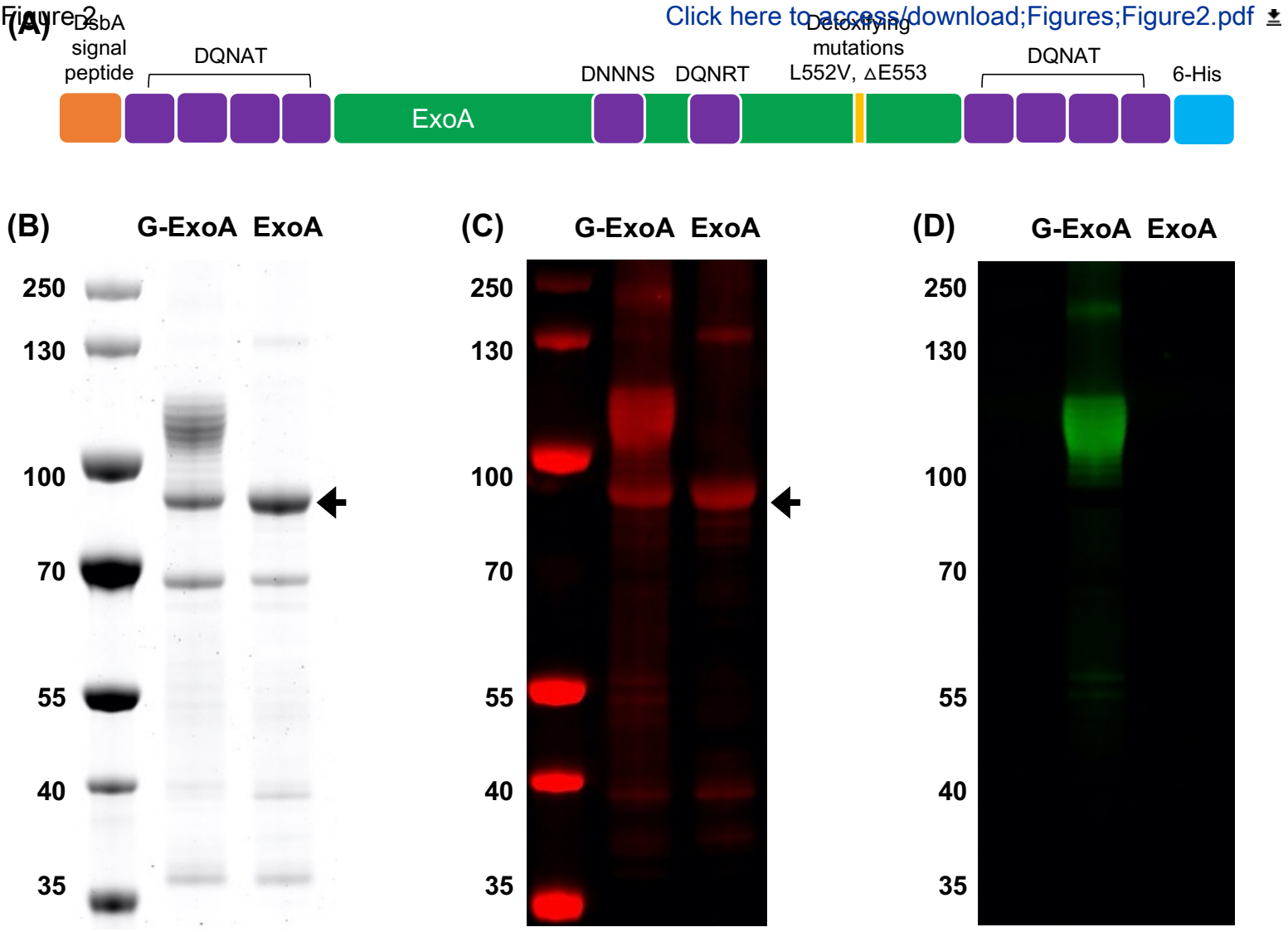

Figure 3

Click here to access/download;Figures;Figure3.eps

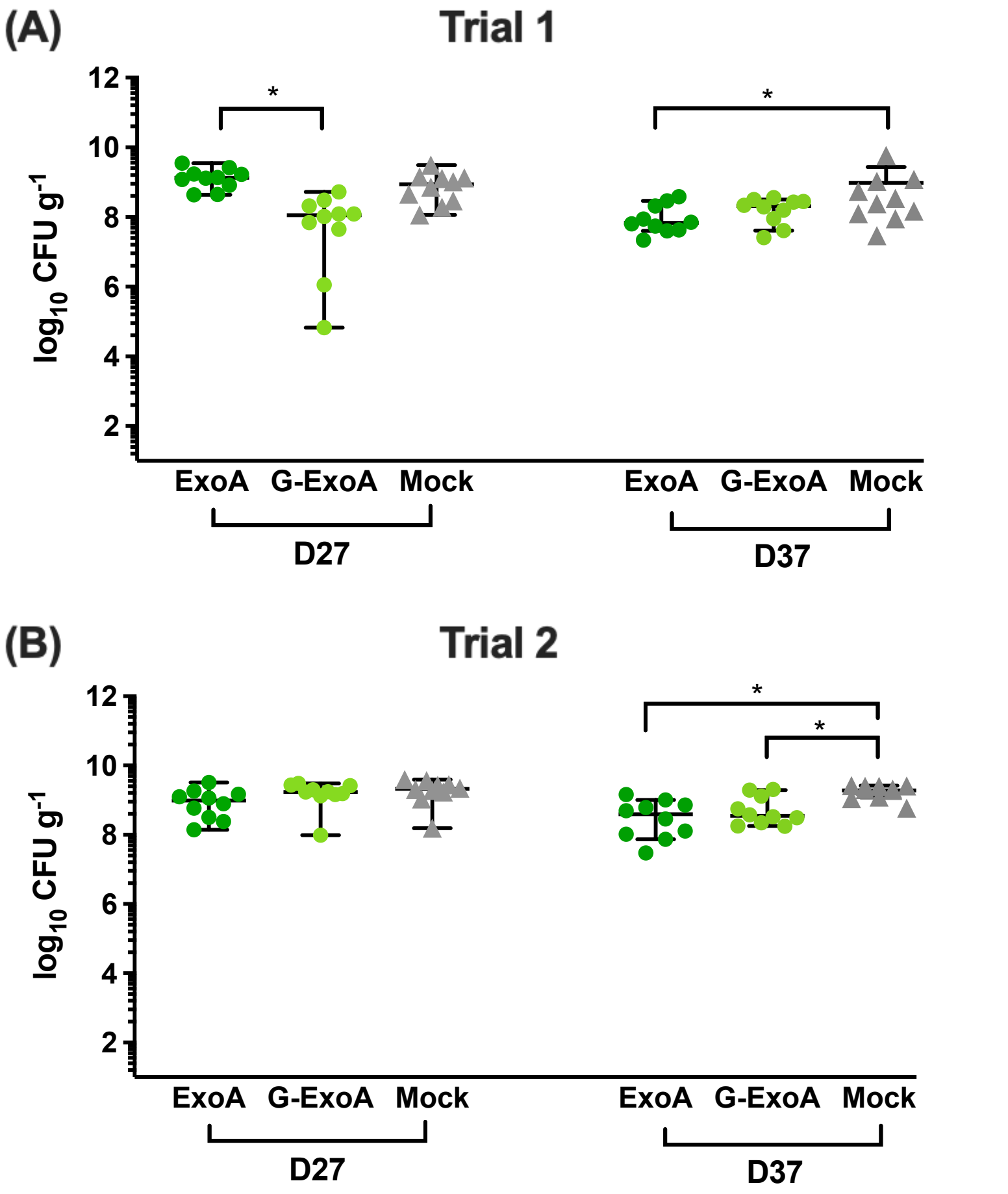

Figure 4

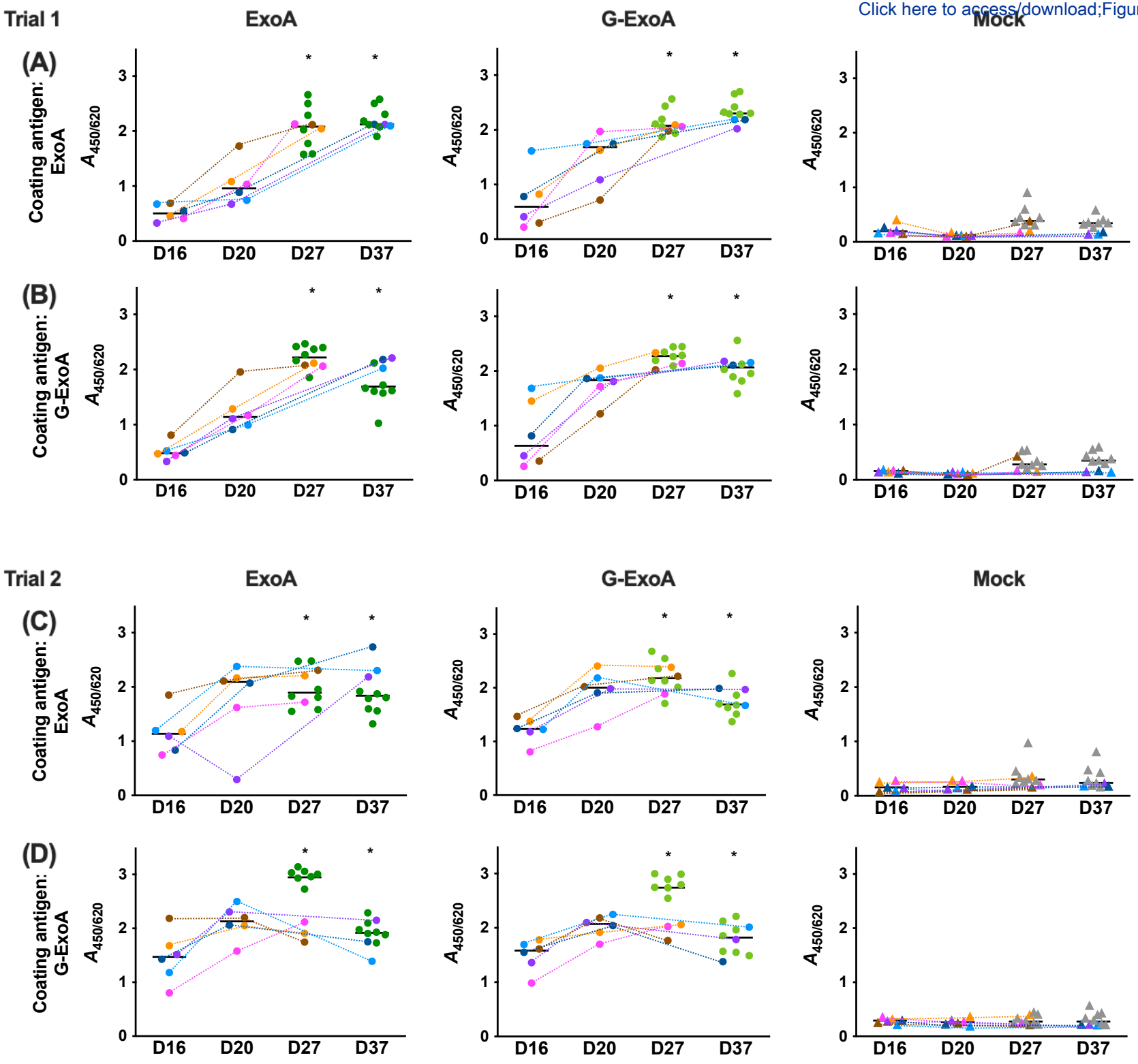

Figure 5

Click here to access/download;Figures;Figure5.eps