Carbohydrate binding and gene expression by *in vitro* and *in vivo* propagated *Campylobacter jejuni* using a modified immunomagnetic separation

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Abstract

Campylobacter jejuni is an important human food-borne intestinal pathogen, however relatively little is known about its mechanisms of pathogenesis or pathogen-host interactions. To monitor changes in gene expression and glycan binding of *C. jejuni* within a common avian host, an immunomagnetic separation technique (IMS) was utilised to directly isolate infecting *C. jejuni* 81116 from a chicken host. An average of 10^5 cells/g was re-isolated from chicken caecal samples by IMS technique. The *in vivo* passaged strains were used successfully in evaluation of carbohydrate binding through the use of a glycan array and were further suitable for transcriptome analysis. The glycan microarray analysis demonstrated differences in binding to negatively charged glycans of laboratory grown strains of *C. jejuni* compared with strains isolated after *in vivo* passage. The *in vivo* passaged strains showed marked up-regulation of chemotaxis receptors and toxin genes. The optimised *Campylobacter* IMS technique described in this study allowed isolation directly from an animal host. Changes in gene expression and glycan binding at an *in vivo* level can also be identified by using this method.

INTRODUCTION

Campylobacter jejuni is a major cause of human bacterial gastroenteritis world wide [1, 2]. It is estimated that approximately 1-10% of individuals globally develop campylobacteriosis or *Campylobacter* related illness each year [3]. *C. jejuni* is a commensal organism of animals such as chickens and colonises these asymptomatically [4]. However, the pathogen is able to cause disease in human hosts with symptoms ranging from a mild, often watery diarrhoea to a more severe inflammatory diarrhoea containing blood and leukocytes accompanied by abdominal cramping, fever, nausea and vomiting [5, 6]. Even though infection with *C. jejuni* is usually self limiting, it can lead to severe complications such as chronic infection leading to immunoproliferative disease [7], or the development of Guillain-Barré Syndrome (GBS), a post-infection polyneuropathy that affects the peripheral nervous system [8, 9].

C. jejuni infection can be established with doses as low as 5-500 orally ingested organisms [10]. However despite its importance as a human infectious agent, the mechanisms of pathogenesis are still poorly understood. Studies have previously identified flagella and motility, along with other surface structures as essential requirements for pathogenesis [11-14]. The genome sequences of *C. jejuni* subsp. *jejuni* NCTC11168 and more recently *C. jejuni* subsp. *jejuni* 81116 (NCTC11828) have provided instrumental information into gene organisation and highlighted *C. jejuni* 81116 as an isolate that is amenable to genetic manipulation, infective for chickens and reported as being relatively genetically stable [15, 16]. It has also been suggested that strain 81116 may prove to be a more stable and reliable candidate strain for *in vivo* experiments [15].

The majority of studies on *C. jejuni* virulence factors are conducted *in vitro* using bacterial/mammalian cell co-culture systems that do not replicate the complex *in vivo* mucosal environment. Culture of *C. jejuni in vitro* with purified human intestinal mucin

revealed up-regulation of multiple pathogenicity genes demonstrating the influence of one host factor [17]. However, to investigate differences between *in vitro* and *in vivo* grown bacteria requires techniques to isolate bacteria without culture steps. Methodologies using immunomagnetic separation (IMS) have been used previously with success to isolate *Campylobacter* spp. from poultry products including chicken carcasses and ground chicken meat [18, 19]. The study presented here demonstrates that IMS can be used to isolate *C. jejuni* from a chicken caeca and verified that isolated bacteria can be used in applications such as a glycan array and quantitative PCR. One major advantage of IMS technique is that gene expression and bacterial characteristics can be evaluated immediately following isolation from the host rather than undergoing the routine 24 to 48 h subculture, whereby up or down regulation of virulence factors may occur. We documented changes in both glycan binding and gene expression, including known pathogenicity genes, by *C. jejuni* within an avian host.

MATERIALS AND METHODS

Bacterial strains and culture conditions

C. jejuni strain 81116, *C. jejuni* 11168-GS sequenced lab strain [20] and 11168-O (the original clinical isolate), were kindly provided by D. G Newell, Centre of Veterinary Laboratories, London, UK. All strains were routinely grown on Columbia agar (Oxoid, Hampshire, UK) supplemented with 5 % (v/v) defibrinated horse blood (IMVS) and 1% (v/v) Skirrow supplement (Oxoid) for 18-48 h at 42 °C or 37 °C under microaerobic conditions (5 % O_2:10 % CO_2:85 % N_2; BOC, Rocklea, QLD, Australia). Cells were harvested in Brucella broth and bacterial concentrations (10^6 to 10^{12} cfu ml^{-1}) were estimated from optical density followed by culturable counts.

Coating of magnetic beads with *Campylobacter jejuni* specific antibody

Dynabead® M-280 Sheep anti-Rabbit IgG 2.8 µm beads (Dynal) were prepared according to the manufacturer's protocol. Briefly an aliquot of M-280 Dynabeads containing 6 x 10^8 beads ml^{-1} were washed with excess phosphate-buffered saline (PBS) pH 7.4 and separated using a Dynal MPC™ magnetic particle concentrator. The washed magnetic beads were resuspended in a round bottom tube and mixed with rabbit anti-*Campylobacter jejuni* polyclonal antibodies (Fitzgerald Industries International, Acton, MA, USA; Cat# 20-CR89) at a concentration of 60 µg (5 mg ml^{-1}) per 6 x 10^8 beads. This bead-antibody complex was incubated for 30 min in a MACSmix™ tube rotator (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) allowing tilt rotation at room temperature followed by overnight tilt rotation at 4 °C to ensure complete antibody coating of magnetic beads. Subsequently, coated Dynabeads were washed three times (30 s) with excess PBS containing 0.1 % bovine serum albumin (BSA) by using magnetic particle separator, resuspended (~6 x 10^8 beads ml^{-1}) in the same buffer and stored at 4 °C; prior to use coated Dynabeads were washed with PBS+0.1%BSA immediately.

Determination of Immunomagnetic Separation (IMS) parameters for recovery of *C. jejuni*

C. jejuni 81116, *C. jejuni* 11168 and 11168-O were resuspended in Brucella broth to variable cell densities and subjected to immunomagnetic separation (IMS) with previously coated Dynabeads M-280. The bacterial cell densities assessed ranged from 10^6 to 10^{12} cfu ml^{-1}. 40 µl of antibody coated Dynabeads was added to 1 ml of bacterial suspension and mixed by tilt rotation for 30 min at room temperature to allow sufficient binding of target bacteria. After that, captured beads loaded with *C. jejuni* cells were washed three times with PBS pH 7.4 containing 0.1% Tween 20 and resuspended in 100 µl Brucella broth.

Efficacy of IMS method was assessed by serial dilution and culturable count enumeration of *C. jejuni* on selective agar. Elution of *C. jejuni* from M-280 Dynabeads was optimised using 0.05% trypsin-0.02% EDTA in Hank's balanced salt solution (Invitrogen), 0.1 M citrate pH 3 (Sigma) and 1,4-Dithiothreitol (DTT) in diethyl pyrocarbonate (DEPC)-treated H_2O (A. G. Scientific). These solutions were assessed and tested for their ability to elute the bacteria from the beads as well as their effect on *C. jejuni* viability. The M-280 Dynabead-*C. jejuni* complex was subject to treatment with 500 µl of eluting solution and mixed by tilt rotation for 3 min. After IMS bacteria containing solution was transferred to a fresh tube, this step was repeated 3 times. The eluted *C. jejuni* were concentrated by centrifugation (10000 *g*, 5 min at room temperature) and serially diluted to determine the number of colony forming units (cfu) per ml in each solution. To ensure counts of bacteria captured by Dynabeads were accurate microscopy was performed on fluorescently labelled bacteria captured by magnetic beads. Microscopy was performed using a Nikon Eclipse E600 fluorescence microscope.

Preparation of *C. jejuni* inoculum and avian host infection

Newly hatched male Ross breed chickens (Barters, Rochedale, Queensland, Australia) were used to assess the efficiency of recovery using IMS of *C. jejuni* from the contents squeezed from the caecum following colonisation. One day after hatching pre-inoculation cloacal samples were obtained and cultured to ensure absence of *C. jejuni*. The following day animals were orally inoculated with 30 µl of Brucella broth containing approximately 10^8 *C. jejuni* 81116 or 11168-O cells. To confirm and monitor host colonisation, cloacal samples were taken and cultured at days 1, 2, 3 and 4. After 5 days post-inoculation animals were sacrificed and the content of the caeca removed and weighed aseptically. Enumeration of bacteria was performed by culturable counts of *C. jejuni* as colony-forming

units per gram of caecal content. Control groups of animals tested negative for *C. jejuni* colonisation.

All animal experiments were approved by the Griffith University Animal Ethics Committee (approval number: MSC/04/08/AEC) and performed as described previously [21, 22].

Isolation of *C. jejuni* from avian host by immunomagnetic separation (IMS)

IMS of *C. jejuni* from chicken caecal content was performed as described above with the following modifications: due to the viscosity of the caecal content chilled Brucella broth was used to dilute the content to a volume of 2 ml to allow dispersion of the beads and maximum efficiency of binding of the *C. jejuni* cells to immunocoated beads. To minimize effects on bacterial gene expression all steps were done on ice. A 5 s centrifugation at 10000 *g* step was introduced to remove large particulate material that could interfere with the movement of the beads, and the supernatant was transferred to a clean tube. IMS was performed using 80 µl beads for 2 ml diluted caecal content and once isolated the bead-*C. jejuni* complex was washed 4-5 times with PBS containing 0.1% Tween 20 to remove mucus and other viscous components present within the caecal content. Bacteria were eluted by treatment with 0.05% trypsin for 30 s prior to the addition of protease inhibitor cocktail (Sigma Aldrich), the suspension was concentrated by centrifugation for 10000 *g* 5 min at 4 °C. This was performed on three separate occasions with biological replicates of six chickens per group with the contents of 12 caeca used. The isolated bacteria from the each of the caecal contents were pooled into one sample prior to splitting in two for RNA or glycan based analysis. The bacterial pellets (between 5 x 10^6 and 10^7 bacteria) were either resuspended in Qiagen RNA lysis solution and stored at -80 ºC for RNA extraction or resuspended in PBS and used immediately for Glycan microarray analysis. Concurrently;

C. jejuni were grown on CBA-Skirrow agar at 42 °C, harvested in Brucella broth and subjected to capture/isolation at the same time of processing the chicken caecal content. These captured/isolated *C. jejuni* was used as controls.

Glycan microarray

The isolated bacteria were pelleted, washed twice in PBS pH 7.4 before resuspension in 500 µl of PBS pH 7.4 containing 25 µM carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE; Molecular Probes). Fluorescent labelling was allowed to proceed for 30 min at 42 ºC, following which cells were collected by centrifugation, washed three times with PBS pH 7.4 and suspended in a final volume of 500 µl. Hybridisation, data acquisition and analysis of glycan binding was performed as previously described [23, 24].

RNA purification for microarray

Independent purification of RNA from *in vivo* isolated *C. jejuni* (81116 and 11168-O) and laboratory grown *C. jejuni* (81116 and 11168-O) was performed using the Qiagen RNeasy® mini isolation kit.

Labelling of total RNA

Total RNA from IMS isolated *C. jejuni* was either directly labelled or amplified using the Ambion Prokaryotic RNA Amplification kit as described in the manufactures protocol.

RNA from *C. jejuni* was labelled for microarray analysis with Alexa Fluor® 555 using the Superscript™ Plus cDNA indirect labelling System (Invitrogen) as described in the manufacturers protocol.

Oligonucleotide design for microarray

The arrays used for transcriptome analysis were prepared and designed in the laboratory of Prof IF Connerton and are based on the genomes of *C. jejuni* 11168, *C. jejuni* RM1221, *C. coli* RM2228 along with any miscellaneous genes and plasmids from other strains available in the database. They are composed of synthetic oligonucleotide 70-mers: Tm 71 °C ± 5 °C, location from 3' end of gene >70 bp, poly (N) tract <8 bp, stem length in potential hairpin <8 bp contiguous bases common to any non-self ORF <20 bp, cross-hyb identity to all other genes <70%.

Microarray preparation and hybridisation

The slides prepared for microarray were γ APS coated, A^+ bar-coded Schott nexterion slides. The slides were printed according to *Campylobacter* dense array gal by *Microgrid* II GOD robot (Digilab) using TAS software version V2.4.03. This had 2500 pins with a 26 x 26 pin grid configuration. The spacing between the spots was 0.165 mm and the slides were rehydrated by incubation at room temperature in a humidity chamber for 30 min followed by a 30 min incubation at 60 ºC to dry. The slides were blocked in a solution containing 100 mM ethanolamine, 1 M Tris (pH 9.0), and 0.1% SDS for 15 min at 50 ºC, then thoroughly rinsed with water and spun dry. The slides contain duplicate sets of oligonucleotides representing the genomes of *C. jejuni* NCTC11168, RM1221 and *C. coli* RM2228.

Microarray slides were pre-scanned at 532 nm before being washed and pre-blocked for hybridisation. Washes were performed in 0.2% SDS PBS and followed by a 1 h blocking step in 5 x saline sodium citrate (SSC), 0.2% SDS, 0.1% BSA. The slides were then rinsed twice with water, once with ethanol and centrifuged dry (100 *g* for 3 min). The slides were

then ready for hybridisation. Microarray hybridisations were performed using cDNA derived from both *in vitro* and *in vivo* isolated *C. jejuni*.

Data acquisition and analysis

Fluorescence intensities of array spots were measured using the ProScanArray® Microarray 4-Laser Scanner (PerkinElmer). Microarray slides were scanned using the software setting for AlexaFlour® 555, green HeNe 543.5 nm excitation laser set to 555 excitation and 565 emission. Glycan array slides were scanned on the FITC setting using the blue argon 488 excitation laser set to 494 excitation and 518 emission. Both sets of arrays were further analysed using the ProScanArray® imaging software ScanArray Express® (PerkinElmer).

RNA purification and cDNA synthesis for quantitative real time PCR

Isolation of RNA was performed as described previously and 40 ng of total RNA was processed into cDNA using the Promega Improm-II™ reverse transcriptase system with a $MgCl_2$ concentration of 3 mM and random primers. The qPCR reactions were set up to a final volume of 20 µl with 2 µl of cDNA, 5 ρmol of forward and reverse gene specific primers (Table 1) and 1x SensiMix SYBR® green PCR master mix (Quantace, UK). The PCR was performed using the Bio-Rad iQ5 system and analysed using the iQ5 software package.

All qPCR reactions were carried out using the same thermal profile conditions, an initial step of 94 ^{o}C for 5 min, followed by 45 cycles of 30 s at 94 ^{o}C, then 30 s at 48 ^{o}C and 72 ^{o}C for 1.5 mins with fluorescence measured during the 72 ^{o}C extension phase. Melt curves were produced for each amplification product and these were measured 80 times over the incremental increases in temperature. Amplification plots and melt curves were analysed by

the Bio-Rad iQ5 optical system software program. Products were reconfirmed by agarose gel electrophoresis. Three independent RNA samples were produced for the qPCR and each PCR was repeated in each RNA sample three times. The cycle threshold C(t) of gene expression in different samples was used to analyse the relative expression corrected to 16S rRNA levels present in each sample (fold regulation). To calculate the fold difference in gene expression, the difference in C(t) in each sample is calculated using the comparative C(t) method [25].

RESULTS

Optimisation of Immunomagnetic Separation (IMS) of *C. jejuni*

To test the efficiency and sensitivity of *C. jejuni* 81116 capture using *Campylobacter*-specific antibody coated magnetic beads (Dynabeads M-280), isolation was optimised for the following parameters: bacterial concentration, Dynabead concentration and incubation time (data not shown). The optimal conditions for *C. jejuni* capture by immunocoated M-280 Dynabeads were 2.4×10^7 Dynabeads for 30 minutes with a bacterial concentration of 1×10^9 (Table 2). An inoculum size between 10^6 to 10^{12} cfu ml^{-1} did not alter the final recovered yield, however, 10^9 cfu ml^{-1} was chosen as this most closely resembles the bacterial load found in the luminal content of the chicken caeca.

Following bacterial isolation with M-280 Dynabeads, an appropriate elution protocol required for downstream evaluation was established. Table 3 shows that trypsin-EDTA was the optimal solution to use for elution with <0.02% of bacteria remaining, on average, attached to the beads. Other solutions tested (citrate and DTT) resulted in little to no elution of the bacteria from the beads (Table 3).

In order to ensure that the capture and elution protocol established using *C. jejuni* strain 81116 can be used for other *Campylobacter* strains, two other commonly used strains with varying colonising and invasive capabilities were assessed. No significant difference was noted between the quantities of isolated cells of various strains of *C. jejuni* using the optimised conditions outlined above ($p>0.05$), with an average 10^5 *C. jejuni* cells for all three strains obtained after isolation and elution (not shown). Freshly coated beads were found to yield up to 1 log more bacteria than aged bead-antibody complexes with reduction of binding capacity of the beads observed over an extended period of time (data not shown).

This isolation technique was assessed in artificially spiked chicken caecal material to evaluate the efficacy of isolation of *C. jejuni* from a chicken GI tract. This allowed the assessment of the isolation protocol in the presence of components of the normal mucosa which could be a potential limiting factor for the binding of the bacteria to the immunocoated Dynabeads and subsequently affect the amount of bacteria recovered. The IMS isolation of *Campylobacter* directly from artificially spiked chicken caecal material indicated that on average 10^5-10^6 cfu ml^{-1} were recovered with approximately 10^5 *C. jejuni* cells eluted from the beads (Table 4), with no significant difference to cell numbers recovered from Brucella broth ($p>0.05$).

The number of bacteria captured per bead was investigated using CFDA-labelled bacteria, using the methodology reported for the glycan microarray experiments, followed by observation under light and fluorescence microscopy. Our results were similar to those reported for *C. jejuni* capture with Dynabeads in previous experiments [19, 26]. This

showed a random assortment of bead-bacteria interactions with typically more than one bead associated with one bacterium but never more than one bacterium per bead or grouping of beads (data not shown). These results indicate that no bias is present in the counts reported for Tables 2-4 and is confirmed by the number of bacteria in culturable counts following removal of the beads (Tables 3-5).

Isolation of *C. jejuni* from colonised chickens

One day old chickens were colonised with *C. jejuni* 81116 and were recovered from the chicken caeca using the optimised IMS technique developed. The rabbit anti-*C. jejuni* polyclonal antibody used to coat the M-280 Dynabeads was assessed prior to *in vivo* experimentation for its binding capacity of normal flora and mucosal components using a dot blot assay, with the results indicating that there is no substantial reactivity with normal flora or mucosal components in the chicken caeca (data not shown).

Colonisation of chickens was established as 1.5×10^9 cfu g^{-1} of caecal content, with other normal flora evident in the caeca identified as *Proteus* spp. After IMS the recovered *C. jejuni* cell number averaged at 10^5 cfu g^{-1} caeca with approximately 10^1 *Proteus* spp. present using the immunocoated M-280 Dynabeads with a high proportion of captured bacteria eluted (Table 5). This level of isolation of *C. jejuni* was not significantly different to the maximum possible cell number isolated in the control *in vitro* isolation of campylobacters using IMS system, whether from broth or spiked caecal contents (Tables 2 and 4).

With successful isolation of *C. jejuni* from chicken caecal content further testing and analysis of the bacteria was performed. Carbohydrate binding analysis and gene expression

analysis using both microarray and qPCR were performed to identify differences between laboratory grown bacteria and those isolated *in vivo*.

Glycan Array

C. jejuni isolated by IMS were assessed for their ability to bind various carbohydrate structures using our previously described glycan array methodology (Day *et al.*, 2009). *C. jejuni* 81116 grown on Columbia 5% horse blood agar plates under standard laboratory conditions at 42 ºC, known core body temperature of chickens, was used as the reference strain to assess the binding of *C. jejuni* directly isolated from the chicken caeca. The binding specificity was then compared to that of *C. jejuni* 81116 isolated from chicken caeca. Only glycan binding found to be significantly different between isolates tested are described (Fig. 1, Table 6). The full data set is available in the Supplementary Material (Table SM1).

In general similar binding was observed for all terminal galactose (Gal) structures with some notable exceptions (Fig. 1a). Binding to disaccharide Galβ1-3/4/6Gal structures was observed to be significantly higher in the laboratory grown strain (e.g. 2B Fig. 1a; $p<0.05$). Binding of the *C. jejuni* 81116 isolated from the chicken host appeared to bind a disaccharide Galβ1-3GlcNAc (1A) significantly greater than for the laboratory grown strain ($p<0.05$). However the addition of any sugars to lengthen this disaccharide structure resulted in a loss of the observed binding.

The binding of the laboratory grown strain to repeating N-acetylglucosamine (GlcNAcβ1-4GlcNAc repeats) containing structures was consistent across a range of repeat lengths. For the *in vivo* isolated strain, a general increase in binding was observed as the number of

GlcNAc repeats increased to a maximum of 4 sugars (4B). Binding of *in vivo* isolated bacteria to GlcNAc structures longer than 4 repeats appeared to decrease (Fig. 1a).

The binding to mannose (Man) structures by *C. jejuni* 81116 was found to be different between the laboratory grown and *in vivo* isolated bacteria. The bacteria isolated from the *in vivo* host showed greater binding to α1-3 linked di-Man (5D) when compared to the binding by the laboratory grown strain, however the laboratory strain favoured binding to α1-4 linked di-Man (5H) (Fig. 1a).

The *in vivo* isolated *C. jejuni* also showed reduced binding to the shorter fucosylated glycans compared with laboratory grown bacteria (7H & M; Fig. 1a) and less binding was also noted to glycans with the branched fucose linked to a non-terminal Gal/Glc (7H, L & M; Fig. 1a). The binding to glycans with subterminal fucose by *C. jejuni* 81116 isolated from chickens was not reduced in the presence of a structures containing GlcNAc or GalNAc (see Supplementary Material; Table SM1).

Differences were observed between laboratory grown and IMS isolated *C. jejuni* in the binding of negatively charged glycans such as sialylated and sulphated structures with significantly more binding observed for laboratory grown bacteria. Although, in general, the binding observed for *C. jejuni* 81116 to sialic acid was lower than the other structures tested. Few structures show significant binding for either isolate, however the biantennary structure 10D (Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc; Fig. 1b) was strongly bound by the laboratory grown strain. Binding to the unbranched structures containing Neu5Acα2-3Galβ1-3/4GlcNAc (10C & 10K) was also

observed for the laboratory grown strain. Carageenans (polysaccharide extracts from *Chondrus crispus*) to glycosaminoglycans (GAG) and various digests of GAG structures were also present on the array. Similarly the binding of the laboratory grown bacteria to negatively charged glycans such as carageenans to glycosaminoglycans (GAG) and various digests of GAG structures was greater than the *in vivo* isolated bacteria (12A-13M; Fig 1b). Binding to negatively charged fucosylated structures such as sulfo-Lewis^a and sulfo-Lewis^x (8A & B; Fig. 1b) was also higher for the laboratory grown *C. jejuni* strain 81116 when compared to *in vivo* isolated bacteria ($p<0.05$).

As shown in Table 5, a *Proteus* spp. was co- isolated at low level along with the *C. jejuni* from the chicken caeca and therefore was also subjected to glycan array analysis to investigate if these bacteria influenced *C. jejuni* binding to the glycan array. No significant binding of glycan structures above background by *Proteus* spp. was observed when using 10^4-10^6 bacteria.

Gene expression analysis of host-passaged *C. jejuni* isolated by IMS

Initial attempts were made to perform transcriptome wide analysis of isolated bacteria using microarrays. For analysis of gene expression, total RNA was extracted from *C. jejuni* strains re-isolated from the chicken host and processed for hybridisation to an oligonucleotide based microarray with and without pre-amplification. The scan of the array showed that the labelled cDNA obtained without pre-amplification was successfully hybridised to the chip with sufficient signal only from the most highly expressed present, therefore no analysis could be performed (data not shown). When pre-amplification was utilised, the RNA from IMS isolated bacteria from the chicken caecal content was not uniformly amplified. No two amplifications produced the same result upon analysis and

therefore no data of value was obtained (data not shown). Unfortunately, no reliable results could be obtained from either approach for complete transcriptome analysis (data not shown). Based on bacteria obtained directly from laboratory cultures, approximately 10^9 bacteria would be sufficient for successful microarray analysis, however, this would require between 1000 and 10 000 IMS isolations to be performed from the caecal content.

Far greater success was achieved with qPCR analysis of the RNA from IMS isolated *C. jejuni*. We chose to look at factors that are involved in virulence including; chemotaxis, toxin and cell surface glycosylation genes. The chemotaxis receptor genes *tlp1, 2, 3, 4, 7* and *10*, the cytolethal distending toxin genes *cdtA, cdtB* and *cdtC,* and genes involved in cell surface glycosylation *waaC* (heptosyltransferase I) and *msbA* (ABC glycoconjugate transporter) were tested with qPCR (Table 7). The chemotaxis and toxin genes were strongly up-regulated after passage through an avian host while the two cell surface glycosylation genes were strongly down regulated (Table 7).

DISCUSSION

This paper describes the development of an optimised IMS method for the rapid isolation of *C. jejuni* from avian host. The IMS procedure allowed for the isolation of sufficient bacteria for the analysis of gene expression by qPCR and for the profiling of glycan binding. Binding to negatively charged glycans was reduced in *C. jejuni* 81116 isolated from the luminal content of chicken caeca when compared to laboratory grown bacteria. qPCR analysis identified the regulation of genes involved in chemotaxis, toxin production and cell surface glycosylation.

The isolation of 6 x 10^5 *C. jejuni* from broth and ~10^5 *C. jejuni* from the caecal content of colonised chicken hosts using our IMS protocol is approximately 10-times greater than that recovered by Yu *et al.* 2001, using IMS to isolate *C. jejuni* from broth and ground poultry products. The number of *C. jejuni* required in the inoculum to yield the maximum isolated bacteria (10^6 cfu g^{-1}) was similar to that reported by Yu *et al*. (2001) but 100-fold lower than that reported by Lamoureux *et al.* (1997). These data indicate that the optimised conditions described in this study compare extremely well with or exceed previous IMS conditions reported for *C. jejuni* [18, 19, 26].

The efficient elution of three different strains of *C. jejuni* from the beads with 0.05% trypsin-EDTA indicated that this methodology is likely to be universally applicable for all *C. jejuni* strains. There was no significant difference in the ability of this IMS system to recover *C. jejuni* cells from *in vitro* cultures or chicken caecal contents, indicating that the presence of gut flora and other mucosal components did not have a major influence on the ability to recover *C. jejuni* cells *ex vivo*. Low levels of *Proteus* spp. (0.01% of total isolated bacteria) were co-isolated with *C. jejuni* from chicken caeca; however this level of contamination is unlikely to have affected the outcomes of either the glycan array or qPCR analysis. Contamination by *Proteus* spp. may be due to the abundance or size of this organism which leads to co-localisation with the M-280-*C. jejuni* complex. Furthermore, contaminating bacteria may be trapped within the mucosal material and remain associated with the beads rather then bound to the beads themselves, and therefore not completely removed by the washing steps used in this study.

Glycans are important structures for the adherence of *C. jejuni* to host cells [24]. The glycan array analysis of bacteria allows interrogation of glycoconjugates potentially

involved in host-bacteria interactions [24]. The analysis revealed a number of differences in overall binding between *in vivo* isolated and laboratory grown bacteria. The main observed differences were in the ability to bind extended glycan structures, and negatively charged sugar structures.

It is interesting to note that the laboratory grown strains bound negatively charged glycans containing groups such as sulphur or sialic acid residues whereas the bacteria recovered from chicken caeca did not. These observations are similar to that reported for *C. jejuni* NCTC 11168 and are consistent with the contention that the binding of sialic acid may be important for initial host-pathogen interaction but not for prolonged colonisation [24]. That is, the *C. jejuni* isolated from chicken caecal content was already present in and adapted to its preferred gastrointestinal niche and so is unlikely to require the ability to recognise sialic acid structures.

IMS isolated *C. jejuni* was also successfully used to isolate whole cell RNA that enabled the identification of genes differentially expressed by *C. jejuni* when colonising chicken caeca. Each of the chemotaxis receptor genes were up-regulated *in vivo,* highlighting the importance of these genes in the infection and colonisation of hosts by *C. jejuni*. The most striking result was the up-regulation of *tlp1* with an almost on/off response between *in vivo* and *in vitro* expression noted. We have previously shown in Hartley-Tassell *et al* (2010) that *tlp1*, the aspartate receptor of *C. jejuni*, is crucial for the prolonged colonisation of chicken hosts [27]. Other chemotaxis receptor genes have been found to be important to colonisation of chickens including *tlp2*, *4* and *7* [28, 29]. *C. jejuni* isolated from the luminal content of the chicken caeca also had increased expression of the *cdt* genes in agreement to analysis performed by Tu *et al.,* (2008), which showed higher *cdt* expression in the

presence of MUC2. The down regulation of *waaC* and *msbA* was unexpected, as lipooligosaccharide (LOS) production and cell surface glycosylation are known to be important for colonisation [30, 31], and *waaC* mutants have decreased adherence/invasion potential [32, 33]. However, considering that regulation of the LOS presented by *C. jejuni* was shown to be dependent on the growth temperature *in vitro* [34], it is possible that LOS within hosts, particularly avian hosts, may be more variable *in vivo*.

In conclusion, the IMS protocol described here was successfully used to isolate and purify *C. jejuni* from *in vivo* host tissue, and can be used to identify factors involved in host bacteria interactions without the loss of potential information by sub-culturing of isolated bacteria. The observed changes in glycan binding and gene expression validate this approach and demonstrate *in vivo* studies requiring recovery of bacteria for analysis can now be utilised in order to fully understand *C. jejuni* biology.

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Table 1. Primers used for qPCR gene expression analysis.

Primer name	5`-3` primer sequence.
Tlp1p F	TTG TTA TCG TTT ACG CTG ATG
Tlp1p R	TGG AAG ATC TTT ATT ATA ATT TTT TAA GGG TTT AA
Tlp2p F	CAT ATG CAA GCA ATT TTT CAT GAA GTT GTG A
Tlp2p R	CTC GAG TTA TTT ATA AAC TGG AGC TTC TAT TTG TT
Tlp3p F	CAT ATG ACC TCA CTA TAT GAA AGC ACT CTT
Tlp3p R	CTC GAG TTA TGC AGC TTT ATA AAT AGG TTT ATT TAT A
Tlp4p F	CTC GAG GAT TCG AGA AAC AAT ACA TAT GAA TT
Tlp4p R	CTC GAG TTA TTG TTT CAT TAA AAT AGA ATT AAC AGC
Tlp7p F	CAT AGT TTT AAA AAT ACT GCC AAT AAA ATG AG
Tlp7p R	CTC GAG TTA AGA TTG ACT GGT TTT GCT TAT ATC
Tlp10p F	CAT ATG AAC TAT TCT TCA TCT AAA GAT AAT AA
Tlp10p R	CTC GAG TTA TTT AAA TAA ATT AGA TTG TTC TAT AGT
CdtB F	ATC TTT TAA CCT TGC TTT TGC A
CdtB R	TTG CGC TAG TTG GAA AAA CCA CT
Therm 1 (16S)	TTA TCC AAT ACC AAC ATT AGT
Therm 2.1 (16S)	GAA GAT ACG GTG CTA TTT TG
CdtA F	ATG CAA AAA ATT ATA GTT
CdtA R	TCA TCG TAC CTC TCC TTG
CdtC F	GGA GAT TTG AAA GAT TTT AGG G
CdtC R	CAT CTT GAC AAG ATT TTG CTC C
Cj0803 F	GGT TTA GCA GGA GGC TTT GAT AAA AAT G
Cj0803 R	CAA CTC TCT TAA ATT TTT AGT ATT AGG AG
Cj1133 F	GAT ATA GAA ATT CAT TGG TTT GTA GAT G
Cj1133 R	CTA CGC TAA AAA TAT CTT GTT TAA AGG C

Table 2. Optimal parameters for efficient *C. jejuni* recovery. *In vitro* cultured *C. jejuni* were incubated at different concentrations of anti-*C. jejuni* coated beads for 15-60 min. Number of bead captured cells was determined by culturable counts.

Concentration of beads	Inoculum (cfu ml-1)	Incubation time (min)	Number of bead captured cells (cfu ml-1)
1.2×10^7	1×10^9	15	$6.0 \pm 0.17 \times 10^5$
		30	$8.3 \pm 0.17 \times 10^5$
		60	$5.0 \pm 0.14 \times 10^5$
2.4×10^7	1×10^9	15	$1.1 \pm 0.14 \times 10^6$
		30	$2.7 \pm 0.06 \times 10^6$
		60	$2.5 \pm 0.09 \times 10^6$
6×10^7	1×10^9	15	$1.0 \pm 0.05 \times 10^6$
		30	$2.9 \pm 0.1 \times 10^6$
		60	$3.1 \pm 0.12 \times 10^6$

N=6 independent experiments; +/- 1 SD

Table 3. Evaluation of different antibody elution solutions for the recovery of *C. jejuni.*

Elution solution	Number of cells captured *	Number of cells eluted *	Cells remaining on beads after elution *
0.1 M citrate	$1.6 \pm 0.08 \times 10^6$	45 ± 5.4	$1.0 \pm 0.12 \times 10^6$
20 mM DTT	$2.4 \pm 0.11 \times 10^6$	15 ± 3.7	$5.7 \pm 0.06 \times 10^5$
50 mM DTT	$2.9 \pm 0.1 \times 10^6$	30 ± 3.4	$1.1 \pm 0.11 \times 10^6 \pm$
0.05% trypsin-EDTA	$1.8 \pm 0.09 \times 10^6$	$6.3 \pm 0.14 \times 10^5$	100 ± 14.7

* cfu ml^{-1}; average +/- 1SD (N=6)

Table 4. Capture and elution of *C. jejuni* cells from artificially spiked chicken caecal content. Laboratory cultures of *C. jejuni* (3 strains) were mixed with caecal homogenates, incubated with anti-*C. jejuni* coated magnetic beads and eluted with trypsin. Bacterial numbers were determined by culturable counts.

***C. jejuni* strain**	**Number of cells captured ***	**Number of cells eluted ***	**Cells remaining on beads after elution ***
81116	$2.4 \times 10^6 \pm 1.2 \times 10^5$	$5.2 \times 10^5 \pm 6.4 \times 10^3$	$1.5 \times 10^2 \pm 12.9$
11168	$8.5 \times 10^5 \pm 3.8 \times 10^4$	$3.9 \times 10^5 \pm 1.0 \times 10^4$	$3.0 \times 10^2 \pm 12.5$
11168-O	$6.3 \times 10^5 \pm 1.7 \times 10^4$	$1.6 \times 10^5 \pm 1.0 \times 10^4$	$2.2 \times 10^2 \pm 12.9$

* cfu ml^{-1}

Table 5. Recovery of *C. jejuni* 81116 from infected avian host. Caecal homogenates were incubated with anti-*C. jejuni* coated magnetic beads and eluted with trypsin. Bacterial numbers were determined by culturable counts.

Organism recovered	**cfu per g caecal content**	**Cells isolated per g caecal content**	**Cells eluted from 2.4×10^7 Dynabeads ***
C. jejuni 81116	1.5×10^9	1.9×10^5	9.8×10^4
Proteus spp.	1.0×10^7	8.6×10^1	1.3×10^1

* cfu ml^{-1}

Table 6. Glycan structures for which significant difference in *C. jejuni* binding were detected.

ID	Glycan name	Glycan structure
		Neutral glycans
1A.	Lacto-N-Biose I	Galβ1-3GlcNAc
1G	Lacto-N-tetraose	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
1H	Lacto-N-neotetraose	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
1J.	Lacto-N-hexaose	Galβ1-4GlcNAcβ1-6(Galβ1-3GlcNAcβ1-3)Galβ1-4Glc
1P.	Linear B-6 Trisaccharide	Galα1-3Galβ1-4Glc
2B.	β1-6Galactobiose	Galβ1-6Gal
2E.	P1 Trisaccharide	Galα1-4Galβ1-4GlcNAc
4A.	N,N'-Diacetyl chitobiose	GlcNAcβ1-4GlcNAc
4B.	N,N',N''-Triacetyl chitotriose	GlcNAcβ1-4GlcNAcβ1-4GlcNAc
4E.	Muramyl disaccharide	GlcNAcβ1-4MurNAc
5B.	Bianntennary N-linked core pentasaccharide	GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Man
5D.	α1-3-Mannobiose	Manα1-3Man
5E.	α1-4-Mannobiose	Manα1-4Man
5H.	α1-3,α1-3,α1-6-Mannopentaose	Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man
7H.	3'-Fucosyllactose	Galβ1-4(Fucα1-3)Glc
7L.	Lactodifucotetraose	Fucα1-2Galβ1-4(Fucα1-3)Glc
7M.	Blood Group B Trisaccharide	Galβ1-3(Fucα1-2)Gal
		Negatively charged glycans
8A.	Sulpho Lewis[a]	SO_3-3Galβ1-3(Fucα1-4)GlcNAc
8B.	Sulpho Lewis[x]	SO_3-3Galβ1-4(Fucα1-3)GlcNAc
10C.	Sialyllacto-N-tetraose α	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc
10D.	Monosialyl, monofucosyllacto-N-neohexose	Galβ1-4(Fucα1-3)GlcNAcβ1-6(Neu5Acα2-6Galβ1-4GlcNAcβ1-3)Galβ1-4Glc
10K.	3'-Sialyllactosamine	Neu5Acα2-3Galβ1-4GlcNAc
12A.	Neocarratetraose-41, 3-di-O-sulphate (Na+)	$C_{24}H_{36}O_{25}S_2Na_2$
12E.	Neocarraoctaose-41, 3, 5, 7-tetra-O-sulphate (Na+)	$C_{48}H_{70}O_{49}S_4Na_4$
12H.	DUA-GlucNS-6S	$C_{12}H_{16}NO_{16}S_2Na_3$
12I.	DUA-2S-GlucNS	$C_{12}H_{16}NO_{16}S_2Na_3$
12N.	DUA-GalNAc-4S (Delta Di-4S)	$C_{14}H_{19}NO_{14}SNa_2$
12O.	DUA-GalNAc-6S (Delta Di-6S)	$C_{14}H_{19}NO_{14}SNa_2$
13G.	Hyaluronan fragment (8mer)	$(GlcAβ1\text{-}3GlcNAcβ1\text{-}4)_n$ (n=8)
13M.	Chondroitin 6-sulfate	$(GlcA/IdoAβ1\text{-}3(±6S)GalNAcβ1\text{-}4)_n$ (n<250)

Table 7. Comparative qPCR analysis of cytotoxin, chemotaxis and glycosylation gene expression in *C. jejuni* 81116 grown in the laboratory and after passage through chicken.

Gene name/number	Fold Change
cdtA/Cj0079c	2272 ± 719
cdtB/Cj0078c	1694 ± 164
cdtC/Cj0077c	209 ± 20
tlp1/Cj1506	10544592 ± 3861139
tlp2/Cj0144	243 ± 40
tlp3/Cj1564	67690 ± 4718
tlp4/Cj0262	529 ± 70
tlp7/Cj0951/52	68 ± 20
tlp10/Cj0019	7.0 ± 2.3
msbA/Cj0803	0.178 ± 0.07
waaC/Cj1133	0.07 ± 0.007

N=3 independent biological repeats performed in triplicate. Relative fold difference is calculated by the $2^{\Delta\Delta Ct}$ between cells isolated from chickens and laboratory grown cells (fold change ± 1 SD).

Figure 1. Glycan structures with significant different binding: comparison of *in vivo* isolated and laboratory grown *C. jejuni* 81116. (a) Fluorescence of binding to neutral glycan structures (terminal galactose, glucose, mannosylated and fucosylated structures), (b) Fluorescence of binding to negatively charged glycans (sialylated, sulphated and GAG structures). Black bar *in vivo* passaged cells, grey bar laboratory grown cells (42ºC) ($p<0.05$).