

Campylobacter Seroreactivity in Adults with Recent Onset of Crohn's Disease

Jacqueline I Keenan, Nina Salm, John F Pearson, Richard B Gearry, Andrew S Day

Jacqueline I Keenan, Nina Salm, Department of Surgery, University of Otago Christchurch, PO Box 4345, Christchurch 8140, New Zealand

John F Pearson, Department of Public Health and General Practice, University of Otago Christchurch, PO Box 4345, Christchurch 8140, New Zealand

Richard B Gearry, Department of Medicine, University of Otago Christchurch, PO Box 4345, Christchurch 8140, New Zealand

Andrew S Day, Department of Paediatrics, University of Otago Christchurch, PO Box 4345, Christchurch 8140, New Zealand

Correspondence to: Jacqueline Keenan, Department of Surgery, University of Otago Christchurch, Christchurch, New Zealand. jacqui.keenan@otago.ac.nz

Telephone: +064-3-3640-570 Fax: +064-3-3641-427

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ABSTRACT

AIM: The rate of campylobacteriosis in New Zealand is amongst the highest in the developed world and is especially prevalent in the 15-29 year old age group, a demographic that is within the peak age group of diagnosis of Crohn's disease in our community. With evidence mounting for a role for acute bacterial enteritis in the aetiology of Crohn's disease, our aim was to determine if a link exists between recent campylobacter enteritis and the first occurrence of this debilitating disease.

METHODS: An immunoassay was developed and validated by assaying sera from 420 community volunteers and 50 people with recently confirmed campylobacter enteritis. Selection of the test population of Crohn's disease patients ($n=126$) was based upon disease diagnosis within 2 years of recruitment. All sera were tested following absorption with *Helicobacter pylori* to reduce risk of false positive responses.

RESULTS: *Campylobacter* IgG seroreactivity was increased in 9.6% of males with recent onset of Crohn's disease, compared to 1.9% of male volunteers from the same population ($p=0.016$). In contrast, only one of 75 females recently diagnosed with Crohn's disease had serological evidence of *Campylobacter* infection. Coincidentally, one quarter of the *Campylobacter*-infected controls failed to mount a significant IgG antibody response to infection.

CONCLUSIONS: Our findings suggest a link between recent *Campylobacter* infection and the onset of CD in males only. The failure to mount an IgG response to infection in a subset of infected individuals warrants further investigation as an independent risk factor for subsequent gastrointestinal disease.

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Key words: Crohn's disease; Campylobacter; ELISA; Immune tolerance

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INTRODUCTION

Crohn's disease (CD) incidence has risen exponentially over the last five decades and the Canterbury province in New Zealand has one of the highest rates reported worldwide^[1]. The aetiology of this disease remains enigmatic but a role for gut flora is strengthened by studies that demonstrate diminished inflammation following antibiotic and probiotic treatment^[2]. Moreover, animal models that provide evidence that gut microbiota play an essential role in triggering a Crohn's-like colitis strengthen this association^[3]. Whether the initial host response is directed at a specific bacterial pathogen or fuelled by a genetically predisposed loss of tolerance by the immune system to the gut microbiota en masse is less clear. However, once mucosal inflammation is triggered, chronic enterocolitis ensues.

Recent data demonstrates that acute campylobacter enteritis can be associated with an increased risk of first occurrence of CD^[4,5]. Consistent with this hypothesis is a growing body of evidence that these bacteria have the potential to cross the normally impermeable gut epithelial barrier^[6,7], and are therefore capable of initiating an inflammatory response that may subsequently develop into IBD in a subset of individuals^[8]. Intriguingly, the rate of campylobacteriosis in New Zealand is amongst the highest in the developed world and is especially prevalent in the 15-29 year old age group^[9], a demographic that is within the peak age group of diagnosis of CD^[1].

Following acute campylobacteriosis there is an increase in antibody responsiveness in most individuals^[10]. Whereas antibody

levels gradually decrease with time, they reportedly remain higher than those seen in uninfected controls as long as 20 months post-infection^[10]. While detection of seropositivity to *Campylobacter* spp. antigens does not prove a causative role in the aetiology of IBD, it does add weight to the hypothesis that infection may increase the risk of disease in some patients. Accordingly, we developed an assay to measure serum IgG antibodies to *C. jejuni* in patients recently diagnosed with CD to determine if a similar association exists in our community.

METHODS

Controls

Sera collected from people randomly selected from the Christchurch electoral roll for an unrelated study^[11] were used as a control population. Samples from equal numbers of males and females ranging in age from 18 to 86 years of age were selected. The samples were grouped, by age, into six groups (18-30, 31-40, 41-50, 51-60, 61-70 and 70+ years) and each group consisted of 70 sera ($n=420$). This group comprised the community volunteers. An additional 50 people with recent campylobacter enteritis (diagnosed by culture and/or PCR) were asked to provide a blood sample approximately 3 weeks after the onset of symptoms^[12]. This group comprised the infection controls. All subjects provided written consent.

Crohn's disease population and serum samples

The study population was selected on the basis of a diagnosis with CD within two years of recruitment from a large population-based cohort of more than 1 400 patients, as described previously^[1]. Using diagnosis based on standard endoscopic, histological or radiological criteria^[13], 126 subjects with recently-diagnosed CD were identified. This group was comprised of 51 males and 75 females with a median age of 43 (range, 18 to 93 years). The range of disease location within this patient cohort included one individual with ileal disease, 57 with ileocolonic and 68 with colonic disease. Fourteen patients with ileocolonic disease and 5 patients with colonic disease also presented with perianal disease. Each patient had provided a blood sample at the time of recruitment and it was these samples that were screened for anti-*Campylobacter* seroreactivity. Again, all individuals provided written consent.

Preparation of *C. jejuni* whole cell sonicate for serology

A pool of ten strains of *C. jejuni* representing the most prevalent disease-causing strains in the Canterbury region was used for preparation of the antigen. These strains, which included the Penner type strains 1, 2, 4, 6, 8, 11, 12, 21, 33, and 44, were provided by Dr Stephen On (Environmental & Scientific Research, Christchurch). Following growth on 5% sheep blood agar at 37°C for 48 h in a CO₂ incubator, the bacteria were harvested into sterile phosphate-buffered saline (PBS), heated at 100°C for 10 min (to kill bacteria), and sonicated with 15-s pulses over a 2-min period^[10]. Debris was removed by centrifugation (12 000 x g, 10 min, 4°C). Sodium azide was added to a final concentration of 8 mg/mL and the protein concentration determined using standards prepared with bovine serum albumin (BSA) in PBS.

Enzyme-linked immunosorbent assay

Sera were examined for IgG antibodies to *Campylobacter* spp. by an indirect, enzyme-linked immunosorbent assay (ELISA), with the optimal concentration of antigen (0.5 µg of *C. jejuni* sonicate per well) and conjugate (1/4000 dilution) determined by checkerboard titration. Briefly, 96-well ELISA plates (Nunc Maxisorp, Roskilde,

Denmark) were coated with antigen for 1 h at room temperature (RT). Unbound antigen was removed by washing three times with PBS-0.1% Tween 20 (PBS-T) and wells were blocked with 2% goat serum in PBS-T overnight at 4°C before sera (diluted 1/1000 in 0.2% goat serum in PBS-T) were added (in duplicate). After incubation for 1 h at RT, a horse radish peroxidase-conjugated anti-human IgG antibody was added to the washed wells and the plate incubated for another hour before addition of TMB+ substrate (Dako, Carpinteria, CA, USA). The reaction was stopped with 2.5 M sulphuric acid and absorbance values were read at 450 nm in a spectrophotometer. All data were normalised to a positive control that was included on every plate

Absorption of sera with *Helicobacter pylori* antigens

To exclude false positives through cross-reactivity with antibodies to the closely related *H. pylori*, all sera were tested for anti-*C. jejuni* activity following absorption with sonicated *H. pylori* bacteria^[14]. Serum samples were diluted 1/1000 in a sonicate prepared from 5 *H. pylori* strains (final concentration 70 µg/mL in 0.2% goat serum in PBS-T) and tested by ELISA, as detailed above.

Statistical analysis

One-way analysis of variance (ANOVA), Fisher's exact test or t-tests were used where appropriate, all tests were considered significant if the two sided *p* value was less than 0.05. Significant ANOVAs were followed by Dunnett's multiple comparison posthoc test against the youngest age group. Linear and logistic regression models were used to assess the relationship between seroreactivity and seropositivity (respectively) and age and gender.

RESULTS

Developing an ELISA to detect serum IgG responses to campylobacter infection

To establish the specificity of the ELISA, the results from the community were averaged, and the standard deviation (SD) from the mean was determined. From this, a level of 2 SD above the mean was calculated at 94.7 arbitrary units and used as the threshold for significant elevation^[10]. Using this threshold, significantly increased *C. jejuni* IgG seropositivity as detected in 16 (3.8%) of the volunteers and in 23 (46%) of the individuals with recently diagnosed campylobacter enteritis, yielding sensitivity (Se) 46.0% (95% CI 31.8%-60.7%) and specificity (Sp) 96.2% (95% CI 93.9%-97.8%). There was evidence for a linear increase in *C. jejuni* IgG seroreactivity of 2.4 arbitrary units per decade after 18, (95% CI 1.0-3.8, $p=0.0009$; Figure 1A) however this did not translate into a significant increase in seropositivity with age.

There was no significant difference in the level of IgG seroreactivity when a subset of the infection control sera was screened against a panel of non-*jejuni* strains (including *C. coli*, *C. lari* and *C. upsaliensis*). This indicates that the immunoassay has the potential to detect infections with species other than *C. jejuni*. It also intimates that infection with non-*jejuni* strains was not the reason for the low sensitivity of the ELISA. Instead, antigenic cross-reactivity with the closely related gastric pathogen *H. pylori* was identified as a contributing factor. When the 420 volunteer sera were retested for *Campylobacter* IgG seropositivity after pre-mixing *H. pylori* antigens, a level of 2 D above the mean was calculated at 59.4 arbitrary units instead of the original 94.7. The significant age-related increase in *C. jejuni* seroreactivity within this group was still evident (1.7 arbitrary units per decade after 18, (95% CI 0.85-2.61, $p=0.0001$; figure 1b) and this was not gender-related (Figure 1c). There was no

evidence of an increase in seropositivity with age ($p=0.9$). However, the lower threshold in the modified assay meant that the number of population control samples that were above the cut-off value (and therefore considered true positives) fell to 2.9%. Inversely, the percentage of infection control samples above the cut-off increased from 44 % to 76 % (38 of 50; figure 2a), yielding sensitivity (Se) 76.0% (95% CI 61.8%-86.9%) and specificity (Sp) 97.1% (95% CI 95.1%-98.5%). No significant age- (Figure 2b) or gender-related (not shown) difference was detected among the infection controls. However, in each age band, a similar proportion (20% to 30%) of the participants failed to develop an IgG response to infection (Figure 2c).

***Campylobacter* IgG seropositivity in patients with recent onset CD**

The Receiver Operator (ROC) curve (Figure 3) showed the modified ELISA to have an acceptable area under the curve (AOC) of 94.1% (95% CI 90.2% - 97.9%).

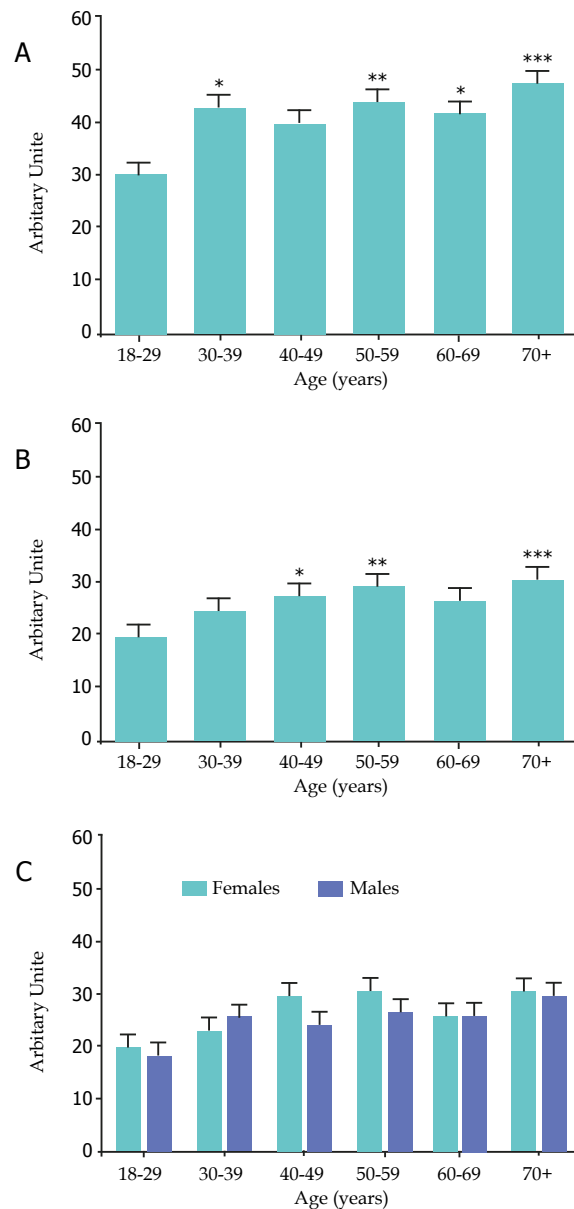


Figure 1 Prevalence of *C. jejuni* IgG seroreactivity in each decade for 420 volunteer controls. *, **, ***; results are statistically significant from the youngest age group ($p<0.05$, 0.01 and 0.001, respectively).

Using this assay, *Campylobacter* IgG seroreactivity was found to be increased in six of the patients with recent onset CD. This was not related to age ($p=0.40$), disease location ($p=0.41$) or duration of symptoms prior to diagnosis ($p=0.07$). Likewise, the distribution

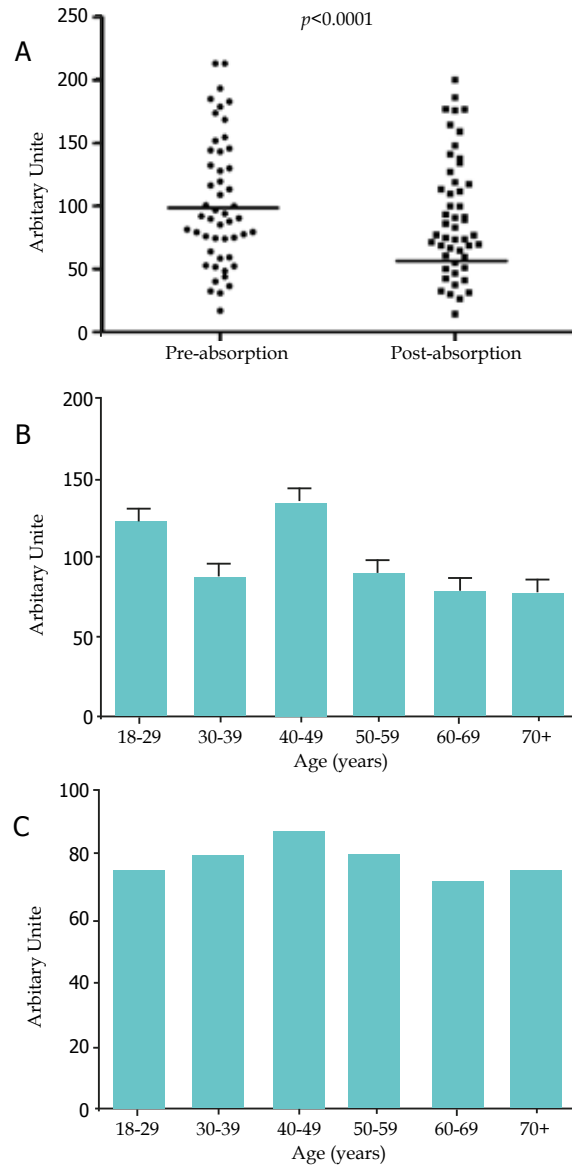


Figure 2 Prevalence of *C. jejuni* IgG in 50 infection controls.

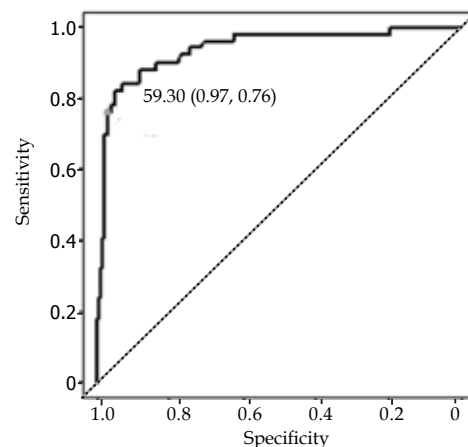


Figure 3 Receiver operator characteristic (ROC) curve of the *Campylobacter* ELISA, based on the values obtained from healthy controls ($n=420$) and *Campylobacter* enteritis patients ($n=50$).

of disease behaviour that included stricturing, penetrating and inflammatory disease was evenly spread across the *Campylobacter*-positive and -negative patients (results not shown). Intriguingly, more males than females with recent onset CD had increased IgG reactivity to *Campylobacter* spp. antigens ($p=0.039$, Fisher's exact test, two-sided). Furthermore, seroreactivity was increased in 9.6% of males with recent onset of CD, compared to 1.9% of healthy male controls from the same population ($p=0.016$, Fisher's exact test, two-sided). In contrast, only one of 75 (1.3%) females recently diagnosed with CD had significantly elevated IgG antibodies to *C. jejuni* (Table 1). This was not significantly different to the female volunteer control group.

Table 1 *Campylobacter* IgG seropositivity in recently diagnosed CD patients ($n=126$) versus community volunteers and infection controls ($n=420$ and 50 respectively).

ALL	Seronegative (%)	Seropositive (%)	OR ¹	<i>p</i> value ²
Volunteers	408 (97.1)	12 (2.9)	1	
Infection controls	12 (24)	38 (76)	107.7	<0.0001
Crohn's Disease	120 (95.2)	6 (4.8)	1.7	0.391
MALES	Seronegative (%)	Seropositive (%)	OR ¹	<i>p</i> value ²
Volunteers	206 (98.1)	4 (1.9)	1	
Infection controls	5 (26.3)	14 (73.7)	144.2	<0.0001
Crohn's Disease	46 (90.2)	5 (9.8)	6.0	0.016
FEMALES	Seronegative (%)	Seropositive (%)	OR ¹	<i>p</i> value ²
Volunteers	202 (96.2)	8 (3.8)	1	
Infection controls	7 (22.6)	24 (77.4)	86.57	<0.0001
Crohn's Disease	74 (98.7)	1 (1.3)	0.34	0.453

¹Odds Ratio; ² values from Fisher exact test.

DISCUSSION

The measurement of serum antibodies is considered a useful tool for identifying recent *Campylobacter* infection^[10,12]. Accordingly, we developed an ELISA to measure *Campylobacter* IgG seroreactivity in patients recently diagnosed with CD as a means to determine if infection is associated with increased risk of disease. In developing and validating the assay we observed an age-related increase in *Campylobacter* IgG seroreactivity in our volunteer population. Whereas this has been reported elsewhere^[15], the high cutoff in our assay led us to consider that seropositivity to antigenically-related^[16] gastric pathogen *H. pylori* (as previously shown to increase with age in these same individuals^[11]) might be artificially inflating *Campylobacter* seroreactivity. This was confirmed when we retested our volunteer cohort using a modified ELISA that included absorption of the sera with *H. pylori* antigens. The sensitivity of the assay increased from 44% to 76%, comparable to other reported studies^[10,12,17].

We chose to measure serum IgG responses to infection as these reportedly remain elevated for longer whereas serum IgM and IgA responses decline more rapidly over time^[18]. Accordingly, our finding that the average level of seropositivity in the CD patient cohort was considerably lower than that in individuals with recently diagnosed infection (67.5±4.6 and 109±6.6, respectively) was not unexpected, given that the blood samples were collected from the patient cohort up to two years post-diagnosis of CD^[10,12]. Using the modified ELISA, we found an association between significant elevations of serum IgG antibodies to *C. jejuni* in males with recent onset of CD compared to females in the same patient cohort, and to healthy male controls from the same geographic location. However, only one of 75 females recently diagnosed with CD had serological evidence of *Campylobacter* infection, whereas the level of seroprevalence in the gender-matched control population was notably higher. It remains to

be determined why the females with CD have lower seroreactivity to *Campylobacter* antigens but it is unlikely to reflect a gender-based difference in exposure since twice as many women in the volunteer cohort had evidence of elevated *Campylobacter* IgG seroreactivity when compared to the men (3.8% and 1.9%, respectively).

Analysis of our data confirmed earlier reports that younger people have notably lower *Campylobacter* IgG seroreactivity than older individuals^[15,17]. This is thought to reflect repeated exposure to *Campylobacter* infections during a lifetime. Interestingly, of the 6 males with CD who demonstrated significantly elevated serum IgG responses in our study, only one was in the youngest age group and three were older than 55 years of age. It remains to be determined if little (or no previous) exposure to *Campylobacter* infection also explains the finding that, despite a confirmed diagnosis of campylobacter enteritis, a subset of individuals fail to develop significantly elevated IgG responses in the weeks following infection^[10,18]. Collectively, these findings highlight that we cannot use the measurement of IgG antibodies as a tool to determine whether prior campylobacter infection was a risk factor in the development of CD in our study^[4,5]. However, they raise the intriguing possibility that the host adaptive response may have a previously underappreciated role in the outcome of infection.

Campylobacter pathogenesis involves the adherence and invasion of gut epithelial cells by these bacteria, triggering a host innate immune response that results in an influx of inflammatory cells such as neutrophils and monocytes to the site of infection^[9]. These cells produce an environment that plays a key role in limiting bacterial invasion through the gut barrier, aided by the production of *Campylobacter*-specific antibodies. Thus, a failure to develop increased antibody titres following acute campylobacteriosis (particularly in younger people) may increase the risk of bacterial persistence, an observation that gains significance when one considers that young people in our community are more likely to develop campylobacter enteritis^[9] and this age group encompasses the peak age for first diagnosis of CD^[1].

It is currently unknown whether people who fail to seroconvert develop persistent infection but this is possible, given evidence of a role for anti-*C. jejuni* antibodies in bacterial clearance. An impaired humoral IgG response is associated with persistent *C. jejuni* colonisation of NF-κB deficient mice^[20]. In vitro studies show opsonised *C. jejuni* bacteria that are coated with specific IgG antibodies are internalised and targeted to lysosomes where they are killed in intestinal epithelial cells. In contrast, non-opsonised bacteria are able to avoid lysosomal delivery and survive following internalisation^[21]. Thus, one clinical outcome of a poor antibody response to *Campylobacter* infection may be an increased risk of bacterial persistence that, in turn, has the potential to compromise the gut epithelial barrier and even promote the translocation of non-invasive bacteria across the intestinal epithelium^[7]. As such, chronic *Campylobacter* infection may prove to be a keystone pathogen in the aetiology of CD^[22]. If so, identifying and treating patients with *Campylobacter* enteritis who fail to seroconvert may reduce this risk.

Our conclusion is that recent *Campylobacter* infection may be a trigger or contribute to the pathogenesis of CD in some individuals. The apparent tolerance of the immune system of young women (who are more likely to develop CD than young men in our community) to *Campylobacter* infection may identify a poor humoral response to infection that augments the subsequent development of chronic inflammation that leads to CD. Prospective studies are planned to investigate this hypothesis.

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Peer reviewer: Judith Collett, Gastroenterologist, Department of Gastroenterology, Kew Hospital, Southern District Health Board, New Zealand, Kew Road, Invercargill, PO Box 828, Invercargill 9840, New Zealand.