Antibody Responses to *Campylobacter* Infections Determined by an Enzyme-Linked Immunosorbent Assay: 2-Year Follow-Up Study of 210 Patients

METTE AAGAARD STRID,1 JØRGEN ENGBERG,1 LENA BRANDT LARSEN,1 KAMILLA BEGTRUP,2 KÅRE MÖLBÅK,1,2 AND KAREN ANGELIKI KROGFELT1,*

Department of Gastrointestinal Infections1 and Department of Epidemiology Research,2 Statens Serum Institut, DK-2300 Copenhagen S, Denmark

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An enzyme-linked immunosorbent assay (ELISA) was adapted to measure immunoglobulin G (IgG), IgM, and IgA classes of human serum antibody to *Campylobacter jejuni* and *Campylobacter coli*. Heat-stable antigen, a combination of *C. jejuni* serotype O:1,44 and O:53 in the ratio 1:1, was used as a coating antigen in the ELISA test. A total of 631 sera from 210 patients with verified *Campylobacter* enteritis were examined at various intervals after infection, and a control group of 164 sera was tested to determine the cut-off for negative results. With a 90th percentile of specificity, IgG, IgM, and IgA showed a sensitivity of 71, 60, and 80%, respectively. By combining all three antibody classes, the sensitivity was 92% within 35 days after infection, whereas within 90 days after infection, a combined sensitivity of 90% was found (IgG 68%, IgM 52%, and IgA 76%). At follow-up of the patients, IgG antibodies were elevated 4.5 months after infection but exhibited a large degree of variation in antibody decay profiles. IgA and IgM antibodies were elevated during the acute phase of infection (up to 2 months from onset of infection). The antibody response did not depend on *Campylobacter* species or *C. jejuni* serotype, with the important exception of response to *C. jejuni* O:19, the serotype most frequently associated with Guillain-Barré syndrome. All of the patients infected with this serotype had higher levels of both IgM (P = 0.006) and IgA (P = 0.06) compared with other *C. jejuni* and *C. coli* serotypes.

Together with *Salmonella* serovars, *Campylobacter* spp. are the most common bacterial enteric pathogens in developed countries, and *Campylobacter jejuni* is now the most recognized antecedent cause of Guillain-Barré syndrome (15, 16, 21). In Denmark, the incidence of registered *Campylobacter* infections has increased markedly since 1992 (from 22 cases per 100,000 inhabitants in 1992 to 78 cases per 100,000 in 1999), and a similar emergence of *Campylobacter* has been observed in other industrialized countries (6). The diagnosis of *Campylobacter* infections is routinely done by stool culturing on selective medium, and *C. jejuni* and *Campylobacter coli* account for 94 and 6%, respectively, of Danish human isolates (17). Furthermore, culturing of stools is not a sensitive method for detection of the bacteria in patients treated with antibiotics or in patients with late reactive complications such as arthritis and Guillain-Barré syndrome or long-lasting intestinal distress (16). In these cases and for epidemiological studies in general, serodiagnosis is valuable. Antibodies to *C. jejuni* and *C. coli* can be detected in several test systems with various sensitivities using a homologous strain or selected reference strains in crude antigen preparations.

Agglutination and complement fixation (24, 25) and immunofluorescence (4) tests have been used for serological diagnosis of *C. jejuni* infection, but these have been limited by low sensitivity or specificity or the need to use homologous isolates. Few attempts on an experimental basis have been made for the development of enzyme immunoassays for detecting antibody response to *C. jejuni* (3, 9, 10, 22, 23). They all found that the quality of a diagnostic test relies mainly on the antigen preparation used.

The objective of the present study was to establish a sensitive and specific diagnostic serologic test for the demonstration of immunoglobulin class-specific antibodies common to the most prevalent strains of *C. jejuni* and *C. coli* in Denmark. Various preparations of antigens from different *C. jejuni* serotypes were tested in an enzyme-linked immunosorbent assay (ELISA). Finally, a mixture of *C. jejuni* heat-stable antigens O:1,44 and O:53 (18) was found to be suitable for the diagnosis of *Campylobacter* infections in Denmark.

MATERIALS AND METHODS

**Study population and serum samples.** The study included 210 stool culture-confirmed cases of *Campylobacter* infection from 1996 to 1997. All patients had gastroenteritis, were from general practice, and had a median age of 33.5 years (range, 10 to 76 years). Each person was asked to give a blood sample at approximately 3 weeks, 3 months, 6 months, and 2 years after onset of symptoms. All patients gave their written acceptance, and the Danish Central Scientific Ethical Committee approved the project. To determine the cut-off for a negative result, we included 162 negative sera from patients submitting blood samples for *Helicobacter pylori* serology testing. As control for cross-reactions, sera from patients found positive for *H. pylori* (n = 39), *Yersinia enterocolitica* O:3 (n = 39), *Salmonella enterica* serovar enteritidis (n = 21), *S. enterica* serovar Typhimurium (n = 9), *S. enterica* serovar Typhi (n = 5), *S. enterica* serovar Paratyphi B (n = 1), and *S. enterica* serovar Manhattan (n = 1), *Legionella pneumophila* (n = 21), and *Escherichia coli* O:157 (n = 4) were examined against the selected *Campylobacter* antigens. All antisera were supplemented with 0.01% sodium azide and stored at −20°C.

**Identification and serotyping of isolates.** Fecal samples were cultured on CCDA substrate (18209 SSI Diagnostica, Hillerød, Denmark) and incubated in a microaerobic atmosphere (85% N2, 6% O2, 3% H2, and 6% CO2) at 37°C and

* Corresponding author. Mailing address: Department of Gastrointestinal Infections, Division of Diagnostics, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark. Phone: 45 3268 3745. Fax: 45 3268 8238. E-mail: kak@ssi.dk.
examined after 2 to 3 days. All isolates were identified as *C. jejuni* or *C. coli* by conventional phenotypic tests (15). *C. coli* was distinguished from *C. jejuni* by a negative sodium hippurate test. Serotyping of *C. jejuni* and *C. coli* was undertaken by passive hemagglutination based on heat-stable antigens in microtiter plates against 47 *C. jejuni* and 19 *C. coli* antisera as previously described (17).

**Preparation of heat-stable antigen.** A number of prevalent *C. jejuni* serotypes in Denmark (17) were considered candidates for the ELISA antigen, including *C. jejuni* O:1,44 (SSI:8133-96), O:2 (SSI:162-96), O:4 complex (SSI:6576-95), and O:53 (SSI:16059-96). In addition, *C. jejuni* O:19 (SSI:2075-96) was examined as candidate antigen. The bacteria were kept in bovine bouillon with 10% glycerol at −80°C until use. They were grown on 10% blood agar plates supplemented with 5% yeast (686 SSI Diagnostica) in an atmosphere of 90% N₂, 5% O₂, and 5% CO₂ at 37°C for 2 days. All were harvested with saline, boiled for 1 h at 100°C, and stored at −20°C.

Following the ELISA procedure described below, 40 selected acute-phase sera from culture-confirmed *Campylobacter* patients and sera from 40 negative controls were used in the identification of the most appropriate antigen. The combination of *C. jejuni* O:1,44 and O:53 antigens gave the best result, exhibiting a difference between acute-phase sera from patients and controls larger than for a single antigen (results available on request). The combined heat-stable antigen had a protein concentration of 1 μg/ml, measured by the Pierce BCA protein assay (reagent 23225, 0194; Pierce, Rockford, Ill.) in the ratio 1:1 between O:1,44 and O:53, and stored at −80°C.

**ELISA procedure.** Polyisorb microwell plates (17106; Nunc, Roskilde, Denmark) were coated overnight at 5°C with 100 μl of a solution of the described antigen in coating buffer (0.1 M sodium carbonate [pH 9.6]) with a total protein concentration of 1 μg/ml. Plates were emptied and incubated for 15 min with blocking buffer (phosphate-buffered saline [PBS] [pH 7.4] with 5% Tween 20) and washed four times with PBS [pH 7.4] containing 0.1% Tween 20.

All test sera were diluted 1:400 in PBS containing 0.01% (wt/vol) sodium azide. Test and control sera were applied in duplicate for 75 min at room temperature, followed by four cycles of washing. Horseradish peroxidase-labeled rabbit antiserum to human IgG (Dako 216; Dako, Glostrup, Denmark), IgM (Dako 215; Dako), or IgA (Dako 214; Dako) was diluted 1:2,000, 1:1,000, and 1:500, respectively, in washing buffer, and 100 μl of tetramethylene benzidine (4380A; Kem-En-Tec, Copenhagen, Denmark) substrate was added and incubated for 10 min. The reaction was stopped by adding 100 μl of 0.2 M H₂SO₄. The optical density (OD) was read as arbitrary units (a.u.) at 450 nm, with background correction at 620 nm.

**Selection of reference sera.** To identify suitable control sera, 40 sera from patients positive for *Campylobacter* antibodies and 164 sera from patients negative for *H. pylori* antibodies were measured against the heat-stable combination antigen. Sera from patients positive for *Campylobacter* antibodies with OD values between 1.2 and 2.2 a.u. were pooled and used as a positive control. Sera from patients negative for *H. pylori* antibodies with OD values below 0.25 a.u. were pooled and used as a negative control.

**Calibration system.** In order to control day-to-day variation, a positive control serum diluted 1:200 in PBS containing 0.01% (wt/vol) sodium azide. Test and control sera were applied in duplicate for 75 min at room temperature, followed by four cycles of washing. Horseradish peroxidase-labeled rabbit antiserum to human IgG (Dako 216; Dako, Glostrup, Denmark), IgM (Dako 215; Dako), or IgA (Dako 214; Dako) was diluted 1:2,000, 1:1,000, and 1:500, respectively, in washing buffer, and 100 μl of tetramethylene benzidine (4380A; Kem-En-Tec, Copenhagen, Denmark) substrate was added and incubated for 10 min. The reaction was stopped by adding 100 μl of 0.2 M H₂SO₄. The optical density (OD) was read as arbitrary units (a.u.) at 450 nm, with background correction at 620 nm.

**Statistical methods.** The mean antibody response following *Campylobacter* infection was modeled in a generalized linear mixed model tailored for the analysis of unbalanced repeated measurements, i.e., longitudinal data with variable intervals between measurements (7). Based on an analysis of model fit, we decided to model square-root-transformed ODs by a separate piecewise linear function with knots at 4.5 months after infection for IgG, 2 months for IgM, and 2.5 months as well as 7 months for IgA. Time since infection, *Campylobacter* species, *C. jejuni* serotype, and age were used as explanatory variables. To account for individual variability, we used a random-effect model, supposing that the antibody response depends on some common level of antibodies and supposing that a linear time trend exists for each person but with a varying interpersonal random intercept. Maximum-likelihood methods were used for the regression analyses by applying the MIXED procedure of the SAS software (SAS Institute, Cary, N.C.), and hypothesis testing was done by likelihood ratio tests.

### RESULTS

**Identification and serotyping of isolates.** From the 210 patients included in the study, a total of 180 *Campylobacter* isolates were available for further analysis; 173 (96%) were *C. jejuni* and 7 (4%) were *C. coli*. The distribution of serotypes is shown in Table 1. Most isolates reacted in only one serum or in a combination of sera comprising well-known complexes, e.g., O:1,44, O:4 complex, and O:6,7. Four *C. jejuni* isolates reacted with two or more antisera, which were not within well-known complexes but were O:2,38, O:3(13,50,65), O:10,44, and

| Serotype | No. of samples | % of total |
|----------|----------------|-----------|
| **C. jejuni** |                |           |
| 1,44      | 20             | 11.6      |
| 2         | 34             | 19.7      |
| 4 complex | 28             | 16.2      |
| 19        | 5              | 2.9       |
| 53        | 3              | 1.7       |
| 1         | 3              | 1.7       |
| 2,38      | 1              | 0.6       |
| 3         | 7              | 4.0       |
| 5         | 6              | 3.5       |
| 6,7       | 7              | 4.0       |
| 9         | 1              | 0.6       |
| 10        | 4              | 2.3       |
| 10,44     | 2              | 1.2       |
| 11        | 3              | 1.7       |
| 12        | 7              | 4.0       |
| 13        | 1              | 0.6       |
| 13,65     | 1              | 0.6       |
| 15        | 1              | 0.6       |
| 17        | 2              | 1.2       |
| 18        | 3              | 1.7       |
| 21        | 5              | 2.9       |
| 23,36     | 2              | 1.2       |
| 27        | 1              | 0.6       |
| 31        | 2              | 1.2       |
| 33        | 1              | 0.6       |
| 35        | 2              | 1.2       |
| 37        | 3              | 1.7       |
| 42        | 1              | 0.6       |
| 44        | 4              | 2.3       |
| 47        | 4              | 2.3       |
| NT        | 4              | 2.3       |
| UK        | 3              | 1.7       |
| **Total** | 173            | 100.0     |

Different OD values corresponding to the 0.05, 0.25, 0.50, 0.75, 0.90, and 0.95 fractiles were evaluated as potential lower cut-off values for positive results. At each different OD value, sensitivity was defined as the percentage of samples in the true positive group that gave a value greater than the cut-off value. The paired sensitivity and specificity, i.e., fractiles for cut-off, estimates were graphically shown in a receiver-operating characteristic (ROC) curve (20).
FIG. 1. Serum antibody response to *Campylobacter* infection in patients. (A) IgG; (B) IgM; (C) IgA. Individual responses of 210 patients over a 2-year period according to immunoglobulin class and the fitted population average (bold line) are shown.
The association between antibody response and Campylobacter species or C. jejuni serotype was assessed by categorizing typing results in five groups. C. coli; C. jejuni (all serotypes); common C. jejuni serotypes (O:1,44, O:2, and O:4 complex); uncommon C. jejuni serotypes; and unclassified serotypes (not typeable and unknown). The assessed antibody response was independent on these major groups (P > 0.4 for all three antibody classes). However, by comparing the response to C. jejuni O:19 against other classified serotypes, the five O:19 patients had higher IgA values (OD, 0.03 a.u.; 95% CI, 0.00 to 0.10; P = 0.06) and IgM values (OD, 0.07 a.u.; 95% CI, 0.01 to 0.22; P = 0.006, adjusted for age). In addition, patients with C. jejuni O:19 had 0.01 a.u. (95% CI, −0.02 to 0.10; P = 0.48) higher IgG values than others. The results were essentially the same when the unclassified types were lumped together with the group of other classified types and included in the analyses.

Cross-reactions. Sera from patients with infection due to other microorganisms causing gastrointestinal infections were assayed for antibodies against C. jejuni using the ELISA. Sera from 39 patients positive for H. pylori were tested for cross-reactions against heat-stable combination antigen, since H. pylori is phylogenetically the most closely related bacterium to Campylobacter. By using the cut-offs described above for IgG, IgM, and IgA, we found one, four, and six positive samples, respectively. Four sera from patients with E. coli O:157 infection were all found negative. Three groups of 39 sera each from patients with Yersinia O:3, Salmonella, and Legionella infections were assayed, and we found zero, eight, and three; two, four, and two; and zero, zero, and three positive sera, respectively, according to the cut-offs.

Determination of sensitivity. By using the 90th percentile of the negative sera as a cut-off and thus obtaining a specificity of at least 90%, Campylobacter infection could be detected with a sensitivity of 71% using IgG, 60% using IgM, and 80% using IgA within 35 days after infection. By combining all three antibody classes, the sensitivity was 92%, and after 3 months (90 days) from infection, the combined sensitivity was 90% (IgG, 68%; IgM, 52%; and IgA, 76%). In Fig. 2, ROC curves representing the diagnostic value (sensitivity and specificity) of our test at different times after infection are presented.

**DISCUSSION**

The aim of this study was to establish an ELISA suitable for a general screening of Campylobacter infections. The assay should be sensitive for the most prevalent C. jejuni and C. coli serotypes and have few or no cross-reactions with other genera.

C. jejuni serotypes O:1,44 and O:53 used as the antigen represent the most common serotypes (18%) and the seventh most common (3%) in Denmark (17). The evaluation of this antigen combination showed that the measured antibody response was independent of the major groups of C. jejuni serotypes and that the assay was also suitable for detecting antibodies against C. coli. The serotype distribution of the 177 isolates in this study was in accordance with earlier studies in Denmark (17). The study included five patients with C. jejuni O:19 infections, and these patients had significantly higher levels of IgM and IgA than others did. C. jejuni O:19 is associated with the most serious disease caused by Campylobacter, Guillain-Barré syndrome (11, 26). The high levels of IgM and IgA in C. jejuni O:19 patients may be related to a higher affinity of the test for antibodies against this serotype than others. However, O:19 was not chosen for the antigen preparation, and it is therefore more likely that the high antibody levels reflect the high immunogenicity of C. jejuni O:19 rather than an increased affinity of the ELISA against this serotype. This view is in line with observations made by Rautelin et al. (19) and warrants further studies. Allos and colleagues have recently shown that C. jejuni O:19 strains, regardless of Guillain-Barré syndrome association, are more serum resistant than...
Our findings corroborate the hypothesis that the elevated immunologic response induced by O:19 strains leads to injury of peripheral nerve structures. None of the five patients with infections caused by 

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serotype O:19 developed Guillain-Barré syndrome.

It was not possible to investigate sera from infections caused by Campylobacter species other than C. jejuni and C. coli, as the current method for the diagnosis of Campylobacter enteritis is culturing of fecal samples on selective medium adjusted to these species. Furthermore, a recent study suggests that C. fetus, C. lari, and C. upsaliensis are minor causes of Campylobacter enteritis in Denmark (8).
The prepared crude antigen showed no cross-reactions based on a 90th percentile towards the phylogenetically most closely related bacterium *H. pylori* and the other bacteria examined. Earlier works have reported cross-reactions between *C. jejuni* and *H. pylori* when using sonicated antigen (12). Cross-reactions between *Salmonella* serovars Typhi and *Paratyphi* and *C. jejuni* have been reported when the antigen used was based on the flagellar protein (13). Finally, cross-reactions of the IgM class between *Legionella pneumophila* and *C. jejuni* based on formalin-treated antigen have been reported (5).

Class-specific antibody response profiles were determined over a period of 2 years based on a large number of patients. The IgA response was associated with acute infection. The IgM response was highest in the younger age groups, and there was a significant association between age at infection and IgM response (Table 2). Thus, serodiagnosis based on IgM response is a particularly useful tool for young patients. It is likely that a considerable proportion of elderly persons have a secondary antibody response without IgM elevation. IgG levels were highly individually variable, and this large variability of IgG response was also reflected by a large variation in IgG levels among the negative controls (data not presented). At a 90% specificity, 71, 60 and 80% of acute infections could be detected by IgG, IgM, and IgA, respectively, in a single convalescent-phase sample. By using a combination of all three antibody classes, sensitivity was 92 and 90% within the first 35 days and within 3 months after infection, respectively. Furthermore, the ROC curves show that approximately 50% of the infections can be detected serologically up to 1 year postinfection.

Blaser and coworkers (3) used a glycerol-HCl-extracted surface antigen of serotypes O:1, O:2, and O:3 in their ELISA. For IgG antibodies, the test had a specificity of 74% and 59% sensitivity. For IgM, the specificity was 68% and the sensitivity was 74%, and for IgA the specificity was 81% and the sensitivity was 76%. Thus, the IgA ELISA was the most specific and sensitive assay in their study to detect an acute infection as measured in an enzyme-linked immunosorbent assay. Infect. Immun. 44:292–408.

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