Improved serodiagnosis of Campylobacter jejuni infections using recombinant antigens

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Campylobacter jejuni is a frequent cause of infectious diarrhoea and is increasingly recognized as a trigger for late-onset complications. The poor standardization of commonly used serological tests might explain the conflicting results regarding the frequency of antecedent C. jejuni infections in defined patient groups. In order to obtain reliable epidemiological data as to the role of C. jejuni in causing late-onset complications, a highly specific and sensitive diagnostic tool for the epidemiological investigation of C. jejuni-associated diseases was developed. It was shown that recombinant proteins encoded by the C. jejuni genes cj0017 (P39) and cj0113 (P18) are specifically recognized by antibodies in sera from patients with C. jejuni enteritis. An ELISA using recombinant P18 and P39 as antigens was 91·9 % sensitive and 99·0 % specific, with positive and negative predictive values of 97·1 % and 99·0 %, respectively, comparing favourably with the 27·0 % sensitivity of a routinely used serological assay.

INTRODUCTION

Campylobacter jejuni is a very important cause of diarrhoeal disease in developing and industrialized countries (Friedman et al., 2004), ranking second in incidence after Salmonella enterica in the USA and Germany (CDC, 2003; Stark & Alpers, 2004). There is an increasing concern that C. jejuni infections might trigger a number of late-onset complications, which so far has been best documented for Guillain-Barré syndrome (Hughes & Rees, 1997) and HLA B27-associated reactive arthritis (Colmegna et al., 2004). Recent data suggest a link between C. jejuni and immunoproliferative small intestinal disease (Lecuit et al., 2004), a primary intestinal lymphoma with poor prognosis that accounts for one-third of primary small intestinal lymphomas in Mediterranean countries (Salem et al., 1986).

The aetiological link between these diseases and C. jejuni infections has mostly been established on the basis of serological data, since cultural diagnosis of C. jejuni after cessation of diarrhoea is rarely successful (Svedhem & Kajser, 1980). Several serological methods have been used to detect C. jejuni-specific antibodies, including complement-fixation assays (CFAs), immunoblotting and ELISAs. So far, these tests have not been standardized with regard to antigens used or to stringent criteria defining seropositivity. Crude antigenic preparations are commonly applied, including acid-glycine extracts (Blaser & Duncan, 1984), heat-stable antigens (Kaldor & Speed, 1984), whole-cell sonicates (Boucquey et al., 1991), outer-membrane protein preparations (Enders et al., 1993) and phenol/water extraction of C. jejuni LPS (Jacobs et al., 1998). This lack of standardization might explain the conflicting results regarding the frequency of preceding C. jejuni infections in patients with Guillain-Barré syndrome (Hughes & Rees, 1997) or reactive arthritis (Bremer et al., 1991; Eastmond et al., 1983; Kosunen et al., 1980; Locht & Krosgfelt, 2002). Moreover, the specificity of these assays might be low due to antigenic cross-reactivities, in particular with the closely related Helicobacter pylori and with other Gram-negative bacteria that are associated with clinical manifestations similar to C. jejuni, such as enteropathogenic Yersinia species (Colmegna et al., 2004). In order to obtain reliable epidemiological data as to the role of C. jejuni in causing late-onset complications, specific serological markers of past C. jejuni infections are essential.

So far only a few immunoreactive components in crude C. jejuni extracts have been identified. Although the immunodominance of flagellin (FlaA) during human infection is well established (Nachamkin & Hart, 1985; Wenman et al., 1985), the diagnostic exploitation of this antigen is hampered by immunodominant epitopes of FlaA being serotype specific (Newell & Nachamkin, 1992). Seroconversions to the major cell-adherence factor Cj0921 (Peb1) and the glycoprotein Cj0289 (Peb3) have been observed in a small number of convalescent patients (Pei et al., 1991), and the putative peptidoglycan-associated protein Cj0113 showed a reaction
with sera from some patients with arthritis of suspected C. jejuni etiology (Burnens et al., 1995). The C. jejuni flagellar hook protein Cj1729c (FlgE2) and the major outer-membrane protein Cj1259 (PorA) are immunogenic in humans (Blaser et al., 1984). However, as for FlaA, the surface-exposed epitopes of FlgE2 seem to be serotype specific (Power et al., 1992) and antibodies reacting with PorA are highly prevalent in the healthy population (Blaser et al., 1984). Taken together, low sensitivity or specificity of FlaA, FlgE2 and PorA limit their use as reliable serological markers for C. jejuni infection.

We show that the proteins encoded by strain NCTC 11168 genes cj0017 (P39) and cj0113 (P18) are valuable antigens for the serological diagnosis of past C. jejuni infections. Using purified recombinant P39 and P18, we established a highly specific and sensitive serological test for C. jejuni infections, which will be a valuable tool for future epidemiological studies on late-onset complications of C. jejuni enteritis.

**Methods**

**Bacterial strains.** The C. jejuni strain NCTC 11168 was obtained from the National Collection of Type Cultures (NCTC, London, UK). Escherichia coli DH10B was purchased from Invitrogen.

For the recombinant expression of C. jejuni DNA we used Epicurian Coli BL21-CodonPlus from Stratagene adapted for the expression of AU- and AGA-rich coding sequences, since a bias regarding these codons seems to be responsible for the reported difficulties in expressing AUA- and AGA-rich coding sequences, we used Epicurian Coli BL21-CodonPlus from Stratagene adapted for the expression of AU- and AGA-rich coding sequences, since a bias regarding these codons seems to be responsible for the reported difficulties in expressing C. jejuni DNA (E. coli; Kletky, 1997).

**Sera.** Patients attending the University Hospital of Göttingen between January 2003 and December 2004 with bloody or watery diarrhoea were routinely tested for C. jejuni in their faces. Campylobacter strains were identified at the species level using a commercial biochemical differentiation kit (API CAMPY, bioMérieux). Post-infection sera from 37 patients (age, mean ± SD, 48 ± 20 years) were collected within 4 weeks (3–24 days) of the cultural diagnosis of C. jejuni enteritis. Follow-up sera were obtained from four patients at various intervals after the onset of diarrhoea. Control sera were sampled from five healthy blood donors.

**Generation of a genomic expression library.** Genomic DNA of the C. jejuni reference strain NCTC 11168 was prepared using Qiagen Genomic-tip 20/G (Qiagen) according to the manufacturer’s instructions and partially digested with Sau3A (New England Biolabs). DNA fragments sized 1–2 kb were gel purified with the MinElute Gel Extraction Kit from Qiagen. The DNA fragments were cloned in-frame into a BamHI restriction site of the pET3a, b or c expression vectors (Novagen) under the control of the inducible T7 promoter. The resulting three genomic pET3 libraries were electropropared into E. coli DH10B. Transformants were selected on LB agar containing ampicillin (50 μg ml⁻¹) and chloramphenicol (30 μg ml⁻¹). Approximately 10,000–15,000 transformants were pooled and plasmid DNA was extracted with the MiniPrep Kit from Qiagen. Plasmid DNA (100 ng) was subsequently transformed into electrocompetent Epicurian Coli BL21-CodonPlus. Transformants were grown overnight on LB agar containing ampicillin (50 μg ml⁻¹) and chloramphenicol (30 μg ml⁻¹).

Ten thousand colonies were pooled in LB broth and aliquots were kept at −80°C until they were used for immunoscreening.

**Colony immunoscreen of the genomic C. jejuni expression library.** The expression library was plated on nitrocellulose membranes (0.22 mm) (Optitran BA-S 85, Schleicher & Schuell) at a density of 500 c.f.u. per membrane, and grown overnight at 37°C on LB agar containing ampicillin (50 μg ml⁻¹) and chloramphenicol (30 μg ml⁻¹) (master membrane). Replica membranes were constructed as previously described (Sambrook et al., 1989). Protein synthesis was induced by exposing the master membrane to 1 mM IPTG for 3 h at 37°C. Bacteria were lysed in situ in TBST, lysozyme (40 μg ml⁻¹) and DNase I (1 μg ml⁻¹) for 2 h at room temperature. After blocking the membranes for 60 min with 5% (w/v) skimmed milk in TBST (Tris-buffered saline with 0.05% (v/v) Tween 20; blocking solution), a colony immunoscreen was performed, as described previously (Sambrook et al., 1989), using pooled convalescent phase sera from five patients with culture-confirmed C. jejuni enteritis (diluted 1:200 in TBST and 1% (w/v) skimmed milk) and an alkaline phosphatase-conjugated rabbit anti-human-IgG secondary antibody (Dianova) diluted 1:5000 in TBST. The immunoreactivity of selected library clones was confirmed by immunoblot analysis, according to the instruction manual of the pET system (Novagen). Briefly, SDS-PAGE of induced bacterial culture was performed under denaturing conditions according to the Laemmli method (Sambrook et al., 1989), followed by immunoblotting on Optitran BA-S 85 nitrocellulose (Schleicher & Schuell) with patient sera as described above.

**Identification, recombinant expression and purification of immunogenic C. jejuni proteins.** The plasmids of immunoreactive library clones were purified (MiniPrep Kit, Qiagen) and the pET3 vector was used as a template for DNA sequencing reactions with the T7-promoter primer. The resulting sequences were blasted against the published C. jejuni NCTC 11168 genome sequence (www.sanger.ac.uk/Projects/C_jejuni/), revealing the coding DNA for the immunoreactive proteins.

For the recombinant expression of the immunoreactive proteins, the genes cj0017, cj0113 and cj1339 were PCR amplified from NCTC 11168 genomic DNA using the KOD Hot Start DNA polymerase (Novagen) and the primer pairs FB7/FB8 (5’-GGATCCCGGTGTAAGATTGATTTAAA-3’/5’-GGGATCCGCCTGTAAGATTTGATTTAAAAGGTTT3’), FBI/FB2 (5’-CGGATCCCGGTGTAAGATTGATTTAAAAGGTTT3’/5’-CGGATCCCGGTGTAAGATTGATTTAAAAGGTTT3’), and FB3/FB4 (5’-GGGATCCCGGTGTAAGATTGATTTAAAAGGTTT3’/5’-GGGATCCCGGTGTAAGATTGATTTAAAAGGTTT3’), respectively. The PCR product was performed in a volume of 50 μl x 1 PCR buffer for KOD polymerase (20 ng of C. jejuni NCTC 11168 genomic DNA; 200 μM each of dATP, dCTP, dGTP and dTTP; 0.3 μM each primer; 1 mM MgSO₄; 1 U of KOD polymerase). PCR conditions were as follows: an initial melting temperature of 95°C for 2 min; 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min; final extension at 72°C for 10 min. The resulting PCR products were cloned in-frame in the pET15b expression vector (Novagen). After transformation into Epicurian Coli BL21-CodonPlus, expression of the 6 × His fusion proteins was induced with 1 mM IPTG following the pET System manual (Novagen). The 6 × His fusion proteins were purified by Ni-NTA affinity chromatography (Ni-NTA agarose, Qiagen) under denaturing conditions according to the manufacturer’s instructions. The immunoreactivity of the purified fusion proteins was confirmed in an immunoblot with post-infection serum samples as described above.

**Recombinant immunoblot and ELISA.** SDS-PAGE of affinity-purified recombinant P18 and P39 was performed under denaturing conditions. Subsequently, the proteins were blotted onto Optitran BA-S 85 nitrocellulose (Schleicher & Schuell) and incubated with sera diluted 200-fold in TBST. Bound antibodies were detected using alkaline

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Identification of immunoreactive C. jejuni proteins

Fourteen out of 21,000 library clones tested expressed a C. jejuni protein that reacted with post-infection sera and were therefore retained for further analysis. Sequencing of the corresponding DNA identified the genes cj0017, cj0113 and cj1339 in four, four and six of these clones, respectively.

The gene cj0017 encodes a putative protein of unknown function, designated P39. The hydropathy profile of the protein sequence reveals several hydrophobic domains in the N-terminal part of the protein, suggesting a membraneous location, which is supported by the analysis with the Transmembrane Hidden Markov model (www.cbs.dtu.dk/services/TMHMM-2.0), predicting the presence of five sequential transmembrane protein domains. The two cysteines in the first periplasmic domain are in a Cys-X-Y-Cys configuration, which is characteristic of the active site of proteins involved in disulphide bond formation (Bardwell et al., 1991). The scanning of the Prosite database (www.expasy.ch/tools/scanprosite/) reveals a putative ATP/GTP binding site (P-loop) within the hydrophilic C-terminal part of the cj0017-encoded protein. Homologues to the cj0017-encoded protein are only found in closely related H. pylori (54 % similarity, 37 % identity) and in Corynebacterium diphtheriae (45 % similarity, 28 % identity), which are both pathogenic to humans. Wolinella succinogenes, a non-pathogenic member of the Campylobacterales, has no homologue to cj0017.

The immunogenic protein P18, which is encoded by cj0113, is homologous to the peptidoglycan-associated protein (Pal) of E. coli (54 % similarity, 36 % identity). Pal-related proteins have been described in many bacterial species (Engleberg et al., 1991; Hemila, 1991; Lazzaroni & Portalier, 1992; Nelson et al., 1988), in which they generally play an important role in the host immune response. In Haemophilus influenzae, the Pal homologue might be an important antigen for the induction of protective immunity (Murphy et al., 1992). The gene cj1339 encodes the structural protein FlaA of flagella. The proteins encoded by the genes cj0113 and cj1339 have been shown to be immunogenic by others also (Burnens et al., 1995; Nachamkin & Hart, 1985), thus confirming the validity of our systematic approach.

P18/P39 ELISA and CFA

On the basis of the immunoblot data, we developed a P18/P39 ELISA. Fig. 3 shows the results of the ELISA with sera from healthy blood donors, patients with positive Y. enterocolitica serology, patients with positive H. pylori serology and post-infection sera from patients with C. jejuni enteritis.
Considering all controls, the accuracy of the IgA-specific ELISA (0.981) was significantly higher ($P < 0.002$) than the accuracy of the IgG-specific ELISA (0.855), which indicates that the IgA-specific ELISA is more appropriate for discriminating between diseased and non-diseased individuals. The cut-off OD$_{490}$ values defining positivity were determined according to the Youden index and were 0.614 for IgG and 0.444 for IgA. The results of the serological tests are combined in Table 2. Specificity and sensitivity were highest in the IgA-specific ELISA (99% and 92%, respectively). Twelve post-infection sera were negative in the IgA-specific ELISA, 10 of which were positive in the IgA-specific assay. Follow-up sera (see below) from two out of four patients analysed indicated that titres of IgA antibodies peaked before IgG antibodies. Most of the IgG-negative sera were sampled in the acute phase and we assume therefore that IgG antibodies had not yet reached detectable levels. The maximum sensitivity of the P18/P39 ELISA (94.6%) for our study group is achieved by combining the data for both antibody classes (Table 2).

Ten post-infection sera (27%) were positive in the standard CFA (titre $>1:30$), with titres above 1:160 in five of these. All of the CFA-positive sera were positive in

![Fig. 1. Coomassie-stained SDS-PAGE of purified recombinant fusion proteins.](image)

![Fig. 2. IgG-specific immunoblot of P18 and P39 with convalescent-phase sera from patients with C. jejuni enteritis and healthy blood donors. Pooled convalescent-phase serum from five patients with culture-confirmed C. jejuni enteritis was used as positive control (K+). Alkaline phosphatase-conjugated rabbit anti-human-IgG was used as secondary antibody (see Methods).](image)

<table>
<thead>
<tr>
<th>Tested antibody</th>
<th>No. of subjects with positive serum</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV† (%)</th>
<th>NPV‡ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls* ($n = 48$)</td>
<td>Patients with C. jejuni enteritis ($n = 27$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG anti-P18</td>
<td>10</td>
<td>20</td>
<td>74-1</td>
<td>79-2</td>
<td>66-7</td>
</tr>
<tr>
<td>IgG anti-P39</td>
<td>6</td>
<td>19</td>
<td>70-3</td>
<td>87-5</td>
<td>76-0</td>
</tr>
<tr>
<td>IgG anti-P18+P39</td>
<td>13</td>
<td>23</td>
<td>85-2</td>
<td>72-9</td>
<td>63-9</td>
</tr>
<tr>
<td>IgA anti-P18</td>
<td>0</td>
<td>11</td>
<td>40-7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IgA anti-P39</td>
<td>0</td>
<td>6</td>
<td>22-2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IgA anti-P18+P39</td>
<td>0</td>
<td>11</td>
<td>40-7</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Sera from 26 healthy blood donors and 22 patients with positive Y. enterocolitica serology.

†Positive-predictive value.

‡Negative-predictive value.
both the IgA- and IgG-specific P18/P39 ELISA. However, CFA titres did not correlate with the OD<sub>490</sub> values measured in the ELISA. None of the control sera was positive in the CFA.

**Table 2. Serological testing with the P18/P39 ELISA**

<table>
<thead>
<tr>
<th>Antibody class</th>
<th>No. of subjects with positive serum</th>
<th>Sensitivity (%)</th>
<th>Specificity* (%)</th>
<th>PPV† (%)</th>
<th>NPV‡ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy blood donors (&lt;i&gt;n&lt;/i&gt; = 57)</td>
<td>Patients with positive <em>Y. enterocolitica</em> serology (&lt;i&gt;n&lt;/i&gt; = 19)</td>
<td>Patients with positive <em>H. pylori</em> serology (&lt;i&gt;n&lt;/i&gt; = 22)</td>
<td>C. jejuni patients (&lt;i&gt;n&lt;/i&gt; = 37)</td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>25</td>
<td>67-6</td>
</tr>
<tr>
<td>IgA</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>34</td>
<td>91-9</td>
</tr>
<tr>
<td>IgG+IgA</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>35</td>
<td>94-6</td>
</tr>
</tbody>
</table>

*Combining all controls.
†Positive predictive value.
‡Negative predictive value.

**DISCUSSION**

*C. jejuni* is a major cause of bacterial diarrhoeal disease (CDC, 2003; Stark & Alpers, 2004) and there is increasing concern that *C. jejuni* is involved in the triggering of numerous other diseases, such as Guillain-Barré syndrome (Hughes & Rees, 1997), reactive arthritis (Kosunen et al., 1980) and malignant disease (Lecuit et al., 2004). The reported frequency of antecedent *C. jejuni* infections in these diseases varies (Bremell et al., 1991; Colmegna et al., 2004; Eastmond et al., 1983; Kosunen et al., 1980; Locht & Krogfelt, 2002), which might reflect not only geographical differences but also the lack of standardized specific assays for *C. jejuni* serodiagnosis. Moreover, the sensitivities and specificities of the various serological assays can be contested since the antigen mixtures used for antibody detection include *C. jejuni* antigens that have been shown either to be strain-specific (Newell & Nachamkin, 1992; Power et al., 1992) or to lack specificity (Blaser et al., 1984).

Therefore, the goal of this study was to develop a reliable diagnostic tool for future serological studies dealing with *C. jejuni* as a potential cause of late-onset diseases. Our first step was to identify and purify conserved antigens that could be used as serological markers of *C. jejuni* infections. In a systematic screen of our *C. jejuni* genomic expression library with post-infection sera from patients with *C. jejuni* enteritis, we identified three immunogenic proteins encoded by the genes *cj0017*, *cj0113* and *cj1339*. We succeeded in purifying the recombinant proteins P39 and P18, encoded by *cj0017* and *cj0113*, respectively. These recombinant proteins were both specifically recognized by antibodies in post-infection

**Time-course of the P18/P39 serological response**

Sequential sera of four patients with *C. jejuni* enteritis were tested with the P18/P39 ELISA. In all patients, specific IgA antibodies (titres > 1 : 128) and IgG antibodies (titres = 1 : 2048) were detected. In all patients the IgA antibody titres dropped below the cut-off within 4 weeks after cultural diagnosis, whereas IgG antibodies remained positive (titres > 1 : 256) for at least 3 months (Fig. 4).
Our immunoblot analysis indicated that the combined use of P18 and P39 increases the sensitivity compared to each protein used alone. Consequently, we combined both proteins as antigens in the ELISA for our serological investigations. The detection of specific IgA antibodies was most discriminatory for a recent C. jejuni infection, reaching a 100% specificity with regard to healthy blood donors and patients with serological evidence of a Y. enterocolitica infection and 96% with regard to H. pylori-infected controls. Taking together the results for all patient sera and controls, the specificity of the IgA-specific ELISA was 99.0%, the sensitivity was 91.9%, and the positive- and negative-predictive values were 97.1% and 97.0%, respectively. Detection of C. jejuni-specific IgG antibodies was also highly specific (92.9%) for a previous C. jejuni infection, but the sensitivity (67.6%) was lower than for IgA, which might be due to the very early sampling of some patient sera. The time-course of the P18/P39-specific antibody response in single patients showed that, in most patients, the specific IgA antibody titre fell below the assay cut-off within 14 days after onset of diarrhoea, whereas IgG antibody titres persisted for at least 3 months. The combined data of both antibody classes maximizes the sensitivity of the assay. In addition, the IgA-specific testing is useful for narrowing down the time-point of infection. To assign the role of C. jejuni in causing late-onset complications, we suggest the combination of data for both antibody classes.

Y. enterocolitica and C. jejuni are both aetiological agents of reactive arthritis and it is therefore noteworthy that the P18/P39 ELISA did not cross-react with sera from patients with serological evidence of a preceding Yersinia infection. The specificity of the assay is further confirmed by the negative test results of sera from patients chronically infected with H. pylori, a species closely related to C. jejuni. The discrimination between C. jejuni and H. pylori infections is particularly relevant considering the high prevalence of chronic H. pylori infection in the general population (Dunn et al., 1997).

In conclusion, we show that the use of selected recombinant C. jejuni proteins improves the serodiagnosis of C. jejuni infection. We have established a recombinant ELISA with excellent specificity and sensitivity for a preceding C. jejuni infection. This assay will enable reliable epidemiological data as to the role of C. jejuni in causing late-onset complications to be generated.

ACKNOWLEDGEMENTS

We thank Professor Dr M. Köhler from the department of Transfusion Medicine of the University of Göttingen for providing sera from healthy blood donors and Friederike Fisher and Regina Schmidt-Ott for the critical reading of the manuscript. The study was approved by the ethics committee of the University of Göttingen (No. 5/9/03).
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