Improved serodiagnosis of *Campylobacter jejuni* infections using recombinant antigens

Ruprecht Schmidt-Ott, Felicitas Brass, Christiane Scholz, Carola Werner and Uwe Groß

1,2Institute of Medical Microbiology 1 and Department for Medical Statistics 2, University of Göttingen, D-37075 Göttingen, Germany

*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 97.0 %, 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 have best been 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 (Bremell et al., 1991; Eastmond et al., 1983; Kosunen et al., 1980; Locht & Krogefelt, 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 AUA- and AGA-rich coding sequences, since a bias regarding these codons seems to be responsible for the reported difficulties in expressing *C. jejuni* DNA in *E. coli* (Ketley, 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 faeces. *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 57 healthy blood donors (age, mean ± SD, 47 ± 12 years), from 19 patients with arthritis and serological evidence for a recent *Yersinia enterocolitica* infection (Y. *enterocolitica* IgG/IgA ELISA, Virotech) (age, mean ± SD, 38 ± 22 years), and from 22 patients who were tested positive for IgG in a *H. pylori*-specific immunoblot (Karvar et al., 1997) (age, mean ± SD, 27 ± 20 years). All sera were stored at −70°C until used.

**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 pET libraries were electroproporated 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 (25 × 25 mm) (Optitran BA-S 85, Schleicher & Schuell) at a density of 500 c.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 (Diagnostic) 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 FB1/ FB8 (5’-GGATCGCCGTGTAAGATTATGTTAAAACA-3’/5’-GGATCGCCGTGTAAGATTATGTTAAAACCA-3’), FB1/FB2 (5’-CCGCGTCCGATATGTTATAGTTGGTGTACCA-3’/5’-CCGCGTCCGATATGTTATAGTTGGTGTACCA-3’/5’-CCGCGTCCGATATGTTATAGTTGGTGTACCA-3’), respectively. The PCR was performed in a volume of 50 μl × 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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phosphatase-conjugated rabbit human-IgG- or IgA-specific secondary antibody (Dianova) diluted 1:5000 in TBST.

ELISA microtitre plates (Greiner) were coated with recombinant affinity-purified P39 and P18. The proteins were pooled in coating ELISA microtitre plates (Greiner) were coated with recombinant antibody (Dianova) diluted 1:5000 in TBST. Phosphatase-conjugated rabbit human-IgG- or IgA-specific secondary antibody was added to each well and the plate was incubated for 1 h at 37 °C. The conjugates were diluted in PBST as follows: anti-human-IgG–HRP (DAKO), 1:6000; anti-human-IgA–HRP (DAKO), 1:4000. Five washing steps with washing buffer (0.05 M Tris/HCl pH 7.4, 0.1% v/v Tween 20) was added to each well for 2 h at 37 °C. Subsequently, 50 μl horseradish peroxidase (HRP)-conjugated secondary antibody was added to each well and the plate was incubated for 1 h at 37 °C. The IgA- and IgG-specific P18/P39 ELISA were compared with an affinity-purified P62. 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 immunoblot

P18, P39 and P62 were fused to 6 X His tags at their N-terminus, omitting N-terminal signal sequences and hydrophobic domains to facilitate subsequent expression and purification of the fusion protein. The fusion proteins P18, P39 and P62, corresponding to the genes cj0113, cj0017 and cj1339, respectively, were recombinitely expressed at high levels in Epicurian Coli BL21-CodonPlus (Fig. 1) and were immunoreactive with post-infection serum samples. Affinity purification of 6 X His-tagged P18 and P39 was successful, whereas the preparation of 6 X His-tagged P62 appeared to be impure (Fig. 1). Prevalence of P18- and P39-specific antibodies in post-infection sera (n = 27) and in control sera from healthy blood donors (n = 26) and patients with positive Yersinia serology (n = 22) was tested in an immunoblot. IgA and IgG antibodies in post-infection sera reacted more strongly and significantly more often with P18 and P39 than those in control sera (Fig. 2, Table 1). Combining the immunoblot data of P18 and P39 for both antibody classes resulted in a sensitivity of 88.8% and a specificity of 72.9%, with positive- and negative-predictive values of 64.9% and 92.1%, respectively.

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.

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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 $\geq$ 1:30), with titres above 1:160 in five of these. All of the CFA-positive sera were positive in

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**Table 1. Immunoblot analysis of recombinant *C. jejuni* proteins with sera of patients and controls**

<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 <em>C. jejuni</em> enteritis ($n = 27$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG anti-P18</td>
<td>10/48</td>
<td>20/27</td>
<td>74.1</td>
<td>79.2</td>
<td>66.7</td>
</tr>
<tr>
<td>IgG anti-P39</td>
<td>6/48</td>
<td>19/27</td>
<td>70.3</td>
<td>87.5</td>
<td>76.0</td>
</tr>
<tr>
<td>IgG anti-P18+P39</td>
<td>13/48</td>
<td>23/27</td>
<td>85.2</td>
<td>72.9</td>
<td>63.9</td>
</tr>
<tr>
<td>IgA anti-P18</td>
<td>0/48</td>
<td>11/27</td>
<td>40.7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IgA anti-P39</td>
<td>0/48</td>
<td>6/27</td>
<td>22.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IgA anti-P18+P39</td>
<td>0/48</td>
<td>11/27</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 OD490 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 (n = 57)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Patients with positive Y. enterocolitica serology (n = 19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Patients with positive H. pylori serology (n = 22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>2</td>
<td>67-6</td>
<td>92-9</td>
<td>78-1</td>
<td>88-4</td>
</tr>
<tr>
<td>IgA</td>
<td>0</td>
<td>91-9</td>
<td>99-0</td>
<td>97-1</td>
<td>97-0</td>
</tr>
<tr>
<td>IgG+IgA</td>
<td>2</td>
<td>94-6</td>
<td>91-8</td>
<td>81-4</td>
<td>97-8</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 sera from the patients used as serological markers of *C. jejuni* enteritis, whereas IgG antibodies remained positive (titres > 1 : 128) for at least 3 months (Fig. 4).
The high seroconversion rate to P18 and P39 in patients presumably infected with different C. jejuni strains indicates that the immunoreactive epitopes of these proteins are conserved in C. jejuni and not restricted to the infecting strain. Conservation of P18 in C. jejuni strains of different geographical origins has been shown previously (Burnens et al., 1995; Pawelec et al., 2000). Taken together, the results suggest that the recombinant proteins P39 and P18 are suitable candidates as antigens for serological studies.

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.

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REFERENCES


