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 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 has 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 & Kaijser, 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; Loch & Krogfelt, 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

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Abbreviations: CFA, complement-fixation assay; ROC, receiver operating characteristic.

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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 *Sau*3A (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 *Bam*HI restriction site of the pET3a, b or c expression vectors (Novagene) under the control of the inducible T7 promoter. The resulting three genomic pET3 libraries were electroporated into *E. coli* DH10B. Transformants were selected on LB agar containing ampicillin ($50\text{ }\mu\text{g ml}^{-1}$) and chloramphenicol ($30\text{ }\mu\text{g ml}^{-1}$). 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 ($50\text{ }\mu\text{g ml}^{-1}$) and chloramphenicol ($30\text{ }\mu\text{g ml}^{-1}$).

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 ($\varnothing$ 82 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 ($50\text{ }\mu\text{g ml}^{-1}$) and chloramphenicol ($30\text{ }\mu\text{g ml}^{-1}$) (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 ($40\text{ }\mu\text{g ml}^{-1}$) and DNase I ($1\text{ }\mu\text{g ml}^{-1}$) 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 (Novagene) and the primer pairs FB7/FB8 (5'-GGGATCCGCTGTAAAGATT AGTTTAA-3'/5'-CGGGATCCGTTAGTTTAAAGTATAAAGCTTG-3'), FB1/FB2 (5'-CCGCTCGAGGTTATTAGTGGTTGTAGCA-3'/5'-CCGCTCGAGTTATCTTGATAATTTAAATTC-3') and FB3/FB4 (5'-CGGGATCCGGGATTTCGTATTAAACACCA-3'/5'-CGGGATCC CTACTGTAGTAATCTTAAAC-3'), respectively. The PCR was performed in a volume of $50\text{ }\mu\text{l}$ 1 $\times$ 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 MgSO_4 ; 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 $\times$ His fusion proteins was induced with 1 mM IPTG following the pET System manual (Novagen). The 6 $\times$ 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

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 buffer (0.015 M Na₂CO₃, 0.035 M NaHCO₃, pH 8.4) to a final concentration of 0.3 µg ml⁻¹ of each. Each well of the ELISA microtitre plates was coated with 100 µl antigen solution (4 °C, overnight). After blocking with 150 µl ELISA blocking reagent (Boehringer) per well for 30 min, 100 µl diluted patient serum [1 : 100 in PBS-0.05 % v/v Tween 20 (PBST)] 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 plates were 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 (0.35 M NaCl, 0.05 M Tris/HCl, pH 7.4, 0.1 % v/v Tween 20) were performed between each step of the ELISA. Substrate was prepared with 14 mg 1,2-phenylenediamine dihydrochloride (DAKO), 12 ml H₂O and 5 µl 30 % H₂O₂. One hundred microlitres of the substrate was added to each well and the plate was incubated for 15 min. The reaction was stopped with 100 µl 0.25 M H₂SO₄, and the optical density (OD) was measured at a wavelength of 490 nm (reference filter, 620 nm). All serum samples were tested in duplicate.

Complement-fixation assay. For the *C. jejuni*-specific CFA a commercial kit (Virion Serion) was used according to the instructions of the manufacturer. As recommended by the manufacturer, sera were tested at a dilution of 1 : 30.

Statistical analysis. To define the diagnostic value of the P18/P39 ELISA, receiver operating characteristic (ROC) curve analyses were performed (Zhou *et al.*, 2002). For the estimation of the accuracy of the P18/P39 ELISA, i.e. the ability to discriminate between individuals with and without disease, the areas under the ROC curves were calculated for IgA- and IgG-antibodies. The accuracy of a test tends to 1 as the ROC curve tends to perfect distinction. The areas under the ROC curves for the IgA- and IgG-specific P18/P39 ELISA were compared with an extension of the Wilcoxon-Mann-Whitney test (Kaufmann *et al.*, 2005) to see if one of the assays has a significantly higher accuracy, i.e. is more appropriate for detecting a preceding infection. Since our intention was to optimize the assay for both sensitivity and specificity, the cut-off values were determined according to the Youden index (Zhou *et al.*, 2002), which maximizes the sum of both.

RESULTS

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 *Campylobacteriales*, 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 immunoblot

P18, P39 and P62 were fused to 6 × 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 recombinantly expressed at high levels in Epicurian Coli BL21-CodonPlus (Fig. 1) and were immunoreactive with post-infection serum samples. Affinity purification of 6 × His-tagged P18 and P39 was successful, whereas the preparation of 6 × 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.

Fig. 1. Coomassie-stained SDS-PAGE of purified recombinant fusion proteins.

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 IgG-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

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).

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

Tested antibody	No. of subjects with positive serum		Sensitivity (%)	Specificity (%)	PPV† (%)	NPV‡ (%)
	Controls* (n = 48)	Patients with <i>C. jejuni</i> enteritis (n = 27)				
IgG anti-P18	10	20	74.1	79.2	66.7	84.4
IgG anti-P39	6	19	70.3	87.5	76.0	84.0
IgG anti-P18+P39	13	23	85.2	72.9	63.9	89.7
IgA anti-P18	0	11	40.7	100	100	75.0
IgA anti-P39	0	6	22.2	100	100	69.6
IgA anti-P18+P39	0	11	40.7	100	100	75.0

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

†Positive-predictive value.

‡Negative-predictive value.

Fig. 3. Scattergrams showing (a) IgG- and (b) IgA-specific OD₄₉₀ values measured in sera with the P18/P39 ELISA. The cut-off values indicated by the dotted lines were determined according to the Youden index (Zhou *et al.*, 2002).

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.

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 $\geq 1:128$) 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 $\geq 1:256$) for at least 3 months (Fig. 4).

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; Loch & 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

Table 2. Serological testing with the P18/P39 ELISA

Antibody class	No. of subjects with positive serum				Sensitivity (%)	Specificity* (%)	PPV† (%)	NPV‡ (%)
	Healthy blood donors (n = 57)	Patients with positive <i>Y. enterocolitica</i> serology (n = 19)	Patients with positive <i>H. pylori</i> serology (n = 22)	<i>C. jejuni</i> patients (n = 37)				
IgG	2	2	3	25	67.6	92.9	78.1	88.4
IgA	0	0	1	34	91.9	99.0	97.1	97.0
IgG+IgA	2	2	4	35	94.6	91.8	81.4	97.8

*Combining all controls.

†Positive predictive value.

‡Negative predictive value.

Fig. 4. Antibody titres measured in the P18/P39 ELISA in follow-up sera from four patients with culture-confirmed *C. jejuni* enteritis. The sera were sampled at various intervals after the onset of diarrhoea.

sera. 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

- Bardwell, J. C., McGovern, K. & Beckwith, J. (1991). Identification of a protein required for disulfide bond formation *in vivo*. *Cell* **67**, 581–589.
- Blaser, M. J. & Duncan, D. J. (1984). Human serum antibody response to *Campylobacter jejuni* infection as measured in an enzyme-linked immunosorbent assay. *Infect Immun* **44**, 292–298.
- Blaser, M. J., Hopkins, J. A. & Vasil, M. L. (1984). *Campylobacter jejuni* outer membrane proteins are antigenic for humans. *Infect Immun* **43**, 986–993.
- Boucqquey, D., Sindic, C. J., Lamy, M., Delmee, M., Tomasi, J. P. & Laterre, E. C. (1991). Clinical and serological studies in a series of 45 patients with Guillain-Barre syndrome. *J Neurol Sci* **104**, 56–63.
- Bremell, T., Bjelle, A. & Svedhem, A. (1991). Rheumatic symptoms following an outbreak of *Campylobacter* enteritis: a five year follow up. *Ann Rheum Dis* **50**, 934–938.
- Burnens, A., Stucki, U., Nicolet, J. & Frey, J. (1995). Identification and characterization of an immunogenic outer membrane protein of *Campylobacter jejuni*. *J Clin Microbiol* **33**, 2826–2832.
- CDC (2003). Preliminary FoodNet data on the incidence of foodborne illnesses – selected sites, United States, 2002. *MMWR Morb Mortal Wkly Rep* **52**, 340–343.
- Colmegna, I., Cuchacovich, R. & Espinoza, L. R. (2004). HLA-B27-associated reactive arthritis: pathogenetic and clinical considerations. *Clin Microbiol Rev* **17**, 348–369.
- Dunn, B. E., Cohen, H. & Blaser, M. J. (1997). *Helicobacter pylori*. *Clin Microbiol Rev* **10**, 720–741.
- Eastmond, C. J., Rennie, J. A. & Reid, T. M. (1983). An outbreak of *Campylobacter* enteritis – a rheumatological followup survey. *J Rheumatol* **10**, 107–108.
- Enders, U., Karch, H., Toyka, K. V., Michels, M., Zielasek, J., Pette, M., Heesemann, J. & Hartung, H. P. (1993). The spectrum of immune responses to *Campylobacter jejuni* and glycoconjugates in Guillain-Barre syndrome and in other neuroimmunological disorders. *Ann Neurol* **34**, 136–144.
- Engleberg, N. C., Howe, D. C., Rogers, J. E., Arroyo, J. & Eisenstein, B. I. (1991). Characterization of a *Legionella pneumophila* gene encoding a lipoprotein antigen. *Mol Microbiol* **5**, 2021–2029.
- Friedman, C. R., Neimann, J., Wegener, H. C. & Tauxe, R. V. (2004). Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In *Campylobacter*, 2nd edn, pp. 121–138. Edited by I. Nachamkin & M. J. Blaser. Washington DC: American Society for Microbiology.
- Hemila, H. (1991). Sequence of a PAL-related lipoprotein from *Bacillus subtilis*. *FEMS Microbiol Lett* **66**, 37–41.
- Hughes, R. A. & Rees, J. H. (1997). Clinical and epidemiologic features of Guillain-Barre syndrome. *J Infect Dis* **176** (Suppl 2), S92–S98.
- Jacobs, B. C., Rothbarth, P. H., van der Meche, F. G., Herbrink, P., Schmitz, P. I., de Klerk, M. A. & van Doorn, P. A. (1998). The spectrum of antecedent infections in Guillain-Barre syndrome: a case-control study. *Neurology* **51**, 1110–1115.
- Kaldor, J. & Speed, B. R. (1984). Guillain-Barre syndrome and *Campylobacter jejuni*: a serological study. *Br Med J (Clin Res Ed)* **288**, 1867–1870.
- Karvar, S., Karch, H., Frosch, M., Burghardt, W. & Gross, U. (1997). Use of serum-specific immunoglobulins A and G for detection of *Helicobacter pylori* infection in patients with chronic gastritis by immunoblot analysis. *J Clin Microbiol* **35**, 3058–3061.
- Kaufmann, J., Werner, C. & Brunner, E. (2005). Nonparametric methods for analysing the accuracy of diagnostic tests with multiple readers. *Stat Methods Med Res* **14**, 129–46.
- Ketley, J. M. (1997). Pathogenesis of enteric infection by *Campylobacter*. *Microbiology* **143**, 5–21.
- Kosunen, T. U., Kauranen, O., Martio, J. & 7 other authors (1980). Reactive arthritis after *Campylobacter jejuni* enteritis in patients with HLA-B27. *Lancet* **i**, 1312–1313.
- Lazzaroni, J. C. & Portalier, R. (1992). The *excC* gene of *Escherichia coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (PAL). *Mol Microbiol* **6**, 735–742.
- Lecuit, M., Abachin, E., Martin, A. & 10 other authors (2004). Immunoproliferative small intestinal disease associated with *Campylobacter jejuni*. *N Engl J Med* **350**, 239–248.
- Locht, H. & Krogfelt, K. A. (2002). Comparison of rheumatological and gastrointestinal symptoms after infection with *Campylobacter jejuni/coli* and enterotoxigenic *Escherichia coli*. *Ann Rheum Dis* **61**, 448–452.
- Murphy, T. F., Nelson, M. B. & Apicella, M. A. (1992). The P6 outer membrane protein of nontypeable *Haemophilus influenzae* as a vaccine antigen. *J Infect Dis* **165** (Suppl 1), S203–S205.
- Nachamkin, I. & Hart, A. M. (1985). Western blot analysis of the human antibody response to *Campylobacter jejuni* cellular antigens during gastrointestinal infection. *J Clin Microbiol* **21**, 33–38.
- Nelson, M. B., Apicella, M. A., Murphy, T. F., Vankeulen, H., Spotila, L. D. & Rekosh, D. (1988). Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. *Infect Immun* **56**, 128–134.
- Newell, D. G. & Nachamkin, I. (1992). Immune responses directed against *Campylobacter jejuni*. In *Campylobacter jejuni: Current Status and Future Trends*, pp. 201–206. Edited by I. Nachamkin, M. J. Blaser & L. S. Tompkins. Washington DC: American Society for Microbiology.
- Pawelec, D. P., Korsak, D., Wyszynska, A. K., Rozynek, E., Popowski, J. & Jagusztyn-Krynicka, E. K. (2000). Genetic diversity of the *Campylobacter* genes coding immunodominant proteins. *FEMS Microbiol Lett* **185**, 43–49.
- Pei, Z. H., Ellison, R. T. & Blaser, M. J. (1991). Identification, purification, and characterization of major antigenic proteins of *Campylobacter jejuni*. *J Biol Chem* **266**, 16363–16369.
- Power, M. E., Alm, R. A. & Trust, T. J. (1992). Biochemical and antigenic properties of the *Campylobacter* flagellar hook protein. *J Bacteriol* **174**, 3874–3883.
- Salem, P., Anaissie, E., Allam, C., Geha, S., Hashimi, L., Ibrahim, N., Jabbour, J., Habboubi, N. & Khalyil, M. (1986). Non-Hodgkin's lymphomas in the Middle East. A study of 417 patients with emphasis on special features. *Cancer* **58**, 1162–1166.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Stark, K. & Alpers, K. (2004). Bakterielle Gastroenteritiden: Situationsbericht 2003. *Epidemiologisches Bulletin* **31**, 251–254 (in German).
- Svedhem, A. & Kaijser, B. (1980). *Campylobacter fetus* subspecies *jejuni*: a common cause of diarrhea in Sweden. *J Infect Dis* **142**, 353–359.
- Wenman, W. M., Chai, J., Louie, T. J., Goudreau, C., Lior, H., Newell, D. G., Pearson, A. D. & Taylor, D. E. (1985). Antigenic analysis of *Campylobacter* flagellar protein and other proteins. *J Clin Microbiol* **21**, 108–112.
- Zhou, X., Obuchowski, N. A. & McClish, D. K. (2002). *Statistical Methods in Diagnostic Medicine*. Wiley Series in Probability and Statistics. New York: Wiley.