Immunogens of interest for the diagnosis of Campylobacter jejuni infections

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Received 12 August 1999; received in revised form 25 November 1999; accepted 29 November 1999

Abstract

In order to identify the Campylobacter jejuni immunogens of interest for the diagnosis of Campylobacter jejuni infections, we analyzed the humoral response of 153 patients by using complement fixation (CF) and western blot assays. A first group of 79 sera was from Campylobacter jejuni infected patients suffering from enteritis (n = 16), Guillain-Barré syndrome (GBS) (n = 40) and arthritis (n = 23). A second group of 49 sera was from healthy blood donors and a third group consisted of 25 sera from children under 4 years old. Using the CF test, 88.6% of the Campylobacter jejuni infected patients were seropositive versus 28.5% of the healthy blood donors and none of the children. The Western blot assay allowed detection of antibodies directed against seven selected antigens ranging from 14 to 67 kDa. Three of these antigens with a molecular size of 29, 37 and 43 kDa were detected by 86.0%, 84.8% and 91.1% of the Campylobacter jejuni infected patients, respectively. These three antigens seem to be good candidates for the development of assays suitable for direct and indirect diagnosis of Campylobacter jejuni infections. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Immunogen; Diagnosis; Campylobacter jejuni

1. Introduction

Campylobacter jejuni and Campylobacter coli have been recognized since the late 1970s as a common cause of sporadic bacterial diarrheal illnesses throughout the world, 85% of them being associated with Campylobacter jejuni and 15% with Campylobacter coli [1,2]. They account for an estimated 2 400 000 cases of infectious enteritis annually in the United States [3]. Infections occur with a summertime incidence peak which may reflect a greater frequency of contamination of animal products used for food, as well as larger infectious doses. Campylobacters are especially transmitted to man through contaminated food, raw milk or water; person to person transmission appears to be rare. Poultry, cattle, swine and sheep constitute the major food reservoir for Campylobacter jejuni. Because of certain dietary customs, meat is frequently undercooked and the frequency of Campylobacter jejuni infections is increasing. Poultry is recognized as the most important source of Campylobacter jejuni infections and it would be of great interest to detect contamination directly in the meat or in the poultry rearings [4].

In developing countries, the highest infection rates are among children under 2 years old, then the incidence decreases with age and older children are often asymptomatic carriers. In developed countries, the incidence of infection shows a bimodal age distribution with the highest values in young infants and in the 20–40 age group [3]. Asymptomatic excreters are estimated to be less than 1%. Incubation period ranges from 2 to 10 days. Then, the diarrhea may range from loose stool to bloody stool and generally last 2 or 3 days but may persist for a week or longer. Further extra-intestinal complications include reactive arthritis located at large peripheral joints (ankle, knee, shoulder, elbow), pancreatitis, endocarditis and neurologic diseases as Guillain-Barré syndrome (GBS) or Miller Fisher syndrome (MFS) [5]. Multivariate analysis have shown that infections by a large variety of infectious agents precede GBS and MFS and that a recent Campylobacter jejuni infection is involved in 32% of the GBS [6,7]. The Lipopolysaccharide (LPS) of Campylobacter jejuni has been suspected to play a major role in GBS because of molecular mimicry of the GM1 and the GD1b gangliosides of neuronal cells [8]. The...
mimicking structures are located at the terminal regions of the oligosaccharide core of the molecule. Penner serotype O:19 is overrepresented in strains associated with GBS but other neuropathogenic serotypes including O:1, O:2, O:12 and O:64 have also been identified [9,10]. Serotype O:10 is the most often associated with MFS, the LPS mimicking the terminal region of human ganglioside GD3 [11].

Diagnosis of a C. jejuni/coli enteritis is usually performed by stool-culture. In GBS and reactive arthritis related to C. jejuni, the symptoms appear 1 week to 3 months after the diarrheal episode. At that time, the bacteria may have been eradicated and, consequently, the etiology of the illness can only be established by serology. A variety of serologic tests have been developed for the detection and/or quantification of antibodies to C. jejuni/coli: serum bactericidal assay [12], agglutination assay [13], immunofluorescent antibody assay [14], enzyme immunoassay [15,16] and complement fixation assay (CF) [17]. The only commercially available test is a CF test which uses a glycine bacterial extract obtained from five Swiss C. jejuni strains as antigen (Institut Virion, Switzerland) [18]. This test has a good specificity but is time consuming (48 h), difficult to perform, and does not distinguish between the two species, C. jejuni and C. coli. The CF test measures the total complement-fixing antibodies. It does not discriminate between IgG and IgM, but most of the antibodies detected are of the IgM class. Thus, the CF test allows the serodiagnosis of acute infection but is not suitable for the diagnosis of late complications of C. jejuni enteritis.

The aim of this study was to identify the major immunogens of C. jejuni which could be purified and used for the development of rapid serodiagnosis assays in order to detect IgG and IgM response developed during C. jejuni/coli infections and late complications. This would also allow the development of direct diagnosis assays for the detection of the bacterium or of its antigens directly in food or in poultry rearings.

2. Patients and methods

2.1. Human sera

Sera were collected from 79 C. jejuni infected patients, 49 healthy blood donors and 25 children under 4 years old. The C. jejuni infected patients consisted of 16 patients with uncomplicated enteritis, 40 patients with C. jejuni-related GBS, and 23 patients with C. jejuni-related reactive arthritis. C. jejuni infection was confirmed by culture of the bacteria for the enteritis patients, and either by culture and/or presence of CF antibodies for the GBS and the arthritis patients. The C. jejuni status of the healthy blood donors and the children was unknown at the beginning of the study.

2.2. CF test

CF antibodies to C. jejuni were detected using the Vi-rion CF test (Institut Virion, Switzerland). The test was conducted according to the manufacturer’s instructions. Each serum sample was assayed from the 1/8 to the 1/128 dilution. A titer of 1/8 or more indicated a previous or an ongoing infection with C. jejuni. A negative test was shown by an antibody titer < 1/8.

2.3. Western-blot

The western-blot was developed using C. jejuni strain 81176. This strain was originally isolated from a 9 year old girl in Minnesota during an outbreak of enteritis [19]. Bacteria were grown at 37°C for 48 h on Mueller Hinton blood agar (bioMérieux, Marcy l’Etoile, France) in a micro-aerobic atmosphere composed of 5% oxygen, 10% carbon dioxide, and 85% nitrogen (Anaerocult C, Merck, Darmstadt, Germany). Three kinds of antigenic extracts were prepared. The whole cell extract was obtained by sonication of bacteria harvested in bidistilled water as previously described [20]. In order to prepare the saline and glycine extracts, bacteria were harvested in 0.15 M NaCl and centrifuged at 4000×g for 15 min. The supernatant, corresponding to the saline extract, was removed. For preparation of glycine extracts, the surface proteins were extracted by stirring the washed cells in 0.2 M glycine hydrochloride buffer, pH 2.2, for 15 min at 37°C. After centrifugation at 11000×g for 15 min, the supernatant corresponding to the glycine extract was neutralized with 1 M NaOH. The three bacterial extracts were dialyzed for 24 h against distilled water and the protein concentration was determined with the BCA protein assay reagent kit (Pierce Chemical, Rockford, IL, USA).

Antigenic extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and using a mini-slab gel apparatus (Bio-Rad Laboratories, Richmond, CA, USA). [21]. Samples were stacked in a 4% acrylamide gel and resolved in a 14% acrylamide mini-gel. Molecular mass standard proteins were purchased from Pharmacia LKB. After migration, proteins were electro-transferred to nitro-cellulose membranes and the Western blot was carried out as previously described, testing the human sera at a 1/100 dilution [22]. Antibodies to C. jejuni were detected using alkaline-phosphatase conjugated rabbit antibodies directed to human IgG (Dako, Copenhagen, Denmark). The position of the immunoreactive bands revealed by the patients sera were assessed with a calibrating curve constructed by plotting the distance of migration of the immunoreactive bands (in millimeters) against the molecular mass of the standard proteins or of immunoreactive bands obtained with monoclonal antibodies directed to antigens of 19, 27, 29, 43 and 67 kDa.
2.4. Statistical analysis

The $\chi^2$ test was used to compare the frequencies of immunoreactive bands of the different patients.

3. Results

The serological status of the 153 patients included in the study was initially established using the commercial CF test. Thirteen of the 16 patients (81.2%) with a C. jejuni enteritis, 40 patients (100%) with a GBS and 17 of 23 patients (73.9%) with a reactive arthritis were seropositive by the CF test. Of the 49 healthy blood donors, 14 (28.5%) were positive by the CF test and none of the 25 children under 4 years old was positive.

Antibody patterns of the 153 patients were established by western-blot. In order to choose the best antigenic bacterial extract to use in the western-blot assay, antibody patterns of a limited number of sera, 15 CF-positive and five CF-negative sera, were firstly analyzed by Western blot using the three bacterial extracts as antigen i.e.: saline, glycine and whole cell extracts prepared from C. jejuni 81176. For each serum, the number and the nature of the immunoreactive bands observed on the blots varied with the C. jejuni extract used to coat the nitrocellulose membrane. Fig. 1 shows the antibody patterns of five CF-positive and three CF-negative sera, blotted to the three antigenic preparations. Using the glycine extract, we could easily distinguish seven immunoreactive bands regularly recognized by the seropositive samples. These bands corresponded to antigens with molecular masses of approximately 67, 43, 37, 29, 24, 18 and 14 kDa. The two other bacterial extracts gave antibody patterns with numerous immunoreactive bands, some of them being difficult to distinguish from each other. Consequently, the glycine extract was chosen to analyze the serologic response of the 153 patients. For each serum, the seven immunoreactive bands previously cited were used to define the antibody pattern.

Of the 25 sera collected from young children, 17 (68%) showed no immunoreactive band and 8 (32%) showed just one immunoreactive band directed to the 67 kDa antigen (Table 1). For the 49 sera from the healthy blood donors, different antibody patterns could be observed. All of them (100%) reacted with the 67 kDa antigen and 29 sera (59.1%) reacted with the 14 kDa antigen. Sera having no more than two immunoreactive bands located at 67 and/or 14 kDa (34.7%) were also negative by the CF test and were thus considered as seronegative. The immune response detected to these two antigens was probably due to a non specific recognition. To confirm this hypothesis, we analyzed by Western blot the anti-C. jejuni reactivity of 103 patients suffering from infections with various Gram negative bacteria including Helicobacter pylori ($n=12$), Salmonella ($n=13$), Yersinia ($n=10$), Legionella ($n=8$), Leptospira ($n=6$), Borrelia ($n=19$), Treponema pallidum ($n=24$), and Prevotella ($n=11$). Analysis of the antibody patterns obtained with the 103 sera showed that all the sera reacted with a 67 kDa antigen of C. jejuni and that 58 sera (56.3%) recognized a 14 kDa antigen (data not shown). Thus, immunoreactive bands located at 67 and 14 kDa could not be considered as C. jejuni specific. Fifteen of the 103 sera (14.5%) showed antibody patterns with antibodies directed to more than three antigens including some located at 43, 37, 29, 24 and 18 kDa. In the

![Fig. 1. Western blot analysis of glycine, saline and whole cell extracts obtained with sera collected from five C. jejuni infected patients (lanes 1–5) and three non infected patients (lanes 6–8).](image-url)
rest of the study only the five immunoreactive bands located between 43 and 18 kDa were considered. They were recognized by nine (18.3%) to 26 (53%) of the healthy blood donors and 18 (36.7%) of them recognized three antigens or more.

Analysis of the humoral response of the 79 C. jejuni infected patients showed diverse antibody patterns. The results are shown in Table 1 and Fig. 2. Of the five selected antigens, three having molecular masses of 43, 37 and 29 kDa were the most frequently detected since they were recognized by 68 (86%), 67 (84.8%) and 72 (91.1%), respectively of the sera from C. jejuni infected patients.

The antigens of 24 and 18 kDa were only recognized by 32 (40.5%) and 30 (37.9%) of the sera from infected patients, respectively. Fourteen (87.5%) sera of the enteritis group, 23 (100%) sera of the arthritis group and 33 (83.3%) sera of the GBS group reacted with at least three of the five selected antigens. The differences of frequency of each immunoreactive band between the three categories of C. jejuni infected patients were not statistically significant except for the 24 kDa antigen which was recognized by 56.2% of the enteritis patients compared with only 30% of the GBS patients (P < 0.05) (Table 1).

4. Discussion

The purpose of this study was to identify the major immunogens of C. jejuni in order to further improve the serologic assays currently available for the diagnosis of the diseases associated with this bacterium. A collection of 153 sera from C. jejuni seronegative and seropositive patients were tested using two serological methods, a commercial CF test and an ‘in-house’ immunoblot. The commercial kit ‘Virion CF test’ is the most often used for C. jejuni coli serology. It uses an antigenc preparation obtained from 5 C. jejuni strains. The CF antibodies react with group-specific antigens, and does not allow a species distinction between C. jejuni and C. coli. It especially detects IgM antibodies and therefore is confined to the diagnosis of acute or recent infections [14,16]. In this study, 81.2% of patients with C. jejuni enteritis and 73.9% of patients with reactive arthritis were seropositive with the CF test. The C. jejuni infection of the other patients was bacteriologically proved. The IgM response of these seropositive patients was probably not yet developed at the time of the sera collection.

Sera of infected and non-infected patients were used in immunoblots with the glycine extract prepared from C. jejuni 81176. This strain has been extensively used before for research purposes and expresses all the major antigens of the C. jejuni species. The glycine extract contains the proteins removable from the bacterial cells by extraction with low pH glycine buffer suggesting that these proteins are exposed to the cell surface [20]. The antibody profiles of the C. jejuni infected patients showed several immunoreactive bands, the most reactive being at 67, 43, 37, 29, 24, 18 and 14 kDa. Surprisingly, none of the children under 4 years old were seropositive, neither by the CF test nor by Western blot. This category of patients is generally considered as one of the two risk groups for C. jejuni infection [3]. However, these children were hospitalized for another reason than a diarrheal illness. They were free from C. jejuni infection and therefore used as negative controls. Of the 49 sera from the healthy blood donors, 28.5% were positive by the CF test and 40.8% showed numerous immunoreactive bands in the Western blot as-

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Table 1
Frequencies of five immunoreactive bands observed in the three groups of patients: Children under 4 years old (CHI), Healthy blood donors (HDB), and C. jejuni infected patients suffering from enteritis (ENT), reactive arthritis (ART) and GBS
say, with at least three bands located between 43 and 18 kDa, and have been considered as seropositive. The Western blot assay detecting antibodies of the IgG class allows the detection of ancient as well as recent infections. This seroprevalence is consistent with previous observations in this age group [3].

Among the *C. jejuni* infected patients, 88.6% were positive with the CF test and on Western blot showed at least three immunoreactive bands located between 18 and 43 kDa. Analysis of the results showed that among the 40 GBS patients, 40 were CF positive and only 33 were Western blot positive, indicating a recent *C. jejuni* infection for seven of these patients (8.2%). Conversely, among the 23 arthritis patients, 17 were CF positive and 23 were Western blot positive. The *C. jejuni* infections of the six CF negative patients (26.1%) were diagnosed by positive stool cultures and the serological results indicated that the IgM response has decreased and may not be detectable by the CF test. Thus, the results are dependent on the time of serum collection relative to the time of the bacterial infection and show the difference in sensitivity and specificity of each method. The CF test is of great interest for the detection of acute *C. jejuni* infection since it detects especially the IgM antibodies. Being positive during only 1 or 2 months, it permits discrimination between infected and non-infected persons [14]. The western blot detecting IgG antibodies is preferable for the diagnosis of late complications of *C. jejuni* enteritis and for epidemiologic studies.

The main antigens recognized by serum antibodies ranging from 14 to 67 kDa. An immunoreactive band located at 67 kDa was recovered in all sera from adult patients and in 32% of the young children. The corresponding antigens may probably be the flagellar protein or/and the GroEl-like heat shock protein, these two proteins having similar molecular masses and being highly conserved amongst Gram negative bacteria [23–25]. In the analysis of the 103 sera from patients infected with various Gram negative bacteria different from *C. jejuni*, we have shown that a second immunoreactive band located at 14 kDa frequently cross reacted with other bacteria. This antigen could be the lipopolysaccharide or/and the GroES-like protein for which cross reactions have frequently been reported between Gram negative bacteria [26–28]. For these reasons, the two immunoreactive bands located at 67 and 14 kDa were disregarded as non specific of *C. jejuni* infection in our analysis.

Among the five antibodies retained as specific of *C. jejuni* infection, those corresponding to the 43, 37 and 29 kDa antigens appeared as major immunogens since they were recognized by more than 80% of the *C. jejuni* infected patients. The 43 kDa antigen may be the major outer membrane protein (MOMP) corresponding to a porin located in the outer membrane [29,30]. It has been shown that this protein represented one of the two major immunogens of *C. jejuni*, the second one being the flagellin. The antigen of 37 kDa is probably the protein identified by Konkel et al. [31] as CadF, a fibronectin-binding protein that promotes the binding of the bacteria to intestinal epithelial cells. It is specific for *Campylobacter* and analysis of the corresponding gene in food products has been reported as a method of detection of campylobacters [32]. The 29 kDa antigen may be the protein described by Burucoa et al. [33] which resembles a member of the ABC transporter family [34]. Some cross-reactions exist with close bacteria, especially with *H. pylori*. Nevertheless, corresponding antibodies could be considered for serodiagnosis of *C. jejuni* infections when that antibodies directed to other specific antigens, i.e. the 43 and 37 kDa proteins, are simultaneously detected.

We concluded that the antigens of 43, 37 and 29 kDa are the major immunogens of the glycine extract prepared from *C. jejuni* strain 81–176. Their detection in both food products and human stools could be useful for diagnostic purposes. These proteins appear to be good candidates for the development of sensitive and specific immunoassays suitable for the serodiagnosis of *C. jejuni* infections. The distinction between IgM and IgG antibodies would be interesting for the discrimination of recently infected patients, and between acute and late complications of *C. jejuni* infections.

Acknowledgements

We thank F. Mégraud and A. Burnens for providing us some human sera. We are grateful to L. Pezennec for technical assistance. This work has been financially supported by the Ministère de l’Enseignement Supérieur et de la Recherche, the University of Poitiers, by AVENTIS Mérieux-Pasteur and the Institut de Recherche des Maladies de l’Appareil Digestif (France).

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FEMSIM 1176 9-2-00