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Characterization of clinical *Campylobacter jejuni* isolates with special emphasis on lipooligosaccharide locus class, putative virulence factors and host response☆

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ABSTRACT

Recent studies have indicated a role of the lipooligosaccharide (LOS) of *Campylobacter jejuni* in the severe neurological Guillain Barré syndrome, as well as in development of more severe symptoms of acute enteritis. We evaluated the role of the LOS locus class in *C. jejuni* infection among 163 enteritis patients. The prevalence of LOS locus classes differed according to the origin of the isolates. Furthermore, LOS locus classes A and B were significantly associated with susceptibility or resistance to ciprofloxacin and doxycycline. However, our results do not corroborate earlier findings that isolates with potential to sialylate LOS might be associated with more severe symptoms of enteritis. Instead, in an infection model, such isolates gave weaker epithelial IL-8 responses than nonsialylated isolates. Absence of the iron transport protein encoded by the gene *ceuE* as well as the putative fucose permease gene *cj0486* was associated with increased in vitro IL-8 secretion.

Introduction

Campylobacter jejuni is the most common cause of bacterial gastroenteritis in the western world, estimated to cause around nine million infections annually in the European Union (EFSA, 2011). However, many aspects of the pathogenesis of *C. jejuni* still remain unknown. Despite the identification of numerous putative virulence factors (Dasti et al., 2010), their clinical relevance is still largely unrevealed. The structure of the lipooligosaccharide (LOS) of *C. jejuni* has been shown to affect pathogenic properties in vitro and in some studies *C. jejuni* strains capable of sialylating their LOS have shown increased adhesion and invasion of cultured intestinal epithelial cells (Louwen et al., 2008; Habib et al., 2009). Furthermore, antigenic mimicry between human gangliosides and sialylated LOS of *C. jejuni* may be associated with the development of severe post-infectious neuropathies (Koga et al., 2006). In a recent study on patients with *C. jejuni* enteritis in Denmark, isolates with LOS locus classes harboring genes for sialylation of LOS were associated with a prolonged duration of diarrheal symptoms and isolates of LOS locus class A were associated with joint symptoms (Mortensen et al., 2009).

A hallmark of *C. jejuni* enteritis is abundant infiltration of neutrophilic granulocytes into the intestinal lumen (Wassenaar and Blaser, 1999). The chemokine IL-8 is an important chemo attractant for neutrophils and is secreted by intestinal epithelial cells in response to infection caused by several enteric mucosal pathogens (Yang et al., 1997). Hickey et al. showed that *C. jejuni* 81–176 triggered IL-8 secretion in INT-407 cells upon infection, in a time and dose dependent manner (Hickey et al., 1999). Some studies suggest that IL-8 secretion requires viable bacteria and de novo protein synthesis, and that it is associated with both bacterial adhesion and invasion of the epithelial cells (Hickey et al., 1999; Watson and Galan, 2005; Johanesen and Dwinell, 2006). Others have found that viable bacteria might not be needed for cell activation (Mellits et al., 2002; Bakhiet et al., 2004; de Zoete et al., 2010). One study suggested the importance of cytolethal distending toxin (CDT) and an intact flagellum for induction of IL-8 release in T84 cells (Zheng et al., 2008), whereas Chen et al. noted that basolateral infection resulted in higher IL-8 secretion, indicating the importance of invasion or subvasion (Chen et al., 2006). Hence, the exact mechanisms of mucosal cell activation and the importance of specific bacterial factors still remain elusive.

We have recently presented data on associations between putative virulence factors among clinical *C. jejuni* isolates and the characteristics of the corresponding patients. One finding

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was that isolates which had the gene *cgtB*, encoding a putative β-1,3-galactosyltransferase involved in LOS biosynthesis, were overrepresented among patients with bloody stools (Feodoroff et al., 2010). The primers used in that study detected the version of the gene *cgtB* encoded by the open reading frame orf6ab1 which is a marker for LOS locus classes A1 and B1 (Parker et al., 2005). One aim of the current study was to determine the LOS locus class of the *C. jejuni* isolates using a PCR screening method in order to find possible associations between specific LOS locus classes and other bacterial, or patient, characteristics. In addition, we analyzed the IL-8 response to infection of INT-407 cells for the same isolates to compare in vitro host responses between isolates of different LOS locus classes as well as between isolates proficient or deficient in six different putative virulence factors.

Materials and methods

Patients and Campylobacter isolates

A total of 163 *C. jejuni* stool culture isolates from Finnish patients with sporadic enteritis (no other bacterial enteropathogens detected) and questionnaire-based background information for the patients were available including age, gender, symptoms of disease, hospitalization, foreign travel etc. (Feodoroff et al., 2009, 2010). An infection was considered to be acquired abroad if the patient had reported travel abroad within two weeks prior to onset of symptoms. All isolates were hippurate positive and identified to the species level by PCR according to the methods described earlier (Linton et al., 1996, 1997; Gonzalez et al., 1997; Denis et al., 1999; Feodoroff et al., 2011). The presence of genes encoding putative virulence factors had been determined for all isolates by PCR or by phenotypic testing (Feodoroff et al., 2010), and the prevalence of each putative virulence factor was as follows: gammaglutamyltranspeptidase (GGT) 15%, the plasmid located virulence gene *virB11* 2.4%, *Campylobacter* invasion antigen B (*ciaB*) 99%, the putative fucose permease gene *cj0486* 49%, the enterochelin periplasmic binding protein *ceuE* 86% and the cytolethal distending toxin genes *cdtABC* 79%. Minimal inhibitory concentration (MIC) testing according to the CLSI guidelines (CLSI, 2006) had shown that 83 isolates were resistant to ciprofloxacin (including one isolate of domestic origin) and 64 isolates (no domestic) were resistant, or had reduced susceptibility to doxycyclin (Feodoroff et al., 2009). The isolates were stored at −70 °C until analyzed.

Infection of INT-407 cells

The human epithelial cell line INT-407 (ECACC, Salisbury, UK) was infected with the 163 *C. jejuni* isolates and IL-8 secretion was measured after 24 h. Two days before the experiment, cells were seeded on to 24 well plates (Nunc, Roskilde, Denmark) at a density of 5×10^5 cells per well in DMEM complemented with 2 mM L-Glutamine, 1% nonessential amino acids, penicillin (60 μg/ml), streptomycin (50 μg/ml) (Swedish National Veterinary Institute, Uppsala, Sweden) and 10% fetal calf serum (FCS) (Invitrogen, New York, USA). One hour before the experiment cells were gently washed with PBS (15 mM/pH 7.2) and 0.9 ml of fresh antibiotic free infection medium (DMEM supplemented with 1% FCS) was added to each well. Bacteria were incubated microaerobically in Brucella broth (Decton Dickinson, Sparks, USA) for 16–18 h at 42 °C. Optical density (OD) was measured at 405 nm and after centrifugation at 8000 × *g* for 5 min, the bacterial concentration was adjusted to 1×10^9 cfu/ml with fresh infection medium. Cells were infected with 100 μl of the bacterial suspension (MOI = 100); each isolate was inoculated into 2 wells and the plate was incubated at 37 °C in 5% CO_2. The bacterial concentration of the inoculum was verified by plate counts after each experiment. Ten isolates were tested at the time in one 24 well cell culture plate on three separate occasions. The *C. jejuni* strain 81–176 was used as a positive control and infection medium as a negative control in each plate. Supernatants were harvested after 24 h, centrifuged at 200 × *g* for 10 min, transferred to a new tube and immediately frozen at −20 °C until further analysis. All infection experiments were performed in duplicates on three separate occasions.

Quantification of IL-8 secretion

Quantification of IL-8 secretion into the supernatant was performed by ELISA using the Quantikine Human CXCL8/IL-8 kit (R&D systems, inc., Minneapolis, USA). Undiluted supernatants (50 μl) from the infected cells or uninfected control were added to each well, together with 100 μl of assay diluent provided. All further steps were performed according to the manufacturer's instructions. To enable comparison between isolates tested at separate occasions, the IL-8 secretion in response to each isolate was related to that of the positive control (strain 81–176). The mean background value from the negative control was subtracted from the mean IL-8 value of the duplicate samples in each experiment. This background corrected value was then expressed as the percentage of the positive control mean IL-8 value from each cell culture plate. The relative IL-8 values from each of the three repeated experiments for each isolate were used to calculate a mean value for each isolate expressed as the percentage of strain 81–176 (% of 81–176).

Determination of LOS locus class

LOS locus class was determined for the 163 isolates by PCR screening as described by Parker et al. (Parker et al., 2005, 2008). The primer names and sequences used in this study are described in Table 2 of Parker et al., 2005. Primers for orf12 (*waaV*) were used to verify successful DNA extraction. The LOS locus classes A and B were identified using primers for orf7ab (*cstII*), orf6ab1 (*cgtB-1*), orf6ab2 (*cgtB-2*) and orf5bII (cgtA2). Class C was identified using primers for orf6c (*cgtB*) and orf7c (*cstIII*). The genetically related classes E, H, O and P were identified by primers for orf26e and orf27e. Primers for orf18df detect isolates of the nonsialylated classes D, F, I, J, K, N, S, or Q and were used to attribute isolates that were untypeable using the primers above. The PCR reaction mixture consisted of 1 × AmpliTaq Gold 360 buffer, 1.25 U of AmpliTaq Gold 360 polymerase (Applied Biosystems, Foster City, USA), 200 μM dNTP (Fermentas, St.Leon-Rot, Germany), 0.2 μM of each primer (Eurogentec, Ougrée, Belgium) and 5 μl of template DNA in a total volume of 25 μl. Cycling conditions for all primer pairs were as follows: 30 cycles of 25 s at 94 °C, 25 s at 52 °C, and 60 s at 72 °C and final extension at 72 °C for 5 min. Bacterial DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Sollentuna, Sweden) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed with GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA) and PASW Statistics 18 (SPSS for Windows, Rel. 18.0.2. 2010. SPSS Inc, Chicago, IL, USA). Mann–Whitney test was used to analyze differences of median IL-8 values between groups and χ^2 or Fisher's exact tests were used for comparison of categorical data. Multivariate analyses were performed with stepwise binary logistic regression models. All tests were two-sided, and a P value < 0.05 was considered significant.