ANALYSIS OF *CAMPYLOBACTER JEJUNI* ISOLATES OF VARIOUS SOURCES FOR LOCI ASSOCIATED WITH GUILLAIN–BARRÉ SYNDROME

P. Amon¹, D. Klein², B. Springer³, S. Jelovcan³, D. Sofka¹ and F. Hilbert^{1*}

¹Department of Farm Animals and Veterinary Public Health, Institute of Meat Hygiene, Meat Technology and Food Science, University of Veterinary Medicine Vienna, Vienna, Austria

²Section of Herd Health Management, Department of Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Vienna, Austria

³Institute of Medical Microbiology and Hygiene, Graz, Austria

Received: January 11, 2012; Accepted: January 13, 2012

Campylobacter jejuni is a major cause of the Guillain–Barré syndrome (GBS) and related diseases. These autoimmune diseases are caused by antibodies cross-reacting with the peripheral (GBS) and central neural tissue (Miller Fisher syndrome – MFS, Bickerstaff’s brainstem encephalitis – BBE), leading to acute polyneuropathy. Recently, specific gene loci in *C. jejuni* have been distinguished which are associated with the onset of GBS, despite a molecular or phenotypic clustering. In this study, we used PCR to analyse *C. jejuni* isolates of different origin (i.e. bovine, poultry, human) for these genes. A total of 196 isolates were tested for *cst-II* and *neuA*. Of these, 101 isolates harboured the *cst-II* locus and 102 the *neuA* locus. Eighty-six isolates (44%) hold both genes. The frequency of *cst-II* in different sources of isolates of bovine, poultry and human isolates did not vary significantly (52, 50 and 52%, respectively). In contrast, the *neuA* locus was less often found in poultry isolates. Two human strains – from a family outbreak of campylobacteriosis (in 1989 in Austria) in which one person developed MFS – harboured both genes. Thus, although only one in more than 3000 patients with *Campylobacter*-associated enteritis develop GBS, about half of *Campylobacter jejuni* strains found in different environments are possibly able to cause GBS. These strains almost equally distributed in bovine, poultry and human isolates. Our results suggest that isolates associated with GBS are not selected by environmental or host-specific factors. Accordingly, this study indicates that host factors such as humoral and cellular immunity are possibly responsible for the development of these autoimmune diseases.

Keywords: *Campylobacter jejuni*, Guillain–Barré syndrome, *cst-II*, *neuA*, cattle, poultry, meat

Introduction

Campylobacter jejuni is the major cause of bacterial food-borne enteric disease in the world. Infection with this particular pathogen most likely represents the major cause of the Guillain–Barré syndrome (GBS), an autoimmune disease caused by antibodies cross-reacting with the peripheral neural tissue leading to acute polyneuropathy [1]. The Miller Fisher syndrome (MFS) is a variant characterized by ataxia and ophthalmoplegia [2]. Bickerstaff’s brainstem encephalitis (BBE) is also hypothesized to be related to this syndrome [3]. Each year, about 6000 cases of *Campylobacter*-associated enteritis are reported in Austria (but only inconclusive data on GBS (around 200–300 cases per year) [4, 5]. The most important source of infection is poultry (up to 70%), but also cattle-related disease outbreaks – via undercooked beef or unpasteurized milk and dairy products – are reported [6, 7]. Strains of *C. jejuni* can be characterized

by molecular methods, for example by MLST (multi locus sequence typing). By using this method, poultry isolates can be clustered versus the cattle isolates [8].

Certain serotypes of *C. jejuni* have more frequently been associated with the development of GBS or MFS [9]. The production of autoimmune antibodies against *C. jejuni* lipo-oligosaccharide (LOS) is responsible for the development of these autoimmune diseases [10]. Various studies indicate that the terminal sugar regions of *C. jejuni*’s LOS is responsible for the production of these autoimmune antibodies. These specific antibodies are involved in attacking human gangliosides [11, 12]. Since Van Belkum and co-workers [13] showed that a sialyltransferase encoded by the *cst-II* gene is associated with *C. jejuni* GBS, other studies have confirmed this correlation [14, 15]. Constructions of the first *Campylobacter* sialic acid mutants showed that another gene *neuA* (ORF10) influenced the reactivity with sera of GBS patients. The enzyme encoded

*Corresponding author: Friederike Hilbert; Department of Farm Animals and Veterinary Public Health, University of Veterinary Medicine Vienna, Veterinärplatz 1, A-1210 Vienna, Austria; Phone: +43-1-25077-3316; Fax: +43-1-25077-3390; E-mail: friederike.hilbert@vetmeduni.ac.at

by *neuA*, i.e. an acylneuraminate cytidyltransferase, is also involved in the construction of the outer residue of *Campylobacter*'s LOS [15].

In this paper, we assess the incidence of GBS-related *cst-II* and *neuA* genes in *C. jejuni* isolates of different origin to determine whether environmental or host-specific factors select for or against GBS-related *C. jejuni* strains.

Materials and methods

Bacterial strains

Campylobacter jejuni were isolated from chicken ($n=83$) and turkey ($n=34$) meat samples from Austria in the years 2002–2010 ($n=117$) and from bovine faecal and beef samples from Austria, in the years 2003–2010 ($n=50$). Human samples ($n=27$) were obtained from the Austrian Campylobacter Centre and were isolated from Austrian patients during 2006–2010. Two additional isolates were included from an Austrian family outbreak of campylobacteriosis in 1989 including one diseased person with Miller Fisher syndrome.

PCR of genes associated with GBS

DNA of isolates was extracted by using standard molecular biological techniques [16]. All isolates were screened by polymerase chain reaction (PCR) for a 400-bp fragment of the bi-functional sialyltransferase *cst-II* gene and for a 500-bp fragment of the neuraminidase gene *neuA*. Concurrently, all isolates were amplified (as a control for DNA extraction and *Campylobacter jejuni* species confirmation) by PCR analysis of the hippuricase gene *hip* [17]. Conditions are given in Table 1.

Sequencing of PCR products

Nine PCR products were gel-extracted and further sequenced for control of the PCR product. Sequencing was done on an ABI PRISM® 310 Genetic Analyzer using Big Dye 3.3 and POP6. For sequencing reactions, 25 cycles

were performed on both strands using the forward – and in a second reaction the reverse primer. Sequence analysis was completed by using the Sequence Analysis Software v5.2; consensus sequences were established from both strands. For alignments, the ClustalW software was used and matched with the database.

Statistical analysis

Statistical analysis was done by SPSS® version 14.0 for Windows®. Statistical differences between isolates of different origins were analyzed using the Mann–Whitney *U* test, dependent binary outcome variable and independent categorical variable analysis.

Results

In each of the 196 isolates, the *hip* gene was detected by PCR. Thus, all isolates could be confirmed as *C. jejuni*. Additionally, this PCR was suitable to control the DNA extraction procedure. Therefore, the extracted DNA of all isolates was useful for PCRs. From a total of 196 isolates, 101 tested positive for *cst-II* and 102 harboured the *neuA* locus. In a total of 86 isolates both genes could be detected whilst 79 isolates gave no positive result for either gene. There was a statistically highly significant correlation ($p < 0.01$) for the incidence of both genes when an isolate harboured one of the associated genes. Nevertheless, in 15 isolates only the *cst-II* gene could be detected by PCR and in 16 isolates only the *neuA* gene was verified.

In 16 of 29 human isolates the *cst-II* gene was detected. Of these, one isolate did not harbour the *neuA* gene. In 21 isolates the *neuA* gene could be detected by PCR and of these six isolates were not positive for *cst-II*. Two isolates from a family outbreak were included in this study. One *C. jejuni* was isolated from a child that came down with MFS and the second from an adult male with symptoms of enteritis only. Both isolates tested positive for *cst-II* and *neuA* by PCR.

Twenty-six isolates of a total of 50 cattle isolates were positive for the *cst-II* gene and six of these did not give a positive PCR for the *neuA* gene, whereas 28 isolates tested

Table 1. Primers and conditions for PCR

Primer name	Sequence	Target gene	PCR-amplicon size	Annealing temp (°C)	Reference
cstII-f	5'-gttattattgctggaatggaccaagt-3'	<i>cstII</i>	400	52	14
cstII-r	5'-acatatagaccctgaggtattcttt-3'				
neuA-f	5'-gctcgtggtgctcaaaggg-3'	<i>neuA</i>	500	52	This study
neuA-r	5'-attgcaccattgctcatata-3'				
HIP400F	5'-gaagagggtttgggtggtg-3'	<i>hip</i>	735	66	17
HIP1134R	5'-agctagcttcgcataataacttg-3'				

positive for the *neuA* gene and in eight of these isolates the *cst-II* gene could not be recognized.

From 117 poultry isolates (chicken and turkey) 51 held both genes associated with GBS. Eight isolates solely harboured the *cst-II* gene and two isolates tested positive for the *neuA* gene only. From 83 chicken isolates 40 harboured the *cst-II*, 35 the *neuA* and 33 both genes. From 34 turkey isolates 19 tested positive for *cst-II* and 18 for *neuA* – all of these 18 tested positive for *cst-II* as well.

In the statistical analysis of the distribution of the two genes among the various origins of the isolates, the two human outbreak strains were excluded as they did not fall into the group of human strains isolated during 2006–2010.

The distribution between the isolates of different origins harbouring both genes (*cst-II* and *neuA*) was almost equal and statistically not different (40% of cattle isolates, 44% of poultry isolates and 48% of human isolates, respectively). However, a significantly lower amount of isolates from chicken meat harboured the *neuA* gene as detected by PCR ($p=0.013$). In cattle and humans *neuA* harbouring isolates were detected in higher numbers than *cst-II* positive isolates. In contrast, in poultry isolates more *cst-II* positive isolates than *neuA* positives were found (Table 2).

Discussion

To date, many studies have analysed *C. jejuni* human isolates for GBS-related genes [10, 13–15, 18–20] only two studies included environmental and chicken meat isolates [21, 22]. Parker and co-workers [22] could detect genes responsible for ganglioside mimics in 35 out of 55 human enteric isolates (64%). This result is similar to those in our human isolates (*cst-II* and *neuA*: 48%; *cst-II*: 52%; *neuA*: 70%). Hardy and co-workers [21] compared their broiler meat isolates with enteric human isolates and to GBS and MFS isolates. They could not detect a difference between the broiler isolates and the enteric human isolates but a significant difference was seen between upper mentioned and GBS (MFS) isolates. Our results are similar. We included all human strains independent of clinical symptoms for statistical analysis and found no difference between human, cattle, turkey or chicken isolates as regards the frequency of *cst-II*. In our study, we relied on the detection of *neuA*, a gene encoding for an enzyme named acylneuraminase

cytidyltransferase. This enzyme is responsible for the activation of sialic acid after which it can be transferred by the sialyltransferase (*cst-II* gene product). In all likelihood, in *C. jejuni* *cst-II* and *neuA* are working together. Human, cattle and turkey isolates did not differ in the distribution of this gene, whereas in chicken isolates a lower number of isolates harbouring *neuA* was detected. For a better understanding, the role of *neuA* needs to be further addressed in (1) the development of GBS and associated disease and (2) adaptation to the chicken-specific gut and/or the chicken meat surface. On the other hand, the equal distribution of *cst-II* in all main human infectious sources of campylobacteriosis – as in chicken, turkey and cattle – and the crucial role of *cst-II* in ganglioside mimicry [23] let us to hypothesize that no selection for or against GBS-associated strains of *C. jejuni* occurs in the environment. Thus, despite the high amount (about 50%) of isolates from infectious sources that bear the risk of post-infectious neuropathy after campylobacteriosis, the individual immune system of the host (humoral and cellular immunity) is responsible for development of GBS and related autoimmune diseases.

Acknowledgements

We thank Dr. G. Feierl for the two family outbreak isolates of *C. jejuni*. This work was supported, in part, by the “Profillinien” program of the University of Veterinary Medicine, Vienna.

References

1. Rees JH, Soudain SE, Gregson NA, Hughes RA: *Campylobacter jejuni* infection and the Guillain-Barré syndrome. *N Engl J Med* 333, 1374–1379 (1995)
2. Nachamkin I, Allos BM, Ho T: *Campylobacter* species and Guillain-Barré syndrome. *Clin Microbiol Rev* 11, 555–567 (1998)
3. Mori M, Koga M, Yuki N, Hattori T, Kuwabara S: Bickerstaff's brainstem encephalitis after an outbreak of *Campylobacter jejuni* enteritis. *J Neuroimmunol* 196, 143–146 (2008)
4. Feierl G, Jelovcan S: Campylobacteriosis in Austria: situation and trends. *Wien Klin Wochenschr* 121, 103–107 (2009)
5. Jelovcan S: Nationale Referenzzentrale für Campylobacter Jahresbericht 2010. http://bmg.gv.at/cms/home/attachments/5/4/2/CH1305/CMS1299587296083/jb_campylobacter_2010_rev.pdf (2011)
6. Sheppard SK, Dallas JF, Strachan NJ, MacRae M, McCarthy ND, Wilson DJ, Gormley FJ, Falush D, Ogden ID, Maiden MC, Forbes KJ: *Campylobacter* genotyping to determine the source of human infection. *Clin Infect Dis* 48, 1072–1078
7. Skirrow MB: Epidemiology of *Campylobacter* enteritis. *Int J Food Microbiol* 12, 9–16 (1991)
8. Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, Fox A, Fearnhead P, Hart CA, Diggle PJ: Tracing the source of campylobacteriosis. *PLoS Genet* 4, e1000203 (2008)
9. Kuroki S, Saida T, Nukina M, Haruta T, Yoshioka M, Kobayashi Y, Nakanishi H: *Campylobacter jejuni* strains from

Table 2. Differences in the distribution of *cst-II* and *neuA* between human, cattle, chicken and turkey meat isolates (p -values)

	Human/ cattle (p -values)	Human/ chicken meat (p -values)	Human/ turkey meat (p -values)
<i>cst-II</i>	0.990	0.741	0.745
<i>neuA</i>	0.220	0.013*	0.167

*Significant difference at the 95% confidence level.

- patients with Guillain–Barré syndrome belong mostly to Penner serogroup 19 and contain beta-N-acetylglucosamine residues. *Ann Neurol* 33, 243–247 (1993)
10. Godschalk PC, Kuijf ML, Li J, St Michael F, Ang CW, Jacobs BC, Karwaski MF, Brochu D, Moterassed A, Endtz HP, van Belkum A, Gilbert M: Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain–Barré and Miller Fisher syndromes. *Infect Immun* 75, 1245–1254 (2007)
 11. Ang CW, Laman JD, Willison HJ, Wagner ER, Endtz HP, De Klerk MA, Tio-Gillen AP, Van den Braak N, Jacobs BC, Van Doorn PA: Structure of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity and clinical features of Guillain–Barré and Miller Fisher patients. *Infect Immun* 70, 1202–1208 (2002)
 12. Prendergast MM, Moran AP: Lipopolysaccharides in the development of the Guillain–Barré syndrome and Miller Fisher syndrome forms of acute inflammatory peripheral neuropathies. *J Endotoxin Res* 6, 341–359 (2000)
 13. Van Belkum A, van den Braak N, Godschalk P, Ang W, Jacobs B, Gilbert M, Wakarchuk W, Verbrugh H, Endtz H: A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. *Nat Med* 7, 752–753 (2001)
 14. Nachamkin I, Liu J, Li M, Ung H, Moran AP, Prendergast MM, Sheikh K: *Campylobacter jejuni* from patients with Guillain–Barré syndrome preferentially expresses a GD(1a)-like epitope. *Infect Immun* 70, 5299–5303 (2002)
 15. Godschalk PC, Heikema AP, Gilbert M, Komagamine T, Ang CW, Glerum J, Brochu D, Li J, Yuki N, Jacobs BC, van Belkum A, Endtz HP: The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain–Barré syndrome. *J Clin Invest* 114, 1659–1665 (2004)
 16. Sambrook JF, Fritsch EF, Maniatis T (1989): *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York
 17. Linton D, Lawson SJ, Owen RJ, Stanley J: PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *J Clin Microbiol* 35, 2568–2572 (1997)
 18. Islam Z, Van Belkum A, Wagenaar JA, Cody AJ, De Boer AG, Tabor H, Jacobs BC, Talukder KA, Endtz HP: Comparative genotyping of *Campylobacter jejuni* strains from patients with Guillain–Barré syndrome in Bangladesh. *PLoS One* 4, e7257 (2009)
 19. Koga M, Takahashi M, Masuda M, Hirata K, Yuki N: *Campylobacter* gene polymorphism as a determinant of clinical features of Guillain–Barré syndrome. *Neurology* 65, 1376–1381 (2005)
 20. Yuki N: *Campylobacter* sialyltransferase gene polymorphism directs clinical features of Guillain–Barré syndrome. *J Neurochem* 103, 150–158 (2007)
 21. Hardy CG, Lackey LG, Cannon J, Price LB, Silvergeld EK: Prevalence of potentially neuropathic *Campylobacter jejuni* strains on commercial broiler chicken products. *Int J Food Microbiol* 145, 395–399 (2011)
 22. Parker CT, Horn ST, Gilbert M, Miller WG, Woodward DL, Mandrell RE: Comparison of *Campylobacter jejuni* lipooligosaccharide biosynthesis loci from variety of sources. *J Clin Microbiol* 43, 2771–2781 (2005)
 23. Kuijf ML, Samsom JN, van Rijs W, Bax M, Huizinga R, Heikema AP, van Doorn PA, van Belkum A, van Kooyk Y, Burgers PC, Luider TM, Endtz HP, Nieuwenhuis EE, Jacobs BC: TLR4-mediated sensing of *Campylobacter jejuni* by dendritic cells is determined by sialylation. *J Immunol* 185, 748–755 (2010)