

## CROSS-REACTIVE EPITOPES PRESENT IN *CAMPYLOBACTER JEJUNI* SEROTYPES ISOLATED FROM ENTERITIS PATIENTS

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**Abstract:** *Campylobacter jejuni* (*C. jejuni*) infection frequently triggers auto-immune-mediated neuropathies, especially the Guillain-Barre syndrome (GBS). The molecular mimicry between the core oligosaccharides of bacterial lipopolysaccharides (LPSs) and the human gangliosides presumably results in the production of anti-neural cross-reactive antibodies which are likely to be a contributory factor in the induction and pathogenesis of GBS. The aim of our study was to determine the presence of cross-reactive epitopes in *C. jejuni* LPSs isolated from enteritis patients and to determine their antigen reactivity. For that purpose we collected stool specimens from 21 patients with enteritis and without neurological symptoms. Seven different serotypes of *C. jejuni* (0 : 27; 0 : 6/0 : 7; 0 : 38; 0 : 3; 0 : 1/0 : 44; 0 : 19; 0 : 37) were detected using the Penner system. Unexpectedly, one serotype from this group was detected as 0 : 19, a serotype rarely isolated from enteritis patient and in close association with GBS. Binding studies using cholera toxin-B subunit and peanut agglutinin, showed the presence of ganglioside-like epitopes in *C. jejuni* strains 0 : 37, 0 : 19 and 0 : 27. Reactivity with sera from patient with GBS, with confirmed previous exposure to *C. jejuni* and with high a titre of anti-ganglioside antibodies, showed that the same three LPSs from *C. jejuni* serotypes 0 : 37, 0 : 19 and 0 : 27 bear cross-reactive epitopes in their LPSs structures. Our results confirm the results from previous studies that LPSs from certain *C. jejuni* serotypes bear cross-reactive ganglioside-like epitopes which might be involved in the induction of GBS after *C. jejuni* infection.

**Key words:** *Campylobacter jejuni*, Guillain-Barre syndrome, lipopolysaccharides, gangliosides.

### Introduction

*Campylobacter jejuni* (*C. jejuni*) is one of the main causes of bacterial diarrhoea and gastroenteritis in humans [1]. Infection with *C. jejuni* is frequently associated with the development of the Guillain-Barre syndrome (GBS), an autoimmune demyelinating polyneuropathy of the peripheral nervous system. Recent studies have shown that one-fourth to one-third of GBS patients had an antecedent *C. jejuni* infection [2], and that only specific *C. jejuni* serotypes are associated with the development of GBS [3]. Serotyping of *C. jejuni* strains by the heat-stable antigenic Penner system distinguishes more than 70 serotypes on the basis of the saccharide structure of the bacterial lipopolysaccharides (LPSs) [4]. Biochemical and serological analysis showed that some *C. jejuni* serotypes bear LPS structures that mimic human gangliosides [5, 6]. Gangliosides are components of the neuronal cell surface and myelin sheath, and they are considered one of the possible target antigens for an autoimmune attack, since serum antibodies against gangliosides are present in the acute phase of GBS subsequent to *C. jejuni* infection [7, 8]. The molecular mimicry between the core oligosaccharides of bacterial LPS and the carbohydrate moiety of the human gangliosides presumably results in the induction of cross-reactive antibodies which are likely to be a contributory factor in the induction and pathogenesis of GBS [9, 10, 11]. Detection of cross-reactive ganglioside-like epitopes present in *C. jejuni* LPSs is usually done using molecular markers such as cholera toxin-B subunit (CT-B) as GM1 ligand and peanut agglutinin (PNA) as marker for Gal $\beta$ 1-3GalNAc structures. A number of studies have shown that the *C. jejuni* serotype 0 : 19 is epidemiologically most commonly associated with GBS, and that this serotype is rarely isolated from gastroenteritis patients [12, 13]. Studies have also reported a presence of epitope mimicry between serotype 0 : 19 LPS and GM1 ganglioside [12, 31, 33]. Other *C. jejuni* serotypes identified in association with GBS include 0 : 2, 0 : 2/44, 0 : 4/59, 0 : 15, 0 : 18, 0 : 21, 0 : 24, 0 : 30, 0 : 37 and 0 : 53 [14, 15, 16]. Previous studies have shown that the acute-phase sera of most patients with *C. jejuni* associated GBS contain a high titre of antibodies to various gangliosides, predominantly to GM1 in approximately 30%, and also antibodies against asialoGM1, GD1b, GT1a, GM3, GD1a and GT1b [17, 18].

The aim of our study was to determine whether cross-reactive epitopes are present in *C. jejuni* serotypes isolated from enteritis patients and to determine their antigenic reactivity.

### Material and Methods

Stool samples from 21 patients with uncomplicated enteritis and no neurological symptoms were obtained in the period June 2008 – April 2009 from the Infectious Diseases Clinic, Skopje, Macedonia. Isolation, growth and bacterial identification of *C. jejuni* was done at the Microbiology and Parasitology Institute, Medical Faculty, UKIM Skopje, according to standard procedures. *C. jejuni* serotype 0 : 19 (ATCC 700297) obtained from the American Type Culture Collection (Rockville, MD, USA) was used as a positive control since its origin is from a GBS patient. The *C. jejuni* isolates were serotyped by the heat-stable (HS) serotyping Penner and Hennessy scheme [4, 19] using a commercial antisera set (*Campylobacter* Antisera Seiken Set, Denka Seiken Co., Japan). This analysis was performed in duplicate and the results were concordant in all patients.

LPSs were extracted from all *C. jejuni* isolates using the hot mini phenol-water technique described by Prendergast [20]. Bacterial cells were scraped into saline, washed in phosphate-buffered saline (PBS), centrifuged, dissolved in water, and an equal volume of 90% phenol was added. Residual phenol was removed by extraction and the combined aqueous layers containing most of the LPS were dialyzed. Characterization of the carbohydrate moiety present in gangliosides GM1 and asialoGM1 (1- $\mu$ g aliquots; Sigma Chemical Co.) and in LPS extracts (5- $\mu$ l aliquots) was done using thin-layer chromatography (TLC) on precoated silica gel 60 glass plates (Merck, Germany). Solvent systems consisting of chloroform–methanol–0.22%  $\text{CaCl}_2 \times 2\text{H}_2\text{O}$  (50 : 45 : 10 [vol/vol/vol]), and n-propanol–water–25%  $\text{NH}_4\text{OH}$  (60 : 30 : 10 [vol/vol/vol]) were used as developers for gangliosides and LPSs, respectively. Separated gangliosides and LPSs were visualized by spraying plates with resorcinol-HCl reagent.

The presence of ganglioside-like structures in isolated LPSs was detected by binding of CT-B and PNA on Western blot [21]. For that purpose GM1 and asialoGM1 gangliosides (1- $\mu$ g aliquots) and LPSs extracts of all *C. jejuni* serotypes (5- $\mu$ l aliquots) were separated on SDS-PAGE and transferred to a nitrocellulose membrane (pore size 0.45  $\mu\text{m}$ ; at 200 mA, Tris-glycine buffer, 4 hours). Subsequently, LPSs and gangliosides were incubated with CT-B peroxidase conjugate diluted in 1 : 1000 and PNA-peroxidase conjugate diluted in 1 : 50 in gelatin-PBS, for one hour.

The cross-reactivity between isolated LPSs, GM1 and asialoGM1 gangliosides was also studied using sera from GBS patient. Sera from the patient (L.G) with confirmed GBS and a high titre of anti-GM1 antibodies was generously given by Professor Slobodan Apostolski, from the Neurology Institute,

Clinical Centre Belgrade, Serbia, where the clinical differentiation stadium was done according to clinical, electrophysiological and laboratory criteria. In our previous work, infection with *C. jejuni* in this patient was confirmed by the presence of a high level of anti-glycoprotein and anti-ganglioside antibodies to a mixture of bacterial acid glycine extracted glycoproteins and LPS [22].

Antigen-reactivity of the isolated LPSs from all *C. jejuni* serotypes was detected by Western blotting [23]. Membranes containing isolated LPSs and gangliosides were overlaid with human serum (GBS patient serum and human control serum at 1: 100 dilutions) in 0.3% gelatine/PBS pH 7.4. Lanes were overlaid with peroxidase-conjugated anti-human IgG or IgM diluted 1: 500 in gel/PBS, incubated and washed as above. Visualization of the blot membrane was done using peroxidase substrate DAB (3,3'-diaminobenzidine tetrahydrochloride), and 20 ml 30% H<sub>2</sub>O<sub>2</sub>.

In this paper we present our data on ligand and antibody binding between gangliosides and LPSs of 7 different *C. jejuni* serotypes isolated from 21 patients with enteritis and no neurological symptoms, where cholera toxin-B subunit (CT-B) was used as GM1 ligand, peanut agglutinin (PNA) as marker for Galβ1-3GalNAc structures and antigen reactivity was tested with sera with a high titre of anti-ganglioside antibodies.

## Results

### *Serotyping*

Results from Penner serotyping of the strains are listed in Table 1. Among the 21 *C. jejuni* patient isolates, seven different serotypes were detected (0 : 27; 0 : 6/0 : 7; 0 : 38; 0 : 3; 0 : 1/0 : 44; 0 : 19; 0 : 37) and one was not detectable. Most frequent was serotype 0 : 3 present in 6 patients accounting for 28.5 % (enteritis patients 04, 07, 08, 12, 15 and 17), serotype 0 : 6/0 : 7 was present in 4 patients or 19.05 % (enteritis patients 02, 06, 14 and 16), serotypes 0 : 38 and 0 : 1/0 : 44 were present in 3 patients or 14.28 %, respectively (enteritis patients 03, 10 and 13 and enteritis patients 05, 09 and 19) and serotype 0 : 27 was present in two patients or 9.52% (enteritis patients 01 and 20). Serotypes 0 : 19 and 0 : 37 were present in one patient (enteritis patient 11 and enteritis patient 21, respectively) and one serotype was not detectable (N.D) (enteritis patient 18, accounting for 4.76%). One of each of the *C. jejuni* serotypes isolated was used for molecular and antigenic characterization.

Table 1

*C. jejuni* serotypes used in this study

Bacterial species	Penner serotype	Strain designation	Source
<i>C. jejuni</i>	0 : 27	IC 6754 IC 9067	Enteritis patients N <sup>o</sup> 01; 20
	0 : 6/0 : 7	IC 6487 IC 9089 IC 6769 IC 6875	Enteritis patients N <sup>o</sup> 02; 06; 14; 16
	0 : 38	IC 8007 IC 8532 IC 9051	Enteritis patients N <sup>o</sup> 03; 10; 13
	0 : 3	IC 9045 IC 6768 IC 6755 IC 4182 IC 4457 IC 5382	Enteritis patients N <sup>o</sup> 04; 07; 08; 12; 15; 17
	0 : 1/0 : 44	IC 4943 IC 6807 IC 4921	Enteritis patients N <sup>o</sup> 05; 09; 19
	0 : 19	IC 6041	Enteritis patient N <sup>o</sup> 11
	N.D.	IC 8751	Enteritis patient N <sup>o</sup> 18
	0 : 37	IC 7668	Enteritis patient N <sup>o</sup> 21
	0 : 19	ATCC 700297	GBS patient

*Thin-layer chromatography of gangliosides and LPSs*

After visualization with a resorcinol-HCL reagent, spots of gangliosides GM1 and asialoGM1 and of all serotypes of isolated *C. jejuni* LPSs were present. This confirmed that the isolation of LPS was successful, indicating the presence of a carbohydrate moiety in their structure (figure 1). The position of the spots was according to the migration of the gangliosides and LPSs dissolved in used developers.

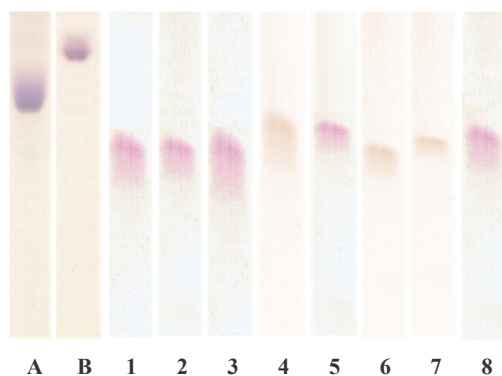


Figure 1 – TLC of gangliosides and LPSs with resorcinol-HCL; Lanes: A – GM1; B – asialoGM1; Lanes 1 to 8 LPSs serotype 0 : 19 (ATCC 700297) (1); 0 : 37 (2); 0 : 27 (3); 0 : 38 (4); 0 : 19 (5); 0 : 1/0 : 44 (6); 0 : 3 (7), 0 : 6/0 : 7 (8)

*Binding of cholera toxin-B subunit, peanut agglutinin and patient sera to gangliosides and isolated LPSs*

LPSs from all *C. jejuni* serotypes were tested with CT-B subunit and PNA ligand in order to detect the presence of ganglioside-like epitopes in those LPSs. Serotypes 0 : 19 (ATCC 700297), 0 : 27 and 0 : 19 showed a positive reaction with CT-B indicating the presence of a GM1-like epitope in their LPSs structures. Serotype 0 : 37 had a positive reaction with PNA ligand showing the presence of Gal $\beta$ 1-3GalNAc epitope in this LPS. The other serotypes did not react with CT-B or PNA (Figure 2).

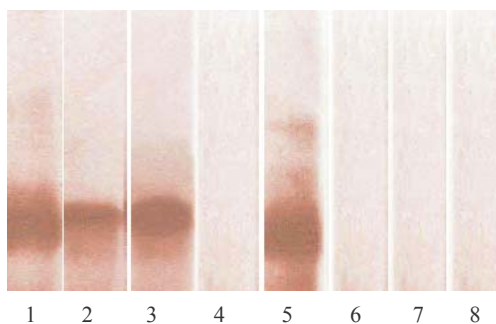


Figure 2 – Binding of cholera toxin-B subunit and peanut agglutinin to isolated *C. jejuni* LPSs by western blot; Lines 1 to 8 LPSs serotype: 0 : 19 (ATCC 700297) (1); 0 : 37 (2); 0 : 27 (3); 0 : 38 (4); 0 : 19 (5); 0 : 1/0 : 44 (6); 0 : 3 (7), 0 : 6/0 : 7 (8). Positive reaction shown in line 1 (binding of serotype 0 : 19 (ATCC 700297) with CT-B subunit), line 2 (serotype 0 : 37 with PNA), line 3 (serotype 0 : 27 with CT-B subunit) and line 5 (serotype 0 : 19 with CT-B subunit)

In general, sera patients showed an IgG positive reaction with gangliosides GM1 and asialoGM1, and with LPSs serotypes 0 : 19 (ATCC 700297), 0 : 27, 0 : 19 and 0 : 37. The IgM response with all antigens was very weak or absent (Figure. 3). In details, GBS patient sera (L.G) gave a very strong IgG reaction only with GM1, and the asialoGM1 reaction was weak. Patient sera showed a strong IgG reaction towards LPS serotypes 0 : 37 and 0 : 19 (ATCC 700297) and had a positive reaction with 0: 27 and 0: 19 serotypes (Figure 3). This patient IgM response was weak to serotypes 0 : 37 and 0 : 19 (ATCC 700297), and absent with other antigens. No reaction between patient sera and serotypes 0 : 6/0 : 7, 0 : 38, 0 : 1/0 : 44 and 0 : 3 LPS was observed. Sera from healthy individuals used as a control did not react either with gangliosides or with isolated LPSs (data not showed).

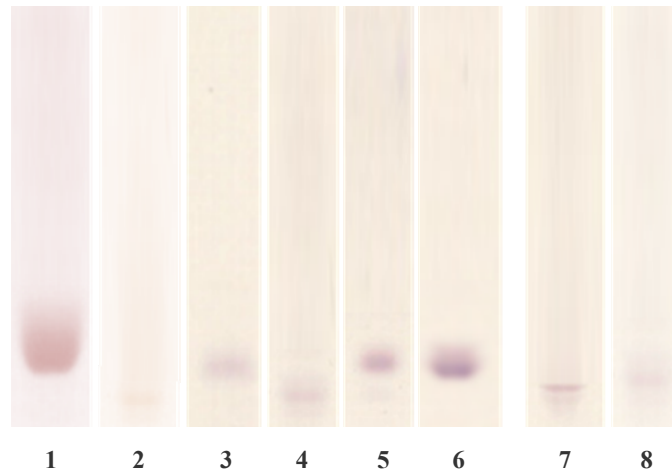


Figure 3 – Reactivity of GBS patient sera (L.G) to gangliosides and isolated *C. jejuni* LPSs by western blot; line 1 to 6 IgG reaction to GM1 (1), asialoGM1 (2), serotypes 0 : 27 (3), 0 : 19 (4), 0 : 37 (5) and 0 : 19 (ATCC 700297) (6); line 7 and 8 IgM response to serotypes 0 : 37 (7) and 0 : 19 (ATCC43446) (8).

### Discussion

The association of GBS with the preceding infection has led to a search for candidate bacterial antigens which may participate in the autoimmune responses in the host. Gangliosides have been extensively studied as possible host antigens for autoimmune diseases since antibodies against gangliosides, especially against GM1, are found during the acute phase of GBS when preceded by *C. jejuni* infection. Molecular mimicry between LPS of certain *C. jejuni* sero-

types associated with GBS and human gangliosides has been confirmed in several studies [24, 25]. Still, little information is available concerning the expression of ganglioside mimicry in isolates from uncomplicated *C. jejuni* enteritis [26]. Important step in elucidating the pathogenesis of the disease is determining the structure of the immunogenic epitopes in ganglioside-mimicking *C. jejuni* LPS. To detect whether ganglioside-like epitopes are limited to a few *C. jejuni* serotypes, our collection of *C. jejuni* serostrains was screened for the ganglioside-like epitope in their LPSs structures. Serological analysis using antiganglioside antibodies and ligands binding has proved a useful approach for analysis of mimicry in *C. jejuni* LPS [27, 28]. Therefore we have investigated ligand cross-reactivity and antibody binding between gangliosides and LPSs of different *C. jejuni* serotypes using cholera toxin-B subunit as a GM1 ligand and peanut agglutinin as a marker for Gal $\beta$ 1-3GalNAc structures, while immune reactivity was tested with sera with high titers of anti-ganglioside antibodies. Detection and characterization of cross-reactive ganglioside-like epitopes present in isolated LPS from *C. jejuni* strain was done using TLC, confirming that isolation of LPS structures was successful and detecting the presence of the carbohydrate moiety in isolated LPSs. The conformation of glycolipids and glycoproteins is hard to preserve, and TLC is an appropriate technique for this purpose.

Our result showed that isolates from our *C. jejuni* collection belonged to 7 different serotypes. The most frequent was serotype 0 : 3, present in 6 out of 21 patients (28.5%). Structural and epidemiological studies showed that this serotype does not contain ganglioside-like epitopes, has not been associated with GBS and is often used as a negative ganglioside-like serotype control [29, 34]. These findings were expected in our results, since isolates came from patients with enteritis and with no neurological symptoms. The other serotypes detected in our *C. jejuni* collection were serotypes 0 : 6/0 : 7, 0 : 1/0 : 44, 0 : 38, 0 : 37 and 0 : 27 (Table 1). From this group of serotypes previous studies reported the association between serotype 0 : 37 and GBS [3, 30, 32]. In our results this serotype showed the presence of Gal $\beta$ 1-3GalNAc structure in its LPS (positive reaction with PNA ligand), and also reacted with sera patients with GBS (Fig. 3, Table 2). Reactivity to ligands (CT-B) and to patient sera was present in serotype 0 : 27, indicating that this serotype was also bearing ganglioside-like epitopes. In our study, the detection of serotype 0 : 19 was unexpected, in only one patient, since this serotype is epidemiologically most associated with GBS. A number of studies show, and our results confirmed, that this serotype bears ganglioside-like epitopes (CT-B positive binding) and also showed positive reaction with patient sera with GB. This result is in agreement with previously reported epitope mimicry between serotype 0 : 19 LPS and GM1 ganglioside [12, 31, 33]. Studies have shown that this serotype is very rarely isolated from an uncomplicated enteritis patient. Unfortunately, we do not have any information or follow-up for this patient, to see whether some neurological



symptoms will develop. Still, this serotype 0 : 19 and serotypes 0 : 37 and 0 : 27 will be the prime focus in our future investigation into *C. jejuni* serotypes in association with GBS. Serotype 0 : 19 (ATCC 700297) used as positive control showed a reaction to CT-B and reacted to sera patientx.

The results of our study are summarized in Table 2. Ligand and/or antibody recognition of epitopes showed that gangliosides GM1 and asialoGM1, and LPS of *C. jejuni* serotypes 0 : 37, 0 : 19 and 0 : 27 shared common epitopes. The serological findings of our study showed that ganglioside-like epitopes are present in *C. jejuni* serotypes isolated from enteritis patients and are not limited only to strains associated with GBS. Whether this mimicry can trigger the production of anti-ganglioside antibodies and can have a potential role in the pathogenesis of neurological diseases, probably involves other bacterial and/or host attributes.

Still, future immunisation studies using serotypes with ganglioside-like epitopes will confirm whether these epitopes can function as cross-reactive antigens *in vivo*, and their potential role in the development of neurological symptoms will be determined.

Table 2

*Reactivity of gangliosides GM1 and asialo GM1 and isolated LPSs C. jejuni serotypes 0 : 37, 0 : 6/0 : 7, 0 : 19, 0 : 38, 0 : 3, 0 : 1/0 : 44, 0 : 27 and 0 : 19 (ATCC 700297) with ligands and with GBS patient sera*

	Gangliosides		<i>C. jejuni</i> LPSs							
	GM1	asialo GM1	0 : 37	0 : 6/0 : 7	0 : 19	0 : 38	0 : 3	0 : 1/0 : 44	0 : 27	0 : 19 ATCC 700297)
<b>CT-B</b>	+++	+	(-)	(-)	++	(-)	(-)	(-)	++	++
<b>PNA</b>	+	++	++	(-)	(-)	(-)	(-)	(-)	(-)	(-)
<b>Patient sera (L.G)</b>										
<b>IgG</b>	+++	+	++	(-)	++	(-)	(-)	(-)	++	++
<b>IgM</b>	(-)	(-)	+	(-)	(-)	(-)	(-)	(-)	(-)	+

Reaction grade: +++ very strong reaction; ++ strong reaction; + weak reaction; +- barely visible reaction – no reaction

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#### Резиме

### ВКРСТЕНО РЕАКТИВНИ ЕПИТОПИ ПРИСУТНИ ВО СОЕВИ НА САМПУЛОБАКТЕР JEJUNI ИЗОЛИРАНИ ОД ПАЦИЕНТИ СО ЕНТЕРИТИС

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Инфекцијата со *Campylobacter jejuni* (*C. jejuni*) често индуцира автоимуну посредувани невропатии, особено Guillain-Barré синдромот (GBS). Молекуларната мимикрија меѓу срцевините олигосахариди од бактериските липолисахариди (LPSs)

и хуманите ганглиозиди, се претпоставува дека резултира со создавање на анти-неврални вкрстено реактивни антители, кои најверојатно учествуваат во развојот и патогенезата на GBS. Целта на нашето испитување беше да се открие присуството на вкрстено реактивните епитопи во *C. jejuni* соевите изолирани од пациенти со ентеритис и да се определи нивната антигенска реактивност. За таа цел беа собрани примероци на фецес од 21 пациент со ентерит, без невролошки симптоми. Седум различни серотипови на *C. jejuni* беа определени користејќи го Penner-от систем на серотипизација (0 : 37; 0 : 6/0 : 7; 0 : 38; 0 : 19; 0 : 1/0 : 44; 0 : 3; 0 : 27;) и еден изолат не можеше да се детектира. Неочекувано, еден од серотиповите во оваа група е детектиран како 0 : 19, серотип ретко изолиран кај пациенти со ентерит и во честа асоцијација со GBS. Студии со врзување на колера токсин Б субединица и лектин од кикирика, покажаа дека серотиповите на *C. jejuni* 0 : 37, 0 : 19 и 0 : 27 имаат LPS кои содржат епитопи слични на ганглиозид. Реактивноста со серум од пациент со GBS, со потврдена претходна инфекција со *C. jejuni* и со висок титар на антиганглиозидни антители, покажа дека истите три изолирани LPSs од *C. jejuni* серотиповите 0 : 37, 0 : 19 и 0 : 27 содржат вкрстено реактивни епитопи во своите LPS структури. Нашите резултати ги потврдија резултатите од претходните студии дека LPSs од некои од *C. jejuni* серотипови имаат епитопи слични на ганглиозид, кои може да дејствуваат како вкрстено реактивни антигени и најверојатно имаат придонес во појавата на GBS по инфекција со *C. jejuni*.

**Клучни зборови:** *Campylobacter jejuni*, Guillain-Barre syndrome, липолисахариди, ганглиозиди.

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