Characterization of *Campylobacter Jejuni* and *Campylobacter Coli* Strains Isolated in the Region of Niš, Serbia

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SUMMARY

Introduction *Campylobacter jejuni* and *Campylobacter coli* represent one of the main causes of bacterial diarrhoea in humans. Although the disease is usually mild and self-limiting, severe chronic sequelae may occur, such as reactive arthritis, Guillain-Barré and Miller Fisher syndromes. Serotyping is used as an epidemiological marker, while post-infective polyneuropathies are associated with several O serotypes.

Objective Strains of *C. jejuni* and *C. coli* were serotyped based on heat stable (HS) and heat labile (HL) antigens, as well as biotypes to determine strain diversity.

Methods Campylobacter spp. was isolated using selective blood media with antibiotics. Differentiation to the species level was done by a combination of biotyping tests and by a PCR-based RFLP test. The isolates were characterised by Penner and Lior serotyping methods.

Results The serotypes showed diversity without predominant serotypes. 24 HS serotypes were detected among 29 *C. jejuni* strains, and seven serotypes among nine *C. coli* strains. HL serotyping method successfully typed 62.5% of strains. Among 16 *C. jejuni* strains 14 serotypes were detected, and three among four *C. coli* strains. A *C. jejuni* strain associated with a patient with Guillain-Barré syndrome was typed as biotype II, O:19.

Conclusion The biotyping and serotyping results have indicated that *C. jejuni* and *C. coli* strains in the region of Niš, Serbia are diverse and could be probably of unrelated sources of origin or reservoirs. The strain associated with the Guillain-Barré syndrome patient was serotype O:19, one of the most common in this post-infective complication.

Keywords: *Campylobacter jejuni*; *Campylobacter coli*; serotyping; biotyping

INTRODUCTION

Campylobacter jejuni (*C. jejuni*) and *Campylobacter coli* (*C. coli*) represent the main cause of bacterial diarrhoea in developed countries [1], and one of the most important causes of enterocolitis in developing countries [2]. Clinical manifestations of illness are diarrhoea, fever, abdominal pain, and in some patients, faecal blood. Subsequent to *C. jejuni* infection, severe chronic sequelae may occur, such as reactive arthritis and post-infective neuropathy, Guillain-Barré and Miller Fisher syndromes (GBS and MFS, respectively) [3]. Most Campylobacter infections are thought to be foodborne, with poultry as the principal source [4]. In industrialized countries, Campylobacter infections are usually sporadic and only a small subset of infected patients is thought to be associated with outbreaks. In characterization of clinical isolates, serotyping still remains the main scheme for the characterization of campylobacters [5]. Some serotypes have been reported to be commonly associated with GBS and MFS [6]. There is a lack of evidence of serotype distribution for some geographical areas, among them for Serbia, as well as for GBS associated strains.

OBJECTIVE

The purpose of this study was to provide information on the serotype distribution of thermophilic *Campylobacter spp.* isolated from clinical cases of human infections in the region of Niš, Serbia.

METHODS

We investigated 38 strains of thermophilic campylobacters isolated in the region of Niš from January 1, 2003 to October 1, 2004, one was a strain isolated from a patient with GBS which was preceded by Campylobacter diarrhoea, while 37 strains were isolated from patients with enterocolitis.

Stool specimens were streaked on the surface of Columbia agar base supplemented with 5% sheep blood and antibiotics (cefoperazone, 1.5 g/L, colistin 106 U, vancomycin 1 g/L, amphotericin B 0.2 g/L), (bioMérieux, Marcy l'Etoile, France). Inoculated plates were incubated at 42°C for 48 hours in a microaerobic atmosphere (gas generating system "Torlak", Belgrade, Serbia). Colonies of *Campylobacter* were presumptively

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identified microscopically by stained (1% carbolfuchsin) slides, with the observation of S- and spiral-shaped bacteria with gull-wing morphology, and by oxidase and catalase tests. Strains were differentiated to the species level by a combination of biotyping tests and using a PCR-based RFLP test.

In biotyping scheme, hippurate hydrolysis, rapid H_2S production and DNA hydrolysis tests were used [7].

In the PCR-RFLP test the primer sequences amplify a 1004-bp fragment within the coding region of the 16S rRNA gene in *Campylobacter*, *Arcobacter*, and *Helicobacter* species. The forward and reverse primers used were CAH 16S 1a (59 AAT ACA TGC AAG TCG AAC GA 39) and CAH 16S 1b (59 TTA ACC CAA CAT CTC ACG AC 39), respectively. For amplicon digestion, restriction endonucleases *DdeI* (Boehringer-Mannheim, Indianapolis, Ind.), *TaqI* (Boehringer-Mannheim), or *BsrI* (New England Biolabs, Inc., Beverly, Mass.) were used. For distinguishing between *C. jejuni* and *C. coli* an additional set of primers was designed to amplify a portion of the hippuricase gene by using forward and reverse primers Hip 1a (59 ATG ATG GCT TCT TCG GAT AG 39) and Hip 2b (59 GCT CCT ATG CTT ACA ACT GC 39), respectively [8].

Heat labile (HL) serotyping according to the Lior system was performed by slide agglutination with live bacteria using crude and absorbed antisera for the detection of heat HL antigens. Briefly, the antisera were prepared using bacterial suspensions containing 10^{10} bacteria/ml. Suspension used to inoculate rabbits were prepared from smooth colonies of reference strains inoculated on Mueller Hintion broth (Oxoid LTD; London, England) containing 1.0 to 1.25% agar (Difco Laboratories, Detroit, Mitch.) and incubated for 48 hrs at 37°C in microaerophilic atmosphere. New Zealand white rabbits were injected intravenously at 4 to 5-day intervals for 4 weeks with increasing doses (0.5 to 2.5 ml) of bacterial suspension in phosphate buffered saline (PBS) pH 7.2 containing 0.5% formalin. Rabbits were exsanguinated 7-10 days after the last injection and the sera preserved with 1:10,000 Merthiolate R at 4°C [9].

Heat stabile (HS) serotyping according to the Penner system was performed using a passive hemagglutination test using erythrocytes sensitized with heat extracted antigens and antisera. Briefly, the antisera were prepared from confluent bacterial growth on two blood agar plates (Columbia agar base [Oxoid]; 7% horse blood), obtained after 48 hrs at 37°C in a CO_2 incubator (Forma Scientific, Marietta, Ohio) set to maintain an atmosphere with 5% CO_2. Bacteria were transferred to 3 ml of saline (0.85% NaCl), washed twice in saline, and resuspended to an optical density of 0.375 at 625 nm (determined with a Spectronic 20 spectrophotometer). After a preimmune bleeding, the New Zealand white rabbits were inoculated intravenously five times over a two-week period. The doses were 1, 2, 2,4 and 4 ml. Blood was taken by cardiac puncture 7 to 10 days after the last injection. Sera were separated and stored at -20°C [10].

RESULTS

In the period from January 1, 2003 to October 1, 2004, there were 214 strains of isolated campylobacters. The speciation of randomly selected *Campylobacter* strains using PCR-RFLP was successful in 100%. For *C. jejuni* strains, a unique RFLP fingerprint pattern was obtained with generation of the 176-bp hippuricase amplicon. In *C. coli* strains that amplicon was missing.

C. jejuni was detected in 29 isolates, and *C. coli* in nine strains. The relative ratio of *C. coli* and *C. jejuni* showed that *C. coli* were less common than *C. jejuni*. Biotyping was performed on all 38 strains. Three biotypes were identified in *C. jejuni* strains; biotype I (15 isolates), biotype II (11 isolates) and biotype III (three isolates). In *C. coli* strains, biotype I was represented by eight strains, and biotype II by one strain.

The HS system was efficient for 100% of the stains; it typed successfully all of the 38 *C. jejuni* and *C. coli* strains. Twenty-four serotypes were detected among 29 *C. jejuni*, and seven serotypes were detected among nine *C. coli* strains. The results of HS serotyping are presented in Table 1 for *C. jejuni* and in Table 2 for *C. coli* isolates.

Table 1. Results of HL and HS serotyping of *C. jejuni* strains

Number of HS/HL serotypes	Number of strains (total 29)	HS serotypes	HL serotypes
1	1	**O:1**	ND
2	2	**O:2**	4
3	1	**O:2**	UT
4	1	O:2, 66	UT
5	2	O:3	1 36; 1 ND
6	1	O:3, 50	UT
7	1	**O:4, 13, 43, 65**	90
8	1	**O:4, 13, 50, 65**	71
9	1	O:6, 57	6
10	1	O:6, 57	UT
11	1	O:8	85
12	1	O:8, 17	90
13	1	O:9, 21, 58	UT
14	1	**O:10**	42
15	2	O:11	82
16	1	O:15	86
17	1	**O:19**	ND
18	1	O:19, 38	UT
19	1	O:33	23
20	1	O:37	28
21	1	O:40, 41	ND
22	1	**O:41**	18
23	3	O:53	1 UT; 2 ND
24	1	O:63	52

ND – not detected; UT – untypable
Bold HS – serotypes that may be involved in GBS pathogenesis

Table 2. Results of HL and HS serotyping on *C. coli* strains

Number of HS/HL serotypes	Number of strains (total 9)	HS serotypes	HL serotypes
1	1	O:4,28, 32,	UT
2	2	O:14, 34	UT
3	1	O:24	110
4	2	O:34	46
5	1	O:34	UT
6	1	O:49	97
7	1	O:64, 66	UT

ND – not detected; UT – untypable

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The HL serotyping was performed on 32 strains. Out of 23 *C. jejuni* and nine *C. coli* strains that were HL serotyped, the HL serotyping scheme successfully typed 20 strains (62.5%); 14 serotypes were detected among 16 *C. jejuni* and three among four *C. coli* strains, as listed in Table 1 for *C. jejuni* and in Table 2 for *C. coli* strains.

The strain associated with GBS was identified as *C. jejuni*, biotype II, HS serotype O:19.

We detected six HS serotypes in *C. jejuni* strains that may be involved in GBS pathogenesis (marked in bold in Table 1).

DISCUSSION

Consistent reports on the characterization of thermophilic *Campylobacter* strains isolated from all over the world are yet to be organized into a global surveillance system. The characterization of thermophilic *Campylobacter* strains is not necessary for routine diagnostic procedures since the disease is often mild and self-limiting without complications. However, some properties of clinical presentation, such as chronic post-infectious sequelae, may be related to a certain HS serotypes.

In this study, biotype I was predominant for both *C. jejuni* and *C. coli*. Similar results were attained in many studies in different locations; Central African Republic [11], Portugal [12], Poland [13] India [14] and Italy [15]. Only one report from Austria in 1987 revealed the predominance of *C. jejuni* biotype II over *C. jejuni* biotype I [16].

The investigation of HS serotypes in *C. jejuni* and in *C. coli* confirmed their clonal diversity, without predominant serotypes. In *C. jejuni* strains, HS serotypes O:2 and O:53 were isolated more frequently and comprised 10.34% of investigated strains, each. However, the size of the analyzed sample was small and results could not be entirely representative, and without cluster analysis clones could not be differentiated with great confidence.

Data related to HS antigen distribution among campylobacters are not available for Central, South and Southeast Europe. The HS serotypes of strains isolated in Serbia were similar to those found in distant geographic areas, although every area is specific according to the prevalence of serotypes.

The dominant serotypes of *C. jejuni* and *C. coli* in Ethiopia were O:34; O:1; O:3, O:8; O:26; O:30; O:51 [17]. In UK, three most common HS serotypes were O:1, O:2 and O:4 [18]. In South Africa the serotyping technique revealed that the most common serotypes were: O:4, O:2, O:12, O:23/36 and O:19 respectively, together comprising 25% of the isolates in *C. jejuni/coli* strains [19]. In Central Australia a total of 46 serotypes was identified, and the predominant serotypes were O:8.17; O:22; O:1.44, and O:19 [20]. In Thailand, 10 HS serotypes were detected with HS antigens 2 and 3 being the most frequent [21]. In Denmark, in two counties, serotyping divided the *C. jejuni* isolates into 38 HS serotypes. The three dominant HS serotypes were serotype 2 (30% of isolates), serotype 4 complex (21%) and serotype 1.44 (10%). In the same study, PFGE analysis confirmed the validity of selected clusters identified by serotyping [22]. In a clinical isolates of *C. jejuni* in children in Greece, the majority of the serotyped strains belonged to serotype HS:2 (14%) followed by HS:(4,13,16,43,50) (9.3%), HS:(1,44) (5.4%) and HS:37 (5.4%) [23].

In this study, a variety of HL serotypes were detected in *C. jejuni* (4, 6, 18, 23, 28, 36, 42, 52, 71, 82, 85, 86, 90) and in *C. coli* (46, 97, 110). Such a substantial number of serotypes found in the investigated population, suggests clonal diversity among the strains. Some of the detected serotypes (4, 28, 36) identified in Serbia , were identified in Tuscany, Italy (1, 2, 4, 11, 28, 36, 53) [24], in Romania (4, 5, 8, 9, 11, 17, 21, 28, 29, 32, 36, 44, 47, 48, 55, 57, 59) [25] and Austria (1, 2, 4, 6, 11, 13, 21, 28, 29, 36) [16]. Serotype 4 was reported from all parts of the world and was also detected in our study. In Bangkok, in the period from 1991 to 2000, the predominant HL serotypes in children were 36, 2, and 4 in *C. jejuni*, and 8, 29 and 55 in *C. coli* [26].

In order to increase the discriminatory power of serotyping, attempts have been made to provide a unique system by combining both HL and HS procedures. In one study some more frequent combinations of HL and HS serotypes were observed; O:2/HL125; O:2/HL121; O:2/HL4; O:2/HL40; O:2/HL100; O:41/HL27 [27]. We also noticed the association between O:2 and HL4 and O:6 and HL6 antigens in *C. jejuni* strains. Additionally, O:57 was present in O:6 isolates.

Many reports confirm that the HS O:19 serotype is associated with GBS [28] as shown by this study as well. Our strain was isolated from a patient with GBS and was associated with campylobacter diarrhoea. The isolate was a *C. jejuni*, biotype II, HS O:19. HS serotypes observed in other GBS patients include O:1; O:2; O:4; O:4-complex (4, 13, 16, 43, 50); O:5; O:10; O:16; O:23; O:37; O:41; O:44 [29], and O:35 and O:13/65 [30]. We did not find any data related to the biotypes of *C. jejuni* isolated in GBS patients with preceding diarrhoea.

In patients who suffered from diarrhoea we detected the presence of O:1, O:2, O:4, O:10, O:41, serotypes of *C. jejuni* that were described as preceding GBS and MFS [28, 29]. Since certain serotypes occur more frequently in GBS patients following diarrhoea caused by *C. jejuni*, these serotypes may serve as markers for the risk of GBS and MFS.

CONCLUSION

The biotyping and serotyping results indicated that *C. jejuni* and *C. coli* strains in Serbia are diverse and could be of unrelated sources of origin or reservoirs. The strain associated with the Guillain-Barré syndrome patient in our study was O:19 serotype, one of the most common in this post-infective complication. Also, among patients suffering from diarrhoea, the presence of serotypes of *C. jejuni* was detected as proceeding GBS and MFS. However, the number of analyzed strains was small, so that this report provides only preliminary data on serotype distribution in *C. jejuni* and *C. coli*.

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NOTE

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Особине врста *Campylobacter jejuni* и *Campylobacter coli* изолованих у региону Ниша, у Србији

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КРАТАК САДРЖАЈ

Увод Бактерије *Campylobacter jejuni* и *Campylobacter coli* су веома важни узрочници дијареје код људи. Мада је ово обољење обично благо и пролази спонтано, након њега могу да се јаве тешке, хроничне секвеле, као што су реактивни артритис, Гијљен–Бареов (*Guillain–Barré*) и Милер–Фишеров (*Miller–Fisher*) синдром. Серотипизација се користи као епидемиолошки показатељ, а постинфекцијске полинеуропатије повезане су са неколико О серотипова.

Циљ рада Да би се утврдиле особине сојева, извршена је биотипизација и серотипизација *C. jejuni* и *C. coli* на основу њихових термостабилних и термолабилних антигена.

Методе рада *Campylobacter spp.* је изолован на селективној крвној подлози са додатком антибиотика. Диференцијација до нивоа врсте вршена је комбинацијом биотипизације и методе *RFLP-PCR*. Серотипизација је вршена методом Пенера (*Penner*) и Лиора (*Lior*).

Резултати Утврђен је већи број серотипова без доминације једног серотипа. Код 29 сојева *C. jejuni* доказана су 24 термостабилна серотипа, док је седам серотипова доказано код девет сојева *C. coli*. Методом термолабилне серотипизације успешно је типизирано 62,5% испитиваних сојева. Код 16 сојева *C. jejuni* доказано је 14 серотипова, а код четири соја *C. coli* доказана су три серотипа. Соj *C. jejuni* који је изолован код болесника са Гијљен–Бареовим сидромом идентификован је као биотип *II*, О:19.

Закључак Резултати биотипизације и серотипизације указују на различитост између сојева *C. jejuni* и *C. coli* у региону Ниша, као и да вероватно воде порекло из извора или резервоара који међусобно нису повезани. Соj изолован код болесника са Гијљен–Бареовим сидромом припада серотипу О:19, једном од најчешћих код ове постинфекцијске компликације.

Кључне речи: *Campylobacter jejuni*; *Campylobacter coli*; серотипизација; биотипизација

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