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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Comparative genomic fingerprinting for the subtyping of *Campylobacter jejuni* and *Campylobacter coli* biotypes

Biljana Miljković-Selimović[1,2], Tatjana Babić[1], Branislava Kocić[1,2], Ljiljana Ristić[1], Tatjana Milenković[1], Dragan Bogdanović[1,4]

[1]Institute for Public Health, National Reference Laboratory for *Campylobacter* and *Helicobacter*, Center for Microbiology, Niš, Serbia;
[2]University of Niš, Faculty of Medicine, Niš, Serbia;
[3]Institute for Public Health, Center for Informatics and Biostatistics in Healthcare, Niš, Serbia;
[4]State University of Novi Pazar, Department of Biomedical Sciences, Novi Pazar, Serbia

SUMMARY

Introduction/Objective Thermophilic campylobacters, especially *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*), are the most important causes of bacterial diarrhea in developed and developing countries. The disease can occur as a sporadic infection or as large and small outbreaks. Phenotyping and genotyping methods are in use to determine similarities between strains as well their possible common origin. The goal of the study was to compare discriminatory power of biotyping tests and comparative genomic fingerprinting (CGF) 40 (100%), as well as a combination of the two tests in detection of clonality or epidemiological relatedness between the studied strains.

Methods We investigated 23 *Campylobacter* strains using biotyping and CGF typing.

Results We found that biotyping was a more discriminatory method for *C. coli*, and CGF for *C. jejuni* strains. In the discrimination of *C. jejuni* strains, CGF had better discriminatory power [Simpson's index of diversity (ID) was 0.879] over the discrimination of *C. coli* strains (Simpson's ID was 0.389).

Conclusion Biotyping and CGF can be complementary methods in detection of similarity, relatedness and possible common origin between strains since the combination of biotyping and CGF methods gives more precise data about diversity within *C. coli* and *C. jejuni* strains.

Keywords: biotyping; molecular typing; multiplex PCR

INTRODUCTION

Campylobacter spp. (predominantly (*C. jejuni* and *C. coli*) are the most frequent causes of enterocolitis in developed and developing world [1]. Enterocolitis usually occurs sporadically. However, detected or not, small house outbreaks are more possible [2]. In order to trace the sources of outbreak or to detect epidemiologically related strains, extended biotyping or serotyping schemes based on heat labile (Lior scheme) or heat stabile (Penner) antigens can be used [3, 4, 5]. Molecular techniques, e.g. polymerase chain reaction- (PCR) based methods, provided more rapid tools for the discrimination between the strains and they are very convenient when used for detection of *Campylobacter* spp. in the specimen. However, molecular methods are not sufficiently reliable because of some *Campylobacter* genus features such as high genetic diversity, weak clonality, and high levels of intraspecies recombination. Consequently, secondary methods for the successful tracking of epidemic strains are necessary [6]. Since clusters of C*ampylobacter* have not been well defined, the detection of unreported outbreaks of food-borne diseases can be more difficult.

There are several genotyping techniques adopted for campylobacters: pulsed-field gel electrophoresis (PFGE) [7]; restriction fragment length polymorphism analysis of the flagellin gene (flaA RFLP) [8]; the DNA sequencing of the flagellin gene short variable region (flaA SVR) [9]; multilocus sequence typing (MLST) [10]; multilocus variable-number tandem repeat analysis (MLVA) – a promising tool, but still without a widely accepted protocol [11, 12]; DNA microarrays [13]; clustered regularly interspaced short palindromic repeat (CRISPR) polymorphism analysis [14]; single nucleotide polymorphism (SNP) typing [15]; and binary gene typing (BGT) [16].

The PFGE with validated protocol for *Campylobacter* spp. is superior in outbreak investigation. Yet, PFGE has numerous disadvantages: it is time-consuming and labor-intensive, and requires high concentrations of a pure culture. Contemporary requirements from a typing method as a microbiological tool are less complicated procedures on a routine basis, rapid results, inexpensiveness, better discrimination and quantitative relatedness between strains, compatibility with PFGE data, preferably automatic and portable equipment, and easy comparison within and between laboratories by the existing databases.

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Correspondence to:
Biljana MILJKOVIĆ-SELIMOVIĆ
University of Niš, Faculty of Medicine
81 Dr. Zorana Đinđića Blvd.
18000 Niš, Serbia
biljanams@medfak.ni.ac.rs

In an effort to establish reproducible, discriminatory, rapid, low cost, and easy performing genotyping method for *Campylobacter*, applicable in molecular epidemiology for *C. jejuni* and *C. coli,* a 40-gene CGF assay (CGF40) at the National Microbiology Laboratory of the Public Health Agency of Canada (Winnipeg) was developed [17]. The basis for CGF is the presence or absence of genes found to be variable in previous comparative genomic studies involving multiple *C. jejuni* isolates [17]. The method involved eight multiplex PCR, each consisting of five reactions assessing alleles at multiple loci and their genetic variability. Used marker genes were those with a distribution indicative of clear presence/absence, classified as unbiased genes, with a representative genomic distribution, and the ability to capture strain-to-strain relationships and were present in two or more of *C. jejuni* genomes [17]. Data do not require querying a centralized data bank. Therefore, this type of genome analysis is exceptionally portable within laboratory networks, and exchange of information is very easy [18].

Control and prevention of disease and outbreaks are complex tasks. Of great importance is not only to develop and implement effective control measures on the identification of the sources of an infection, but also to choose an efficient microbiological tool. Nowadays, in Serbia, there are no consistent programs for surveillance and monitoring of food-borne infections and outbreaks and infections caused by enteric bacteria as well as by *C. jejuni* and *C. coli*. The methods for bacterial typing with more discriminatory power for clonality investigation can provide information on epidemiologically related strains that are more accurate.

The aim of the study was to (a) compare discriminatory power of biotyping tests commonly used in microbiological laboratories and CGF40 (100%), as well as a combination of the two tests in detection of the strains isolated in small house outbreaks, and (b) to determine the similarity, clonality or epidemiological relatedness of the strains.

METHODS

We have investigated 23 thermophilic *Campylobacter* spp. strains designated in Arabic numerals from 1 to 23, from patients with enterocolitis isolated in 2011 in Serbia. Available clinical and epidemiological data provided strain selection, and the investigation of suitability of CGF40 was conducted in relevance to epidemiology of the strains. Among investigated strains, 11 pairs (22 strains) of *Campylobacter* were identified as isolated at the same time, with the same geographical distribution and the same pattern of sensitivity to antimicrobials. We presumed that strain pairs belonged to the same species; i.e. biotype and CGF type had the same clonal pattern. Strain pairs were designated from A to K with the belonging strains as: A) 1, 2; B) 3, 4; C) 5, 6; D) 7, 8; E) 13, 14; F) 19, 15; G) 22, 23; H) 9, 10; I) 11, 12; J) 20, 16; K) 21, 17.

Strain identification and biotyping

Strains sent to the Reference Laboratory for *Campylobacter* and *Helicobacter* in Amies medium were cultured in Columbia agar [Columbia blood agar with 5% sheep blood (CBA), Liofilchem, Roseto degli Abruzzi, Italy] and *Campylobacter* agar with 5% sheep blood (CA), Liofilchem, brain heart infusion broth (BHI), (Blood agar base heart infusion, Biolife Italiana S.r.l., Milan, Italy) and Bolton medium (Fluka Chemie GmbH, Buchs, Switzerland) with 10% laked horse blood (Oxoid ltd., Basingstoke, UK), and subcultured on CBA and CA after 48 hours in the same conditions.

Previously isolated strains, stored in BHI with 15% glycerol at -70°C, were thawed at room temperature and plated on the same media at same conditions. The media were incubated for 48 hours, in a microaerobic atmosphere with 9% CO_2 at the temperature of 37°C in an incubator (pCO_2 inkubator, BINDER Inc., Bohemia, NY, USA). Colonies of *Campylobacter* were presumptively identified microscopically by stained (1% carbol-fuchsin) slides (presence of S and spiral-shaped bacteria with gullwing morphology), and by oxidase and catalase tests.

A combination of biotyping and the PCRbased RFLP test provided *Campylobacter* differentiation to the species level. In the biotyping scheme, hippurate hydrolysis, rapid H_2S production, and DNA hydrolysis tests were used [7].

In the PCR-RFLP test, in *Campylobacter*, *Arcobacter*, and *Helicobacter* species, the primer sequences amplify a 1004-bp fragment within the coding region of the 16S rRNA gene. 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. Restriction endonucleases *DdeI* (Boehringer Mannheim Corp., Indianapolis, IN, USA), *TaqI* (Boehringer Mannheim Corp.), or *BsrI* (New England Biolabs Inc., Ipswich, MA, USA) were used for amplicon digestion. Distinguishing between *C. jejuni* and *C. coli* required an additional set of primers designed to amplify a portion of the hippuricase gene by using forward and reverse primers Hip 1a (5' ATG ATG GCT TCT TCG GAT AG 3') and Hip 2b (5' GCT CCT ATG CTT ACA ACT GC 3'), respectively [19].

CGF analysis

To generate CGF40, eight multiplex PCRs were performed on each isolate using forty primer sets [13]. Used loci were the following: (1) Cj0298c, Cj0728, Cj0570, Cj0181, Cj0483; (2) Cj0057, Cj0860, Cj1431c, Cj0733, Cj1427c; (3) Cj0297c, Cj1727c, Cj0264c, Cj0008, Cj1585c; (4) Cj1550c, Cj1329, Cj0177, Cj1334, Cj0566; (5) Cj0421c, Cj0033, Cj0486, Cj0569, Cj0625; (6) Cj0755, Cj0736, Cj096, Cj1141, Cj1136; (7) Cj1306c, Cj1552c, Cj1439c, Cj1721c, Cj1679; (8) Cj1294, Cj1551c, Cj0307, Cj1324, Cj0035c. Designations of multiplex PCR were 1, 2, 3, 4, 5, 6, 7, and 8, respectively. All CGF types were given in a binary format. Detected clusters were designated in Arabic numerals as 1–9 [13]. PCR reaction and its analysis were performed as described by Taboada et al. [17].

Statistical analysis

To determine discriminatory ability of typing systems, we used Simpson's index of diversity (Simpson's ID). This index indicates the probability of two strains sampled randomly from a population belonging to two different types at a 95% CI [20]. The strength and directionality of the congruence between the biotyping and CGF was assessed using the Wallace coefficient (Wi, expected Wallace coefficient value in the case of independence) according to the methods of Carriço et al. [21]. Wallace coefficients provide an estimation of how much additional information is yielded by a secondary typing method. Calculations of Simpson's ID and Wallace's coefficients were performed using an online tool at the Comparing Partitions website (http: //www.comparingpartitions.info) [17].

RESULTS

In 23 investigated *Campylobacter* strains, biochemical and molecular identification revealed the two most common species – *C. jejuni* (14 strains) and *C. coli* (nine strains), represented with three and two biotypes, respectively. All the strains belonged to nine CGF clusters.

In *C. coli*, five strains belonged to biotype I and four to biotype II (Table 1). The investigation of 14 *C jejuni* strains subdivided the isolates into three biotypes: two strains were of biotype I, eight strains of biotype II, four strains belonged to biotype III (Table 1).

C. coli clustered together: *C. coli* biotype I all fell into CGF cluster number 1 (Table 1), while *C. coli* biotype II were slightly more diverse and fell into clusters 1 and 2 (Table 1). CGF subtyping of *C. jejuni* biotype I, *C. jejuni* biotype II, and *C. jejuni* biotype III revealed that strains belonged to clusters 2, 5, and 1, respectively. While C. *jejuni* biotype I (CGF clusters 3 and 4) and *C. jejuni* biotype II were more diverse (clusters 4–8), *C. jejuni* biotype III assemble only into cluster 9 (Table 1).

Table 1. Comparative genomic fingerprinting (CGF) and cluster distribution among investigated *Campylobacter* strains

Species and biotype	N° of strains	Designations of CGF clusters	Distribution of CGF clusters
C. coli I	5	1	1
C. coli II	4	1, 2	2
C. jejuni I	2	3, 4	2
C. jejuni II	8	4, 5, 6, 7, 8	5
C. jejuni III	4	9	1

Simpson's index of diversity for biotyping of *C. coli* and *C. jejuni* strains was 0.556 and 0.615, respectively. In *C. coli strains*, typed by CGF, Simpson's ID were 0.389, while 14 *C. jejuni* strains revealed seven clusters with Simpson's ID of 0.879 (Table 2).

The two methods, biotyping and CGF of genus *Campylobacter*, gave Simpson's ID of 0.913, and in *C. coli* revealed Simpson's ID of 0.667 (Table 3). Biotyping and CGF in *C. jejuni* strains provided Simpson's ID of 0.89, while subtyping of *C. coli* I, *C. coli* II, *C. jejuni* I, *C. jejuni* II, *C. jejuni* III gave Simpson's ID of 0, 0.667, 1, 0.857, and 0, respectively (Table 3).

Assessment of congruence among applied methods revealed that the Wallace coefficient (Wi, expected Wallace coefficient value in the case of independence) for *C. coli* I it was 1 (complete congruence), for *C. coli* II 0.333 (low congruence), for *C. jejuni* I 0 (no congruence), for *C. jejuni* II 0.143 (almost no congruence), and for *C. jejuni* III it was 1 (complete congruence).

Speciation and biotyping revealed seven pairs (A–G) of *Campylobacter* spp., which were identified as being clonally related (Table 4).

Table 2. Simpson's index of diversity calculated for biotyping and CGF of *Campylobacter jejuni/coli* strains

Microorganism method	No. of strains	Method	No. of partitions	Simpson's ID	CI (95%)	CINA (95%)
Campylobacter spp.	23	Biotyping	5	0.798	0.725–0.872	0.709–0.888
		CGF	10	0.874	0.789–0.958	0.778–0.969
C. coli	9	Biotyping	2	0.556	0.482–0.629	0.375–0.736
		CGF	2	0.389	0.081–0.697	0.060–0.718
C. jejuni	14	Biotyping	3	0.615	0.433–0.798	0.412–0.819
		CGF	7	0.879	0.794–0.964	0.764–0.994

CGF – comparative genomic fingerprinting – for this analysis the online tool at the Comparing Partitions website was used (http://www.comparingpartitions.info/); ID – index of diversity; CI – confidence interval; CINA – non-approximated confidence interval

Table 3. Simpson's index of diversity calculated for CGF and biotyping in *Campylobacter jejuni/coli* strains

Microorganism	No. of strains	No. of partitions	Simpson's ID	CI (95%)	CINA (95%)
Campylobacter spp.	23	11	0.913	0.860–0.966	0.846–0.980
C. coli	9	3	0.667	0.446–0.888	0.403–0.930
C. coli I	5	1	0	0.000–0.000	0.000–0.000
C. coli II	4	2	0.667	0.667–0.667	0.258–1.000
C. jejuni	14	8	0.89	0.796–0.985	0.770–1.000
C. jejuni I	2	2	1	1.000–1.000	0.000–1.000
C. jejuni II	8	5	0.857	0.704–1.000	0.641–1.000
C. jejuni III	4	1	0	0.000–0.000	0.000–0.000

CGF – comparative genomic fingerprinting – for this analysis the online tool at the Comparing Partitions website was used (http://www.comparingpartitions.info/); ID – index of diversity; CI – confidence interval; CINA – non-approximated confidence interval

Table 4. Clonality of isolated A–G strain pairs as determined by speciation, biotyping, and comparative genomic fingerprinting (CGF) clustering

Date of isolation	Pair designation/ strain pairs	Species, biotype	CGF cluster
4/11/2011	A) 1, 2	*C. jejuni* III	both strains: cluster 9
11/21/2011	B) 3, 4	*C. jejuni* II	strain 3: cluster 7 strain 4: cluster 5
5/5/2011	C) 5, 6	*C. jejuni* II	both strains: cluster 8
7/6/2011	D) 7, 8	*C. coli* II	strain 7: cluster 1 strain 8: cluster 2
11/29/2011	E) 13, 14	*C. jejuni* II	both strains: cluster 6
4/19/2011	F) 19, 15	*C. coli* I	both strains: cluster 1
4/18/2011	G) 22, 23	*C. jejuni* III	both strains: cluster 9

However, CGF typing revealed some differences among related isolates: pairs A, C, E, F, and G showed homogenicity by CGF typing. Pair B, identified as *C. jejuni* ssp. *jejuni* II, was subdivided into clusters 7 and 5; pair D, identified as *C. coli* II, was subdivided into clusters 1 and 2. Strains of pair D differ in only one allele form of the cj1427c gene, while strains of pair B differ in 15 alleles: Cj0298c, Cj1431c, Cj1727c, Cj0264c, Cj1550c, Cj0033, Cj0486; Cj0569, Cj0755, Cj0736, Cj1306c, Cj1552c, Cj1439c, Cj1721c, and Cj1294. Expression of the gene is represented by green color squares, and the absence of expression with red squares. If same-color squares are positioned one above the other, strains either possess a particular gene or they do not (Figure 1). Strain numbers are shown at the far left of the figure, and identified species are listed at its far right.

Pairs of strains from H to K did not express species, neither biotyping nor CGF homogeneity.

DISCUSSION

In this study, we performed biotyping and CGF on 23 *Campylobacter* strains: nine *C. coli* and 14 *C. jejuni* isolates. Biotyping alone of *C. coli* and *C. jejuni* strains gave Simpson's ID of 0.556 and 0.615, respectively, while CGF typing alone of *C. coli* and *C. jejuni* gave Simpson's ID of 0.389 and 0.879, respectively. Thus, biotyping was a more discriminatory method for *C. coli*, whilst CGF was more discriminatory for *C. jejuni* strains.

The results obtained by the combination of biotyping and CGF methods indicated that application of both procedures had better discriminatory power in *C. jejuni* over *C. coli* strains.

Speciation, biotyping and CGF of investigated *Campylobacter* spp. revealed Simpson's ID of 0.913 expressing high diversity among investigated strains.

In considered *Campylobacter* species, information on temporal and spatial relatedness using biotyping revealed seven pairs of strains (14 isolates) as related. Additional CGF typing revealed that five pairs of strains also belong to the same cluster. Two closely related clusters, 1 and 2, represented one pair (*C. coli* II), which means a possible evolution of one strain. Another pair of strains (*C. jejuni* II) differs in several alleles and represents two distinct clusters: cluster 7 and cluster 5. We did not expect to find differences between pairs considering their temporal and spatial distance [22]. The presence of two pairs of clonally related strains subtyped by CGF was surprising, although it is possible that one strain underwent genetic changes, having in mind that campylobacter is an extremely genetically variable bacterium [23]. CGF expressed better discriminatory power than biotyping in determination of clonality, which can be used in investigation of outbreaks.

Using the CGF method, we found high index of diversity for the species, indicating different sources of the *C. jejuni*. Through future investigation of animal isolates, it could be answered which one of many food animal sources are in question. For the species of *C. coli*, the index of diversity was somewhat lower (0.667), indicating higher similarity between strains, and perhaps a common origin. Therefore, within one year, strains may not have much variability.

A combination of biotyping and CGF methods gave more precise data about similarity between *C. coli* and *C. jejuni* strains, having in mind that congruence between the methods as determined by Wi was 0.143 for *C. jejuni* II and 0.333 for *C. coli* II, allowing association of these two methods. These properties suggest that methods based on comparative genomics represent a better alternative to biotyping.

Detection of an epidemic strain or investigation applied in population biology of bacterial strains are an important task for microbiologists. As it was seen in this investigation, the alone application of serotyping on a strain collection can show great diversity without predominant types, when strains are selected randomly [24]. Although a disadvantage of serotyping is that many strains can be untypable, an investigation of epidemic strains may give

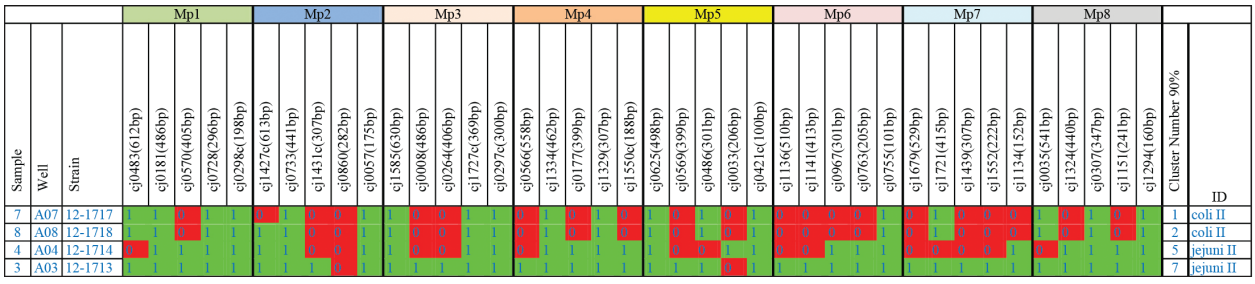

Figure 1. Algorithm of *C. coli* II (pair B) and *C. jejuni* I (pair D) with differences in gene expression; Mp1–8 – multiplex PCR 1–8; cj0483-cj1294, gene loci; ID – identification

representative and reproducible data, as in an outbreak described by DeFraites et al. [25], who detected the Lior serotype 5 in accessible isolates. The authors applied serotyping only and did not find any diversity among strains, which is possible when some subtyping methods or molecular typing methods are used.

To resolve epidemic strains, short variable regions of *C. jejuni* isolates successfully replaced serotyping [9]. One of the contemporary approaches is the multiplex PCR method for determining the capsule types of *C. jejuni*, which correlates with the Penner typing. The multiplex PCR showed sensitivities and specificities ranging 90–100% using strains of known Penner type [26]. A combination of the two methods, when primary typing method was CGF40, suggests that CGF and MLST are highly concordant. However, isolates with identical MLST profiles are composed of isolates with distinct but highly similar CGF profiles [17]. Our investigation showed that CGF and biotyping can be complementary methods in assessing clonality among *Campylobacter* spp. In addition, sequencing of the flaA gene short variable region (flaA SVR sequence typing) could supplement the CGF, with or without subsequent MLST [14].

In one investigation, several typing methods for use in the monitoring of *Campylobacter* spp. were compared [27]. The authors observed that the most discriminative combination with a Simpson's ID of 0.992 for both *C. jejuni* and *C. coli* was obtained by combining MLST with flaA-RFLP, which is feasible for short-term monitoring of *Campylobacter* spp. In our investigation, two methods, biotyping and CGF, revealed a Simpson's ID of 0.667 in *C. coli* and 0.89 in *C. jejuni* strains.

The goal of all typing and subtyping systems is a precise and efficient tracing method of infection sources. Therefore, it is a necessity to employ molecular typing approaches to quantify the contribution of different sources of human *Campylobacter* infections on the national level. Thus, it seems that the CGF method relying on the presence/absence of unbiased genes could fulfill the criteria for a modern typing method alone or in combination with other techniques.

CONCLUSION

Application of CGF alone or in combination with biotyping could reveal the clonal relationship between the strains, e.g. their participation in the same epidemic, especially when an outbreak is suspected. In the absence of the data on the outbreak, the method could reveal relatedness between the strains that could help in the outbreak detection. Introducing CGF could significantly improve investigation of clonal relatedness between strains and therefore contribute to the improvement in investigation of outbreaks. However, testing more samples will obtain more reliable results.

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Примена методе компаративног фингерпринтинга генома за суптипизацију биотипова *Campylobacter jejuni* и *Campylobacter coli*

Биљана Миљковић-Селимовић[1,2], Татјана Бабић[1], Бранислава Коцић[1,2], Љиљана Ристић[1], Татјана Миленковић[1], Драган Богдановић[1,4]

[1]Институт за јавно здравље, Национална референтна лабораторија за *Campylobacter* и *Helicobacter*, Центар за микробиологију, Ниш, Србија;
[2]Универзитет у Нишу, Медицински факултет, Ниш, Србија;
[3]Институт за јавно здравље, Центар за информатику и биостатистику у здравству, Ниш, Србија;
[4]Државни универзитет у Новом Пазару, Одсек за биомедицинске науке, Нови Пазар, Србија

САЖЕТАК

Увод/Циљ Термофилни кампилобактери, посебно *Campylobacter jejuni* (*C. jejuni*) и *Campylobacter coli* (*C. coli*) најчешћи су узрочници бактеријске дијареје и у развијеним земљама и у земљама у развоју. Болест може да се јави у виду спорадичне инфекције, мале кућне или велике епидемије.
За одређивање сличности између сојева као и њиховог евентуалног заједничког порекла могу да се користе фенотипске и генотипске методе. Циљ рада је био да се упореде дискриминаторна моћ биотипизације и компаративног фингерпринтинга генома (КФГ) 40 (100%), као и комбинације ова два теста у детекцији клоналности или епидемиолошке повезаности између испитиваних сојева.

Методе Испитивали смо 23 соја бактерије *Campylobacter* применом биотипизације и типизацијом на основу КФГ.
Резултати Утврђено је да је биотипизација дискриминаторнија метода за *C. coli,* а КФГ дискриминиторна за сојеве *C. jejuni*. Дискриминација *C. jejuni* применом КФГ има већу снагу (Симпсонов индекс различитости износио је 0,879) у односу на сојеве *C. coli* (Симпсонов индекс износио је 0,389).
Закључци Биотипизација и КФГ могу бити комплементарне методе приликом детекције сличности, повезаности или могућег заједничког порекла сојева, пошто њихова комбинација даје прецизније податке о разноликости унутар врста *C. coli* и *C. jejuni*.
Кључне речи: биотипизација; молекуларна типизација; мултиплекс *PCR*