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Original Article / Оригинални рад

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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. Disease can occur as a sporadic infection or large and small outbreaks.
Phenotyping and genotyping methods are in use to determine a similarity between strains as well their possible common origin.
The aim 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 more discriminatory method for *C. coli* and CGF for *C. jejuni* strains. In discrimination of *C. jejuni* strains, CGF had better discriminatory power (Simpson's index of diversity (ID) were 0.879) over discrimination *C. coli* strains (Simpson's ID were 0.389).
Conclusion Biotyping and CGF can be complementary methods in detection of similarity, relatedness and possible common origin between strains since combination of biotyping and CGF methods gave more precise data about diversity within *C. coli* and *C. jejuni* strains.
Keywords: biotyping; molecular typing; multiplex PCR

САЖЕТАК

Увод/Циљ Термофилни кампилобактери, посебно *Campylobacter jejuni* (*C. jejuni*) і *Campylobacter coli* (*C. coli*) су најчешћи узрочници бактеријске дијареје и у развијеним 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 њихово евентуално заједничко порекло могу да се користе фенотипске и генотипске методе.
Циљ рада је био да се упореде дискриминиторна моћ биотипизације и компаративног фингерпринтинга генома (*CGF*) 40 (100%), као и комбинације ова два теста у детекцији клоналности или епидемиолошке повезаности између испитиваних сојева.
Методе Ми смо испитивали 23 соја бактерије *Campylobacter* применом биотипизације и типизацијом на основу *CGF*.
Резултати Утврђено је да је биотипизација дискриминаторнија метода за *C. coli,* а *CGF* за сојеве *Ц. јејун.* Дискриминација *C. jejuni* применом *CGF* има већу снагу (Симпсонов индекс различитости износио је 0,879) у односу на сојеве *Ц. цоли* (Симпсонов индекс износио је 0,389).
Закључци Биотипизација и *CGF* 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 ПЦР

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 [3] or serotyping schemes based on heat labile (HL, Lior sheme) [4] or heat stabile (HS, Penner) antigens [5] can be used. Molecular techniques, e.g. PCR based methods provided more rapid tools for 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 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 disease 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]; a multilocus sequence typing (MLST) [10]; multi-locus variable-number tandem repeat analysis (MLVA) [11] (a promising tool, but still without widely accepted protocol [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 outbreaks 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 microbiological tool are less complicated procedures on a routine basis, rapid results, inexpensiveness, better discrimination, and quantitative relatedness between strains, compatibility with PFGE data, preferable automatic and portable equipment and easy comparison within and between laboratories by the existing databases.

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* genome [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 the great importance is not only to develop and implementation of effective control measures on the identification of sources of infection, but also to choose efficient microbiological tool. Nowadays, in Serbia, there are not consistent programs for surveillance and monitoring of food borne infection 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, (b): to determine the similarity, clonality or epidemiological relatedness of the strains.

METHODS

We have investigated 23 thermophilic *Campylobacter* spp. strains designated in Arabic numbers 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 same geographic distribution and same pattern of sensitivity to antimicrobials. We presumed that strain pairs belonged to the same species; biotype and CGF type i.e. 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 (RL) for *Campylobakter* and *Helicobacter* in Amies medium were cultured in Columbia agar (Columbia blood agar with 5% sheep blood (CBA), Liofilchem, Italy) and *Campylobacter* agar with 5% sheep blood (CA), Liofilchem, Italy), brain heart infusion broth (BHI), (Blood agar base heart infusion, Biolife, Italy) and Bolton medium (Fluka, Suisse) with 10% laked horse blood (Oxoid, UK), and subcultured on CBA and CA after 48h in the same conditions.

Previosly isolated strains, stored in BHI with 15% glycerol at -70^{o}C, were defrozen at room temperature and plated on the same media in the same conditions. Media were incubated for 48h, in microaerobic atmospfere with 9% CO_2 at the 37^{o}C in the incubator (pCO_2 inkubator BINDER, USA). Colonies of *Campylobacter* were presumptively identified microscopically by stained (1% carbol-fuchsin) slides (presence of S- and spiral shaped bacteria with gull-wing morphology), and by oxidase and catalase tests.

A combination of biotyping and PCR-based 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, Indianapolis, Ind.), *TaqI* (Boehringer-Mannheim), or *BsrI* (New England

Biolabs,Inc., Beverly, Mass.) were used for amplicon digestion. Distinguishing between *C. jejuni* and *C. coli required* an additional set of primers which were 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, the eight multiplex PCRs were performed on each isolate using the forty primer sets [13]. Used loci were: (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 binary format. Detected clusters were designated in Arabic numbers as 1-9 [13]. PCR reaction and its analysis were performed as described by Taboada et al. in 2012 [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 CI 95 [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 and co-workers [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 the 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* (9 strains), represented with three and two biotypes, respectively. All strains belonged to nine CGF clusters.

In *C. coli*, five strains belonged to biotype I and four to biotype II. The investigation of 14 *C jejuni* strains subdivided isolates into all 3 biotypes: two strains were of biotype I, eight strains of biotype II, four strains belonged to the biotype III (Table 1).

C. coli clustered together: *C. coli* biotype I, all fell in CGF cluster number 1, while C. *coli* biotype II, were slightly more diverse and fell into clusters 1 and 2. CGF subtyping of *C. jejuni* biotype I, *C. jejuni* biotype II, and *C. jejuni* biotype III revealed that strains belonged to two, five and one cluster, 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 number 9 (Table 1).

Table 1. CGF and clusters distribution among investigated *Campylobacter* strains.

Species and biotype	N° of strains	Designations of CGF cluster	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).

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

Microorganism method	N° of strains	Method	N° 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 – 95% confidence interval; CINA – 95% nonapproximated confidence interval.

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 gave 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, 0, respectively (Table 3).

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 – 95% confidence interval; CINA – 95% nonapproximated confidence interval.

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

Speciation and biotyping revealed 7 pairs (A-G) of *Campylobacter* spp. which were identified as being clonally related (Table 4).

However, CGF typing revealed some differences among related isolates: pairs A, C, E, F and G showed homegenicity by CGF typing. Pair B, identified as *C. jejuni* ssp. *jejuni* II was subdivided into

Table 4. Clonality of isolated strain pairs A-G as determined by speciation, biotyping and 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

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 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 absence of expression with red squares. The position of the same color squares one above the other, either strains posses or not particular gene (Figure 1). At the very left side the figure, there are the numbers of strains, and at the very right end, there are identified species.

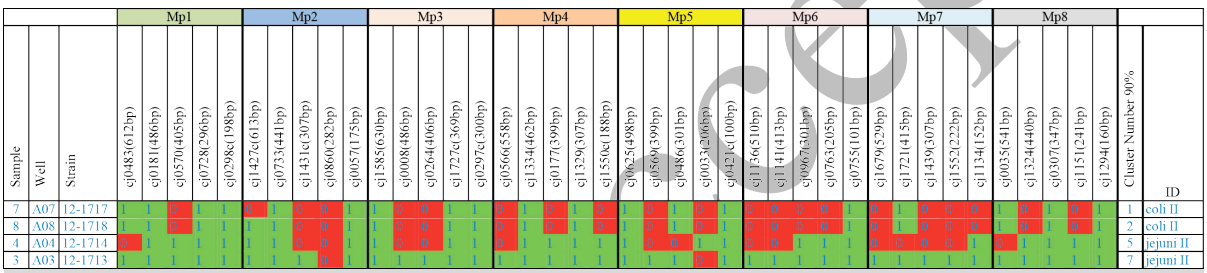

Figure 1. Algorithm of *C. coli* II (pair B) and *C. jejuni* I (pair D) with differences in gene expression. Mp1-8, Multiplex PCR 1-8; cj0483-cj1294, gene loci; ID – identification.

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 a 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, one and two, represented one pair (*C. coli* II), which means a possible evolution of one strain. Other pair of strains

(*C. jejuni* II) differs in several alleles and represents two distinct clusters: cluster seven and cluster five. 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 that were subtyped by CGF was surprising, although, it is possible that one strain underwent genetic changes having in mind that campylobacter is extremely genetically variable bacterium [23]. CGF expressed better discriminatory power than biotyping in determination of clonality, which can be used in outbreaks investigation.

Using the CGF method, we have found high index of diversity for the species, indicating different sources of the *C. jejuni*. Through the future investigation of the animal isolates, it could be answered which one of many food animal sources are in question. For the species of *C. coli* index of diversity was somewhat lower (0.667), indicating higher similarity between strains, and perhaps a common origin. Therefore, in 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 methods as determined by Wi, was for *C. jejuni* II 0.143, and for *C. coli* II 0.333, allowing association of those two methods. These properties suggest that methods based on comparative genomics represent a better alternative to biotyping.

Detection of epidemic strain or investigation applied in population biology of bacterial strains are the important task for microbiologists. The application of only serotyping on strain collection can show great diversity without predominant types, when strains are selected randomly [24], as it was seen in this investigation. Although disadvantage of serotyping is that many of strains can be untypable, investigation of epidemic strains may give representative and reproducible data e.g. in an outbreak described by DeFraites and co-workers. They detected Lior serotype 5 in accessible isolates [25]. Authors applied only serotyping and they did not find any diversity among strains, as it could be possible when some subtyping method or molecular typing method were used.

To resolve epidemic strains, the short variable regions (SVRs) SVRs of *C. jejuni* isolates successfully replaced serotyping, [26]. One of the contemporary approaches is the multiplex PCR method for determination of capsule types of *C. jejuni,* which correlates with Penner typing. The multiplex PCR showed sensitivities and specificities ranging from 90 to 100% using strains of known Penner type [27]. 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 comprised 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. 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 in *C. coli* revealed Simpson's ID of 0.667 *and* in *C. jejuni* strains gave Simpson's ID of 0.89 [28]

The goal of all typing and subtyping systems is a precise and efficient tracing 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 criteria for 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 outbreak, the method could reveal relatedness between the strains that could help in outbreaks detection. Introducing CGF could significantly improve investigation of clonal relatedness between strains and therefore contribute to the improvement in outbreak investigation. However, testing more samples will obtain more reliable results.

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