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Research article

TYPING OF INDIGENOUS *CAMPYLOBACTER* spp. FROM SERBIA BY m-PCR AND RAPD

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Campylobacteriosis is an infectious human disease caused by thermophilic *Campylobacter* species, mainly *C. jejuni* and *C. coli*. It is the leading cause of human gastroenteritis today with the number of cases surpassing the number of *Salmonella* poisoning cases. The epidemiology of the agent is not completely clear, but a number of investigations indicate an important role of broiler meat in human infections. The purpose of this study was to investigate the diversity of *Campylobacter jejuni* strains present in the Republic of Serbia and to determine a fast and reliable system for the confirmation and typing of the isolated strains. Samples taken at slaughterhouses, broiler farms, as well as two human isolates of *Campylobacter* species have been investigated. Strain identification was performed by multiplex-PCR. Genotyping was performed by Random Amplified Polymorphic DNA (RAPD) with multiple primers. Using several unusual primers and a newly designed one (DJP17), we report on the RAPD types of indigenous *Campylobacter* species. RAPD profiles showed different levels of discrimination between the isolates, depending on the primer: SPH1 and AG15 were informative only in part and better results were obtained with AP10, AK16 and DJP17.

Key words: broilers, *Campylobacter* spp., epidemiology, multiplex-PCR, RAPD

INTRODUCTION

Campylobacter species have been recognised as important pathogens of humans and animals [1,2]. Thermotolerant species as *C. jejuni* and *C. coli* cause gastroenteritis in humans and colonize the intestines of poultry, pigs and cattle. During the past few decades bacteria from this genus became the most common cause of food-borne enteritis in developed and developing countries, surpassing the number of *Salmonella enteritis* cases [3]. Approximately 9 million cases of campylobacteriosis occur in the EU

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yearly [4]. These data emphasizes *Campylobacter* species (especially *C. jejuni*) as emerging food-borne pathogens along with Norovirus and *Listeria monocytogenes* [2,5].

The disease usually occurs with symptoms of enteritis. The illness is self-limited, the symptoms cease approximately in a week without treatment. Complications as Guillian-Barr syndrome, arthritis or septicemia in immune-compromised individuals, are possible, but rare.

The epidemiology of *Campylobacter* spp. is not quite clear yet, but it is known that major reservoirs of the agent are poultry, pigs and cattle [6, 7]. The agent normally occurs in the intestine of those animals, providing no symptoms [8]. Commercial flocks of broilers and laying hens are considered as the main reservoir of the agent in nature [9]. Current epidemiological studies suggest that poultry meat is one of the vehicles for transfer of the infection to humans [10,11].

The isolation of the agent is difficult, time consuming and expensive. Problems may occur if bacterial cells are damaged (isolation can be unsuccessful) and traditional bacteriological isolation techniques are laborious and slow. Nowadays investigators tend to rely more on genetic tools both for detection and typing of *Campylobacter*s [12,13]. Identification of species can be performed by biochemical tests, but the verification of the biochemistry is often needed. This verification is performed by multiplex-PCR (m-PCR) method, using different combinations of primer pairs specific for *C. jejuni* and *C. coli*, the main pathogens from the genus [14].

Appropriate typing methods are necessary in order to conduct epidemiological studies. Numerous investigators recommend the use of the RAPD method for *Campylobacter* typing [12,13]. Møller Nielsen et al. [15] concluded that RAPD and PFGE had a better discriminatory power in *Campylobacter* species comparing to *fla*A-RFLP, *fla*A-DGGE, rybotyping and Penner serotyping. Trajkovska-Dokić et al. [16] compared Penner serotyping and RAPD for typing of *C. jejuni* isolates and concluded that RAPD can be used for this purpose.

The aim of this study was to investigate the diversity of *Campylobacter jejuni* strains among isolates from poultry farms and slaughterhouses in the Republic of Serbia by employing m-PCR and RAPD methods.

MATERIALS AND METHODS

Bacterial isolates

Culture collection used in this study consisted of 55 isolates obtained from a broiler farm and slaughterhouses in the region of Pomoravlje. All isolates were biochemically confirmed as *Campylobacter* spp. Two human feces isolates were included in the study. Isolates were stored in Tryptic Soy broth (Oxoid, UK) with 20% of glycerol (Centrohem, Serbia) at -80°C. Subset of 30 isolates was used for molecular analysis (Table 1).

Referent strains were *Campylobacter jejuni* ATCC 33291 (R50) and *Campylobacter jejuni* ATCC 29428 (R51).

Detection of Campylobacter species

The method used for detection of *Campylobacter* spp. was EN/ISO 10272-1:2006 – Microbiology of food and animal feedstuffs – Horizontal method for detection and enumeration of *Campylobacter* species – Part 1: detection method and the nutrient media used were produced by Oxoid, UK.

DNA extraction

The isolates were grown on Columbia blood agar (Oxoid, UK) prior to DNA extraction. Colonies were suspended in 200 μl TE (Tris-EDTA) buffer, heated for 10 min at 95°C and rapidly cooled to -20°C for 5 min for cell lysis, centrifuged at 13 000 rpm for 5 min. Amount of 50 μl of supernatant was transferred into 500 μl TE buffer and stored on -20°C pending investigation. Supernatant containing DNA (1 μl) has been used in each PCR reaction as the template.

Multiplex-PCR

For identification of *C. jejuni* and *C. coli*, m-PCR reaction was carried out according to the protocol described by Denis et al. [14], modified by using only primers for *map*A and *ceu*E gene.

RAPD

Amplification was conducted in 25μl total volume containing DreamTaqGreen Master Mix (ThermoScientific, Lithuania), 1μl of DNA template and 0.1μM of primers. The amplification conditions were as described earlier: for the primers OPA8, OPA10 [17], AG15, AF14, BC318, AX16, AK16 [18], SPH1 [19], and AP10 [20]. The amplification condition for the primer DJP17 (GTGCGCATCAGGCCGTA), designed in this study, included initial denaturation at 95°C for 5 min, 35 cycles each consisting of 1 min at 94°C, 1 min at 57°C, 2 min at 72°C and a final extension step of 5 min at 72°C. All products of amplifications were separated by gel electrophoresis on 1.5% agarose gel in 0.5x Tris borate EDTA buffer at 5V/cm and visualized under UV light.

Cluster analysis

Cluster analysis was performed by unweighted pair group arithmetic average-linkage algorithm (UPGMA) and statistically analyzed by STATISTICA 7 software.

RESULTS

Biochemical and m-PCR analysis

Total of 30 out of 55 isolates were confirmed as *Campylobacter* spp. by biochemical tests (Table 1). Hippurate hydrolysis test revealed that nine of tested isolates were

C. jejuni, and 20 *C. coli*. One of the isolates (B6) could not be classified as neither of the species. Results of biochemical testing of human isolates confirmed one of the isolates as *C. jejuni* and one as *C. coli*.

Table 1. Source of *Campylobacter* spp. isolates and identification and typing results

Sampling location	Sample category	Isolate	Biochemical tests	m-PCR	RAPD pattern
Slaughterhouse A	Carcass swab	A1	*C. coli*	*C. coli*	a
		A2	*C. jejuni*	*C. coli*	a
		A4	*C. coli*	*C. coli*	a
		A43	*C. jejuni*	*C. jejuni*	b
		A46	*C. jejuni*	*C. jejuni*	b
		A53	*C. jejuni*	*C. jejuni* and *C. coli*	/
		A54	*C. coli*	*C. coli*	a
		A55	*C. coli*	*C. coli*	a
		A56	*C. coli*	*C. coli*	a
		A57	*C. coli*	*C. coli*	a
		A58	*C. jejuni*	*C. jejuni*	b
Slaughterhouse B	Carcass swab	B6	*C. lari*	Ø	/
	Neck skin	B13	*C. jejuni*	*C. jejuni*	c
		B15_	*C. jejuni*	*C. jejuni*	d
Slaughterhouse C	Carcass swab	C23	*C. coli*	*C. coli*	e
		C24	*C. coli*	*C. coli*	e
		C25	*C. coli*	*C. coli*	e
		C26	*C. coli*	*C. coli*	e
		C34	*C. coli*	*C. jejuni*	f
		C35	*C. coli*	*C. jejuni*	g
		C36	*C. coli*	*C. jejuni*	h
		C38	*C. coli*	*C. jejuni*	i
		C59	*C. jejuni*	*C. jejuni*	b
		C60_	*C. jejuni*	*C. jejuni*	j
Slaughterhouse W	Carcass swab	W52	*C. coli*	*C. jejuni*	k
Public Health Institute V	Human feces	V16	*C. coli*	*C. jejuni*	l
		V17	*C. coli*	*C. coli*	m
Farm P	Caecum	P27	*C. coli*	*C. coli*	n
		P29	*C. coli*	*C. coli*	n
		P30	*C. coli*	*C. coli*	n

All of the 30 isolates of *Campylobacter* were analyzed by primers for *map*A and *ceu*E genes [14]. Amplicons of different size specific for *C. jejuni* and *C. coli* (589 bp and 462 bp, respectively) were obtained (Fig. 1a and 1b). Summary of the results revealed 13 isolates of *C. jejuni*, and 15 isolates of *C. coli*. One of the isolates yielded both products (A53) and one did not give any amplification (B6).

Results obtained by m-PCR slightly differed from biochemical testing. Sample A2 was characterized as *C. jejuni* by biochemical tests and as *C. coli* by m-PCR. Samples C34, C35, C36 and C38, characterized as *C. coli* by biochemical tests were characterized as *C. jejuni* by m-PCR. Sample A53 produced both bands specific for *C. jejuni* and *C. coli*. Test was repeated three times and the results were identical.

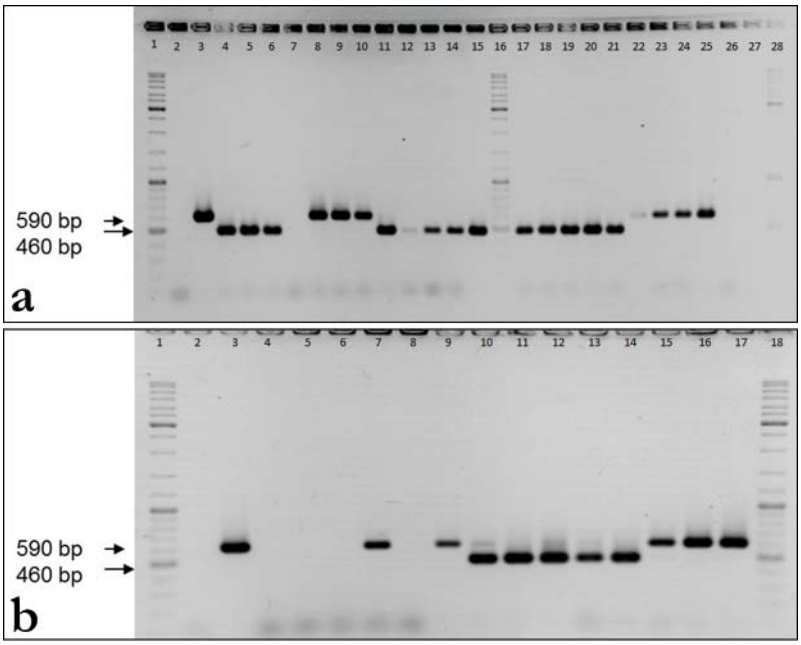

Figure 1. m-PCR of *C. jejuni* i *C. coli* isolates based on *map*A and *ceu*E genes. **a)** Lanes 1, 16 and 28. M - GeneRuler DNA Ladder Mix SM0331 (ThermoScientific, Lithuania); Lanes 2, 26 and 27. blank; Lane 3. *C. jejuni* ATCC strain R51; Lanes 4-15. Isolates: A1; A2; A4; B6; B13; B15; V16; V17; Z20; Z21; C23; C24; Lanes 17-27. Isolates: C25; C26; P27; P29; P30; C34; C35; C36; C38; **b)** Lanes 1 and 18. M - GeneRuler DNA Ladder Mix SM0331 (ThermoScientific, Lithuania); Lanes 2, 4, 5 and 6. blank; Lane 3. *C. jejuni* ATCC strain R51; Lanes 7-17. Isolates: A43; A46; W52; A53; A54; A55; A56; A57; A58; C59; C60

RAPD analysis

RAPD was performed with 10 primers. Some of those primers were formerly used for *Campylobacter* testing, e.g. OPA8 and OPA10, while others were used with different bacterial genomes or for plant genome analysis. One primer was designed for this study and named DJP17. Results obtained with primer SPH1 were less informative. Primers BC318, AF14, AK16 (data not shown) and OPA8 could not differentiate isolates within the species, but could distinguish *C. jejuni* from *C. coli*. Primers OPA10 and AX16 did not produce any amplification with the investigated isolates under recommended conditions. Discriminatory potential of the primer DJP17 specifically designed for this study has been tested and obtained patterns contained 3 to 10 different product sizes (Fig. 2).

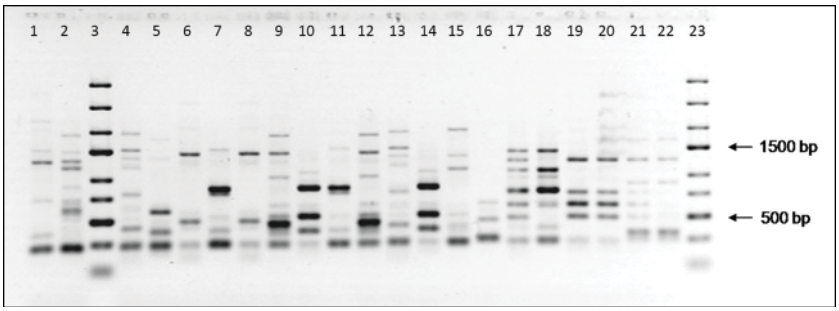

Figure 2. Representative RAPD patterns of *C. jejuni* and *C. coli* isolates according to DJP17 primer. Lanes 1 and 2. *C. jejuni* ATCC strains: R50, R51; Lanes 3 and 23. M- GeneRuler Express DNA Ladder SM1551 (ThermoScientific, Lithuania); Lanes 4-15. *C. jejuni* isolates: A43; A58; B13; B15; W52; C34; C35; C36; C38; C59; C60; V16; Lane 16. A53; Lanes 17-22. *C. coli* isolates: P29; V17; A1; A2; C25; C26.

Primers AP10, AG15 and DJP17 produced profiles with multiple product size and enabled typing of all the isolates (Fig. 3). Cumulative RAPD analysis was performed according to results obtained with primers OPA8, SPH1, AG15, AP10 and DJP17 and 14 different patterns were obtained: 10 for *C. jejuni* and 4 for *C. coli* (Table 1). *C. jejuni* isolates are clearly distinguished from *C. coli* isolates forming separate clusters. *C. jejuni* cluster shows significant differences on the isolate level, while differences among *C. coli* isolates are not very expressed (around 18%).

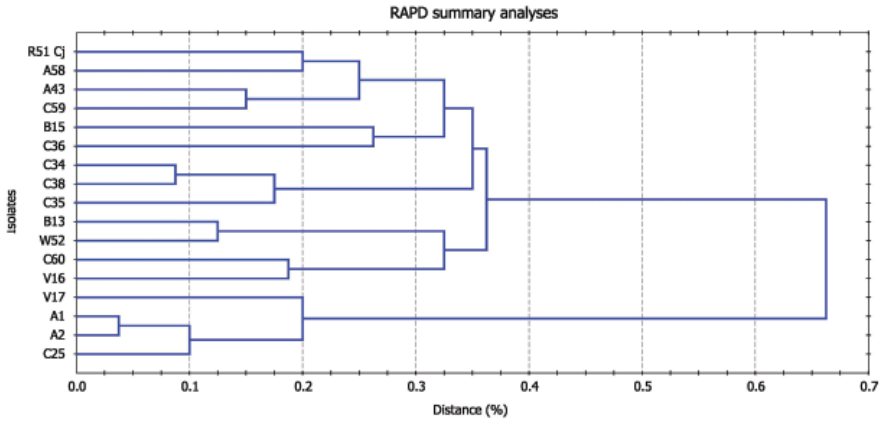

Figure 3. Cumulative RAPD analysis of representative *C. jejuni* and *C. coli* isolates using primers OPA8, SPH1, AG15, AP10 and DJP17.

DISCUSSION

The purpose of this study was to investigate diversity of *C. jejuni* strains present in the Republic of Serbia and to determine a fast and reliable system for the confirmation of

the isolated strains. As it is sometimes difficult to identify isolates to the species level, the combination of biochemical tests and m-PCR provides an excellent solution to this problem. Comparison of biochemical tests results and m-PCR reveals few differences: there were some isolates negative on hippurate hydrolysis test which were classified as *C. jejuni* by m-PCR, as well as one isolate which was hippuricase-positive, but identified as *C. coli* in m-PCR. These results are similar to the results obtained by Al Amri et al. [21] who also obtained false negative results - isolates were hippuricase-negative, but belonged to *C. jejuni* species and false positive results - isolates were hippurate positive, but m-PCR aligns them into *C. coli*. Authors concluded that possible explanation of this finding is that *C. coli* possess an enzyme whose substrate is similar to hippurate, so it disassembles it creating a false positive reaction and false negatives are present when hippuricase gene is not active in the isolate [22,23].

One of the tested isolates yielded amplicons with both sets of primers in m-PCR (isolate A53). In consecutive investigation with RAPD primers it behaved as *C. jejuni* (primers AG15, OPA8) or *C. coli* (primers SPH1, AP10). This finding indicates a mixed infection in broilers. The cases of mixed cultures in the flock or herd were already documented. Al Amri et al. [21] detected mixed infection in feces of broilers and humans. Boes et al. [24] investigated the presence of *C. jejuni* and *C. coli* in pig herds on strictly pig farms and mixed farms (pigs / poultry, pigs / cattle). The incidence of *C. coli* was higher than 90% regardless of farm type (strictly pigs or mixed). In mixed farms *C. jejuni* predominated in cattle and poultry and *C. coli* in pigs, but there were evidence of mixed infection in pigs – *C. jejuni* positive pigs often excreted *C. coli* as well.

RAPD investigation described in this article was conducted in three steps. The first step included primes that were already used by other investigators (OPA8, OPA10) and gave discriminative results for *Campylobacter* [17]. We could not repeat these results – primer OPA10 did not produce and OPA8 produced poor amplicons under recommended conditions. Primer OPA8 detects minor differences among *C. jejuni* isolates, and all *C. coli* isolates produce the same profile. In the second step the following primers (SPH1, AP10, AK16, AG15) were used for RAPD typing. Primer DJP17 was synthesized for the third step of this investigation.

Cluster analysis of cumulative RAPD clearly separated *C. jejuni* from *C. coli*; clusters were mutually different 56%. *C. coli* isolates were mutually similar, they differ maximally 20%. Poultry isolates were highly similar (90%), while human isolate V17 was 20% different from isolates originating from poultry. These findings suggest that poultry was not the source of *C. coli* infection for humans, but extensive investigation is needed. *C. jejuni* cluster consisted of 2 subclusters. Isolates C34, C35, C36 and C38, belonging to the first subcluster, originated from the same sampling in one slaughterhouse. They were characterized as *C. coli* by biochemical tests and as *C. jejuni* by m-PCR. RAPD analysis confirmed m-PCR findings separating these isolates into distinct cluster of *C. jejuni* isolates and clarified biochemical test results. *C. jejuni* isolates A43 and C59 originating from different sampling cluster together in the second subcluster.

We obtained 14 different RAPD patterns, 10 for *C. jejuni* and 4 for *C. coli*. Isolates with distinctive origin were clustered together in the single and cumulative RAPD analysis, while isolates obtained in the same sampling were divided into different clusters, subclusters or branches. These results suggested that RAPD can be used as a typing tool in epidemiological investigations to clarify the sources of infection and way of transmission. RAPD analysis is already used in *Campylobacter* studies. Açik and Çetinkaya [12] used this method in the characterization of *C. jejuni* and *C. coli* isolated from healthy cattle and sheep. They used primer OPA11 and obtained 37 profiles of *C. jejuni* and 5 profiles of *C. coli* in cattle compared to 21 *C. jejuni* and 24 *C. coli* profiles in sheep. RAPD have been used in the genetic variability analysis of *C. jejuni* and *C. coli* from commercial broiler farms in Italy [25]. Giacomelli and associates used RAPD primer AP4 for characterization of isolates obtained from cloacal swabs. They obtained 11 different RAPD patterns, 8 for *C. jejuni* and 3 for *C. coli* isolates.

Campylobacter characterization by RAPD can be useful in the case of a mixed infection with bacteria from other genera. In Serbia, the influence of *Salmonella enterica* infection in chickens on the outcome of campylobacteriosis in experimental conditions has been investigated on the basis of morphological, cultural and biochemical properties [26]. The field strain of *C. jejuni* used in their study may be compared with isolates from our study by RAPD analysis using primers selected in this work.

Primer DJP17, used for diversity screening of *C. jejuni* in Serbia for the first time, was very informative in distinguishing indigenous *C. jejuni* and can help connecting them to the potential source of infection. Before recommending DJP17 for wider use it is necessary to expand the research and apply this primer to a larger number of isolates from Serbia and abroad.

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Authors' contributions

DJ carried out the molecular genetic studies, participated in the sequence alignment, participated in the design of the study and performed the statistical analysis and conceived of the study, drafted the manuscript and coordination to draft the manuscript. JP conceived of the study, performed the statistical analysis and participated the design of the study and coordination and helped to draft the manuscript. OB, ZL, RP, ZR, VK conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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TIPIZACIJA IZOLATA *CAMPYLOBACTER* SPP POREKLOM IZ REPUBLIKE SRBIJE PRIMENOM M-PCR I RAPD METODA

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Kampilobakterioza je infektivna bolest ljudi izazvana termofilnim bakterijama roda *Campylobacter*, uglavnom vrstama *C. coli* i *C. jejuni*. Jedan je od vodećih uzroka gastroenteritisa, pri čemu broj obolelih premašuje broj infekcija *Salmonella* vrstama. Epi-

demiologija uzročnika nije u potpunosti rasvetljena, ali je u mnogim istraživanjima naglašena uloga pilećeg mesa u infekciji ljudi. Cilj ovog istraživanja bio je ispitivanje diverziteta *Campylobacter* vrsta prisutnih u Republici Srbiji i uspostavljanje brzog i pouzdanog sistema za potvrdu i tipizaciju dobijenih izolata. Ispitivani su uzorci uzeti sa farmi brojlera i iz klanica živine, kao i 2 izolata poreklom od ljudi. Identifikacija izolata vršena je primenom multiplex-PCR-a. Genotipizacija je vršena primenom analize nasumično umnožene polimorfne DNA (RAPD) sa više prajmera. Primenom prajmera neuobičajenih za ispitivanje *Campylobacter* vrsta i jednog novodizajniranog (DJP17), dobijeni su RAPD profili autohtonih *Campylobacter* izolata. Stepen diskriminacije izolata zavisio je od primenjenih prajmera: prajmeri SPH1 i AG15 bili su delimično informativni, dok su prajmeri AP10, AK16 i DJP17 dali bolje rezultate.