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Identification of *Campylobacter* Species Isolates with Phenotypic Methods and Polymerase Chain Reaction

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SUMMARY
Introduction *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are the most common bacterial causes of enterocolitis in humans. However, identification of the species level is not always possible using standard biochemical tests.
Objective Therefore, the goal of this study was to identify these microorganisms by both phenotyping and polymerase chain reaction (PCR) technique.
Methods A total of 153 species of thermophilic campylobacters were examined with standard biochemical tests and PCR technique to prove *hipO* genes of *C. jejuni* and *asp* genes of *C. coli*.
Results Standard biochemical tests enabled the speciation of 121 strains of *C. jejuni*, while application of PCR detected 126 C. jejuni strains.
Conclusion PCR technique allowed not only identification of hippurate-positive *C. jejuni,* but also hipurat-negative strains of *C. jejuni* which otherwise would be detected as *C. coli* if only biochemical tests were applied.
Keywords: *Campylobacter jejuni*; *Campylobacter coli*; identification; PCR

INTRODUCTION

Campylobacter jejuni (*C. jejuni*), and closely related organisms *Campylobacter coli* (*C. coli*), *Campylobacter lari* (*C. lari*) and *Campylobacter upsaliensis* (*C. upsalienis*) are recognized as the major cause of bacterial enterocolitis in developed countries as well as in non-industrialized ones with substantial economic burden [1, 2]. Although there are similar clinical presentations of infection with *C. jejuni* and *C. coli,* which is characterized by a sudden onset of fever, abdominal cramps, and diarrhea with blood and leukocytes, they enormously differ in epidemiological characteristics and sensitivity to antimicrobials [3]. Apart from intestinal, more serious extraintestinal infections may appear, such as transient bacteremia and localized infections including septic arthritis, meningitis, peritonitis, cholecystitis, hepatitis, pancreatitis, and abscesses. In addition, fulminant sepsis and myocarditis may also occur [4]. Rarely, infection with *C. jejuni* results in severe consequences on the peripheral nervous system manifested by flaccid paralysis in Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS) [3]. Diseases of the musculoskeletal system triggered by *C. jejuni* include reactive arthritis, tendinitis, enthesopathy, bursitis or Reiter's syndrome (oligoarthritis, conjunctivitis, and urethritis in men or cervicitis in women) [5]. *C. jejuni* associated diseases of the intestinal tract are inflammatory bowel disease (IBD) and immunoproliferative small intestinal disease (IPSID) [6, 7].

Although thermophilic *Campylobacter* species are ubiquitous in most environments, food of animal origin is substantial reservoir of human infection. The most common source of infection is poultry and pork, and even shellfish. Of all thermophilic *Campylobacters*, *C. lari* and *C. upsaliensis* are less frequently isolated. The primarily source of *C. lari* is see-gulls, while *C. upsaliensis* is most commonly isolated from dogs, kittens, and puppies, especially with diarrhea [8].

Campylobacter organisms were assumed as a cause of human infection even before disclosure of successful isolation technique; however they have been recognized for a long period only in veterinary medicine. Filtration techniques and development of the selective media were major progress in detection of Campylobacter in stool specimens [9]. To identify *Campylobacter* species, biochemical tests are performed in routine diagnostic laboratories. However, detection, identification and quantification of these pathogens, transmitted commonly through food, might be often misleading due to a small number of microorganisms present in the specimen or some metabolic alterations which unable their identification. Indeed, at least variable enzymatic activity of *C. jejuni* hippuricase and reduced sensitivity to nalidixic acid cause difficulties and misleading in identification. Namely, some of *C. jejuni* strains, although possessing gene for enzyme synthesis, do not hydrolyze hippurate [10]. Moreover, false positive reactions are described [11]. Strain resistance to

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nalidixic acid refers to *C. lari*, but nowadays, in some geographic areas, more than 90% of *C. jejuni* and *C. coli* strains are resistant to this antimicrobial diminishing the value of the test [12].

Certain improvements in the molecular-genetic methods (polymerase chain reaction – PCR), real-time PCR (RT-PCR), multiplex PCR, nested PCR, blotting methods (i.e., Western blot, Southern blot), microarray analysis etc. [13, 14] facilitate the diagnosis of *Campylobacters* and represents basic techniques that are recently used in many diagnostic laboratories. Moreover, in some cases, the PCR based methods can be applied directly to the specimens, without cultivation, which endows a rapid diagnosis [15]. There are several features such as a high sensitivity, specificity, and amplification of very small samples that makes PCR an irreplaceable method in almost every microbiological laboratory [16].

Precise identification of *C. jejuni* and *C. coli* is necessary for surveillance of their migration through ecological niches, as well as for a more complete understanding of the pathogenesis of the disease, development of drug resistance, epidemiological characteristics and risk factors for infection and application of proper treatment.

OBJECTIVE

The objective of the study was the identification of the isolated bacteria of the genus *Campylobacter* using classical methods and PCR technique and identifying potential problems associated with the identification.

METHODS

The tested strains

The study involved 153 *Campylobacter* strains of enteric origin from the collection of the Serbian National Reference Laboratory (NLR) for *Campylobacter* and *Helicobacter*. The strains were isolated from stool specimens at the Centre for Microbiology, Public Health Institute, Niš, and in other laboratories in Serbia which send cultures of *Campylobacters* to NLR for confirmation and identification. Selective *Campylobacter* Agar with 5% Sheep Blood (Liofilchem, Italy) were used for the isolation and cultivation of *Campylobacter*; inoculated plates were incubated at 42°C in microaerophilic atmosphere (9-10% CO_2) in the pCO_2 incubator (BINDER, USA) for 48 hours.

All cultures of *Campylobacters* in an Amies Transport Medium (Oxoid, UK) submitted to the NLR for *Campylobacter* and *Helicobacter* were processed immediately upon arrival by inoculating on Columbia Agar plate (Columbia blood agar, CBA) containing 5% sheep blood, *Campylobacter* agar (CA) with 5% Sheep Blood (Liofilchem, Italy), Brain Heart Infusion (BHI) Broth (Blood agar base heart infusion, Biolife, Italy), and *Campylobacter* Enrichment Broth (Bolton's) (Fluka, Suisse) with lysed horse blood (Fluka, Suisse) for the selective enrichment of *Campylobacter* spp. Enriched isolates were subcultured on CBA and CA plates for further investigation.

Biochemically based identification

Phenotypic *Campylobacter* species identification method includes typical appearance of colonies, a distinctive microscopic feature when stained with carbol-fuchsin, spiral rod morphology (spiral, S-shaped or in pairs showing seagull-wings-shape), oxidase test (Himedia, India), catalase test, the test for hippurate-hydrolysis (Rosco, Danmark), hydrolysis of indoxyl acetate (Rosco, Danmark), susceptibillity to cephalothin (30 μg disc) and nalidixic acid (30 μg disc) (Rosco Diagnostica, NeoSensitabs, Denmark) [17]. Also, API (Appareils et procédés d'identification) Campy test (bioMérieux, France), which consists of 11 enzymatic and 9 assimilation and inhibition tests were performed.

PCR method

DNA was isolated by PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, California, USA) kit according to the manufacturer's instructions. PCR assay was performed in a total volume of 50μl. The primers sets used were: 0.4 μM *hipO* (*hipO*-F: 5'-GACTTCGTGCAGATATGGATGCTT and *hipO*-R: 5'-GCTATAAC TATCCGAAGAAGCCATCA) for the species identification of *C. jejuni* [18], and 0.8 μM *asp* (CC18F: 5'-GGT ATG ATT TCT ACA AAG CGA G and CC519R: 5'-ATA AAA GAC TAT CGT CGC GTG) (Invitrogen, Carlsbad, CA, USA) for *C. coli* [19]. Commercial kit used for amplifications consisted of *Taq* DNA polymerase, buffer with dNTP and $MgCl_2$ (AmpliTaq Gold PCR Master Mix, Applied Biosystems, California, USA). Of the total volume of isolated DNA, 5μl was used for PCR. PCR reaction was carried out in a PCR thermocycler (Eppendorf, Germany). The PCR amplification cycles included initial denaturation at 95°C for 6 min, followed by 35 cycles of denaturation at 94°C for 50 sec, annealing at 57°C for 40 sec and extension at 72°C for 50 sec. The final stage was an extension cycle at 72°C for 3 min. The amplified PCR products were stained with ethidium bromide (1%). Following 1.5% gel electrophoresis, amplicons were visualized and documented with system for professional gel documentation (BioDocAnalyze, Biometra, Göttingen, Germany). The sizes of PCR amplicons of 344 bp were estimated as *C. jejuni* and of 500 bp as *C. coli*. To determine the size of the fragment, pUC19DNA – Sau3A I Digested DNA molecular weight size marker (Ambion, Austin, Texas, USA) was used.

RESULTS

Identification was performed on 153 isolates. By phenotyping, 121 strains that hydrolyzed hippurate were identified as *C. jejuni*, while 32 strains failed to give positive

Table 1. Phenotypic *Campylobacter* spp. identification

Microorganism	Number of strains (%)
C. jejuni	121 (79)
C. coli	27 (17.7)
Other hippurate-negative *Campylobacter* spp.	5 (3.3)
Total	153 (100)

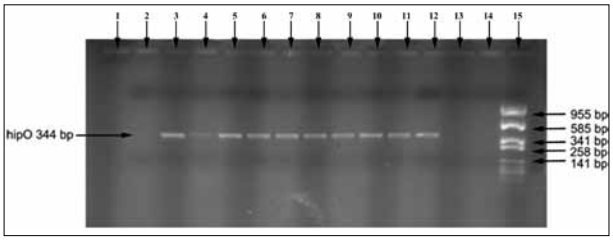

Figure 1. Agarose gel electrophoresis of PCR products from *C. jejuni* using *hipO* primers. Lines 1 and 2: hippurate negative *Campylobacter* spp.; lines 3-11: *C. jejuni;* line 12: *C. jejuni* NCTC 11351 positive control; line 13: *C. coli* ATCC 33559, negative control; line 14: negative control; line 15: DNA size markers.

Table 2. PCR *Campylobacter* spp. identification

Microorganism	Number of strains (%)
C. jejuni	121 (79)
Hippurate-negative *C. jejuni*	5 (3.3)
C. coli	22 (14.4)
Other hippurate-negative *Campylobacter* spp.	5 (3.3)
Total	153 (100)

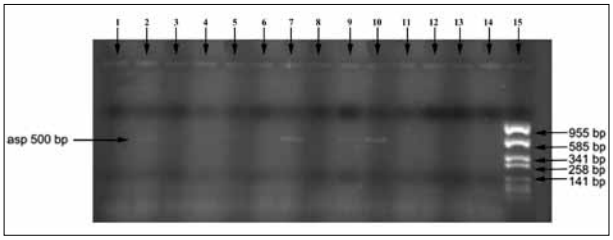

Figure 2. Agarose gel electrophoresis of PCR products from *C. coli* using *asp* primers. Lines 2, 5, 7, 9, 10: *C. coli*; lines 1, 3, 4, 6, 8, 11, 12: *asp* negative *Campylobacter* spp.; line 13: *C. jejuni* NCTC 11351, negative control; line 14: negative control; line 15: DNA size markers.

reaction (Table 1). Twenty-seven strains with negative hippuricase activity, but indoxyl acetate, oxidase and catalase positive were identified as *C. coli*.

Having in mind a possible presence of co-infection with both strains, PCR for *C. jejuni* and *C. coli* was performed on all investigated strains. PCR technique enabled the identification of 126 *C. jejuni* strains (Figure 1); 121 hippurate-positive and five hippurate-negative ones. Twenty two isolates were identified as *C. coli* (Table 2, Figure 2).

Other five hippurate-negative *Campylobacters* have been described by API Campy as *C. lari. C. lari* differ from *C. coli* in negative indoxyl acetate reaction and resistance to nalidixic acid. *C. upsaliensis* give positive indoxyl acetate test but were referred to as the catalase-negative/catalase weak (CNW) group sensitive to cephalothin and nalidixic acid. In this study, we did not detect any *C. upsaliensis*, which is not frequently isolated pathogen from clinical samples, since cephalothin is often used in *Campylobacter* spp. selective media. The method of choice for isolation of *C. upsaliensis* would be filtration technique which could be the reason for suboptimal detection of these bacteria.

DISCUSSION

Accurate identification of *C. jejuni* and precise discrimination of two the most frequently isolated human enteropathogen campylobacters, *C. jejuni* and *C. coli* are essential for microbiological and epidemiological studies. Discrimination among thermophilic strains is based only on biochemical hippurate-hydrolysis test. There are, however, some *C. jejuni* strains that harbor the hippuricase gene but do not express hippuricase activity and are therefore false negative by the hippurate-hydrolysis test, which may lead to misinterpretation of the test results. Indeed, the performance of biochemical tests, in bacteria that have weak biochemical activity, are sensitive to environmental conditions, require special growth and reproduction conditions, is often time- and labor-consuming as not being always efficient.

Enzyme activity and results interpretation depend on the applied methodology and variations which can influence the final outcome of reaction [20]. Therefore, the application of molecular techniques for the determination of genetic sequences characteristic for *C. jejuni* and *C. coli* made their identification significantly accurate.

In this work, on the examination of biochemical characteristics of 153 thermophilic campylobacters, it was found that 121 (79%) isolates hydrolyzed hippurate and were identified as *C. jejuni*. Thirty-two strains (21%) lacked the ability to hydrolyze hippurate. Species that gave a negative reaction were hippurate-negative *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*.

All strains that were identified by conventional biochemical tests were also subjected to PCR. In all 121 strains, *hipO* gen was determined and *C. jejuni* was confirmed. Out of 32 hippurate-negative strains, in five strains the presence of *hipO* gen was also detected endorsed *C. jejuni* species, while 22 strains were confirmed as *C. coli*. Five tested strains, however, were not verified by PCR and two sets of primers, but they were subsequently identified as *C. lari* using API Campy system (bioMérieux, Marcy l'Etoile, France).

Since the introduction of PCR techniques in the early '80s of the last century, the method has become widely used in the identification of microorganisms [21]. In the early '90s, methods based on PCR were developed for the detection and identification of *Campylobacter* spp. targeting distinctive sequences. The fact that existence of the gene does not necessarily mean the simultaneous mRNA synthesis and subsequent enzymes production, PCR technique also enables the identification of hippurate-negative *C. jejuni*, as was confirmed in this study.

The target genes used for the PCR identification of *Campylobacter* spp. are *C. coli*-specific *asp*, *hipO* gene in *C. jejuni* [22], species specific sequences of *ceuE* gene [11], *cadF* [23], 16S rRNK [24], *cdt* [25], and *lpxA* [26]. Wang et al. [15] used *hipO* gene for *C. jejuni*, and *glyA* for

synthesis of serine hydroximethyl-transferase for *C. coli* identification.

Rautelin et al. [20] examined 28 hippurate-negative thermophilic *Campylobacter* isolates, from human stool specimens in Finland. Re-testing of negative strains, using standard biochemical tests, revealed that 10 isolates hydrolyse hippurate, and 18 remained negative. PCR method and primers for variable region of *ceuE* gene allowed accurate molecular identification of *C. jejuni* and *C. coli*: all 10 hippurate-positive strains were confirmed as *C. jejuni*, while out of the remaining hippurate-negative 18 isolates, five were identified as *C. jejuni*, and 13 as *C. coli*

Nakari et al. [27] investigated 240 human isolates (95 hippurate-positive and 145 hippurate-negative) originated from Finland collection of enterocolitis strains, using standardized hippurate-hydrolyze test. Two PCR sets of primers were utilized: *ceuE* gene primers specific for *C. jejuni* and for *C. coli,* and *hipO* gene for *C. jejuni* subsp. *jejuni* and *glyA* gene for *C. coli.* Gene *hipO* was detected in all *C. jejuni* hippurate-positive strains. In hippurate-negative strains, 93 (64%) was diagnosed as *C. coli*, and *hipO* gene of *C. jejuni* was proved in 46 (32%) hippurate-negative strains [27].

During investigation of a Danish collection involving 2,382 phenotypic identified *Campylobacter* strains from poultry, 108 *C. jejuni* and 351 „non-jejuni" isolates were randomly selected and further investigated by PCR using several sets of primers (Hip-PCR and VS-PCR for *C. jejuni*, CS-PCR and CeuE-PCR for *C. coli*, COL/JUN-PCR for *C. jejuni* and *C. coli*) [28]. Out of 108 isolates identified by biochemical tests as *C. jejuni*, 103 were verified, while four were identified as *C. coli*. In one case, there were mixed culture of *C. jejuni/C. coli*. Out of 309 „non-jejuni" strains, 97 strains were confirmed as *C. jejuni* by PCR. Hippurate-hydrolysis retesting of these strains showed that even 84 strains were hippurate-positive, while 13 *C. jejuni* were hippurate-negative [28].

Our results, as well as outcomes of other authors, confirmed the variability of biochemical testing results, especially hippurate-hydrolysis, and that correct identification is possible using PCR technique and specific primers. Although negative hippurate-hydrolysis indicates the presence of *C. coli* and hippurate-negative *C. jejuni*, test alone may be insufficiently reliable, questioning accuracy of phenotyping identification scheme of *C. jejuni*.

Now, Restriction Fragment Length Polymorphism (RFLP), PCR-RFLP on 23S rRNA gene [29], PCR/ELISA (PCR-Enzyme Linked Immuno Assay) for *glyA* gene [30], RT-PCR on *hipO* and *glyA* gene [31] can be used to identify *C. jejuni* and *C. coli*. Al Amri et al. [32] as a target sequence used genus specific *cadF* gene (*Campylobacter* adhesion to fibronectin) and species specific genes, *hipO* gene (for *C. jejuni*) and *asp* (a gene encoding aspartokinase of *C. coli*) for RT-PCR. Also, in the identification of *Campylobacter*, it is possible to apply a very sophisticated techniques such as microarray detection of *fur, glyA, cdtABC, ceuB* – E and *fliY* gene [33].

CONCLUSION

Although the biochemical identification can discriminate the two most common species of *Campylobacters*, *C. jejuni* and *C. coli* in most cases, the PCR method, obtaining rapid results, has the advantage in the identification of *C. jejuni* strains that posses but do not express the hippuricase gene and that by using only phenotypic methods would be misdiagnosed.

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Препознавање изолата *Campylobacter Spp.* применом фенотипских метода и ланчане реакције полимеразе

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КРАТАК САДРЖАЈ

Увод *Campylobacter jejuni* (*C. jejuni*) и *Campylobacter coli* (*C. coli*) се сматрају најчешћим бактеријским узрочницима ентероколитиса код људи. Због њихове сличности није их могуће јасно препознати применом биохемијских тестова.

Циљ рада Циљ рада је био да се препознају ови микроорганизми и фенотипским тестовима и техником ланчане реакције полимеразе (*PCR*).

Методе рада Испитана су 153 соја термофилних кампилобактера стандардном методом и техником *PCR* за доказивање *hipO* гена *C. jejuni* и *asp* гена *C. coli*.

Резултати Класичном методом је утврђено да 121 соју припада врсти *C. jejuni*, а *PCR* техником да овој врсти припада 126 сојева.

Закључак Техника *PCR* је омогућила препознавање не само хипурат-позитивних, већ и хипурат-негативних сојева *C. jejuni*, који би класичним биохемијским методама били препознати као *C. coli*.

Кључне речи: *Campylobacter jejuni*; *Campylobacter coli*; идентификација; ланчана реакција полимеразе (*PCR*)

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