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Multiplex PCR Assay for Identification and Differentiation of *Campylobacter jejuni* and *Campylobacter coli* Isolates

Maria R. Pavlova[1], Elina G. Dobreva[1], Katucha I. Ivanova[1], Galina D. Asseva[1], Ivan N. Ivanov[1], Peter K. Petrov[1], Valeri R. Velev[2], Ivelina I. Tomova[2], Maida M. Tiholova[2], Todor V. Kantardjiev[1]

[1] National Center of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria
[2] Hospital for Infectious and Parasitic Diseases (HIPD), Medical University of Sofia, Bulgaria

Correspondence: Maria Pavlova, National Center of Infectious and Parasitic Diseases (NCIPD), Sofia, BULGARIA
E-mail: mimipavlova@mail.bg
Tel.: +359 898 639 929

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Introduction: *Campylobacter spp*. are important causative agents of gastrointestinal infections in humans. The most frequently isolated strains of this bacterial genus are *Campylobacter jejuni* and *Campylobacter coli*. To date, genetic methods for bacterial identification have not been used in Bulgaria. We optimized the multiplex PSR assay to identify *Campylobacter spp*. and differentiate *C. jejuni* from *C. coli* in clinical isolates. We also compared this method with the routinely used biochemical methods.

Aim: To identify *Campylobacter spp*. and discriminate *C. coli* from *C. jejuni* in clinical isolates using multiplex PCR assay.

Materials and methods: Between February 2014 and January 2015 we studied 93 stool samples taken from patients with diarrheal syndrome and identified 40 species of *Campylobacter spp*. in them. The clinical material was cultured in microaerophilic atmosphere, the isolated strains being biochemically differentiated (hydrolysis of sodium hippurate for *C. jejuni*, and hydrolysis of indoxyl acetate for *C. coli*). DNA was isolated from the strains using QiaAmp MiniKit (QIAGEN, Germany). Twenty strains were tested with multiplex PCR for the presence of these genes: *cad*F, characteristic for *Campylobacter spp*., *hip*O for *C. jejuni* and *asp* for *C. coli*.

Results and discussion: The biochemical tests identified 16 strains of *C. jejuni*, 3 strains of *C. coli*, and 1 strain of *C. upsaliensis*. After the multiplex PCR assay the capillary gel electrophoresis confirmed 16 strains of *C. jejuni*, 2 strains of *C. coli* and 2 strains of *Campylobacter spp*. – because of the presence of the gene *cad*F. *C. jejuni* has the gene *hip*O, and it is possible that this gene may not be expressed in the biochemical differentiation yielding a negative reaction as a result. In comparison, we can conclude that the genetic differentiation is a more accurate method than the biochemical tests.

Conclusion: The multiplex PCR assay is a fast, accurate method for identification of *Campylobacter spp*. which makes it quite necessary in the clinical diagnostic practice.

INTRODUCTION

Campylobacterioses are the most common gastrointestinal bacterial infections in the developed and developing countries with children under the age of five being the most affected population. They are caused by *Campylobacter* which is a genus of Gram-negative, motile, non-spore forming bacteria of which *Campylobacter jejuni* and *Campylobacter coli* are the most frequent isolates (80-85% and 10-15% of all campylobacterioses, respectively). The source of infection is most often contaminated food products – poultry meat, unpasteurized milk, and other sources; domesticated animals such as dogs and cats are also potential sources of campylobacteriosis. The resulting infection may vary from mild watery diarrhoea to a bloody profuse diarrhoea with abdominal pain and high fever; this infection is clinically indistinguishable from gastroenteritis caused by *Salmonella*, *Shigella* or other enteropathogenic bacteria.[1-4,12,15] Most patients recover from the infection without specific treatment - the infectious process is self-limiting and the symptoms are resolved within one week but the infection is recurrent in 20% of patients. Just like other any

other enteropathogen, *Campylobacter* bacteria and especially *C. jejuni* cause post-infection complications of which the Guillain-Barre syndrome, an acute demyelinating disease affecting the peripheral nervous system, is the most prominent.[5,6]

The genus *Campylobacter* are thermophilic, exigent bacteria which die if they stay too long in the presence of oxygen, and this is quite an obstacle when it comes to isolating them. Their cultivation requires microaerophilic conditions (5% O_2 + 10% CO_2 + 85% N_2) at 42-43°C for 48-72h. Biochemical tests have been used most commonly to isolate the *C. jejuni* and *C. coli* – reaction to sodium hippurate hydrolysis and indoxyl acetate hydrolysis. However, the phenotypic differentiation is not always accurate.[7-10,16] To address this shortcoming we optimized a genetic method – the multiplex PCR – to identify the two species, which is more accurate and reliable than the phenotypic test. We used primers for detecting conservative genes: *cad*F - for all types of *Campylobacter* genus, *hip*O – for *C. jejuni* and *asp* for *C. coli*. Analysis of the results of multiplex PCR assay, compared with those of the biochemical tests, confirmed the accuracy of the genetic method and the need for its application in diagnosing campylobacteriosis.

AIM

To develop, optimize and use multiplex PCR assay to identify and discriminate the species of bacteria - *C. jejuni* and *C. coli* - that are the most frequent causes of campylobacteriosis in human population.

MATERIALS AND METHODS

We examined 93 samples of clinical material taken from patients with diarrheal syndrome between January 2014 and January 2015.

1. Bacterial strains and culture conditions.

The strains were isolated by conventional techniques - the clinical samples were grown on blood agar plates (BulBio- NCIPD, LTD, Sofia, Bulgaria) using the membrane method with a nitrocellulose membrane filters (Sartorius Stedim Biotech GmbH 37070 Goettingen, Germany); they were cultured in microaerophilic atmosphere (5% O_2, 10% CO_2 and 85% N_2) at 42-43°C for 48-72 h. Forty out of 93 strains were isolated and these were biochemically differentiated – positive reaction to sodium hippurate hydrolysis and indoxyl acetate hydrolysis for *C. jejuni*, and positive reaction to indoxyl acetate hydrolysis for *C. coli*. The bacterial culture was collected and stored at -20°C for subsequent PCR-analysis.

2. DNA extraction.

The DNA of the isolated strains was extracted with Kit (QIAamp DNA Blood Mini Kit-QIAGEN, Germany) according to the manufacturer's instructions.

3. Multiplex PCR analysis.

Multiplex PCR assay was used to detect conservative genes: the *cad*F ($16S_p$DNA) gene, which is typical for *Campylobacter spp*, *hip*O – the hippuricase gene characteristic for *C. jejuni* and the *asp* - aspartokinase gene characteristic for *C. coli*. The primers we used and their sequences are shown in **Table 1**.[17,18] We also included two reference strains: 814 (*C. coli*) and ATCC 33560 (*C. jejuni*) as positive controls for the PCR analysis and ddH_2O as negative control. The multiplex PCR assay was optimized to function in a final reaction volume of 25 µl with these primers and under these conditions: 1x Taq DNA polymerase buffer; 4 mM $MgCl_2$; 0.2 mM dNTP; 0.03 U/µl Superhot Taq DNA polymerase (set Applichem GmbH, Germany); 0.6 µM *cad*F-F/R; 0.2 µM asp-F/R; 0.2 µM hipO-F/R. The assay was performed using IQ5TM Real Time PCR System (BIO RAD) under the following cycling conditions: initialization step - denaturation at 94°C for 5 min; then 35 cycles with denaturation at 94°C for 45

Table 1. Sequences of the used primers and their amplicon sizes

Gene	Sequence	Amplicon size (bp)	Source
*cad*F - F	**TTGAAGGTAATTTAGATATG**	400	Nayak et al., 2005 [17]
*cad*F- R	**CTAATACCTAAAGTTGAAAC**		
*hip*O– F	**GAAGAGGGTTTGGGTGGTG**	735	Linton et al., 1997 [18]
*hip*O– R	**AGCTAGCTTCGCARAATAACTTG**		
asp – F	**GGTATGATTTCTACAAAGCGAG**	500	Linton et al., 1997 [18]
asp - R	**ATAAAAGACTATCGTCGCGTG**		

seconds each, hybridization at 52°C for 45 second and elongation at 72°C for 60 seconds; the final step was elongation at 72°C for 2 min. The sizes of the produced amplicons were 735 bp, 500 bp, and 400 bp belonging to *Campylobacter spp.*, *C. coli* and *C. jejuni*, respectively.

The amplicons were visualized using capillary electrophoresis (QIAxcel, DNA QIAGEN sample Handbook, and Assay Technologies, 2008).

RESULTS AND DISCUSSION

We isolated 40/93 (43.01%) strains of *Campylobacter spp.* **(Fig. 1).** The phenotypic test identified 25 strains of *C. jejuni* - positive reaction to sodium hippurate hydrolysis **(Fig. 2)**; two strains of *C. coli* - positive reaction to indoxyl acetate hydrolysis **(Fig. 3)** and 13 strains of *Campylobacter spp*.

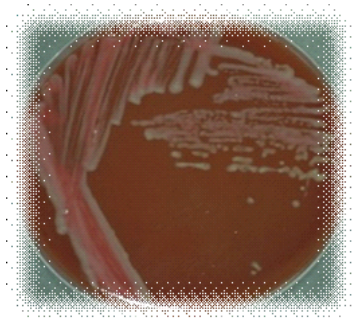


Figure 1. Campylobacter spp. grown on blood agar plates (BulBio- NCIPD,LTD, Sofia, Bulgaria).

Figure 2. Hydrolysis of sodium hippurate (positive reaction in blue).

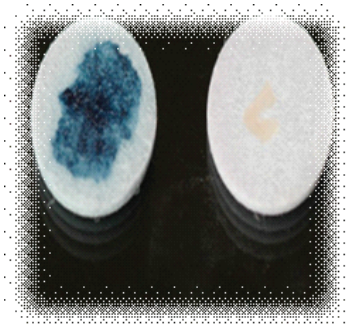
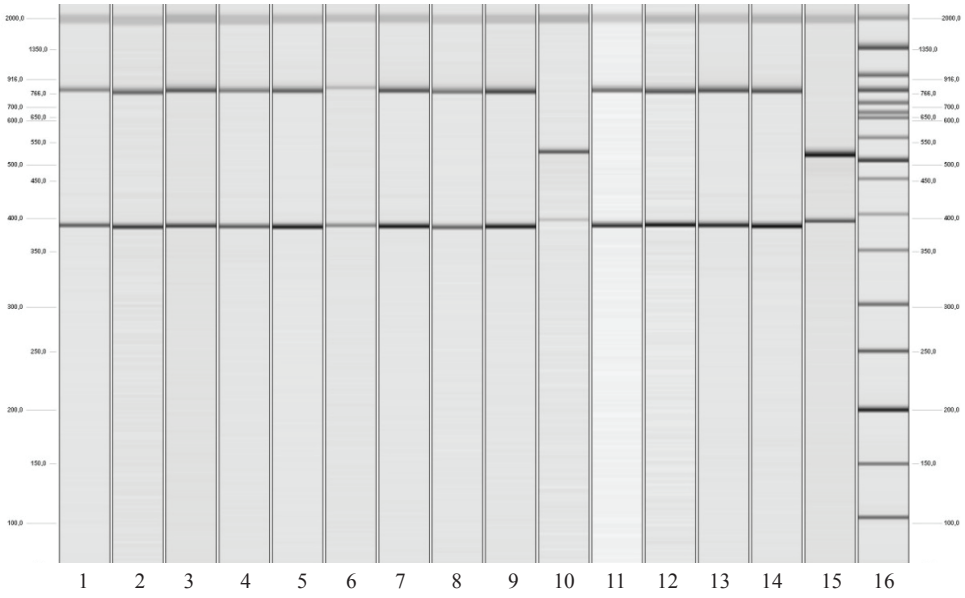


Figure 3. Hydrolysis of indoxyl acetate (positive reaction in blue).



Starts: 14 - *C. jejuni* (+ control); 15 - *C. coli* (+ control); 16 – molecular marker (- control);
Starts: 1-5, 7-9, 11-13 - *C. jejuni*; 10 - *C. coli*: culture grown clinical isolates;
Starts: 6 - *C. jejuni*, identified phenotypically as *C. coli*.

Figure 4. Results of the multiplex PCR assay.

Thirteen strains were randomly selected for genetic differentiation: 11 strains of *C. jejuni* and 2 strains of *C. coli.*

This assay provided a specific PCR product for each of the *C. jejuni* and *C. coli*, and each of the amplicons was sufficiently distinguished by the difference in size by capillary gel electrophoresis that confirmed 12 strains of *C. jejuni* and only one strain of *C. coli*. The second strain, which biochemically was identified as *C. coli*, was identified in the multiplex PCR assay as *C. jejuni* because of the presence of the gene hipO (start 6 in **Fig. 4**). *C. jejuni* has the gene hippuricase - hipO, but this gene might not be expressed, and the reaction in the phenotypic differentiation may be false positive.[19,20] The multiplex PCR assay was initially developed and optimized using two *Campylobacter* reference strains (*C. jejuni* ATCC 33560 and *C. coli* Denmark WHO 814). To evaluate the effectiveness of the multiplex PCR assay, all *Campylobacter* isolates previously identified by conventional biochemical-based identification were tested.

Although these biochemical-based assays successfully identified 12 of 13 isolates, 13 isolates required additional PCR assay to compensate for the low differential power of the biochemical-based assays. The results of the additional PCR assays and the biochemical-based ones are shown in Table 2. Sequencing analysis of 16S rRNA gene PCR products was performed on 12 *C. jejuni* isolates, and one *C. coli*. The genetic differentiation is the more reliable method **(Table 2)**.

Table 2. Results of multiplex PCR assay and biochemical identification of *Campylobacter spp*. isolates

Lab No	Sex	Age yrs	Hospital	Biochemical test Ind	Ac	Hip	A species identified biochemically	Result from PCR assay	A species identified by PCR assay
710	female	1	TH**	-		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
711	male	4	HIPD*	+		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
713	female	4	HIPD	-		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
714	female	2	HIPD	+		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
719	male	3	TH	+		-	*C. coli*	*cadF hipO*	*C. jejuni*
722	male	2	HIPD	+		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
734	male	2	HIPD	+		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
757	male	3	TH	+		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
829	male	1	TH	+		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
840	female	7	TH	-		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
845	male	2	TH	+		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
848	male	-	TH	+		+	*C. jejuni*	*cadF hipO*	*C. jejuni*
814(RS***)	-	-	Denmark WHO	+		-	*C. coli*	*cadF asp*	*C. coli*

* HIPD - Hospital for Infectious and Parasitic Diseases, Medical University of Sofia.
** TH – Tokuda Hospital, Sofia.
*** Reference strain.

Campylobacters are the most common bacterial agents that cause gastroenteritis worldwide.

The estimates of incidence in the developed and developing countries show that Campylobacter related cases are more than the Salmonella-caused infections and these are particularly common in children under the age of two occasionally having lethal outcome. The accurate identification of the infectious agent is of utmost importance. The species of clinical significance that are most frequently isolated are *C. jejuni* and *C. coli*.[11-14,21] They are usually discriminated on the basis of some phenotypic tests which are time-consuming and labor-intensive. Moreover, these tests are not always accurate because some strains of *C. jejuni* with hippuricase gene - *hip*O may not express this gene and the reaction to the sodium hippurate hydrolysis can yield false positive results when the strains are biochemically differentiated. The strain then may be inaccurately identified as *C. coli*.[19,20]

This method will be further optimized to specifically identify *C. jejuni* and *C. coli* directly from feces which will reduce the time and cost of the diagnostic process in Bulgaria where the high incidence of gastrointestinal infections is still an important clinical and socio-economic problem.

CONCLUSIONS

The results of our study suggest that the multiplex PCR assay gives accurate results in the identification process and differentiation of the species that are the most frequent causes of campylobacterioses induced by *C. jejuni* and *C. coli* which makes it useful in the clinical diagnostic process.

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Мультиплексный PCR-анализ в целях идентификации и дифференцирования изолятов Кампилобактер еюни (*Campylobacter jejuni*) и Кампилобактер коли (*Campylobacter coli*)

Мария Р. Павлова[1], Елина Г. Добрева[1], Катуша И. Иванова[1], Галина Д. Асева[1], Иван Н. Иванов[1], Петар К. Петров[1], Валери Р. Велев[2], Ивелина И. Томова[2], Майда М. Тихолова[2], Тодор В. Кантарджиев[1]

[1] Национальный центр инфекционных и паразитарных болезней (NCIPD), София, Болгария
[2] Больница инфекционных и паразитарных заболеваний (ГИПП), Медицинский университет София, Болгария

Переписка: Мария Павлова, Национальный центр инфекционных и паразитарных заболеваний (NCIPD), София, Болгария

Электронная почта: mimipavlova@mail.bg
Тел.:+359 898 639 929

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Введение: Campylobacter spp. являются важным возбудителем желудочно-кишечных инфекций у людей. Чаще всего изолированные штаммы данного вида бактерий включают Campylobacter jejuni и Campylobacter coli. На сегодняшний день в Болгарии не используются генетические методы идентификации бактерий. Мы осуществили оптимизацию мультиплексного PCR-анализа, направленного на идентификацию Campylobacter spp. и дифференцирование C. jejuni от C. coli в клинических изолятах. Кроме того, нами было проведено сравнение данного метода с используемыми обычно биохимическими методами.

Цель: Идентификация Campylobacter spp. и дифференцирование C. coli от C. jejuni в клинических изолятах с помощью мультиплексного ПЦР-анализа.

Материалы и методы: В период времени с февраля 2014 г. по январь 2015 г. нами было проведено исследование 93 проб клинического материала, взятого у пациентов с синдромом диареи, и выявлено 40 разновидностей Campylobacter spp. Клинический материал был инкубирован в микроаэрофильной атмосфере, при этом было осуществлено биохимическое дифференцирование изолированных штаммов (гидролиз гиппурата натрия на наличие C. jejuni и гидролиз индоксил ацетата на наличие C. coli). В штаммах было изолировано ДНК с помощью набора QiaAmp MiniKit (QIAGEN, Германия). С помощью мультиплексного ПЦР-анализа было протестировано двадцать штаммов на наличие следующих видов: cadF, характерный при наличии Campylobacter spp., hipO – при C. jejuni и asp – при C. coli.

Результаты и обсуждение: При проведении биохимических тестов было идентифицировано 16 штаммов C. jejuni, 3 штамма C. coli и 1 штамм C. upsaliensis. После проведения мультиплексного ПЦР-анализа капиллярного гель-электрофореза было подтверждено наличие 16 штаммов C. jejuni, 2 штаммов C. coli и 2 штаммов Campylobacter spp. – ввиду наличия гена cadF. C. jejuni содержит ген hipO и существует вероятность того, что данный ген не экспрессирует при биохимическом дифференцировании, в результате чего может быть вызвана негативная реакция. Для сравнения можно сделать заключение, что генетическое дифференцирование является более точным методом, чем биохимические тесты.

Заключение: Мультиплексный ПЦР-анализ представляет собой быстрый и точный метод идентификации Campylobacter spp., что делает его достаточно необходимым в клинической диагностической практике.