UPDATING THE ROUTINE SPECIATION OF ROMANIAN *CAMPYLOBACTER* STRAINS

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ABSTRACT

Introduction. Diarrhea caused by *Campylobacter* spp., especially *Campylobacter jejuni* and *Campylobacter coli*, is one of the most frequently reported gastrointestinal infection raising public health concern in many European countries.

Objectives. In order to improve the speciation protocol of *Campylobacter* strains for optimal disease surveillance, a PCR approach was introduced for species specific detection of *C. jejuni* and *C. coli* at reference laboratory level.

Methods. A collection of 70 *Campylobacter* strains previously identified at species level based on the hippurate hydrolysis biochemical test was used to test a three-gene multiplex-PCR-based method. Previously published primers were used to target the hippuricase gene (*hipO*) characteristic of *C. jejuni*, aspartokinase gene (*asp*) characteristic of *C. coli*, and a universal 16S rDNA gene sequence serving as an internal positive control.

Results. The phenotypic and genotypic methods were found to be 90% in concordance as six strains initially identified as *C. coli* by the biochemical tests proved to be *C. jejuni* in the PCR tests.

Conclusion. The established multiplex PCR approach can be used for routine diagnosis as well as epidemiological purposes.

Keywords: *Campylobacter jeuni*, *Campylobacter coli*, PCR identification

REZUMAT

Introducere. Diareea produsă de speciile *Campylobacter jejuni* si *Campylobacter coli* este printre cele mai frecvente infecţii gastrointestinale, care ridică probleme de sănătate publică în multe ţări europene.

Obiective. Pentru a îmbunătăţi identificarea speciilor de *Campylobacter*, necesară pentru supravegherea optimă a infecţiei, la nivelul laboratorului de referinţă a fost introdus un protocol de analiză bazat pe PCR.

Metode. O colecţie formată din 70 tulpini de *Campylobacter*, iniţial identificate la nivel de specie cu ajutorul testului de hidroliză a hipuratului, a fost utilizată pentru a pune la punct un protocol bazat pe reacţia PCR multiplex. S-au folosit primeri preluaţi din literatură, pentru amplificarea genei pentru hipuricază (*hipO*), caracteristică speciei *C. jejuni*, genei pentru aspartokinază (*asp*), specifică pentru *C. coli* şi genei pentru ARNr 16S, care a servit drept control intern.

Rezultate. Concordanţa dintre metoda fenotipică şi genotipică a fost de 90%, şase dintre tulpinile identificate biochimic drept *C.coli* dovedindu-se a fi, la testarea prin PCR, *C. jejuni*.

Concluzie. Protocolul bazat pe PCR multiplex poate fi aplicat atât în scop diagnostic, cât şi epidemiologic.

Cuvinte-cheie: *Campylobacter jejuni*, *Campylobacter coli*, identificare prin PCR

INTRODUCTION

The increasing body of literature dedicated to campylobacteriosis, the collective name of the infectious diseases caused by members of the bacterial genus *Campylobacter*, reflects the path of these pathogens from obscurity to notoriety in the field of public health [1]. Typically, the infections present as diarrhea sometimes bloody with cramps, fever and pain that cannot be distinguished from those

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caused by other enteric pathogens. Usually, the symptoms resolve without antimicrobial therapy but occasionally they can persist and result in hospitalization. Although rare, local or systemic complications can occur in some patients. The Guillain-Barre´ syndrome, an acute demyelinating disease of the peripheral nervous system, is the most severe clinical entity linked to a preceding *Campylobacter* infection [2].

Campylobacter jejuni and *Campylobacter coli* are the most frequently isolated Campylobacter species from human disease [3]. Of them, *C. jejuni* makes the greatest contribution, but it seems that the impact of other, less prevalent species has largely been ignored. The understanding of the epidemiology of *Campylobacter* infections relies on the systematic study of the different species and subtypes resulted from the routine characterization of clinical strains. The isolation and identification of *Campylobacter* species are not easy and always successful procedures. Both culture-dependent and culture-independent present flaws, so there is no single gold standard method for *Campylobacter* identification [4]. As in many clinical laboratories molecular typing tools are widely applied in the identification of bacterial strains, this approach is also used for the differentiation of *Campylobacter* at species and strain level.

This report describes the first results obtained by using a PCR-based protocol adopted in the laboratory in order to complement the time consuming and sometimes difficult to interpret biochemical test based on hippurate hydrolysis traditionally used for discriminating between *C. coli* and *C. jejuni*.

MATERIALS AND METHODS

Strains and growth conditions

This study focused on 70 *Campylobacter* strains, isolated from stool specimens. Five *Campylobacter* strains of know species, namely three *C. jejuni* and two *C. coli* were added as controls. All strains were grown on 7% (v/v) sheep blood Columbia agar (prepared in house), under microaerobic conditions generated by Campygen gas generating system (Oxoid) with CampyGen™ sachet (5% O_2, 10% CO_2, and 85% N_2), for 48 h at 42ºC. The template DNA for PCR was prepared from each culture using Pure Link Genomic DNA kit (Invitrogen), following the manufacturer's instruction.

Biochemical tests

Glucose fermentation, catalase, and nitrate reduction were tested by routine laboratory procedures [5]. Oxidase test and hippurate hydrolysis test were performed according to the manufacturers' instructions. Antimicrobial susceptibility testing was performed using a disk diffusion test for nalidixic acid and cephalotin. No zone of growth inhibition was defined as "resistant" while any zone of inhibition was defined as "susceptible".

Multiplex PCR

Bacterial DNA template was prepared using Pure Link Genomic DNA kit (Invitrogen) according to the manufacturers' instructions. Previously published primers targeting hippuricase (*hipO*) and putative aspartokinase (*asp*) genes were used for the molecular identification of the species *Campylobacter jejuni* and *coli*, combined with universal primers used to amplify a fragment of the 16S rDNA gene, serving as PCR internal positive control [6]. The assay was performed with a total volume of 25 µl containing 5 µl of template DNA and the following: 1x PCR buffer with 1.5 mM $MgCl_2$, 200 mM (each) dATP, dCTP, dGTP, and dTTP, 0.4 µM of *asp*-primers, 0.2 µM *hipO*-primers, 0.05 µM 16S rDNA primers, and 1.25 U of Taq DNA polymerase (Promega).

RESULTS AND DISCUSSION

All *Campylobacter* strains recovered from fecal cultures were first subjected to the phenotypic analysis which indicated 53 of them as reactive in the hippurate hydrolysis test and 17 hippuricase-negative.

For the routine diagnosis purpose, this biochemical test is of paramount importance for the differentiation of *C. jejuni* and *C. coli*, the former displaying hipurricase activity by contrast with the latter. However, reports have already showed that it may not be entirely

reliable due to the problems caused by either the strains with an atypical phenotype, such as hippuricase-negative variants of *C. jejuni* as well as the sensitivity limitations leading to inability to detect low-level producers of hippuricase or the influence of a low inoculum size [7, 8]. Therefore, as for other microorganisms, the use of PCR-based genotypic methods helps to overcome the problematic phenotypic identification. To date, the literature indicates that different molecular strategies and genetic targets relying on the PCR technique have been applied for the identification of Campylobacter to species level [9-13].

We decided to implement a multiplex PCR protocol which allowed the co-identification of *C. jejuni* and *C. coli* based on the detection of the *hipO* gene, characteristic of *C. jejuni*, and *asp* gene specific for *C. coli*.

The assay has been designed to also amplify the 16S rDNA gene in order to provide a more intense validation with respect to sensitivity [6]. To optimize the PCR protocol, we initially used five well-characterized *Campylobacter* strains received in the laboratory with the occasion of an External Quality Assessment (EQA).

All the strains originated from the EQA panel gave the same and expected results by both phenotypic and genotypic methods. Afterwards, when the *Campylobacter* clinical collection was tested, a 90% concordance was obtained between the methods used as six strains lacking the hippuricase activity and initially identify to *C. coli* species proved to be *hipO*-positive and *asp*-negative in PCR (Fig. 1).

The PCR assay identified more *C. jejuni* strains than the classical biochemical test by detecting those that most probably did not express hippurate hydrolysis activity *in vitro*.

Overall, the percentage of *C. jejuni* members among the human strains of campylobacter sampled in this study was 84% and even though the aim was not to provide epidemiological data, we could not help noticing that the prevalence of *C. jejuni* was greater compared to the one reported in a previous Romanian study [14].

This finding deserves further investigation to ascertain the trend of *C. jejuni* infections in locals.

The established multiplex PCR approach can be used for routine diagnosis as well as epidemiological purposes.

Conflict of interests: No conflict of interest to declare.

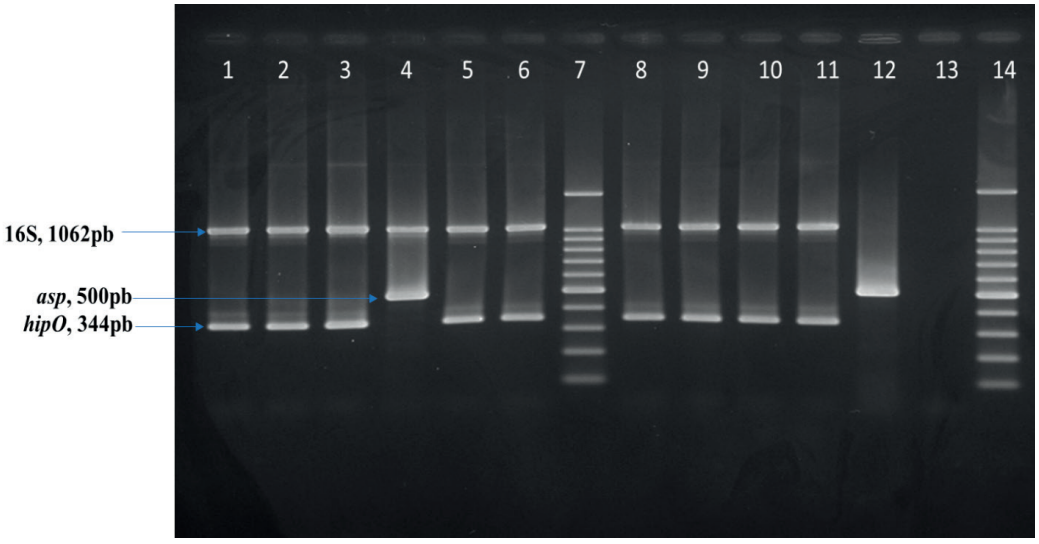

Fig. 1. Multiplex PCR on DNA extracted from Romanian strains of Campylobacter: lanes 1-3 and 5, 6, 8 -11, *Campylobacter jejuni* **strains; lanes 4 and 12,** *Campylobacter coli* **strains; lane 13, DNA negative control (H_2O); lane 7, 14, 100 bp DNA marker.**

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