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Species Differentiation of Thermotolerant *Campylobacters* Based on Distinctive Banding Patterns Obtained by Multiplex PCR

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Abstract

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The differentiation of thermotolerant *Campylobacter* spp. on the species level (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) was provided. Identification is based on different banding patterns obtained for individual species during simple multiplex PCR where regions within the 23S rRNA gene are amplified using newly designed specific forward primers.

Keywords: Campylobacteriosis; alimentary infection; 23S rRNA

Alimentary infections caused by various food-borne pathogens generally pose a threat to public health. Gram-negative thermotolerant bacteria of the genus *Campylobacter* (especially *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) are recognised as leading food-borne pathogens and cause acute enteritidis called campylobacteriosis. Since 2007, in the Czech Republic this disease has been significantly more frequent than the similarly well-known intestinal infection salmonellosis (NIPH 2013). This trend is the same as in all other developed countries around the world (EFSA 2013).

Significant reservoirs of thermotolerant *Campylobacters* are intestinal tracts of domestic warm-blooded animals farmed for meat (especially poultry, pigs, cattle and sheep) as well as intestinal tracts of wild warm-blooded animals. However, many other sources are also known (e.g. sewage, tap and environmental water, raw milk, pets, seafood, insects etc.). From all these sources its dissemination into a food chain or to an immediate proximity of human beings is possible and therefore the risk of infection becomes more real (Allos 2001; Šabatková *et al.* 2004; Moore *et al.* 2005; Humphrey *et al.* 2007).

Although unpleasant, typical symptoms such as fever, headache, acute diarrhoea, abdominal pain and muscle weakness do not generally require any treatment and are self-limiting. An antibiotic treatment is usually indicated in the cases of a severe course of the disease, for pregnant women, HIV positive or immunodeficient patients (Ketley 1997; Wassenaar & Blaser 1999; Allos 2001; Butzler 2004; Moore *et al.* 2005). A serious problem associated with *Campylobacters* is a possibility of developing various post-infectious complications e.g. reactive arthritis, urticaria or erythema nodosum. But the most serious sequels are Guillain-Barré (GBS) and Miller-Fisher (MFS) syndromes which manifest themselves as acute polyneuropathies affecting the peripheral nervous system leading to an ascending paralysis (Mori *et al.* 2012; Hersalis Eldar & Chapman 2014). Especially the infection caused by *C. jejuni* is a common trigger of these diseases (Allos 2001; Butzler 2004; Godschalk *et al.* 2006; Humphrey *et al.* 2007; Drenthen *et al.* 2011).

Considering general reluctance of *Campylobacters* to grow under laboratory conditions and narrow spectrum of their biochemical activity it is very complicated to distinguish between individual species. Especially when some of the test results are difficult to properly interpret, which is for example the case of acquired *C. jejuni* and *C. coli* resistance to antibiotic

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nalidixic acid or inability of certain *C. jejuni* strains to utilise hippuric acid under laboratory conditions (Totten *et al.* 1987; Endtz *et al.* 1991; Rautelin *et al.* 1999; Allos 2001; Caner *et al.* 2008). For reliable risk assessment, proper incidence evaluation or swift sample analysis regarding individual thermotolerant *Campylobacter* species, a demand for simple and rapid method for their distinguishing is obvious. Nowadays there are more options which can be tested for this purpose. Molecular genetic methods, however, appear to be the most reliable and specific. It is common knowledge that PCR in various platforms is now a very well-established, time-saving, culture-independent and feasible technique for which the majority of laboratories are usually technically equipped.

Recently, the possibility of *Campylobacter* identification by MALDI-TOF MS (matrix-assisted laser desorption ionisation time-of-flight mass spectrometry) has been introduced as well. However, some of the main drawbacks linked with this technique have not yet been possible to overcome. The most important ones include for example the inability to analyse mixed samples, the fact that growth conditions (medium, incubation temperature, and time) can strongly influence further identification, limited number of *Campylobacter* strains included in the reference library (only 11 strains of *C. jejuni*, 4 strains of *C. coli*, 5 strains of *C. lari*, and 5 strains of *C. upsaliensis* in Biotyper 3.0 database; Bruker Daltonics, Bremen, Germany) or the cost (Kolínská *et al.* 2008; Bessede *et al.* 2011; Martiny *et al.* 2011, 2013).

This work proposing a protocol of multiplex PCR enabling to identify four thermotolerant *Campylobacters* (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) at once based on obtained banding patterns was triggered by a study dealing with an identical issue (Fermer & Engvall 1999). In the above-mentioned study, detection of four thermotolerant *Campylobacters* was ensured by specific amplification of 491 bp long part of the 23S rRNA gene and further species differentiation was accomplished by PCR product cleavage using two specific restriction endonucleases – AluI and Tsp509I, which resulted in a set of restriction fragments specific to each species. Although our research group completely followed the proposed protocol, we were not able to accomplish the second part of the analysis and restriction with the latter endonuclease (Tsp509I) failed every time. Neither various optimization steps nor inclusion of more *Campylobacter* strains changed the results. In order to keep the idea to distinguish individual species based on different sets of banding patterns, we utilised the set of primers previously designed for *Campylobacter* detection (genus level), which provided a PCR product of 491 bp and also designed four new forward primers which were complementary to the sequence within this PCR product and served for species identification (Table 1).

MATERIAL AND METHODS

In silico analyses. Designing and chemical characterisation (melting temperature, GC% etc.) of primers were performed using FastPCR molecular biology software (Kalendar *et al.* 2009). This software was used for PCR product and banding patterns prediction as well. The following sequences of 23S rRNA gene were used for analyses: GenBank numbers – *C. jejuni* X66616, X66765, X67767, and Z29326; *C. coli* X67764, X67770, and U09611.1; *C. lari* X67769, AB287303.2, and Y11764; *C. upsaliensis* X67763 and X67774. Additional comprehensive analysis for all newly designed primer specificity verification was conducted using Primer-BLAST tool at National Centre for Biotechnology Information (NCBI). As a database query "Genome (chromosome of all organ-

Table 1. List of primers used in a multiplex assay

Forward primer	Primary specificity	Sequence 5' → 3'	Amplicon size (bp)
F1	*C. jejuni*	TCTTCGGTATAAGGTGTGGTTAGC	186
F2B	*C. coli*	AAGTGGTTCGCTTCGTATTA	173
F3	*C. lari*	AAGGACGCTTAGGGCTAAGCAA	461
F4	*C. upsaliensis*	GTTACAACAAAGAGTCCCTCCCGA	129
THERM1*	thermotolerant *Campylobacter* spp.	TATTCCAATACCAACATTAGT	491
THERM4 (reverse)*	thermotolerant *Campylobacter* spp.	CTTCGCTAATGCTAACCC	–

*Fermer and Engvall (1999)

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Table 2. Banding patterns for different *Campylobacter* species

Species	Amplicon sizes (bp)	
	genus identification	species-specific set
C. jejuni	491	129, 186
C. coli	491	129, 173, 186
C. lari	491	129, 173, 186, 461
C. upsaliensis	491	129

isms)" was selected and as an organism query was selected "bacteria (taxid: 2)".

Bacterial strains and culture conditions. *Campylobacter* strains used for experimental protocol optimisation were obtained both from the Czech Collection of Microorganisms in Brno, Czech Republic (*C. jejuni* CCM 6212, *C. coli* CCM 6211, *C. lari* CCM 4897) and the American Type Culture Collection in Virginia, USA (*C. upsaliensis* ATCC 43954). Fourteen human clinical isolates collected in the Czech Republic (Thomayer Teaching Hospital, Prague, Czech Republic) were included as well. *Campylobacters* were cultured in Park and Sanders enrichment broth (HiMedia, Mumbai, India) for 24–48 h at 42°C under microaerobic atmosphere. All strains used in this study were also previously taxonomically determined and biochemically characterised in accordance with standardised microbiological methods for thermotolerant *Campylobacters* (ISO 10272-1:2006 – Microbiology of food and animal feeding stuffs – horizontal method for detection and enumeration of *Campylobacter* spp. Part 1: Detection methods).

Nucleic acid extraction. DNA was extracted by thermal lysis (protocol designed and adjusted in the investigator's laboratory). Briefly, from pure bacterial cultures DNA was extracted from the volume of 1 ml. Suspension was centrifuged for 10 min at 10 000 *g*. Supernatant was discarded and the pellet was resuspended in 1 ml of physiological saline and then centrifuged for the second time under the same conditions. Supernatant was discarded and the pellet was resuspended in 100 µl of nuclease-free water (Promega, Madison, USA). Lysis was performed at 95°C for 20 minutes. Cell lysate was immediately cooled on ice, shortly vortexed and centrifuged for 3 min at 10 000 *g*. The extracted DNA was present in the supernatant (if needed for further experiments, the DNA was stored at –20°C).

Experimental PCR assay. For detection on the genus level, previously published (Fermer & Engvall 1999) primers THERM1 and THERM4 were used. Sequences of newly designed oligonucleotide primers (Metabion, Martinsried, Germany) for individual species identification are listed in Table 1. The PCR mixture contained (total volume 25 µl): 1× Mg free buffer, 3 mM $MgCl_2$, 0.1 mM dNTPs (both Promega, Madison, USA), 0.25 µM of each primer, 0.6 U a*Taq* DNA polymerase (Promega, Madison, USA) and 2 µl of extracted DNA. Temperature cycling was performed on a DNA Engine Peltier Thermal Cycler (Bio-Rad, Munich, Germany) as follows: initial denaturation step at 94°C for 3 min followed by 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min for 35 cycles and final extension at 72°C for 5 minutes. PCR products were visualised by end point horizontal agarose-gel electrophoresis.

RESULTS AND DISCUSSION

The assay for identification and differentiation of four thermotolerant *Campylobacters* provided by Fermer and Engvall (1999) is based on PCR amplification of 491 bp long part of the 23S rRNA gene followed by its digestion with two specific restriction endonucleases AluI and Tsp509I. Cleavage with the former endonuclease gives a unique combination of fragments for *C. jejuni* and *C. lari*, however, certain *C. upsaliensis* strains may give two possible patterns where one of them can be the same as that for *C. coli*. In this case differentiation between *C. coli* and *C. upsaliensis* is achieved by digestion with the

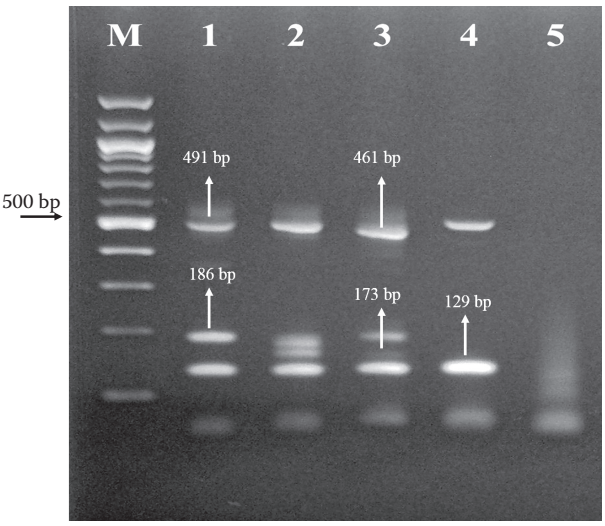

Figure 1. Banding patterns generated by multiplex PCR

Lanes: M – 100 bp DNA Ladder; 1 – *C. jejuni* CCM 6212; 2 – *C. coli* CCM 6211; 3 – *C. lari* CCM 4897; 4 – *C. upsaliensis* ATCC 43954; 5 – non-template control

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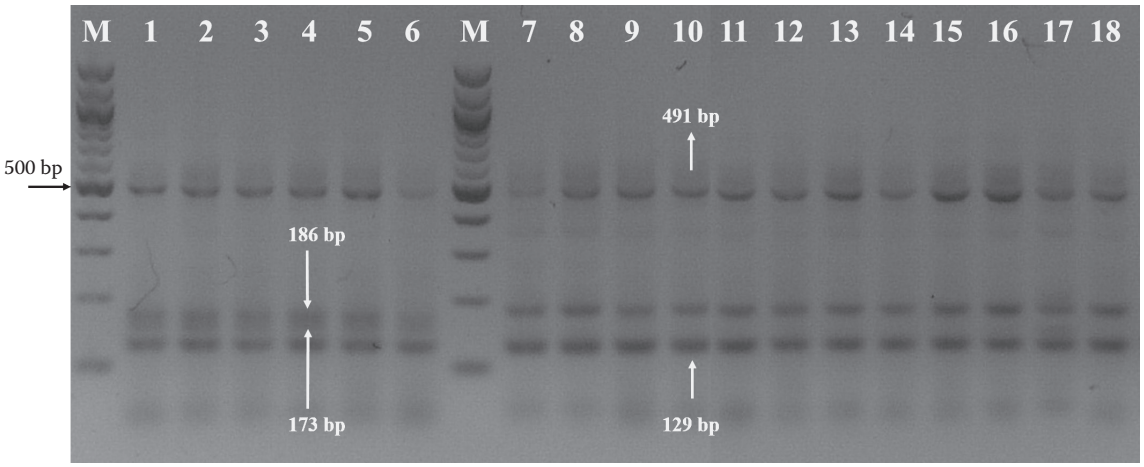

Figure 2. PCR identification of previously characterised clinical isolates

Lanes: M – 100 bp DNA; ladder: 1–6 – *C. coli*; 7–18 – *C. jejuni*)

latter endonuclease. When applied in our laboratory conditions, all strains were positively identified as thermotolerant *Campylobacter* spp. (amplification of 491 bp PCR product). Digestion with AluI occurred as expected, however digestion with the second enzyme failed in any case and after many protocol modifications expected fragments were obtained only in the case of *C. lari*.

In order to accomplish the original idea, when the species determination was based on different sets of obtained DNA fragments, we designed four new forward primers which can be used together in multiplex platform and which anneal within the sequence of the PCR product specific to thermotolerant *Campylobacters* (genus level). Such setting eventually provided different banding patterns (Table 2) for individual species like in the case of digestion with restriction endonucleases. Experimental optimization was performed with collection strains (Figure 1) and afterwards fourteen previously characterized clinical isolates (6 *C. coli* and 8 *C. jejuni* strains) were analysed as well (Figure 2).

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