Species level identification of thermotolerant campylobacters

I. Kolackova, R. Karpiskova

Centre for the Hygiene of Food Chains, National Institute of Public Health, Brno, Czech Republic

ABSTRACT: The aim of this study was to compare the phenotypic and genotypic based methods for species identification of thermotolerant campylobacters of human and food origin from the Czech Republic. Phenotypic methods are time-consuming and sometimes lead to intermediate results, therefore replacement by more specific and rapid methods are needed. Out of a total of 911 campylobacter strains tested, 800 human isolates were received from the clinical bacteriology laboratories from 5 regions and 111 foodstuff isolates (raw chicken and pork meat from retail market) originated from the routine examination in our laboratory. Based on the PCR method 85.1% of these strains were identified as *C. jejuni*, 12.5% as *C. coli* and 2.3% as mixed cultures of *C. jejuni* and *C. coli*. When species determination of campylobacters was based on conventional methods (hippurate hydrolysis test), 28.5% of the isolates were not identified correctly. The mixed cultures of campylobacters have not been detected without further subculturing of strains, which takes several days and enormously extends the identification process. The use of the PCR method showed to be a useful tool for species identification of *Campylobacter* spp.

Keywords: *C. jejuni*; *C. coli*; food products; biochemical tests; food borne disease; PCR

Thermotolerant species (namely *C. jejuni* and *C. coli*) are the second highest cause of food borne disease in the Czech Republic (EPIDAT, 2005). Similar epidemiological situation is also reported from other European countries and the public health burden of campylobacteriosis is increasing (Tauxe, 2001; Coker et al., 2002). Various risk factors for transmission of campylobacters have been identified, including raw food and products of animal origin such as raw milk, raw chicken, pork and beef meat, seafood or inadequately treated potable water (Butzler and Oosterom, 1991; Skirrow, 1991; Kapperud et al., 1992, 2003; Shane, 2000). Most developed countries report *C. jejuni* as predominant species, but in some less developed countries *C. coli* accounts for up to 50% of human cases (Taylor, 1992; Skirrow, 1994; Rautelin and Hanninen, 2000; DeWit et al., 2001; Gillespie et al., 2002; Siemer et al., 2005).

The accurate identification of campylobacter isolates provides important data for surveillance and risk assessment studies on which intervention strategies can be based (On and Jordan, 2003).

Conventional procedures to identify thermotolerant campylobacters are based on selective plating and biochemical identification, but campylobacters are slow growing, fastidious organisms and are considered biochemically inert (Lai-King et al., 1997). These characteristics show the limited potential of conventional procedures. Recently, methods based on PCR have been developed for the detection and identification of *Campylobacter* spp. in food, clinical and environmental samples (Oyofo et al., 1992; Denis et al., 1999; Engberg et al., 2000). The differentiation of the two species most implicated in food poisoning (*C. jejuni* and *C. coli)* is usually based on the hippuric acid hydrolysis. Inadequate buffering of the reaction mixture or low inoculum size can

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lead to false negative results (On and Holmes, 1991; On, 1996; Gorkiewicz et al., 2003).

The aim of this study was to compare the phenotypic and genotypic based methods for species level identification of thermotolerant campylobacters of human and food origin from the Czech Republic.

MATERIAL AND METHODS

The *Campylobacter* spp. strains were isolated from food products (chicken and pork meat) using the ISO 10 272 guideline. Samples were homogenised and directly plated on Charcoal Cefoperazone Desoxycholate Agar (CCDA), (Oxoid, Basingstoke, UK) and cultivated in microaerophilic condition using Gas generating kit Campylobacter system (Oxoid, Basingstoke, UK) at 42°C for 48 hours. Human isolates were received on cotton swabs in transport Amies medium (Meus, Piove di Sacco, Italy) from the clinical bacteriology laboratories from 5 regions of the Czech Republic. Suspect colonies from CCDA were confirmed by biochemical tests according to ISO 10 272 guidelines (Gram staining, catalase, oxidase and hippuric acid hydrolysis).

Reference strains CCM 6214 *C. jejuni* subps. *jejuni*, CCM 6211 *C. coli*, purchased from the Czech Collection of Microorganisms (Brno, Czech Republic), were used as positive controls.

DNA isolation method

Target DNA was isolated according to Engberg et al. (2000) using Chelex 100 (Bio-Rad, Hercules, USA) and multiplex PCR was used using three pairs of primers, one species-specific for *C. jejuni*, one for *C. coli* and that specific for the thermotolerant members of campylobacters also served as an internal control for PCR (Table 1, Figure 1). PCR was performed in 25 µl volumes. The PCR mixture contained 1 µl of template DNA, 10mM Tris-HCl, 50mM KCl, 2.5mM $MgCl_2$, 200µM dATP, dCTP, dGTP, dTTP, 1.25 U *Taq* purple DNA polymerase (Top-Bio, Prague, Czech Republic), 1µM of each oligonucleotide (Generi Biotech, Hradec Kralove, Czech Republic). The PCR was performed on a PTC-200 thermocycler (MJ Research, Watertown, USA) with the following programme: initial denaturation 5 min 95°C, 2 × (1 min 94°C, 1 min 64°C, 1 min 72°C), 2 × (1 min 94°C, 1 min 62°C, 1 min 72°C), 2 × (1 min 94°C, 1 min 60°C, 1 min 72°C), 2 × (1 min 94°C, 1 min 58°C, 1 min 72°C), 2 × (1 min

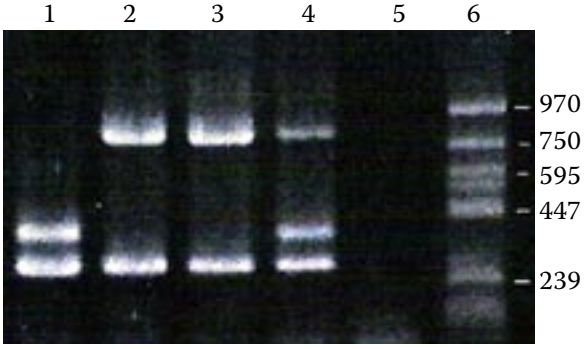

Figure 1. Results of optimised PCR protocol detecting *C. jejuni* and *C. coli* including genus specific product (internal control)

1 – CCM 6213 *C. coli* (364 bp, 287 bp)
2 – CCM 6214 *C. jejuni* (773 bp, 287 bp)
3 – sample 1 *C. jejuni*
4 – sample 2 mixed culture of *C. jejuni* and *C. coli*
5 – negative control
6 – molecular weight standard (155–970 bp, Top-Bio, Prague, Czech Republic)

Table 1. PCR primers used in this study

Target species	Target gene (amplicon size in bp)	Primer sequence	References
C. coli	Random (364)	5′ AGG CAA GGG AGC CTT TAA TC 3′ 5′ TAT CCC TAT CTA CAA ATT CGC 3′	Vandamme et al., 1997
C. jejuni	Random (773)	5′ CAT CTT CCC TAG TCA AGC CT 3′ 5′ AAG ATA TGG CAC TAG CAA GAC 3′	Vandamme et al., 1997
Campylobacter spp.	16S *rRNA* (287)	5′ CTG CTT AAC ACA AGT TGA GTA GG 3′ 5′ TTC TGA CGG TAC CTA AGG AA 3′	Lubeck et al., 2003

Table 2. Discrimination of campylobacters by PCR and hippurate hydrolysis

Species	No. of strains identified by PCR (%)	Hipurate positive reaction (%)
Human isolates		
C. jejuni	723 (90.4)	717 (99.2)
C. coli	62 (7.7)	14 (23.3)
C. jejuni and *C. coli*	14 (1.8)	12 (85.7)
C. lari	1 (0.1)	0
Foodstufss isolates		
C. jejuni	52 (46.8)	48 (92.3)
C. coli	52 (46.8)	9 (18.0)
C. jejuni and *C. coli*	7 (6.4)	5 (71.4)

94°C, 1 min 56°C, 1 min 72°C), 30 × (1 min 94°C, 1 min 54°C, 1 min 72°C), final extension step 10 min 72°C (Vandamme et al., 1997). The PCR products were visualized by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (1 µg/ml) and viewed under UV light.

RESULTS

In total 911 campylobacter strains were tested (Table 2), 800 of human and 111 of food origin. Based on the PCR method 775 strains were identified as *C. jejuni* (85.1%), 114 strains were identified as *C. coli* (12.5%) and 21 strains (2.3%) were mixed cultures of *C. jejuni* and *C. coli* (both the species-specific fragments were detected simultaneously). One strain was not identified on the species level by this PCR, but the target gene for *Campylobacter* genus was detected and using another set of primers it was identified as *C. lari* (not published).

If discrimination of campylobacters was based on the traditionally used hippurate hydrolysis test, 31.3% of human isolates and 25.7% of food isolates were mis-identified. Also, the mixed cultures of campylobacters will not be observed.

DISCUSSION

Species determination of campylobacters by conventional methods is problematic (Fermer and Engvall, 1999). For their accurate identification it is needed to provide up to 67 phenotypic tests (Gorkiewicz et al., 2003). The standardization of such tests is also problematic, in that the results are affected by different parameters, for example by the quantity of inoculum (On and Holmes, 1991) or the growth phase. The use of conventional identification tests is limited by the occurrence of atypical reactive strains (On, 1996). It is possible to find examples of non-specific reactions of hippurate hydrolysis test (Harmon et al., 1997; Steinbrueckner et al., 1999) or about catalase negative strains of *C. jejuni* (Owen et al., 1990).

The principle of hippurate hydrolysis test consists of cleaving N-benzoylglycine (hippuric acid) into glycine and benzoic acid catalyzed by N-benzoylglycine amidohydrolase (hippuricase). Glycine formation is detected by using a ninhydrin-based reagent system with creating of a violet stain. The hippuricase gene (*hipO*) is specific for *C. jejuni* and it was not detected in any other species (Hani and Chan, 1995; Burnett et al., 2002; Kulkarni et al., 2002). The deviation of the assay in hippurate negative *C. jejuni* is caused either by a defect in this gene, the nature of which has not yet been established (Hani and Chan, 1995) or this gene is present but it is not transcribed. Positive reaction by *C. coli* strains is probably a consequence of the acting of other amino acids or peptides which are transported from the culture media or produced during the incubation (Denis et al., 1999).

Comparing the results of both identification methods, PCR was determined to be more specific and rapid than biochemical tests, and did not show any intermediate results. The mixture of *C. jejuni* and *C. coli* strains was revealed by the PCR method. To differentiate mixed cultures using the conventional assays is possible only during further sub-

culturing of strains, which takes several days and enormously extends the identification process.

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Corresponding Author

Dr. Ivana Kolackova, National Institut of Public Health, Centre for the Hygiene of Food Chains, Palackeho 3a, 612 42 Brno, Czech Republic
Tel. +402 549 256 649, e-mail: kolackova@chpr.szu.cz