Optimisation of the PCR Method for the Detection of *Campylobacter jejuni* and *Campylobacter coli* in Samples of Ready-to-Eat Chicken Meals

Zdeňka Šabatková¹,², Kateřina Demnerová¹ and Jarmila Pazlarová¹

¹Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology, Institute of Chemical technology in Prague, Prague, Czech Republic; ²Department of Biomagnetic Techniques, Institute of Systems Biology and Ecology, Academy of Science of the Czech Republic, České Budějovice, Czech Republic

Abstract


This work compared the use of polymerase chain reaction (PCR) and the conventional CSN/ISO/10272 culture-based methods in the detection of *Campylobacter* species in ready-to-eat meals made from chicken meat. PCR was carried out with the primers specific to *C. jejuni*, *C. coli*, *C. lari*, and was modified with an internal control. The detection of campylobacters by PCR was performed on both untreated and spiked samples of real food purchased in local stores.

For PCR, the detection limit was 2 CFU/g after 48 h enrichment in Park and Sanders broth. Duplex PCR proved to be highly reliable in the detection of campylobacters in different food types. Without extra spiking, samples from a global fast food chain exhibited positive amplification of the PCR product while but negative results were obtained from the cultivation of the same samples.

Keywords: polymerase chain reaction; internal control; *Campylobacter* spp.

In the last few decades, new food-borne pathogens have been identified. *Campylobacter*, a food-borne bacteria, is one of the leading causes of diarrhea illness throughout the world (Friedman et al. 2000). The genus *Campylobacter* comprises 16 closely related species and 6 sub-species of gram-negative bacteria, all of which are capable of colonising the gastrointestinal tracts of a wide variety of host species (Volokhov et al. 2003). Epidemiological data show that the most significant of these *Campylobacter* pathogen species are the thermotolerant *C. jejuni* and *C. coli* (Mead et al. 1999). As these bacteria are currently part of the microflora in farmed animals (poultry, pigs, cattle), contaminated foods and water appear to be the most common vehicles of transmission to humans. However, *Campylobacter* is also hosted in wild birds (Glunder et al. 1992). The conventional methods for the detection and differentiation of *Campylobacter* species are supported by the Institute of Systems Biology and Ecology AS CR, Research Plan AV0Z60870520, and by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 6046137305.
tedious and time consuming, usually taking five days to produce a negative result and up to seven days to confirm a positive result. In recent years, numerous molecular diagnostic approaches for the detection and identification of Campylobacter spp. have been developed, including various PCR-based assays (Chuma et al. 1997; Fermer & Engvall 1999; Lübeck et al. 2003a; Sabatková et al. 2004). PCR methods have several advantages because they are faster and more sensitive and specific than the cultivation-based procedures. However, thus far few PCR-based studies have aimed at differentiating between species.

Our goal was to develop a PCR-based rapid screening method for the detection of campylobacters in ready-to-eat foods. The method consisted of two steps: (i) elimination of false negatives obtained in the detection of thermotolerant species of Campylobacter spp.; (ii) species identification.

MATERIALS AND METHODS

Bacterial strains. Campylobacter jejuni subsp. jejuni CCM 6212 and Campylobacter coli CCM 6211 (Czech Collection of Microorganisms, Masaryk University Brno, Czech Republic), were used for testing PCR detection limits and for the spiking of food samples.

Food samples. Four samples of ready-to-eat meals (chicken pieces in jelly; chicken sausage with cheese; chicken baguette; fried chicken pieces) were purchased from local stores.

Cultivation and enumeration of bacteria. The Campylobacter strains were grown either on Karmali agar (Hi-media, Mumbai, India) or in Park and Sanders broth (Hi-media, Mumbai, India) to which sheep blood was added. They were incubated in a microaerophilic atmosphere at 42°C for 24–48 hours. For the cell enumeration, cell suspensions were serially diluted 1:10 in 0.85% NaCl solution. For each dilution, the cell number (CFU/ml) was determined by plating on Karmali agar.

Preparation of food samples. 25-g portions of each food matrix were homogenised at 1:10 (225 ml) with Park and Sanders broth in a stomacher for 1 min to macerate them. To contaminate the samples, the first parts of each food mixture were spiked with approximately 10³ CFU of C. jejuni or C. coli per g of food. The second parts of the mixtures were spiked with approximately 10⁵ of C. jejuni or C. coli per g of food. The third parts of the mixtures were incubated unspiked as controls. All mixtures were incubated in a microaerophilic atmosphere at 37°C for 4 h, and then at 42°C for further 48 hours. After 24 h of enrichment, 1 ml aliquots of each mixture were extracted for PCR analysis only. After 48 h, 1 ml aliquots of each mixture were extracted for both PCR and standard microbiological analyses (CSN ISO 10272). Following the enrichment, the standard microbiological approach (CSN ISO 10272) was followed to determine whether or not the spiked and unspiked samples contained Campylobacter spp. All 1-ml portions (24 h, 48 h) were centrifuged at 10 000 g for 5 minutes. The resulting pellets were kept at −20°C for later DNA analysis; DNA was extracted from the food samples by the use of three rapid methods: (i) extraction by boiling, (ii) extraction by treatment with proteinase K, (iii) resin based extraction, details in Sabatková et al. (2004). Prior to the use of each extraction method, the sample pellet was allowed to thaw at 4°C, washed with 0.1M Tris buffer (pH 8) and centrifuged at 7000 rpm for 5 min.

Preparation of PCR internal control. With some modifications, the PCR internal control (IC) was prepared according to the procedure outlined by Sachadyn and Kur (1998). The following sequences were used in the preparation of the internal control: for the forward primer – 5-CTG CTT AAC ACA AGT GTA GCT TTT GA-3'; for the reverse primer – 5-TTC CTT AGG TAC CGT CAG AAA AGA TCA-3'. The PCR reaction was carried out in 50 μl mixtures containing the following reagents: reaction buffer (Invitrogen, Carlsbad, USA); 2.5mM MgCl₂ (Invitrogen, Carlsbad, USA); 0.2mM of each nucleotide (Promega, USA); 0.4mM of each primer (Generi Biotech, Hradec Králové, Czech Republic); 100 pg/μl of pUC19 (Fermentas, Burlington, Canada); and 0.7 unit of thermostable Platinum Taq DNA polymerase (Invitrogen, Carlsbad, USA). The amplification was carried out for 40 cycles, each of which consisted of the following temperature program: 95°C for 15 s; 48°C for 15 s; 72°C for 30 seconds. The resulting product of the internal control was purified by a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The concentration of the purified product was cleaned using a Quant-it™ PicoGreen® ds DNA Assay Kit (Molecular Probes-Invitrogen, Carlsbad, USA) and then measured by
a LightCycler® 2.0 Instrument (F. Hoffmann-La Roche Ltd., Basel, Switzerland).

**PCR with internal control.** To identify the thermodurable species of Campylobacter spp. present in the food samples (24 h, 48 h), PCR was carried out following the procedure described by Sabatková et al. (2004), with some modifications to the primer concentrations and with the addition of the internal control. The primers used were specific to C. jejuni, C. coli, and C. lari. The primer concentrations selected were 0.88µM for the forward primer and 0.96µM for the reverse primer. The concentration of the internal control was selected to be 0.05 pg of IC per reaction. The determination of PCR sensitivity was performed using DNA extracted by three rapid methods (Sabatková et al. 2004) from the serial dilutions (in the range of 10⁰ to 10⁵ CFU/ml) of the strain C. jejuni CCM 6212. The presence of the thermodurable species of Campylobacter spp. (Figure 1) is demonstrated by 287 bp product, and the presence of the internal control is demonstrated by 147 bp product on agarose gel (Bio-Rad, New Orleans, USA) stained with ethidium bromide (Fluka, Buchs, Switzerland).

**Duplex PCR.** Duplex PCR was used to differentiate between the thermodurable species, C. jejuni, C. coli.

It has been shown that the primers based on the sequence for putative oxidoreductase enable the specific detection of C. jejuni (Winters & Slavík 1995). In terms of the specific detection of C. coli, Linton et al. (1997) have described the use of primers containing the 3 end of the putative aspartokinase gene and a downstream short open reading frame (ORF) encoding a gene of unknown function. Optimal PCR conditions were established by testing various parameters, including: different annealing temperatures; different DNA polymerases (in different concentrations); and different concentrations of MgCl₂. A final reaction volume of 25 μl was created by the addition of the following components: 2.5 μl of sample; 0.4μM of each primer (Generi Biotech, Hradec Králové, Czech Republic); 2mM MgCl₂ (Invitrogen, Carlsbad, USA); 0.2mM of each deoxynucleotide (Promega, Madison, USA); and 0.65U of Platinum Taq DNA polymerase (Invitrogen, USA). The amplification was initiated with DNA denaturation at 95°C for 3 min, followed by a 40-cycle reaction (95°C for 1 min; 57°C for 1 min; 72°C for 1 min), and extension at 72°C for 3 min. Amplicons were detected in 1% (w/v) agarose gel electrophoresis stained by ethidium bromide. The presence of C. jejuni was demonstrated by 159 bp product and the presence of C. coli by 500 bp product (Figure 2). For the determination of sensitivity duplex PCR were carried out with all the described DNA extractions from the serial dilutions of the strain C. jejuni subsp. jejuni CCM 6212 and E. coli CCM 6211 ranging from 10⁰ to 10⁴ CFU/ml.

**RESULTS AND DISCUSSION**

**Detection of campylobacters using PCR with internal control (IC)**

It is necessary to select an such IC concentration that is able to produce a visible band on agarose gel while, at the same time, not reducing the intensity of the target product. On the basis of IC titration, 0.05 pg of IC per reaction was chosen for the detection of Campylobacter (data not shown). Because the original primer concentrations (0.44µM and 0.48µM) produced only weak bands of PCR products in the presence of the internal control, it was necessary to double them. After this modification, the detection limit for the DNA extracted from the suspension of the pure CCM 6212 strain was found to be the same as the detection limit previously found by Sabatková et al. (2004) using PCR. The detection limit ranged from 10⁰–10¹ CFU/ml for DNA extracted with proteinase K (Figure 1).
differentiation between C. jejuni and C. coli using duplex PCR

The sensitivity of the use of duplex PCR with pure cultures was determined using the minimum number of Campylobacter cells that could be amplified. The results of the optimised procedure are shown in Figure 2; the detection limit of C. jejuni and C. coli being $10^2$ CFU/ml for DNA extracted with proteinase K, resin-based extraction (data not shown), and $10^3$ CFU/ml (data not shown) when extracted with boiling lyses.

Examination of spiked food samples

All Campylobacter detections were qualitative. The results summarised in Table 1 were obtained by screening PCR from four independent spiked food matrices. The occurrence of Campylobacter spp. in all spiked food samples was proven using the standard microbiological method of plating on Karmali agar. The detection limit prescribed by the cultivation method, of 1 cell per 25 g of food (0.04 CFU/g) for either 24 h or 48 h enrichment, was not achieved in our experiments. The best detection limit achieved was 2 CFU/g after 48 h enrichment (Table 1), which was obtained for DNA extracted by the resin-based method.

The poorest results were obtained with DNA extracted by boiling. With respect to this finding, Mohran et al. (1998) suggested that within Campylobacter populations is a subset that does not release PCR-detectable DNA upon boiling in water, which could explain why no amplicons were obtained for the two food samples extracted by boiling (Table 1). We suggest that the negative results obtained using duplex PCR were caused by the lower sensitivity of the method, as well as by the complex nature of the food matrix after only 24 h enrichment.

Table 2. Results of optimised Duplex PCR protocol detecting C. jejuni and C. coli

![Image of an agar plate with bands of DNA]
mixed results (Table 2). With the exception of the fried chicken pieces, negative PCR results were obtained with all food samples, and were confirmed by the cultivation method used. In the case of the positive PCR results obtained with the fried chicken pieces, no variance was found between the extraction methods used, and this positive finding was not confirmed by the cultivation method used. This suggests that, while the bacterial cells were destroyed during the food preparation process, a sufficient amount of DNA remained that could be amplified by PCR.

The main limitation of PCR methods in the examination of food samples is the frequent presence of inhibiting compounds that can interfere with the amplification reaction and, consequently, result in either a negative or a false negative analyses. An internal control (IC) should be used in PCR procedures to prevent false negative results, particularly when food samples are to be examined. As evident
from previous studies, the IC can be developed in several different ways (Sachadyn & Kur 1998; Cubero et al. 2002; Lübeck et al. 2003b). The advantage of the IC used in this study is its simplicity, accessibility, and universality. As the amplification of one product may influence that of another, and as the band intensity depends on the amounts of the target DNA and control DNA, it is necessary to find the appropriate ratio of IC DNA to target DNA experimentally. To obtain reliable results, it is necessary to store the IC in a highly concentrated form, because when stored at low concentrations, it may be degraded and lead to irreproducible results (Sachadyn & Kur 1998).

PCR inhibition can be partially overcome by the use of a suitable DNA extraction protocol (Cubero et al. 1999). The rapid extraction methods used in this study are cheap, fast, and undemanding, but their capacity to remove inhibitors is not efficient enough for all types of food matrix. This is why it is important to use an IC in the application of PCR methods.

Our detection limit (2 CFU/g after 48h enrichment) did not reach the level of the ISO norm (0.04 CFU/g). After 24h enrichment, our detection limit was comparable with the limit obtained by Magistrado et al. (2001), who, after 17 h enrichment of chicken rinse, detected 31.7 CFU/g. As our detection limit was determined in highly complex matrices, such as chicken sausage with cheese, we can assume that the use of a simpler matrix, in which inhibiting compounds are not present, would enable us to achieve a detection limit similar to the ISO norm. We plan to investigate this in a future study.

**References**


CSN ISO 10272 Microbiology of food and animal feeding stuffs – Horizontal method for detection of thermo-tolerant *Campylobacter*.


Fermer C., Engvall E.O. (1999): Specific PCR identification and differentiation of the thermophilic campylobacters, *Campylobacter jejuni*, *C. coli*, *C. lari* and

### Table 2. Results obtained by the use of PCR on unspiked ready-to-eat chicken meal samples (both enriched and nonenriched) extracted by three different methods

<table>
<thead>
<tr>
<th>Food sample</th>
<th>Extraction method</th>
<th>PCR results – enrichment</th>
<th>0 h (without)</th>
<th>48 h (with)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PCR – IC</td>
<td>Duplex PCR</td>
</tr>
<tr>
<td>Chicken pieces in jelly</td>
<td>with proteinase K</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<tr>
<td></td>
<td>resin-based</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>by boiling</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<tr>
<td>Chicken sausage with cheese</td>
<td>with proteinase K</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<tr>
<td></td>
<td>resin-based</td>
<td>–</td>
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<td>ND</td>
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<tr>
<td></td>
<td>by boiling</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<tr>
<td>Chicken baguette</td>
<td>with proteinase K</td>
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<td>ND</td>
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<tr>
<td></td>
<td>resin-based</td>
<td>–</td>
<td>ND</td>
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<tr>
<td></td>
<td>by boiling</td>
<td>–</td>
<td>ND</td>
<td>–</td>
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<tr>
<td>Fried chicken pieces</td>
<td>with proteinase K</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td></td>
<td>resin-based</td>
<td>(+)</td>
<td>(+)</td>
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<tr>
<td></td>
<td>by boiling</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
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</table>

*C. jejuni*; + strong band, (+) weak band; – negative band, ND not done

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Corresponding author:
Prof. Ing. Kateřina Demnerová, CSc., Vysoká škola chemicko-technologická, Fakulta potravinářské a biochemické technologie, Ústav biochemie a mikrobiologie, Technická 3, 166 28 Praha 6, Česká republika
tel.: +420 220 443 025, fax: +420 224 355 167, e-mail: katerina.demnerova@vscht.cz