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

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Abstract

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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

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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; Sabatkova *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 the respective mixtures after homogenisation were spiked with approximately 10^1 CFU of *C. jejuni* or *C. coli* per g of food. The second parts of the mixtures were spiked with approximately 10^0 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 Sabatkova *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 TGA GTA GCT CTT 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 $MgCl_2$ (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 thermotolerant species of *Campylobacter* spp. present in the food samples (24 h, 48 h), PCR was carried out following the procedure described by Sabatkova *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 (Sabatkova *et al.* 2004) from the serial dilutions (in the range of 10^0 to 10^2 CFU/ml) of the strain *C. jejuni* CCM 6212. The presence of the thermotolerant 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).

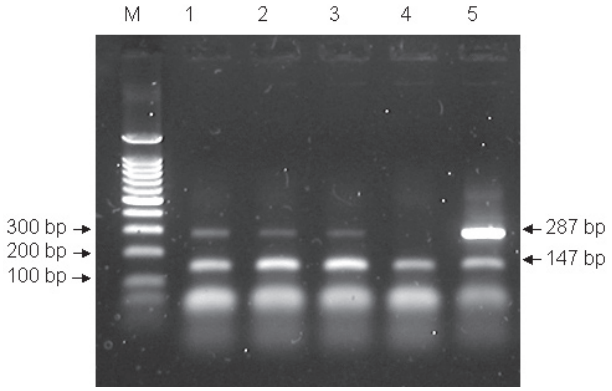
Duplex PCR. Duplex PCR was used to differentiate between the thermotolerant species, *C. jejuni*, *C. coli*.

It has been shown that the primers based on the sequence for putative oxidoreductase enable the specific detection of *C. jejuni* (Winters & Slavik 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 $MgCl_2$. 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 $MgCl_2$ (Invitrogen, Carlsbad, USA); 0.2mM of each deoxynucleotide (Promega, Madison, USA); the reaction buffer (Invitrogen, Carlsbad, 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 10^0 to 10^4 CFU/ml.

RESULTS AND DISCUSSION

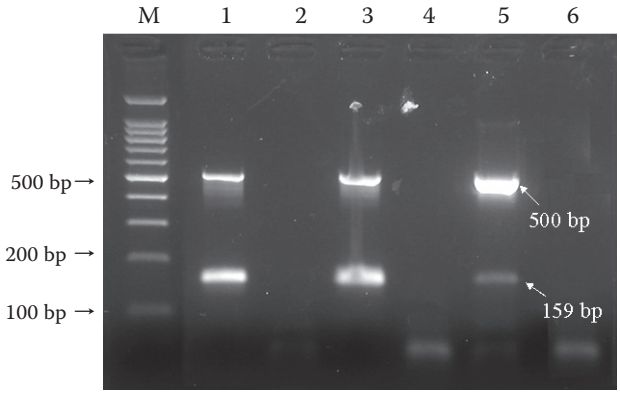
Detection of campylobacters using PCR with internal control (IC)

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 Sabatkova *et al.* (2004) using PCR. The detection limit ranged from 10^0–10^1 CFU/ml for DNA extracted with proteinase K (Figure 1)



Lane M – marker: 100 bp DNA ladder, lanes 1 to 5, DNA extracted by treatment with proteinase K from different amounts of *C. jejuni* in CFU/ml, 10^2 CFU/ml (lane 1), 10^1 CFU/ml (lane 2), 10^0 CFU/ml (lane 3), DNA free (lane 4), 10^5 CFU/ml (lane 5)

Figure 1. PCR products generated from the target sequences in *Campylobacter* spp. (287 bp) and internal control (0.05 pg/μl) (147 bp)



Lane M – marker: 100 bp DNA ladder, CCM 6211 *C. coli* (lane 1), CCM 6212 *C. jejuni* (lane 2), mixed culture of *C. jejuni* and *C. coli* (lane 3), DNA free (lane 4)

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

and resin-based extraction (data not shown). The detection limit for DNA extracted with boiling lyses, a less sophisticated method, was 10^2 CFU/ml (data not shown).

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.

(A) The first analysis was conducted after 24 h of the enrichment cultivation. At this stage, diverse results were obtained by the extraction methods used. For the spiked matrices of approximately 10^1 CFU/g, negative PCR results were obtained during the analyses of the two food matrices extracted by boiling (Table 1). However, using duplex PCR, *C. jejuni* and *C. coli* were detected in three of the four food matrices extracted by the resin-based method. After 24 h enrichment, we were able to detect at least 20 CFU/g in the chicken products, which is similar to the results published by Magistrado *et al*. (2001). For spiked food matrices of approximately 10^0 CFU/g, positive PCR results were obtained in the analyses of the two food matrices 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.

(B) The second analysis was conducted after 48 h cultivation. Positive PCR results, indicating the presence of the thermotolerant group of *Campylobacter* spp., were obtained for all spiked samples. *C. jejuni* and/or *C. coli*. were identified in all four samples using duplex PCR (Table 1). In the case of the spiked chicken pieces in jelly of approximately 10^1 CFU/g, duplex PCR was not carried out for the DNA extracted by the treatment with proteinase K because these species had previously been satisfactorily identifed after 24 h of enrichment. In the case of the spiked chicken sausage with cheese of approximately 10^0 CFU/g, positive PCR results were obtained only when using the extraction by the resin-based method. The species determined using duplex PCR on the DNA extracted from the food samples corresponded to the species used for spiking the samples.

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.

(C) In parallel to the analytical experiments described above (A, B), the control analysis was also conducted on the unspiked food samples with

Table 1. Results obtained by the use of PCR on spiked ready-to-eat chicken meal samples extracted by three different methods after enrichment

Food sample	Extraction method used	No. of *Campylobacter* cells added (CFU/g)	PCR results* – enrichment for			
			24 h		48 h	
			PCR IC	Duplex PCR	PCR IC	Duplex PCR
Chicken pieces in jelly	with proteinase K	50[b]	+	+[b]	+	ND
		5[b]	(+)	–	+	+[b]
	resin-based	50[b]	+	+[b]	+	+[b]
		5[b]	(+)	(+)[b]	+	+[b]
	by boiling	50[b]	+	+[b]	+	+[b]
		5[b]	–	–	+	+[b]
Chicken sausage with cheese	with proteinase K	20[a]	(+)	-	+	+[a]
		2[a]	–	–	-	-
	resin-based	20[a]	(+)	–	+	+[a]
		2[a]	–	–	(+)	(+)[a]
	by boiling	20[a]	–	–	+	+[a]
		2[a]	–	–	-	–
Chicken baguette	with proteinase K	40[a]	(+)	–	+	+[a]
		4[a]	-	–	+	+[a]
	resin-based	40[a]	(+)	(+)[a]	+	+[a]
		4[a]	–	–	+	+[a]
	by boiling	40[a]	–	–	+	+[a]
		4[a]	–	–	+	+[a]
Fied chicken pieces	with proteinase K	40[a]	+	(+)[a]	+	+[a]
		4[a]	+	(+)[a]	+	+[a]
	resin-based	40[a]	+	+[a]	+	+[a]
		4[a]	+	(+)[a]	+	+[a]
	by boiling	40[a]	+	(+)[a]	+	+[a]
		4[a]	+	(+)[a]	+	+[a]

[a]*C. jejuni*; [b]*C. coli*; + strong band, (+) weak band; – negative band, ND not done

*All PCR analyses were done in two independent series. As the results were identical, each column presents only one symbol.

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

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

Food sample	Extraction method	PCR results – enrichment			
		0 h (without)		48 h (with)	
		PCR – IC	Duplex PCR	PCR – IC	Duplex PCR
Chicken pieces in jelly	with proteinase K	–	ND	–	ND
	resin-based	–	ND	–	ND
	by boiling	–	ND	–	ND
Chicken sausage with cheese	with proteinase K	–	ND	–	ND
	resin-based	–	ND	–	ND
	by boiling	–	ND	–	ND
Chicken baguette	with proteinase K	–-	ND	–	ND
	resin-based	–	ND	–	ND
	by boiling	–	ND	-	ND
Fried chicken pieces	with proteinase K	(+)	(+)[a]	+	(+)[a]
	resin-based	+	(+)[a]	+	(+)[a]
	by boiling	(+)	(+)[a]	(+)	(+)[a]

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

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.

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