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UNIVERSITÀ DEGLI STUDI DI TORINO

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Abtract

Campylobacter-contaminated food products are currently the cause of the highest number of gastroenteritis cases in developed countries. Apart for biosafety measures at the primary production level, no other official control measures are currently in place for its control. This is partly due to the lack of quantitative data regarding the prevelance and contamination level of different food products by *Campylobacter* spp. that does not allow for quantitative risk assessment. PCR-based methods, applied without prior enrichment, in food samples circumvent limitations associated with the quantification of foodborne pathogens by traditional, culture-dependent methods. In this study, we report the development of a protocol, based on the amplification of the *rpoB* gene of *Campylobacter jejuni*, by quantitative PCR (qPCR), directly in food samples. The quantification limit of the protocol was determined to be in the order of 10 colony forming units (cfu)/g or ml of food sample. The optimized protocol was applied for the survey of *C. jejuni* in naturally contaminated poultry samples. In parallel, traditional sampling was also performed. A high percentage of samples (87%) resulted to be positive by qPCR, while no *C. jejuni* was detected by traditional analysis. Furthermore, important differences were observed in the detection by qPCR between samples before and after enrichment.

Keywords: *Campylobacter jejuni*, quantitative PCR, direct analysis, poultry

1. Introduction

Campylobacter spp. is currently the bacterial foodborne pathogen causing the highest number of gastrointestinal disease in developed countries, according to the World Health Organization (www.who.int). The main vehicle, responsible for human infections, is broiler meat, either through consumption of inadequately cooked chicken meat or through cross-contamination of ready to eat foods. *C. jejuni* is the most important species with the highest rate of isolation from human cases of gastroenteritis. In Europe in 2007, the proportion of positive poultry meat samples ranged from 4.3 % to 67.1 % and *C. jejuni* accounted for the majority of the isolates (Anonymous, 2009). A number of factors make it a problematic agent to control: (i) it is a zoonotic agent, that is asymptomatic in broilers (as well as other birds), (ii) available sampling and testing methods for its detection and quantification give variable results, thus surveillance data from different sources may not be comparable, (iii) compared to other foodborne pathogens, little is known regarding its physiology and its ability to survive common food processing conditions. Furthermore, the previous belief that thermophilic campylobacters are sensitive to conditions they encounter outside their host seems not to be supported by the epidemiological data (Humphrey et al., 2007).

The application of molecular methods to rapidly and inequivocally detect and identify foodborne pathogens in foodstuffs is offering a valid alternative to traditional microbiological testing. The challenge nowdays is the application of these methods, primarily based on amplification of a target DNA (or RNA) sequence by PCR, without previous enrichment or culturing, in order to quantify foodborne pathogens directly in foods. Such an approach has already been employed for other pathogens like *Listeria monocytogenes* (Rantsiou et al., 2008). Methods to detect and quantify *C. jejuni* in foods until recently have been applied after an enrichment step (Josefsen et

al., 2004; Nogva et al., 2000; Oliveira et al., 2005; Sails et al., 2003; Yang et al., 2003), while direct quantification of *C. jejuni* in chicken rinses has been performed (Debretsion et al., 2007).

In this paper we focused on the development of a protocol for the detection and quantification of *C. jejuni* in foods. The protocol is based on the amplification, by quantitative PCR, of the *rpoB* gene, encoding the β-subunit of the RNA polymerase of *C. jejuni*. The ultimate goal was to apply the protocol to naturally contaminated poultry samples obtained from the retail market in Italy.

2. Materials and methods

2.1. Strains and media.

Strains of *C. jejuni* belonging to the Laboratory of Microbiology of the University of Ghent, Belgium (strain LMG8842) and to the Collection of Institute Pasteur, France (strain CIP70.2T), as well as 3 strains, with codes 119, 592 and 221/05, isolated from bovine feaces the first and poultry the latter two, were employed in this study. In addition, *Campylobacter coli* DSMZ 4689, *Campylobacter upsaliensis* DSMZ 5365 and *Campylobacter lari* DSMZ 11375 were also used. All strains of *Campylobacter* spp. were propagated either in Brain Heart Infusion (BHI) supplemented with 5% horse blood (BHIB) (Oxoid, Milan, Italy) or in *Campylobacter* blood-free selective medium, CCDA (Oxoid) in microaerophilic conditions (CampyGen, Oxoid) at 37 ºC. Moreover, strains of food related bacteria were selected and used in this study in order to determine the specificity of the assay. These were: *Lactobacillus sakei*, *Staphylococcus xylosus*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovvi*, *Listeria seeligeri* and *Listeria welshimeri*. At least 2 strains for each species were

tested. The strains belonged to the culture collection of the University of Turin, Italy (Rantsiou et al., 2008) and were rutinelly propagated in Brain Heart Infusion (BHI) medium (Oxoid, Milan, Italy).

2.2. DNA extraction.

For strains grown in pure culture, DNA was extracted as previously described by Rantsiou et al. (2008). For the extraction of DNA from food samples the Master-Pure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) was used according to the manufacturer's instructions and to previous application for DNA extractions from foodstuffs (Rantsiou et al., 2008).

2.3. Quantitative PCR amplification.

Quantitative PCR was performed by using the SYBR Green chemistry. To amplify part of the *rpoB* gene of *C. jejuni*, primers Cj_rpoB1 (5'-GAGTAAGCTTGGTAAGATTAAAG-3') and Cjs_rpoB2 (5'-AAGAAGTTTTAGAGTTTCTCC-3') were designed by multiple sequence alignment (ClustalW2 algorithm, EBI, UK, available at: http://www.ebi.ac.uk/Tools/clustalw2/index.html). Amplification was carried out using the FluoCycle SYBR Green Mix of Euroclone (Euroclone Spa., Milan, Italy) in the Chromo4 Real-Time PCR Detection System (Biorad, Milan, Italy). The reaction was performed in 25 µl final volume containing 12.5 µl of the 10X mix, 400 nM of Cj_rpoB1 primer, 60 nM of Cjs_rpoB2 primer and 100 ng of DNA extracted from pure cultures or 1 µl of DNA extracted from food samples. The optimized cycle was: 95 ºC for 30 seconds, 62 ºC for 30 seconds and 72ºC for 30 seconds, followed by the fluorescence reading. The cycle was repeated 50 times and was preceded by a denaturation step at 95 ºC for 10 minutes. In each run, a positive and negative control was used. The positive control was represented by DNA extracted from a pure culture of *C. jejuni* LM8842, standardized to 100 ng/µl. In a total reaction volume of 25 µl, 1

µl of DNA was added. For the negative control, 1 µl of sterile, PCR-grade DNA (Sigma-Aldrich, Italy) was added. A melting curve analysis was carried out at the end of each run.

2.4. Construction of calibration curves.

Calibration curves were constructed in milk, poultry skin and poultry meat. For the construction of calibration curves in food samples, the strain of *C. jejuni* LM8842 was used. The strain was streaked in BHIB plates and after 24 hours of incubation, the cells were scraped off the agar surface with 1 ml of Ringer's solution (Oxoid, Milan, Italy) and the use of a plastic, sterile spreader. The cell suspension was serially diluted in Ringer's solution and counted on BHIB plates incubated at 37°C for 24-48 hours. Each dilution was inoculated in 10 g (for poultry skin or poultry meat) or 10 ml (for milk) of food sample. Then, 40 ml of Bolton broth (Oxoid, Milan, Italy), with supplement (Oxoid, Milan, Italy) but without blood, were added. Solid samples (poultry skin and meat) were homogenized, using a stomacher (PBI, Milan, Italy) for 2 minutes at maximum speed, while liquid samples (milk) mixed well. Subsequently, one ml was recovered and mixed with 9 ml of Ringer's solution and 1 ml of the diluted sample was used for DNA extraction as described above (DNA at t=0). The DNA extraction was also performed on the samples after 24 hours of incubation at 37 ºC in Bolton Broth. Also in this case, 1 ml of a ten-fold dilution in Ringer's solution was processed (DNA at t=24). One µl of the t=0 DNA (in triplicate) was used in qPCR amplifications and calibration curves were constructed plotting the threshold cycle against the colony forming units (cfu)/g or ml. Similarly, the t=24 DNA was used in amplification to determine the detection limit after overnight enrichment. The efficiency of the reactions was calculated according to Rutledge and Cote (Rutledge and Cote, 2003). Standard curves were contructed at least three times from three independent experiments.

2.4. Sampling.

Fourty eight samples of individually packaged poultry were purchased from super markets in the Piedmont region, in the Northwest part of Italy. The packaging was removed under a laminar flow hood. From each package, ten g of either meat (for samples without skin) or skin were aseptically cut and mixed with 40 ml of Bolton broth (Oxoid, Milan, Italy), with supplement (Oxoid, Milan, Italy) but without blood, and homogenized as above. One ml of the homogenate was mixed with 9 ml of Ringer's solution and 1 ml of this mix was used for DNA extraction (t=0). At the same time, the homogenate, the 1 to 10 and 1 to 100 dilutions of it were plated in CCDA medium. Plates were incubated at 37 ºC for 48 hours in microaerophilic conditions by using the Anaerogen system (Oxoid, Milan, Italy). The homogenate was also incubated for 18 hours at 37 ºC and DNA extraction was carried out from a ten-fold dilution in Ringer's solution, while a loopfull of the enriched homogenate was streaked in CCDA and incubated at 37 ºC for 48 hours (t=24). *Campylobacter* spp. suspected colonies from the CCDA plates were isolated in BHIB and subjected to qPCR amplification as described above.

3. Results and Discussion

The method of detection and quantification of *C. jejuni* presented in this paper is based on the amplification of partial *rpoB* gene. Primers, intended to be specific for *C. jejuni*, were designed based on all sequences of *Campylobacter* spp. available in GeneBank. By multiple sequence alingment (ClustalW2 algorithm, EBI, UK, available at: http://www.ebi.ac.uk/Tools/clustalw2/index.html), regions were identified that *in silico* were specific. Subsequently, the specificity of the amplification was optimized *in vitro* using as target, DNA extracted from pure cultures of different *Campylobacter* spp. Furthermore, a set of bacteria commonly

found in foods was also used. Several conditions of amplifications, focusing mainly on the temperature of annealing and concentration of the primers were tested, in order to obtain amplification signals only when *C. jejuni* DNA was added as template in the reaction. The conditions described in the materials and methods resulted to be specific for *C. jejuni*. Sequences of the *rpoB* gene of phylogenetically close species, namely *Helicobacter* and *Arcobacter* spp., were also used in an *in silico* analysis to check for possible annealing sites of the primers used in this study. Sequence heterogeneity was high and the primers described here did not align to *Helicobacter* or *Arcobacter* sequences.

In order to quantify, in terms of cfu/g or ml, *C. jejuni* in foods, appropriate calibration curves were constructed. For the DNA extraction, a determining factor for the success of such applications in foodstuffs, a commercial kit (Epicentre) was used which has been previously proven adequate for qPCR in foods (Rantsiou et al., 2008). Its applicability was further confirmed in this study, during the optimization phase. Three food matrices were chosen, based on available information from the literature regarding *C. jejuni* contamination (Anonymous, 2009; Heuvelink et al., 2009; Horrocks et al., 2008). These were: milk and poultry (meat and skin). The calibration curves are presented in Figure 1. The efficiencies calculated for the three matrices were: 100% for the milk, 79% for the poultry meat and 83% for the poultry skin. The R^2 values were 0.96, 0.90 and 0.99 respectively. As can be seen in Figure 1, for all three matrices a linearity range that spanned 5 logarithms was obtained and the quantification limit was in the order of 10 cfu/g or ml. The results obtained, in terms of efficiency, R^2 and limit of quantification were comparable when three independent experiments were performed (data not shown). After the 18 hours overnight enrichment (t=24) at 37 ºC, also matrices inoculated with concentrations of 1-10 cfu/g or ml could be detected.

The qPCR based protocol was applied, in parallel to traditional microbiological analysis, to detect and quantify *C. jejuni* in poultry samples. Fourty-eight samples were taken from large super markets in the Piedmont region in the Northwest of Italy. Samples were parts of chicken and turkey that were pre-packaged and stored at 4 ºC. A qPCR analysis was performed on DNA extracted at the time of sampling (t=0), as well as after an overnight enrichment (t=24). In parallel, traditional microbiological analysis was performed, both at t=0 and at t=24. The results obtained by the two approaches are summarized in Table 1. By direct qPCR analysis on the DNA extracted at t=0, 42 of the 48 (87%) samples resulted positi ve for the presence of *C. jejuni*. Apart from one sample, for which the concentration of *C. jejuni* was determined to be 24 cfu/g, all other positive samples had concentrations below the quantification limit. Melting curve analysis at the end of each run, resulted in melting temperatures for the positive samples, similar to those of *C. jejuni* used as control in the qPCR (data not shown). By traditional analysis, for 33 of 48 samples the *Campylobacter* spp. populations were below the detection limit, while for the remaining 15 samples, counts were in the order of 10^2-10^3 cfu/g. When suspected *Campylobacter* spp. colonies, randomly selected from the CCDA plates, were subjected to DNA and qPCR analysis, none of them resulted to be *C. jejuni*. Most likely, the colonies belonged to other *Campylobacter* spp. that are capable of growing on this medium. It is possible that the lack of correlation observed between the results obtained by traditional method and qPCR is due to the very low concentration of *C. jejuni* and the presence of cells in the samples that were injured or stressed and therefore did not develop on the selective medium.

Surprisingly, at t=24, the percentage of positive samples, obtained by qPCR, decreased compared to the situation at t=0. Thirteen of the 48 samples (27%) gave a positive signal by qPCR. This observation may be attributed either to the presence of

DNA of dead cells of *C. jejuni* in the samples at t=0, or to the presence of stressed and/or injured cells of *C. jejuni* that are not able to propagate in the selective (and stringent) environment of the enrichment broth used. The latter explanation is supported also by the data of the traditional analysis: none of the *Campylobacter* populations counted at t=0 was detected at t=24. Such a behaviour has already been reported for *Campylobacter* in Bolton enrichment broth (Habib et al., 2008). These authors also concluded that lower *Campylobacter* concentrations had lower likelihood of giving a positive result after enrichment compared to direct plating (Habib et al., 2008). It should also be noted that the conditions used for enrichment in this study (incubation at 37 ºC, Bolton Broth without blood), have recently been shown to have no negative effect on the recovery of campylobacters (Williams et al., 2009). Furthermore, in a previous study, Bolton enrichment broth without blood detected more samples positive for thermophilic *Campylobacter* spp. than did Preston enrichment broth (Paulsen et al., 2005). However, it is possible that in these conditions, competing microflora has better chances of outnumbering *Campylobacter*. In fact, it has been previously reported that non-*Campylobacter* species, after enrichment in Bolton broth, can generate in some cases colonies very similar to those produced by *Campylobacter* on selective plating medium (Baylis et al., 2000; Habib et al., 2008, Jasson et al., 2009). Only one sample (P7, Table 1), was negative at t=0 and positive at t=24. It is probable that a *C. jejuni* population below the quantification limit was present in the sample and it was able to produce a signal in qPCR after enrichment.

The use of the qPCR protocol developed in this study accurately detected *C. jejuni* in a high percentage of poultry samples, while the traditional analysis failed to do so. Additionally, the results obtained highlighted important aspects of the physiology of

C. jejuni in food and in enrichment media, commonly used for its isolation, that require further investigation.

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Table 1. Results of microbiological and molecular analysis on poultry samples

Sample Code	Type of Sample	Results t=0[1]		Results t=24[2]	
		Traditional analysis (cfu/g)	qPCR analysis (cfu/g)	Traditional analysis	qPCR analysis
C1	Meat (chicken)	<50	**Positive**	Negative	Negative
PT1	Meat (chicken breast)	<50	**Positive**	Negative	Negative
PT2	Meat (chicken breast)	<50	Negative	Negative	Negative
C2	Meat (chicken)	$6x10^{2}$	**Positive**	Negative	**Positive**
C3	Meat (chicken)	<50	**Positive**	Negative	Negative
GC	Meat (turkey)	<50	**Positive**	Negative	Negative
1C	Meat (chicken)	<50	**Positive**	Negative	Negative
2C	Meat (chicken)	<50	**Positive**	Negative	Negative
3C	Meat (chicken)	<50	**Positive**	Negative	Negative
4C	Meat (chicken)	$2x10^{2*}$	**Positive**	Negative	**Positive**
5C	Meat (chicken)	$7x10^{2*}$	**Positive**	Negative	Negative
6C	Meat (chicken)	$3,5x10^{2*}$	Negative	Negative	Negative
10 PT A	Meat (chicken breast)	<50	**Positive**	Negative	Negative
10 PT B	Meat (chicken breast)	<50	**Positive**	Negative	Negative
PT3 A	Meat (chicken breast)	<50	**Positive**	Negative	Negative
C5	Meat (chicken)	<50	**Positive**	Negative	**Positive**
AC1	Meat (chicken wing)	<50	**Positive**	Negative	Negative
C6	Meat (chicken)	$1x10^{3*}$	**Positive**	Negative	Negative
Ca	Meat (chicken)	<50	**Positive**	Negative	Negative
Cc	Meat (chicken)	<50	**Positive**	Negative	Negative
Cb	Meat (chicken)	<50	**24**	Negative	**Positive**
C7	Meat (chicken)	<50	**Positive**	Negative	Negative
GC2	Meat (turkey)	<50	**Positive**	Negative	**Positive**
C8	Meat (chicken)	<50	**Positive**	Negative	Negative
CS1	Meat (chicken leg)	<50	**Positive**	Negative	**Positive**
CA	Meat (chicken)	<50	**Positive**	Negative	Negative
CB	Meat (chicken)	<50	**Positive**	Negative	Negative
CS2	Meat (chicken leg)	<50	Negative	Negative	Negative
CS3	Meat (chicken leg)	<50	**Positive**	Negative	Negative
CS4	Meat (chicken leg)	<50	**Positive**	Negative	**Positive**
P8	Skin (chicken)	$2x10^{3*}$	**Positive**	Negative	Negative
P1	Skin (chicken)	<50	Negative	Negative	Negative
P2	Skin (chicken)	<50	**Positive**	Negative	Negative
P3	Skin (chicken)	<50	**Positive**	Negative	Negative
GP	Skin (turkey)	<50	Negative	Negative	Negative
7SP	Skin (chicken leg)	<50	**Positive**	Negative	Negative
8SP	Skin (chicken leg)	<50	**Positive**	Negative	**Positive**
P5	Skin (chicken)	$7x10^{2*}$	**Positive**	Negative	Negative
AP1	Skin (chicken wings)	$3,5x10^{2*}$	**Positive**	Negative	Negative
P6	Skin (chicken)	$2x10^{2*}$	**Positive**	Negative	Negative
Pa	Skin (chicken)	$4x10^{3*}$	**Positive**	Negative	**Positive**
Pb	Skin (chicken)	$5x10^{2*}$	**Positive**	Negative	**Positive**
Pc	Skin (chicken)	$4,5x10^{2*}$	**Positive**	Negative	**Positive**
P7	Skin (chicken)	$2x10^{3*}$	Negative	Negative	**Positive**
GP1	Skin (turkey)	$3x10^{3*}$	**Positive**	Negative	Negative
PA	Skin (chicken)	<50	**Positive**	Negative	Negative
PB	Skin (chicken)	<50	**Positive**	Negative	Negative
PS2	Skin (chicken leg)	$6x10^{2*}$	**Positive**	Negative	**Positive**

[1] Results at t=0 are reported as cfu/g as determined by the direct counts on CCDA plates (*Campylobacter* spp. suspected colonies in the traditional analysis) or based on the C(t) values obtained for the sample and the appropriate calibration curve. If the C(t) value falls out of the quantification limit of the calibration curve, the sample is reported as positive but without a quantification value. [2] Results at t=24 are reported as positive or negative. In the traditional analysis, positive refers to the presence of suspected colonies while in the qPCR analysis it refers to a fluorescence signal obtained during the amplification. [*] Randomly selected and isolated *Campylobacter* spp. suspected colonies from the CCDA plates were not confirmed as *C. jejuni* by qPCR.

Figure legends

Figure 1. Calibration curves of *C. jejuni*, serially diluted and inoculated in different food matrices. DNA samples, extracted as described in the materials and methods, corresponding to each *C. jejuni* dilution, were loaded in triplicate in a qPCR reaction. For each *C. jejuni* dilution, the mean and standard deviation of the C(t) values obtained were calculated and used to contsruct the calibration curve. Three independent calibration curves were constructed for each food matrix but only one is shown here. Figure 1a: calibration curve in milk. Figure 1b: calibration curve in poultry meat. Figure 1c: calibration curve in poultry skin.

Figure 1a.

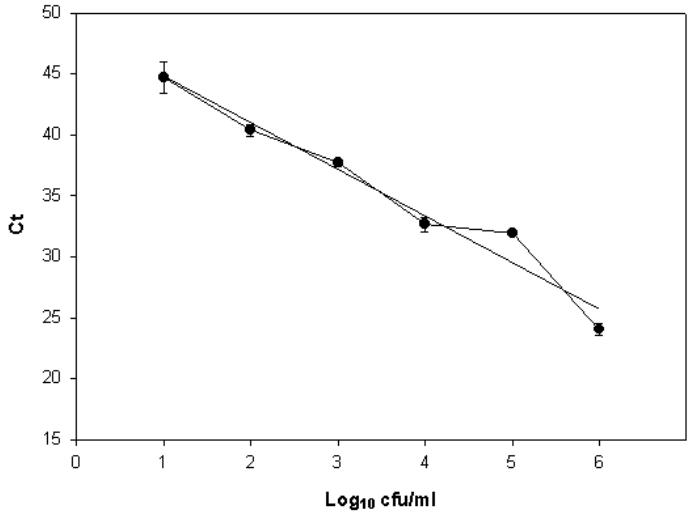

Figure 1b.

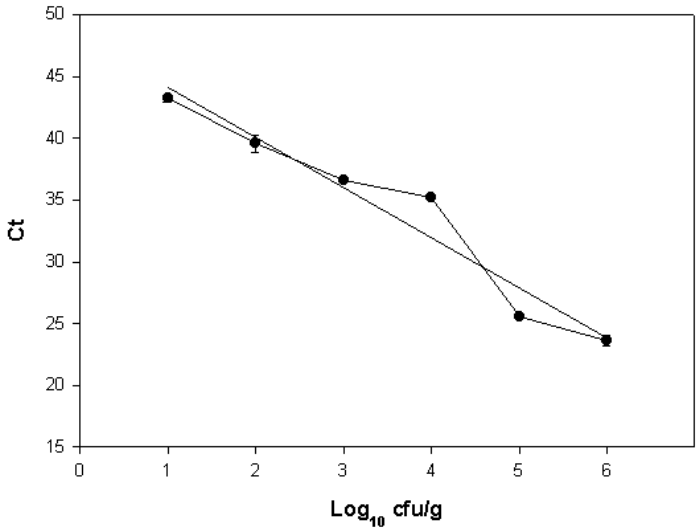

Figure 1c.