Detection, identification and quantification of *Campylobacter jejuni*, *coli* and *lari* in food matrices all at once using multiplex qPCR

Lucie Vondrakova*, Jarmila Pazlarova and Katerina Demnerova

**Abstract**

**Background:** Thermotolerant *Campylobacter jejuni*, *coli* and *lari* are recognized as leading food-borne pathogens causing an acute bacterial enteritis worldwide. Due to narrow spectrum of their biochemical activity, it is very complicated to distinguish between individual species. For reliable risk assessment, proper incidence evaluation or swift sample analysis regarding individual species, a demand for simple and rapid method for their distinguishing is reasonable. In this study, we evaluated a reliable and simple approach for their simultaneous detection, species identification and quantification using multiplex qPCR.

**Results:** Species specific primers and hydrolysis probes are directed to hippuricase gene of *C. jejuni*, serine hydroxymethyltransferase gene of *C. coli* and peptidase T gene of *C. lari*. Efficiencies of reactions were 90.85% for *C. jejuni*, 96.97% for *C. coli* and 92.89% for *C. lari*. At 95.00% confidence level and when cut off is set to 38 cycles, limits of detection are in all cases under 10 genome copies per reaction which is very appreciated since it is known that infectious doses are very low.

**Conclusions:** Proposed assay was positively validated on different food matrices (chicken wing rinses, chicken juice and homogenized fried chicken strips). No inhibition of PCR reaction occurred. Assay was evaluated in accordance with MIQE handbook.

**Keywords:** Thermotolerant *Campylobacter* spp, Multiplex qPCR, Quantification, MIQE

**Background**

Alimentary infections caused by various food-borne pathogens generally pose a threat to public health which has to be determined and, if possible, prevented or eliminated.

Thermotolerant bacteria belonging to *Campylobacter* genus (especially *C. jejuni*, *C. coli*, *C. lari* and marginally *C. upsaliensis*) are recognised as leading human food-borne pathogens causing an acute gastrointestinal disease called campylobacteriosis. Since 2007, the incidence of campylobacteriosis in Czech Republic is significantly higher than incidence of similar well known disease salmonellosis [1]. However, this trend is also apparent in all developed countries worldwide [2].

Digestive tracts of domesticated animals farmed for meat (especially poultry, pigs, cattle and sheep) and wild warm-blooded animals are significant reservoirs of thermotolerant *Campylobacters*. However, many other sources are also known (e.g. sewage, both drinking and environmental water, raw milk, pets, various kinds of seafood, insects etc.). From all these sources they are able to spread into a food chain or to an immediate proximity of human beings and can cause the infection [3-6]. Leaving aside typical clinical symptoms [3-5,7], another significant problem is the possibility of developing various post-infectious complications e.g. reactive arthritis, urticaria or erythema nodosum. The most serious sequel is the Guillain-Barré syndrome (GBS) which manifests as an acute polyneuropathy affecting peripheral nervous system leading to a typical ascending paralysis. Especially infection caused by *C. jejuni* is a common trigger of this disease and it is estimated that its infection proceeded to about 30% of all GBS cases [3,4,8].

Although normative methods for detection and enumeration of *Campylobacter* spp. e.g. [9] show relatively
high degree of specificity, their main disadvantages are apparent. These procedures are based on selective enrichment of desired bacteria, which consequently does not enable their quantification in original sample, followed by their isolation from background microflora, biochemical characterization and phenotypic or serotype identification. Considering Campylobacters’ special requirements for optimal growth, the detection according to standardised methods may take up to 7–10 days. Another problem appears when identification of individual species is required. This is caused mainly because of their relatively narrow spectrum of biochemical reactivity. However, also increasing numbers of nalidixic acid resistant C. jejuni and C. coli strains, or C. jejuni strains which are not able to hydrolyse hippuric acid under laboratory conditions (some of the biochemical tests used for species differentiation) make this situation more complicated as well [3,10-14]. Another issue linked with classical microbiological methods, which should be mentioned, is their inability to detect viable but non-cultururable bacteria (VBNC). Especially C. jejuni is known for its capability to enter this state when stressed, starved or physically damaged. Since this phenomenon has not yet been properly explored, it is assumed that such bacteria may be able to regenerate and therefore become infectious again [15,16].

With a regard to already mentioned problems it is evident that an availability of reliable alternatives for rapid detection, identification and quantification, especially in the food and agricultural industry, is undoubtedly an issue of the day. Over the last several years, various campylobacter-focused studies implementing real-time PCR approach, which seems to be suitable to provide appropriate solution meeting all of the above requirements, have been proposed. However, only very few of them were carried out in a platform of multiplex quantitative real-time PCR (qPCR), which enables complex analysis of a given sample. Main drawbacks of proposed studies are either that they are not focused on all above-mentioned main thermotolerant Campylobacters, and/ or enable detection without species identification or quantification e.g. [17-25], or include undesired pre-enrichment step [26-28]. Also one of the most interesting studies [28] dealing with this issue suffers from several shortcomings. Briefly, C. coli identification is based on amplification of a gene encoding one of the subunits of a cytolethal distending toxin (cdtA), which is one of its virulence factors. Since cdtA is not housekeeping gene, is not essential and its predisposition to mutate is higher, some publications reporting data concerning its mutations and deletions in campylobacter genome have been already published and also cdtA negative strains are known as well [29-33]. Also the achieved detection sensitivity of the assay (about 38 genome copies per reaction), should be higher and improved by further optimization.

Moreover, during our extensive research on other assays concerned real-time PCR released after 2009, we surprisingly found no publication which would be written in accordance with so called MIQE handbook (Minimum information for publication of quantitative real-time PCR experiments), which we consider unfortunate. MIQE is very detailed set of guidelines describing the minimum information which are necessary and should be provided every time when qPCR experiments are evaluated [34-37]. Although MIQE is not obligatory, there is no doubt that such checklist helps to assure the quality of obtained results and for this reason the present study is written in accordance with it.

Methods

Bacterial strains and culture conditions

Bacterial strains used for experimental analyses in this study are listed in Table 1. Before further handling, all Campylobacter strains were incubated in Park and Sanders enrichment broth (HiMedia, India) for 24–48 h at 42°C under microaerobic atmosphere (5% O2, 10% CO2 and 85% N2; O2/CO2 incubator MCO-18, Sanyo, USA). Non-campylobacter bacterial strains were aerobically grown in BHI broth (brain heart infusion; Merck, Germany) for 24 h at 37°C.

Design of qPCR assay

Material and methods section is only a brief extract from the very complex MIQE checklist in Additional file 1 where are provided all available details about experimental design and procedures.

DNA extraction

Genomic DNA from pure bacterial cultures (Table 1) was extracted by thermal lysis. Concentration and purity of the DNA were determined spectrophotometrically using NanoPhotometer™ (Implen, Germany). Only samples whose A260/A280 ratio ranged from 1.7 to 2.1 were used for further analyses. Real genome copy number was determined using the formula:Genome copies/μl = (C × N_A × 10^−12)/ (genome length(bp) × M_w), where C is a measured concentration of extracted DNA (ng/μl), N_A is Avogadro’s constant (6.02 × 10^23 molecule/mole) and M_w is molecular weight of 1 bp which is 660 Da [28].

In silico analyses

Primers and hydrolyses probes selection was based on previously published studies [18,28]. Although previously published, selected primers and probes (Table 2) have not been used together in one reaction and therefore it was necessary to perform additional in silico analyses. Their specificity was tested against 30 bacterial genomes (Table 3) using both basic nucleotide BLAST at NCBI (Basic local alignment search tool; National centre
for biotechnology information) and FastPCR molecular biology software [38]. Additional more comprehensive analysis for primer pair specificity checking was conducted using Primer-BLAST tool at NCBI. As a database query “Genome (chromosome of all organisms)” was selected and as an organism query was selected “bacteria (taxid: 2)”. In order to enable proper optimization of qPCR protocol for a multiplex platform, necessary determination of primers and probe chemical characteristics was also carried out using FastPCR molecular biology software [38].

Empirical primers’ specificity screen

In addition to in silico analyses experimental primers’ specificity verification was implemented. Horizontal agarose-gel electrophoresis and melt curve analysis utilizing SYBR Green fluorescent dye (2×Power SYBR Green PCR Master Mix, Applied Biosystems, USA) were performed. All target and non-target Campylobacter species listed in Table 1 were used for these analyses.

| Species                        | Strain   | Source            | Real-time PCR specificity |
|--------------------------------|----------|-------------------|---------------------------|
| C. jejuni subsp. jejuni        | CCM 6212 | Human blood       | +                         |
| C. jejuni subsp. jejuni        | NCTC 11168 | Human faeces     | +                         |
| C. jejuni subsp. jejuni        | 81-176   | Human faeces      | +                         |
| C. jejuni                      | 1 K      | Chicken meat      | +                         |
| C. jejuni                      | 667/C4   | Human clinical isolate | +            |
| C. jejuni                      | 681/C5   | Human clinical isolate | +            |
| C. jejuni                      | 2517     | Human clinical isolate | +            |
| C. jejuni                      | 3316     | Human clinical isolate | +            |
| C. jejuni                      | 13       | Wastewater treatment plant | +          |
| C. jejuni                      | 53G      | Turkey breasts    | +                         |
| C. coli                        | CCM 6211 | Pig               | +                         |
| C. coli                        | 549/6    | Wastewater treatment plant | +          |
| C. coli                        | 490/3    | Wastewater treatment plant | +          |
| C. coli                        | C253     | Human clinical isolate | +            |
| C. coli                        | C254     | Human clinical isolate | +            |
| C. coli                        | C226     | Human clinical isolate | +            |
| C. coli                        | 2463     | Human clinical isolate | +            |
| C. coli                        | 2521     | Human clinical isolate | +            |
| C. coli                        | 2530     | Human clinical isolate | +            |
| C. coli                        | 52       | Chicken breasts   | +                         |
| C. lari                        | CCM 4897 | Herring gull, cloacal swab | +         |
| C. fetus subsp. fetus          | CCM 6213 | Human blood       | -                         |
| C. upsaliensis                 | ATCC 43954 | Animal faeces     | -                         |
| Arcobacter butzleri            | 2013/43  | Chicken thigh     | -                         |
| Arcobacter cryaerophilus       | 2012/1   | Wastewater treatment plant | -        |
| Arcobacter skirrowii           | 2013/34  | Cow teat          | -                         |
| Bacillus cereus                | DBM 3035 | Not found         | -                         |
| Bacillus megaterium           | CCM 2007 | Not found         | -                         |
| Bacillus subtilis subsp. spitzeri | CCM 1999 | Not found         | -                         |
| Cronobacter sakazakii          | ATCC 2954 | Child’s throat    | -                         |

Table 1 List of experimentally included Campylobacters and other bacterial species (Continued)

| Species                        | Strain   | Source            | Real-time PCR specificity |
|--------------------------------|----------|-------------------|---------------------------|
| Enterobacter cloacae           | CCM 1903 | Plasma            | -                         |
| Enterococcus faecalis          | CCM 4224 | Urine             | -                         |
| Escherichia coli               | CCM 4517 | Human faeces      | -                         |
| Escherichia coli               | 485      | Raw milk          | -                         |
| Listeria innocua               | CCM 4030 | Cow brain         | -                         |
| Listeria ivanovii              | CCM 5884 | Sheep             | -                         |
| Listeria monocytogenes         | CCM 5576 | Guinea pig mesenteric lymph node | - |
| Listeria monocytogenes EGDe   | ATCC BAA-679 | Animal tissue     | -                         |
| Pseudomonas aeruginosa         | CCM 1968 | Not found         | -                         |
| Pseudomonas aeruginosa         | 49       | Turkey meat       | -                         |
| Rhodococcus equi               | CCM 3429 | Lung abscess of foal | -          |
| Salmonella enterica subsp. enterica | CCM 7205 | Animal tissue     | -                         |
| Staphylococcus aureus subsp. aureus | CCM 3953 | Clinical isolate  | -                         |
| Yersinia enterocolytica        | CNCTC 7252 Y41/73 | Human faeces     | -                         |
| Yersinia ruckeri               | CCM 6093 | Rainbow trout     | -                         |

ATCC – American Type Culture Collection.
CCM – Czech Collection of Microorganisms.
CNCTC – The Czech National Collection of Type Cultures.
DBM – Department of Biochemistry and Microbiology, ICT Prague.
NCTC - National Collection of Type Cultures.
Singleplex qPCR

Choice of probes and primers concentrations for the first experiments was based on our research on previously published assays where the most common final concentrations were 0.40 μM and 0.20 μM for primers and probes respectively e.g. [22,24,39-42]. Before standard curves were generated, one experiment was carried out with all target and non-target Campylobacter species as well as with other bacterial strains (Table 1) in order to experimentally verify also specificity of probes. After the positive verification only three reference target Campylobacter species (C. jejuni CCM 6212, C. coli CCM 6211 and C. lari CCM 4897) were used for further experiments. Standard curves for singleplex qPCR platform were generated as described in Additional file 1. All samples were run in duplicates and non-template (NTC) and positive controls were included.

Multiplex qPCR

Multiplex qPCR was evaluated in two phases. In the first phase, reaction was performed with all components (primers and probes) with DNA from one strain in a tube in order to find out whether undesirable inhibition caused by interaction between components occurs or not. Standard curves were generated and quantification cycles (Cq), y-intercepts, slopes, efficiencies (E), standard deviations (SD), correlation coefficients (R²) and linear ranges were determined using 7500 Software (Applied Biosystems, USA, version 2.0.5), Microsoft Office Excel 2007 (Microsoft, USA, version 2007) and GenEx software (MultiD Analyses AB, Sweden, version GenEx 5 Enterprise).

In the second phase, reaction mixture contained all chemical components as well as mixed DNA sample from all target Campylobacter strains. Standard curves were generated and abovementioned parameters determined using the same approach. In accordance with obtained results the concentrations of individual components and reaction conditions were optimized until satisfying values were obtained (Additional file 1).

Data analysis

Output data were analysed with instrument compliant 7500 Software (Applied Biosystems, USA, version 2.0.5), Microsoft Office Excel 2007 (Microsoft, USA, version 2007) and GenEx software (MultiD Analyses AB, Sweden, version GenEx 5 Enterprise).

Assay validation on food samples

Sample collection and processing

Three different types of food matrices were chosen for empirical assay validation – raw chicken wings (local butchery, Prague, Czech Republic), whole frozen chicken without giblets (local hypermarket, Prague, Czech Republic) and fried chicken strips (fast food restaurant, Prague, Czech Republic). All samples were processed immediately after the purchase as described in Additional file 1, and obtained chicken wing rinses, chicken juice and homogenate from fried chicken strips were further examined.

Artificial contamination of food samples

Each sample was divided into four aliquots. One remained unspiked and the rest was artificially contaminated with pure culture of individual reference Campylobacter strain and then serially diluted in order to achieve the range of approximately 10^3-10^5 CFU/ml. The number of Campylobacter cells used for the spiking was determined on Karmali agar with Campylobacter selective supplement (sodium pyruvate, vancomycin, cefoperazone and cycloheximide;
Table 3 Bacterial strains for in silico specificity screen of primers and probes

| Bacterial strain                  | NCBI genome accession number |
|----------------------------------|------------------------------|
| Campylobacter jejuni 81 - 176    | NC_008787.1                  |
| Campylobacter jejuni 81116       | NC_009839.1                  |
| Campylobacter jejuni NCTC 11168  | NC_002163.1                  |
| Campylobacter lari J20           | AER01000001.1                |
| Campylobacter lari RM2100        | NC_012039.1                  |
| Campylobacter concisus 13826     | NC_009802.1                  |
| Campylobacter curvus S25.92      | NC_009715.1                  |
| Campylobacter fetus subsp. fetus 82-40 | NC_008599.1                  |
| Campylobacter hominis ATCC BAA-381 | NC_009714.1                  |
| Arcobacter butleri RM4018        | NC_009850.1                  |
| Arcobacter nitrofigilis DSM 7299 | NC_014166.1                  |
| Bacillus cereus ATCC 10987       | NC_003909.8                  |
| Bacillus subtilis subsp. subtilis str. 168 | NC_009964.3                  |
| Cranobacter sakazakii ATCC BAA-894 | NC_009778.1                  |
| Enterobacter aerogenes KCTC 2190 | NC_015663.1                  |
| Enterobacter cloacae subsp. cloacae ATCC 13047 | NC_014121.1                  |
| Escherichia coli ATCC 8739       | CP000094.6                   |
| Escherichia coli BW2952          | NC_012759.1                  |
| Helicobacter pylori 26695        | NC_000915.1                  |
| Listeria monocytogenes 08-5578   | NC_013766.1                  |
| Listeria monocytogenes EGD-e     | NC_003210.1                  |
| Pseudomonas aeruginosa UCBPP-PA14 | NC_008463.1                  |
| Salmonella bongori NCTC 12419    | NC_015761.1                  |
| Salmonella enterica subsp. enterica serovar Typhimurium strain LT2 | NC_003197.1                  |
| Shigella boydii CDC 3083-94      | NC_010658.1                  |
| Shigella dysenteriae 5d197       | NC_007606.1                  |
| Shigella flexneri 2002017        | CP001383.1                   |
| Shigella sonnei Ss046            | NC_007384.1                  |
| Staphylococcus aureus subsp. aureus NCTC 8325 | NC_007795.1                  |
| Yersinia enterocolitica subsp. enterocolitica 8081 | NC_008800.1                  |

Oxoid, UK) using a drop plate method [43], by our qPCR as well as via genome copy number determination in pure cultures used for spiking [28].

DNA extraction and qPCR

DNA was isolated from 750 μl of each food sample using commercial PrepSEQ® Spin Sample Preparation Kit with Protocol (Applied Biosystems, USA) in accordance with manufacturer’s recommendations.

qPCR reaction was performed as described in Additional file 1. Briefly, standard curves were constructed using 5 μl of mixed DNA extracted from target Campylobacters. Used DNA concentrations ranged approximately from 10⁰ to 10⁶ genome copies (CFU equivalent) per well for C. jejuni and from 10¹⁰ to 10⁵ for C. coli as well as for C. lari. In the case of food samples 10 μl of DNA were added into reaction. Simultaneously, all food samples were examined for presence of Campylobacters using drop plate method [43].

Results and discussion

qPCR assay

For implementation of multiplex qPCR assay three species specific target genes (Table 2) were selected based on research of previously published studies e.g. [23,27,28,44-49]. Considering the very short length and high similarity [50] of campylobacters genomes (from 1.5 to 1.7 Mbp), it was necessary to carefully choose such primers and probes which interact exclusively with its target gene, do not form any secondary structures and also have similar chemical characteristics in order to allow the co-amplification of multiple targets in one tube without any competition or inhibition. Regarding obtained results, C. lari specific probe, previously designed by another research group [28], was additionally internally modified with propynyl at two cytosines (Eastport, Czech Republic) because of its shorter length and therefore lower melting temperature in comparison with the others. Our modification increased its melting temperature by 5°C and therefore its utilization in multiplex platform was possible.

Primers’ specificity was experimentally verified with simple horizontal agarose-gel electrophoresis and melt curve analysis performed with SYBR Green fluorescent dye. DNA from target and non-target Campylobacter species (Table 1) was used. As expected, only specific melting peaks of amplified products were obtained. Nonspecific amplicons of different lengths or primer-dimers did not form. Amplification of non-target DNA (C. fetus subsp. fetus CCM 6213 and C. upsaliensis ATCC 43954) did not occur as well.

Singleplex qPCR

First singleplex qPCR served for specificity screen of hydrolysis probes. As a sample DNA isolated from all bacteria listed in Table 1 was used. Three different combinations of primers and probes final concentrations in reaction were tested as follows: 0.40±0.20, 0.30±0.10 and 0.20±0.05 μM respectively. There were no significant differences between Cq values, therefore for further experiments in singleplex platform the combination of concentrations 0.40±0.20 μM were used. No fluorescent signal was detected when non-target DNA was used as a sample. When specificity of probes was positively verified standard curves for target Campylobacters were generated. Quantification cycles and efficiencies were 18.54 and 84.56% for Campylobacters, respectively.
high Cq value for C. coli when compared with the others, another combinations of primer and probe concentrations were tested as follows: 0.40×0.20 to 0.50, 0.50×0.20 to 0.50 and 0.80×0.80 μM. However, no significant differences were observed. Another in silico analysis showed one non-complementary base at 3’ end of the forward primer, which was not issue when used in original study [18] where only duplex qPCR was evaluated and quantification cycles ranged between 18.80-23.00 (when 10⁶ genome copies per well used). Therefore 3’ end of original primer was amended by adding a two bases which increased a stability and specificity of annealing step (Additional file 1). Our adjustment caused significant decrease in Cq value to 15.72 (when 10⁶ genome copies per well used) and slight increase in efficiency as well (89.24%) when concentrations of primers and probes in reaction were 0.40×0.20 μM (Table 4). Due to the fact that singleplex platform was mainly performed in order to verify the functionality of the reaction, we proceeded directly to the multiplex without further optimization in order to improve obtained values.

**Multiplex qPCR**

In the first experiment, all components were present in reaction mixture at the same concentration as in the singleplex, but DNA sample in each reaction originated only from individual species (not mixed sample). Considering greater number of components when multiplexing, reaction volume was increased from 25 μl to 30 μl. Results showed that there is no inhibition of the reaction caused by interaction between components. Serial dilutions of DNA were in the range of 10⁰-10⁷ genome copies (CFU equivalents) per well. Quantification cycles and efficiencies were 23.80 and 91.35% for C. jejuni, 23.41 and 92.12% for C. coli and 21.53 and 92.08% for C. lari when 10⁵ genome copies per well used (more details in Additional file 1).

In second phase multiplex with mixed DNA sample was evaluated. First experiment was carried out under the same conditions as the singleplex. Serial dilutions of mixed DNA were in the range of 10⁰-10⁷ genome copies of each strain per well. Although Cq values and efficiencies for C. coli and C. lari were comparable with previous multiplex results (DNA from single strain), it was unambiguous that strong inhibition of amplification occurred in the case of C. jejuni because of a complete disappearance of its PCR product. Therefore conventional multiplex PCR (all three pairs of primers and three probes) with end point horizontal agarose-gel electrophoresis was conducted with two possible combinations of DNA present in sample (C. jejuni×C. coli; C. jejuni×C. lari) in order to determine in which case the problem occurs. Based on results it was found that when all components are present in reaction with DNA sample mixed of C. jejuni and C. lari the amplification of C. jejuni target is affected and the typical PCR product does not form. Having regard to the fact that there was no inhibition due to competition for other reaction components when multiplex with DNA sample from each strain individually was performed, this indicated that there was some interaction between C. jejuni and C. lari DNA even though the trend of C. lari reaction was not affected at all. Considering this fact, another optimization was necessary and various concentrations of C. jejuni and C. lari specific primers and probes were tested (results not shown).

Fully optimized reaction mixture consisted of 0.80 μM C. jejuni, 0.40 μM C. coli and 0.05 μM C. lari primers and 0.20 μM of each probe. Eight points of ten-fold serial dilutions in the range of 10⁰-10⁷ genome copies of each strain per well were used to generate standard curves. Values of quantification cycles and efficiencies are 22.99 and 90.85% for C. jejuni, 20.77 and 96.97% for C. coli, 20.04 and 91.05% for C. lari, when 10⁵ genome copies per well used (Table 4). All reactions were linear over seven orders of magnitude in the range 10¹-10⁷ with potential to cover wider range in higher orders. Detection limits of this assay were determined to be between 6.62-16.10 genome copies/well for C. jejuni, 5.13-6.30 genome copies/well for C. coli and 4.87-5.23 genome copies/well for C. lari. All other parameters are provided in Additional file 1.

**Food sample analyses**

For empirical assay evaluation on food samples, three different food matrices which were likely to be naturally contaminated with *Campylobacter* species were examined (chicken wing rinses, chicken juice and homogenate prepared from fried chicken strips). Sample aliquots were artificially contaminated with individual target *Campylobacters*. Unspiked samples were tested for natural

---

**Table 4 Comparison of results for singleplex qPCR and optimized multiplex qPCR with pure cultures**

| Platform   | Singleplex Efficiency (%) | Slope | y-intercept | R²  | Multiplex Efficiency (%) | Slope | y-intercept | R²  |
|------------|---------------------------|-------|-------------|-----|---------------------------|-------|-------------|-----|
| C. jejuni  | 84.560                    | −3.757| 41.308      | 0.999| 90.848                    | −3.565| 42.227      | 0.998|
| C. coli    | 89.235                    | −3.610| 37.393      | 1.000| 96.973                    | −3.399| 40.040      | 0.998|
| C. lari    | 77.930                    | −3.996| 40.234      | 0.993| 92.888                    | −3.506| 39.548      | 0.998|

R² correlation coefficient.
contamination as well. Plate counting method [43] and proposed qPCR assay were simultaneously compared.

Using qPCR, quantification of target Campylobacters was possible in all tested food samples even when the highest dilutions were used for spiking. Quantification by plate counting was always possible in the range of $10^3$ to $10^5$ CFU/ml however, for some target Campylobacters failed when higher dilutions were used for spiking (Table 5). Quantification of C. jejuni by plate counting, regardless of the sample analysed, always failed with the dilution corresponding to $10^2$ CFU/ml and higher in which case no growth on the plates was observed even after prolonged incubation for 72 hours. Therefore this concentration seems to be the detection limit for this specie when the plate counting is used. All the unspiked food samples were determined to be Campylobacter free by plate counting. On the contrary, the unspiked chicken juice was determined to be naturally contaminated by C. jejuni using qPCR (Table 5). Also one of the unspiked chicken rinses was reliably determined to be naturally contaminated by C. coli however, its numbers were below quantification limit. As mentioned above, there is a possibility that food samples or Campylobacter cultures used for spiking contained certain numbers of dead or VBNC cells, which were detected and quantified with qPCR but did not grow on plates. However, considering fact that cultures were fresh and under no stress, it is highly unlikely that number of such cells would be significant.

Conclusions
In conclusion, we provided a reliable method for detection, identification and quantification of three most abundant thermotolerant Campylobacters. The main advantage of this approach over normative methods for their characterization is a possibility to exclude the pre-enrichment step. Exclusion of this part dramatically reduces the time required for analysis. Also the possibility to identify all three species at once is appreciated, since cases of co-contamination and co-infection with more than one Campylobacter specie are relatively common [51-53]. Also this publication is written in accordance with the MIQE handbook [35,36] which introduces a very good way to establish a consensus on how best to perform and interpret qPCR experiments in order to facilitate cooperation between laboratories, comparability and reproducibility of obtained results, and generally serves for higher standardization of real-time PCR experiments.

Additional file

Additional file 1: Supplementary text 1.

Competing interests
Authors declare that they have no competing interests.

Authors’ contributions
LV, JP and KD participated in the design of the study. LV performed experiments, collected and analysed data. All authors provided ideas, comments and prepared a draft manuscript and approved the final manuscript.

Acknowledgements
Financial support provided from specific university research MSMT No 21/2013 and MSMT No 21/2013.

Received: 3 February 2014 Accepted: 4 May 2014
Published: 9 May 2014

References
1. NIPH: Infectious diseases in Czech Republic. Nat Int Pub Health 2013, [http://www.szu.cz/publikace/data/infece-v-cr]
2. EFSA: EFSA explains zoonotic diseases. Campylobacter European Food Safety Authority 2013, [www.efsa.europa.eu/]
3. Allos BM: Campylobacter jejuni infections: Update on emerging issues and trends. Clin Infect Dis 2001, 32:1201–1206.
4. Humphrey T, O’Brien S, Madsen M: Campylobacters as zoonotic pathogens: A food production perspective. Int J Food Microbiol 2007, 117:237–257.
5. Moore JE, Corcoran D, Dooley JS, Fanning S, Lucey B, Matsuda M, McDowell DA, Mégraud F, Millar BC, O’Mahony R, O’Riordan L, O’Rourke M, Rao JR, Rooney PJ, Sails A, Whyte P: Campylobacter. Vet Res 2005, 36:351–382.
6. Sabatková Z, Pazlarova J, Demnerova K: Sample processing effect on polymerase chain reaction used for identification of Campylobacter jejuni. Folia Microbiol 2004, 49:693–697.
7. Butzler JP: Campylobacter, from obscurity to celebrity. Clin Microbiol Infect 2004, 10:868–876.
8. Drenthen J, Yuki N, Meulstee J, Maathuis EM, van Doorn PA, Visser GH, Blok JH, Jacobs BC: Guillain-Barre syndrome subtypes related to Campylobacter infection. J Neurol Neurosurg Psychiatry 2011, 82:300–305.
9. Anonymous: Microbiology of food and animal feeding stuffs - horizontal method for detection and enumeration of Campylobacter spp. Part 1: Detection methods. International organization for Standardization 2006, ISO 10272–1:2006.
10. Caner V, Cokal Y, Cetin C, Sen A, Karagenc N: The detection of hipO gene by real-time PCR in thermophilic Campylobacter spp. with very weak and

| Table 5 Comparison of food sample analyses results obtained by plate counting and qPCR |
|---------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Food sample                     | Wing rinse A | Wing rinse B | Wing rinse C | Chicken juice | Fried strips homogenate |
| CFU/ml                          | 10^1 | 10^2 | qPCR | 10^1 | 10^2 | qPCR | 10^1 | 10^2 | qPCR | 10^1 | 10^2 | qPCR |
| C. jejuni                       | -    | -    | -    | -    | -    | -    | -    | -    | -    | -    | 7.1x10^2 ± 1.6x10^2 | -    | -    |
| C. coli                         | -    | +    | -    | +    | -    | +    | -    | -    | -    | -    | -    | -    |
| C. lari                         | +    | +    | -    | +    | -    | +    | -    | -    | -    | +    | -    | -    |

- negative quantification.
+ positive quantification.

*quantification was not possible, because cell numbers were below quantification limit of qPCR.
negative reaction of hippurate hydrolysis. Antonie Van Leeuwenhoek 2008, 94:277–282.

11. Rautehn H, Jusufovic J, Hinnenin M-L. Identification of hippurate-negative thermophilic campylobacters. Diagn Microbiol Infect Dis 1999, 35:9–12.

12. Totten PA, Patton CM, Tenover FC, Barrett TJ, Stamm WE, Steigerwalt AG, Lin JY, Holmes KK, Brenner DJ. Prevalence and characterization of hippurate-negative Campylobacter jejuni in King County, Washington, J Clin Microbiol 1987, 25:1477–1478.

13. Endz HP, Ruji GI, Vanklinkeun B, Jansen WH, Vandereyden T, Mouton RP: Quinolone resistance in Campylobacter isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J Antimicrob Chemother 1991, 27:209–208.

14. Aarestrup FM, Engberg J. Antimicrobial resistance of thermophilic Campylobacter, Vet Res 2001, 32:311–313.

15. Rollins DM, Colwell RR: Viable but nonculturable stage of Campylobacter jejuni and its role in survival in the natural aquatic environment. Appl Environ Microbiol 1986, 52:531–538.

16. Silva J, Lete D, Fernandez M, Mena C, Gibbs BA, Teixeira P: Campylobacter spp. as a foodborne pathogen: a review. Front Microbiol 2011, 2:200.

17. Hong J, Jung WK, Kim JM, Kim SH, Koo HC, Ser J, Park YH: Quantification and differentiation of Campylobacter jejuni and Campylobacter coli in raw chicken meats using a real-time PCR method. J Food Prot 2007, 70:2015–2022.

18. LaGier MJ, Joseph LA, Passaretti TV, Musser KA, Cintin NA: A real-time multiplex PCR assay for rapid detection and differentiation of Campylobacter jejuni and Campylobacter coli, Mol Cell Probes 2004, 18:275–282.

19. Leblanc-Maridor M, Beaudou F, Seegers H, Denis M, Belloc C: Rapid identification and quantification of Campylobacter coli and Campylobacter jejuni by real-time PCR in pure cultures and in complex samples. BMC Microbiol 2011, 11:13.

20. Toplak N, Kovar M, Piksensik M, Mozina SS, Jerek B: Detection and quantification of Campylobacter jejuni and Campylobacter coli using real-time multiplex PCR. J Appl Microbiol 2011, 12:752–764.

21. Bonjoch X, Calvo L, Soler M, Ruiz-Rueda O, Garcia-Gil LJ: A new multiplexed real-time PCR assay to detect Campylobacter jejuni, C. coli, C. lari, and C. upsaliensis. Food Anal Methods 2010, 3:40–46.

22. Debretasn A, Halzemariam T, Wilson S, Nganwa D, Yehualaeshet T: Real-time PCR assay for rapid detection and quantification of Campylobacter jejuni on chicken rinses from poultry processing plant. Mol Cell Probes 2007, 21:177–181.

23. Nogva HK, Bergh A, Holck A, Rudi K: Evaluation and development of methods for quantitative detection of Campylobacter jejuni 321. Appl Environ Microbiol 2007, 73:1061–1067.

24. Tang DD, Nielsen EM, Schuetz F, Pedersen K, Handberg K, Madsen M: PCR detection of seven virulence and toxin genes of Campylobacter jejuni and Campylobacter coli isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. J Appl Microbiol 2003, 94:1003–1014.

25. Martinez I, Mateo E, Churuca E, Girbau C, Aloros R, Fernandez-Astorga A: Detection of cdtA, cdtB, and cdtC genes in Campylobacter jejuni by multiplex PCR. Int J Med Microbiol 2006, 296:65–68.

26. Samonsouk W, Asakura M, Yoshida E, Taguchi T, Nishimura K, Eannooklap B, Phromwisan V, Chaimayuma W, Yamasaki S: Evaluation of a cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the identification of Campylobacter strains isolated from poultry in Thailand. Microbiol Immunol 2007, 51:909–917.

27. Fernandez M, Mena C, Silva J, Teixeira P: Study of cytolethal distending toxin (cdt) in Campylobacter coli using a multiplex polymerase chain reaction assay and its distribution among clinical and food strains. Foodborne Pathog Dis 2010, 7:103–106.

28. Bustin SA: Why the need for qPCR publication guidelines?-The case for MIQE. Methods 2010, 50:217–226.

29. Bustin SA, Bealeou JF, Huggett J, Jargi R, Kibenge FSB, O'Sullivan PA, Pennning LC, Toegel S: MIQE precis: Practical implementation of minimum guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol 2010, 11:74.

30. Bustin SA, Benes V, Ganson JA, Hallemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaff MW, Shipley GL, Vandesompele J, Wittwer CT: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009, 55:611–622.

31. Taylor S, Walems M, Dijkman G, Abspa M, Nguyen M: A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines. Methods 2010, 50:51–58.

32. Kaplansherry A, Van Dyke MJ, Hutt J: Long-ampliquon propidium monoazide-PCR enumeration assay to detect viable Campylobacter and Salmonella. J Microbiol Methods 2012, 91:173–177.

33. Banihashemi A, Van Dyke MJ, Hub PM: Long-ampliquon propidium monoazide-PCR enumeration assay to detect viable Campylobacter jejuni and Campylobacter coli directly from poultry faecal samples. Res Microbiol 2012, 163:64–72.

34. Flekna G, Schneeveiss W, Smulders FJM, Wagner M, Hein S: Real-time PCR method with statistical analysis to compare the potential of DNA isolation methods to remove PCR inhibitors from samples for diagnostic PCR. Mol Cell Probes 2007, 21:282–287.

35. Rotheroch CJ, Cook KL, Bolster CH: Comparative quantification of Campylobacter jejuni from environmental samples using traditional and molecular biological techniques. Can J Microbiol 2009, 55:633–641.

36. Caputo CT, Nace GW, Irwin PL: A 6 x 6 10 plate method for simultaneous colony counting and MPN enumeration of Campylobacter jejuni, Listeria monocytogenes, and Escherichia coli. J Microbiol Methods 2003, 55:475–479.

37. Engelen MD, Fedoroka-Cray PJ: Evaluation of a commercial diagnostic PCR for the identification of Campylobacter jejuni and Campylobacter coli. Lett Appl Microbiol 2002, 35:353–356.

38. Flekna G, Stefanic P, Wagner M, Smulders FJM, Mozina SS, Hein I: Insufficient differentiation of live and dead Campylobacter jejuni and Listeria monocytogenes cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res Microbiol 2007, 158:405–412.

39. Linton D, Lawson AJ, Owen RI, Stanley J: PCR detection, identification to species level, and fingerprinting of Campylobacter jejuni and Campylobacter coli directed from diarrheic samples. J Clin Microbiol 1997, 35:2568–2572.

40. Wang H, Farber IM, Malini R, Sanders G: Improved PCR detection of Campylobacter jejuni from chicken rinses by a simple sample preparation procedure. Int J Food Microbiol 1999, 52:39–45.

41. Yamazaki-Matsune W, Taguchi M, Seto K, Kawaihara R, Kawai J, Kudema Y, Kitazato M, Nukina M, Misawa N, Tsukamoto T: Development of a multiplex PCR assay for identification of Campylobacter coli, Campylobacter fetus, Campylobacter hyointestinalis subsp hyointestinalis, Campylobacter jejuni, Campylobacter lari and Campylobacter upsaliensis. J Med Microbiol 2007, 56:1467–1473.

42. Yang CB, Jiang Y, Huang KH, Zhu CQ, Yin YL: Application of real-time PCR for quantitative detection of Campylobacter jejuni in poultry, milk and environmental water. FEMS Immunol Med Microbiol 2003, 38:265–271.

43. Chang NC, Taylor DE: Use of pulsed-field agarose-gel electrophoresis to size genomes of Campylobacter species and to construct a Soll map of Campylobacter jejuni UASS. J Bacteriol 1990, 172:5211–5217.
51. Koene MGJ, Houwers DJ, Dijkstra JR, Duim B, Wagenaar JA: Simultaneous presence of multiple Campylobacter species in dogs. J Clin Microbiol 2004, 42:819–821.

52. Lawson AJ, Logan JMJ, O’Neill GL, Desai M, Stanley J: Large-scale survey of Campylobacter species in human gastroenteritis by PCR and PCR-enzyme-linked immunosorbent assay. J Clin Microbiol 1999, 37:3860–3864.

53. Richardson JF, Frost JA, Kranier JM, Thwaites RT, Bolton FJ, Wareing DRA, Gordon JA: Coinfection with Campylobacter species: an epidemiological problem? J Appl Microbiol 2001, 91:206–211.