Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

Optimization and validation of a triplex real-time PCR assay for thermotolerant Campylobacter species associated with foodborne disease

Maria Karlsson

Department of microbiology
Master´s thesis • 30 hec • Second cycle, A2E
Agriculture Programme - Food Science • Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi: 2013:2• ISSN 1101-8151
Uppsala 2013

Optimization and validation of triplex real-time PCR assay for thermotolerant Campylobacter species relevant for foodborne disease

Maria Karlsson

Supervisor: Stefan Roos, Swedish University of Agricultural Sciences, Department of Microbiology

Assistant Supervisor: Karin Jacobsson, National Food Agency, Science department, division of Microbiology

Examiner: Hans Jonsson, Swedish University of Agricultural Sciences, Department of Microbiology

Credits: 30 hec
Level: Second cycle, A2E
Course title: Independent project degree in Food Science
Course code: EX0425
Programme/education: Agriculture Programme - Food Science

Place of publication: Uppsala
Year of publication: 2013
Title of series: Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi no: 2013:2
ISSN: 1101-8151
Online publication: http://stud.epsilon.slu.se

Keywords: thermotolerant *Campylobacter*, multiplex real-time PCR, 16S rRNA, *hipO*, *cadF*

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences
Uppsala BioCenter
Department of Microbiology

Abstract

The genus *Campylobacter* is globally recognised as the leading bacterial cause of human foodborne gastroenteritis. Every year around 8000 Swedes are infected by *Campylobacter*. Most people are infected by thermotolerant *Campylobacter* species, commonly *C. jejuni* and *C. coli*. In this study a triplex real-time PCR has been developed in order to be a last step in a qualitative method for identification of thermotolerant *Campylobacter*, originating from food and water suspected to have caused human gastroenteritis. PCR is preceded by enrichment and isolation on selective medium and cultivation microaerophilically at 41.5 °C. The developed triplex real-time PCR was based on a combination of previously published primers and probes targeting the *hipO*, *cadF*, and 16S rRNA genes. In total 115 strains, representing 17 different *Campylobacter* species and additionally 10 non-*Campylobacter* species, genetically related to *Campylobacter* or commonly causing human gastroenteritis, were tested for inclusivity and exclusivity. Species-specific PCR products were produced to distinguish *C. coli* and *C. jejuni*. Thereto an amplicon of the 16S rRNA gene was produced for *C. coli*, *C. jejuni*, *C. lari*, two of seven *C. upsaliensis*, and *C. insulaenigrae*. No PCR product was obtained from any non-*Campylobacter* strains. Thereby, the method sufficiently met the needed requirements. The aim was also to compare two different methods for DNA extraction, where the InstaGene™ kit with a slight modification was preferred to simple boiling. Also the inhibition of the PCR assay if bacterial colonies were grown on blood agar or modified charcoal cefoperazone deoxycholate agar (mCCDA) was compared, with no differences found between the culturing medias.

Keywords: thermotolerant *Campylobacter*, multiplex real-time PCR, 16S rRNA, *hipO*, *cadF*.

Table of contents

Abbreviations

BA	Blood agar
BHI	Brain heart infusion agar
bp	Base pair
CCUG	Culture Collection University of Göteborg
DNA	Deoxyribonucleic acid
EURL	European Union Reference Laboratory
IPC	Internal positive control
mCCDA	Modified charcoal cefoperazone deoxycholate agar
NCBI	National Center for Biotechnology Information
NFA	National Food Agency, Sweden
NTC	No template control, negative control
PCR	Polymerase chain reaction
RFU	Relative fluorescence unit
SMI	Swedish Institute for Communicable Disease Control
SVA	National Veterinary Institute, Sweden
WHO	World Health Organization

1 Introduction

Campylobacter

Campylobacter are found worldwide and can be transmitted between animal and human, thus they are zoonotic (SMI, 2010). Globally *Campylobacter* is recognised as the leading bacterial cause of human foodborne gastroenteritis (Botteldoorn et al., 2008). In man or animals, *Campylobacter* are usually found on the reproductive organs, intestinal tract and oral cavity. Animals can be asymptomatic carriers, while most species are pathogenic for humans (Humphrey et al., 2007). The only species that have not been isolated from human infections are *C. helveticus* and *C. mucosalis* (Garrity et al., 2005).

Classification of the genus *Campylobacter* is primarily based on phylogenetics (Garrity et al., 2005), however some morphological and physiological traits are common for *Campylobacter*. The genus is defined as Gram negative, non-sporeforming, 0.2-0.8 μm wide and 0.5-5 μm long cells. They are mostly oxidase positive (except *C. gracilis* and sporadic isolates of *C. concisus* and *C. showae*) and most species are nonhemolythic and most do not hydrolyse hippurate. Cells can have one or more helical turns giving *Campylobacter* their characteristic corkscrew shape (Figure 1). Old cells may turn coccoidal, a resting stage. A polar flagellum can appear at one or both ends of the cell.

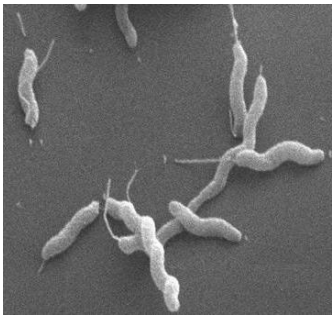

Figure 1. *Characteristic look of Campylobacter. www.health-pic.com*

All *Campylobacter* can grow under microaerobic conditions, requiring an O_2 concentration of 3-15 % and a CO_2 concentration between 3-5 %. Several species can also grow under aerobic or anaerobic conditions. Growth is optimal at 30-37 °C and does not occur at 4 °C. Depending on the growth media, *Campylobacter* form round white, grey or metal glistening colonies. The genus is generally defined via identification to species level.

Thermotolerant Campylobacter

Thermotolerant *Campylobacter* have the ability to grow in temperatures up to 42 °C. Sometimes the term thermophilic is used. The taxonomy of thermotolerant *Campylobacter* is however problematic, and the definition varies among researchers. The definition used in this study is by Bergey's Manual of Systematic Bacteriology (Garrity et al., 2005) which includes *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus* as thermotolerant *Campylobacter*. All thermotolerant *Campylobacter*, except *C. helveticus*, can in low numbers cause human gastroenteritis, and are therefore of special interest in this study. The thermotolerant *Campylobacter*, all except *C. helveticus*, including *C. insulaenigrae* are cluster together in a phylogenetic tree based on the 16S rDNA (Figure 2).

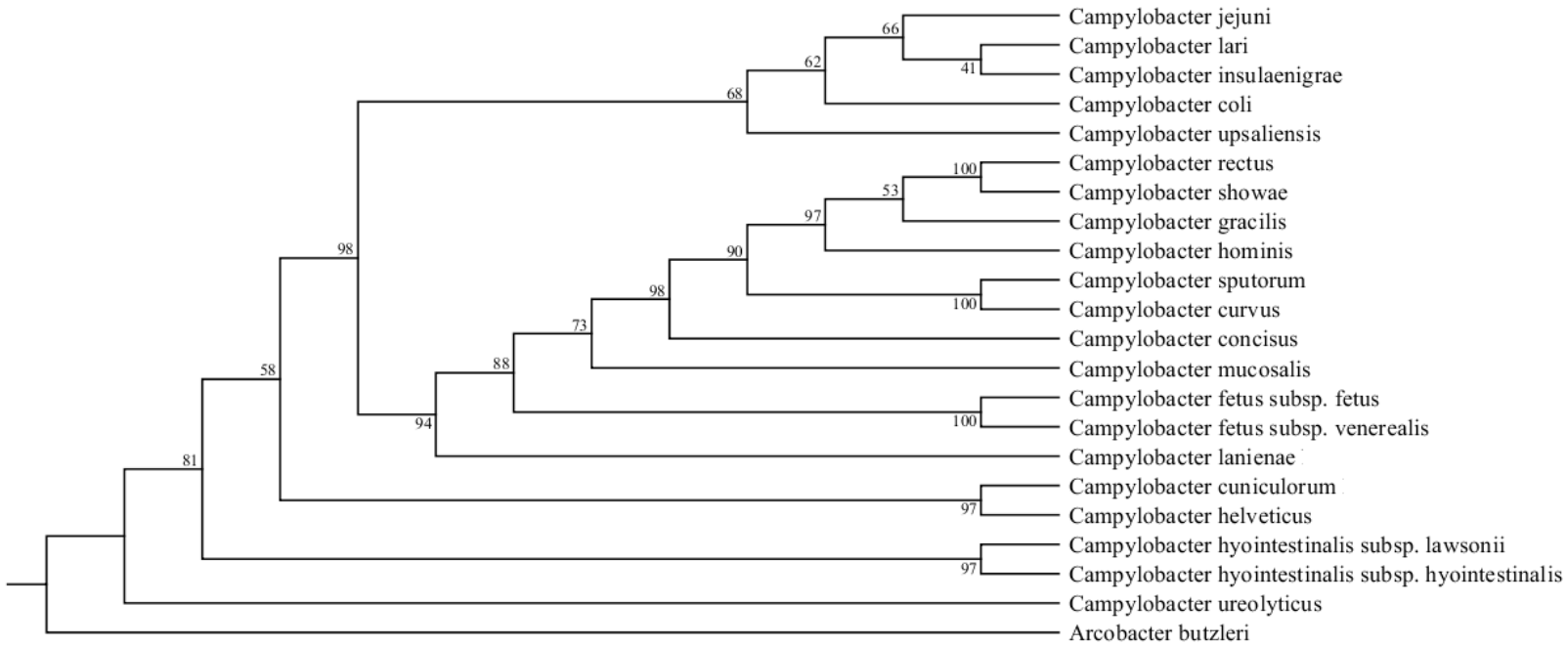

Figure 2. *A neighbour joining phylogeny of the family* Campylobacteraceae*, organized according to similarity in 16S rRNA gene sequence, with root set above* Acrobacter butzleri *using CLC main workbench. The tree was evaluated with the bootstrap resembling technique, 100 replicates, values indicated at nodes.*

Campylobacteriosis

Campylobacter is estimated to cause 5-14 % of all diarrhoea worldwide (WHO, 2012) with most cases caused by *C. jejuni* (80-85 %), *C. coli* (10-15 %) and *C. lari* (Moore et al., 2005). *Campylobacter jejuni* is regarded as the worldwide most common bacterium causing human gastroenteritis. Factor associated with an increased risk for campylobacteriosis is consumption of poultry and contact with domestic pets, such as cats and dogs (Adedayo & Kirkpatrick, 2008; Keramas et al. 2003). Most common sources of infection are meat (predominantly chicken), unpasteurised milk, and water (SMI, 2010). Outbreaks with *Campylobacter* often occur sporadically (SMI, 2008). The number of Swedes diagnosed with *Campylobacter* infections has increased during the last few years and are now around 8000 cases per year (SMI, 2010). During the same period, 2002-2010, direct consumption of fresh and frozen poultry has increased from 13.5 to 16.7 kg per person per year (Jordbruksverket, 2008). In Sweden, about 65 % are infected when travelling abroad. A drastic increase in outbreaks occur during June, July, and August (Figure 3). Incubation time is generally between 1-3 days but can vary between 1-10 days (SMI, 2010). Classical symptoms of a *Campylobacter* infection are diarrhoea, abdominal pain, nausea, vomiting, fever, and headache (Adedayo & Kirkpatrick, 2008). *Campylobacter* can occasionally cause abortion in humans (Garrity et al., 2005). Infections by *C. jejuni* may cause Guillain-Barré syndrome with severe symptoms like muscle weakening and paralysis. A method that can distinguish between *C. coli* and *C. jejuni* is of importance to identify the source of *Campylobacter* (food, water, feed, animals, and patients) and map different routes of infection or contamination (Toplak et al., 2012).

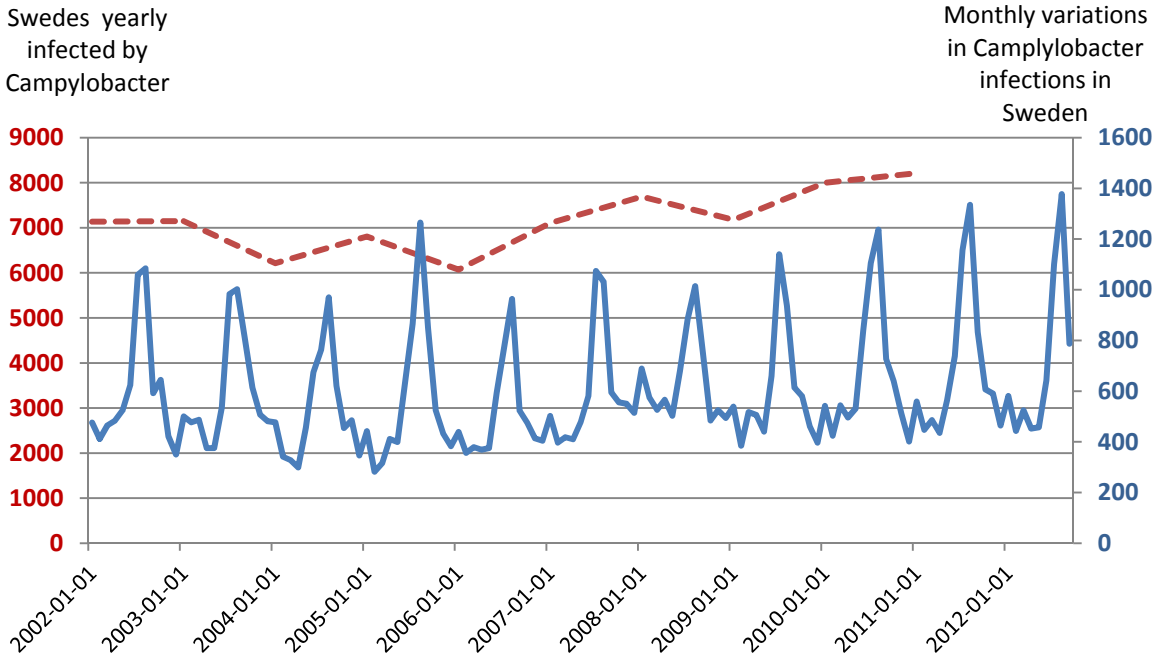

Figure 3. *Total number of identified human* Campylobacter *cases found in Sweden between 2002 and 2012. Statistics are presented in total cases per year (dashed line) and per month (solid line) with a clear seasonal variation. (SMI, 2012).*

Methods for identification

There is no "golden standard" method for detection, species identification or strain characterisation (Anonymous, 2012). Due to the particular growth requirements and phenotypical similarities between *Campylobacter* species, isolation and identification can be difficult (Toplak et al., 2012). There are two main methodologies for identification; phenotypic and genotypic. Methods used in this study are briefly described below but important to note is that there are other methods available for identification. For instance, in order to trace the source and identify the route of transmission in a foodborne outbreak it is important to have a method that distinguishes different strains of the same species, which will not be possible with the methods described below.

Phenotypic methods

Classical detection methods for *Campylobacter* are laborious, time consuming, and sometimes unreliable, but can be used to give an indication of the species. Phenotypical methods can be preceded by culturing on selective agar and incubation in specific climates.

Microscopy

Campylobacter are motile and have a typical corkscrew appearance and are easily recognized in a phase contrast microscope.

Hippurate hydrolysis

Bacteria with the enzyme hippuricase (aminoacylase) are able to hydrolyse hippurate to glycine and benzoic acid (VetBact, 2009). After incubation ninhydrin is added. If free amines are present ninhydrin reacts, giving a purple (Ruhemann's purple) product, while lack of enzyme results in no free amines, giving a colourless product.

To discriminate between *C. jejuni* and all other *Campylobacter* species, a hippurate hydrolysis test is performed in which *C. jejuni* is positive and all others are negative. Though, some strains of *C. jejuni* subsp. *jejuni* give a negative result and 14-50 % of *C. curvus* is positive and can be misinterpreted (Garrity et al., 2005). The hippurate hydrolysis is the only biochemical test to discriminate between *C. jejuni* and *C. coli* (Amri et al., 2007).

Indoxyl acetate hydrolysis

Hydrolysis of indoxyl acetate is analysed by the shift in colour when colony material is added to a disc impregnated with indoxyl acetate (Popovic-Uroic et al., 1990). Hydrolases from the bacteria release indoxyl from the indoxyl acetate and in presence of oxygen indoxyl shifts from colourless to purple.

Strains of *C. coli*, *C. helveticus*, *C. jejuni*, *C. rectus* and *C. upsaliensis* are positive and 60-93 % of *C. curvus* and *C. gracilis* are positive (Garrity et al., 2005). Indoxyl acetate hydrolysis can distinguish *C. lari* from other thermotolerant *Campylobacter*.

Oxidase

An oxidase test is performed to investigate if bacteria has the enzyme cytochrome oxidase in its electron transport chain and thereby can transfer electrons to oxygen. Presence of cytochrome oxidase is detected by application of colony material to an oxidase disc A positive result is indicated by the disc shifting in colour from colourless to blue/purple.

All *Campylobacter* are oxidase positive, except *C. gracilis*, and only 60-93 % of *C. concisus* and 14-50 % of *C. showae* are positive (Garrity et al. 2005).

Genotypic methods

With knowledge of the shortcomings of traditional detection methods the interest for developing new methods based on molecular tools has increased. A molecular based method is of particular interest in food safety analysis where a fast and reliable method is needed.

PCR

Polymerase chain reactions (PCR) are methods based on amplification of a specific DNA fragment, the specific region exponentially amplified varies depending on the purpose of the method. Crucial for amplification during thermal cycling is presence of a DNA polymerase, primers (oligonucleotides) and deoxyribonucleotides. The PCR assay developed in this study was based on the methods by Toplak et al. (2012) and Lübeck et al. (2003). Toplak et al. (2012) suggests that the *cadF* and *hipO* genes are suitable to amplify in order to distinguish between *C. coli* and *C. jejuni* and other *Campylobacter* spp. The *hipO* gene is among previously developed PCR methods the most often used gene for discriminating between *C. jejuni* and other *Campylobacter* (Amri et al., 2007). Lübeck et al. (2003) developed a PCR method by using the ribosomal RNA encoding gene 16S rRNA as a target to detect *Campylobacter*. Besides ribosomal genes, methods are described targeting functional and so called “housekeeping” genes, which are essential for maintenance of vital cellular functions (Bonjoch et al., 2010).

In real-time PCR, the target DNA is detected by an increase in fluorescence measured throughout the analysis, giving the possibility to quantify the amount of DNA in a sample. A multiplex real-time PCR amplifies more than one DNA target in each reaction giving the opportunity to detect different species in a single run (Eberle & Kiess, 2012). In order for a multiplex PCR to work with high efficiency it is recommended to have amplicons of approximately the same length and primers with the same annealing temperature.

Probes give additional specificity to a reaction (Tevfik Dorak, 2006). To generate a signal correct binding of both primer and probe are required. Many PCR instruments of today offer up to six unique excitation/emission filter combinations. This can allow up to six gene assays to be measured within one single multiplex reaction. A prerequisite though is the use of six different reporter dyes, all read by different filters, emitting light at different wavelengths.

Aim

The aim of this study was to optimize and validate a triplex real-time PCR assay for identification of thermotolerant *Campylobacter*, specifically *C. coli* and *C. jejuni*. The triplex real-time PCR is developed in order to be a last step in a qualitative method for identification of thermotolerant *Campylobacter*, originated from food and water suspected to have caused human gastroenteritis. PCR is preceded by enrichment and culturing on selective medium in microaerobic atmosphere at 41.5 °C. The aim was also to compare two different methods for DNA extraction and compare inhibition on the PCR assay if bacterial colonies were grown on blood agar (BA) or modified charcoal cefoperazone deoxycholate agar (mCCDA).

2 Methods

Bacterial strains

Bacterial strains (n = 115) were used to test the inclusivity and exclusivity of the method (Appendix 1). Well characterised strains from the National Food Agency (NFA), *Campylobacter coli* SLV 271, *C. jejuni* SLV 540, and *C. lari* SLV 559, were used as positive controls and included in every PCR assay. *Campylobacter jejuni* strains originating from a previous study by the NFA (Campy-SET, 2005) were tested for inclusivity. A number of different *Campylobacter* species (20 *C. jejuni*, 18 *C. coli*, and 11 *C. lari* strains), two *Helicobacter,* and three *Arcobacter*, including CCUG strains (Culture Collection, University of Göteborg, Sweden), were kindly provided by Eva Olsson Engvall and Boel Harbom, National Veterinary Institute (SVA), European Union Reference Laboratory (EURL) for *Campylobacter*. For exclusivity, purified DNA from pathogens likely to be found in a foodborne outbreak were also included. Lastly, some additional *Campylobacter* strains of unknown origin found in NFA's collections, representing different thermotolerant *Campylobacter* were included.

Culture conditions and media

Pure cultures of bacterial strains were dispersed in brain heart infusion broth (BHI; 237500, Becton Dickinson) containing glycerol (20 % v/v) and stored at -70 °C.

All *Campylobacter* were cultured on BA (CM0331, Oxoid) and some were additionally grown on mCCDA (CM0739, Oxoid). Agars were prepared according to the manufacturer's instructions. All bacterial strains were cultured for a minimum of 48 hours. Strains previously identified as *C. coli*, *C. jejuni*, *C. lari* or *C. upsaliensis* were cultured microaerobically in an atmosphere of 5 % O_2, 10 % CO_2 and 85 % N_2 at 41.5 °C (Jouan IG750 3 Gas/CO_2 incubator with RH control, Thermo Fisher Scientific). Strains of *C. concisus, C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C. helveticus*, *C. hyointestinalis*, *C. insulaenigrae*, *C. mucosalis*, *C. showae*, *Arcobacter butzleri*, *A. cryaerophilus*, *Heliocobacter cineadi* and *H. pullorum* were cultured micoraerobically (CampyGen

CN0025A, Oxoid) at 37 °C (B7395, Termarks). *Campylobacter curvus*, *C. gracilis*, *C. hominis, C. lanianae* and *C. rectus* were cultured anaerobically (AnaeroGen AN0025A, Oxoid) at 37 °C. *Arcobacter skirrowii* was cultured microaerobically (CampyGen CN0025A, Oxoid) at 30 °C (B2324, Termarks).

Phenotypic methods

Nine *Campylobacter* strains (C91, C96, CRL 117-08, CRL 127-08, CRL 143-08, CRL 263-08, CRL 337-08, Cb 7250-04, and Cb 7252-04) were tested for phenotypical traits, to ensure that they had been correctly identified. Included as positive controls were also three reference strains.

Microscopy

All strains were motile and had the characteristic corkscrew appearance under the microscope (BX41, Olympus).

Hippurate hydrolysis

A large amount of colonies were dispersed in 0.4 ml sodium hippurate broth (820648, Merck) and incubated for 4 hours at 37 °C. Thereafter, 0.2 ml ninhydrin (106762, Merck) was cautiously added, followed by an additional incubation for 10 min at 37 °C. Positive result was indicated by a dark blue/purple colour, while negative result gave a light blue or colourless liquid.

Indoxyl acetate hydrolysis

A loop full of fresh colony material was transferred to a disc impregnated with indoxyl acetate (13500, Sigma) and 1-2 drops of distilled water were added. A positive result could after 10 min be read as a shift in colour to blue/green, while a negative result gave no change in colour.

Oxidase

Fresh colony material was transferred to a Bactident® Oxidase strip (113300, Merck). After 20-60 sec the strip changed colour to blue /purple if positive, while a negative result gave no change in colour.

Template preparation

Two methods of DNA extraction were compared; a commercial kit InstaGene™ (Bio-Rad Laboratories Inc., USA) and simple boiling. An isolated colony was resuspended in 50 µl MilliQ water and centrifuged at 10 000 rpm for 1 min. The supernatant was removed and the pellet was dissolved in 200 µl InstaGene™ matrix and incubated at 56 °C for 15 min. The sample was vortexed at high speed for 10 sec and placed in a heating block at 100 °C for 8 min. The sample was vortexed, and spun at 10 000 rpm for 3 min. To avoid chelate beads in the PCR assay, 100 µl of the upper layer of the supernatant were transferred to a new eppendorf tube. To prepare the boiled sample, an isolated colony was resuspended in

50 µl MilliQ water and boiling and spinning was performed as described above. Samples were stored at -20 °C.

DNA purification

Pure colonies of reference strains *C. coli* SLV 271, *C. jejuni* SLV 540 and *C. lari* SLV 559 were resuspended in 10 ml BHI broth and cultured microaerobically at 41.5 °C over night. BHI broths were transferred to Falcon tubes and centrifuged for 15 min at 8000 g. Purification of DNA was performed by DNeasy® Tissue Kit (Qiagen) according to the manufacturer's instructions with some minor changes. Instead of 4 µl of RNase, 10 µl were added to the samples and incubation was extended from 2 to 15 min.

The purity of the DNA was analysed by gel electrophoresis (2 % 0.5 × TBE-agarose gel) and spectrophotometically using a NanoDrop® (ND-1000 Spectrophotometer, Thermo Scientific). By spectrometry the NanoDrop® also measured the concentration of DNA in a sample. Based on the genome size of *Campylobacter* (*C. coli* 1.7 Mb, *C. jejuni* 1.7 Mb and *C. lari* 1.6 Mb) and weight of double stranded DNA (660 g/mole/base) the number of genome copies was calculated (see equation) and the DNA diluted to 10^6 genomes per 5 µl. Pure DNA of known concentration was used as a positive control in all PCR assays.

$$\frac{\text{mass (in grams)} * \text{Avogadro´s number}}{\text{average mol. wt. of a base} * \text{template length}} = \text{molecules of DNA}$$

Primers and probes

The PCR product for thermotolerant *Campylobacter* (including *C. coli*, *C. jejuni*, *C. lari* and some *C. upsaliensis*) was based on amplification of a 287 base pair (bp) sequence of the 16S rRNA gene (Lübeck et al., 2003). Toplak et al. (2012) designed specific primers and probes for *C. coli* and *C. jejuni* targeting the *cadF* and *hipO* genes respectively (Table 1). Primers and probes were purchased from Eurofins MWG Operon, except for the CC_cadF_probe which was from Biosearch Technologies, Inc.

Table 1. Overview of primers and probes included in the triplex real-time PCR

Target gene	Primer or probe	Primer or probe sequence (5' → 3')	Size (bp)	Reference
16S rRNA	OT1559	CTGCTTAACACAAGTTGAGTAGG	287	Lübeck et al. 2003
	18-1	TTCCTTAGGTACCGTCAGAA		
	CT_16S_ probe	FAM-TGTCATCCTCCACGCGGCGT TGCTGC-BHQ2		
cadF	CC_cadF_F	GAGAAATTTTATTTTTATGGTTT AGCTGGT	103	Toplak et al. 2012
	CC_cadF_R	ACCTGCTCCATAATGGCCAA		
	CC_cadF_ probe	CalFluorRed610- CCTCCACTTTTATTATCAAAAGCG CCTTTAGAAA-BHQ2		
hipO	CJ_hipO_F	AATGCACAAATTTGCCTTATAAAAGC	123	Toplak et al. 2012
	CJ_hipO_R	TNCCATTAAAATTCTGACTTGCTAAATA		
	CJ_hipO_ probe	HEX-ACATACTACTTCTTTATTGCTTG-BHQ1		

Optimizing of triplex real-time PCR

The PCR method was based on the optimized duplex PCR method developed by Toplak et al. (2012), in combination with primers and a probe for thermotolerant *Campylobacter* (Lübeck et al., 2003). Primer and probe concentrations optimized by Toplak et al. (2012) were kept as in the original article and concentrations for primers and probe for thermotolerant *Campylobacter* were varied between 0.1-0.8 µM (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 µM) and probe concentrations between 0.1-0.25µM (0.1, 0.15, 0.20, 0.25 µM). Thereafter, the annealing and extension temperature was varied between 53-63 °C to identify optimal results. Results were analysed using the software Bio-Rad CFX Manager, version 1.6 (Bio-Rad Laboratories).

Purified DNA from *C. coli* SLV 271, *C. jejuni* SLV 540 and *C. lari* SLV 559 were diluted to concentrations ranging from 10^1 to 10^5 genomic copies per reaction (per 5 µl). By plotting the obtained threshold cycle (C_t) against the logarithm of the number of genomes in each reaction the linearity (slope) of each PCR-reaction was given. The correlation

coefficient (R^2) and efficiency (E) for each PCR was calculated by the Bio-Rad CFX Manager using the equation: $E = (10^{(-1/\text{slope})} - 1) \times 100$.

After optimization, the reaction mixture contained 5 µl template DNA; 0.6 µM OT1559 and 18-1 primers; 0.3 µM CC_cadF_F and CC_cadF_R primers; 0.8 µM CJ_hipO_F and CJ_hipO_R primers; 0.2 µM of each probe in 2 × Perfecta® Multiplex qPCR Supermix (Quanta BioScience) in a final volume of 25 µl.

After optimization, the program consisted of denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 sec and annealing and extension at 58 °C for 1 min using Bio-Rad CFX96 (Bio-Rad Laboratories). A negative control (NTC) of MilliQ water was included in each run. Samples were analysed in duplicates.

Inhibition

To investigate the PCR inhibitory effect of different agar media (BA and mCCDA), this was analysed using an internal positive control (IPC). The template was prepared from *C. coli* SLV 271, *C. jejuni* SLV 540 and *C. lari* SLV 559 cultured on both medias as described above. A reaction mixture was prepared with 5 µl template DNA; 0.6 µM OT1559; 0.6 µM 18-1; 0.2 µM CT_16S_probe; 2.25 µl primers and probes for IPC (50X Exo IPC DNA 0.4 ng/ml, product code 4304662, Applied Biosystems) and 0.5 µl IPC DNA in a final volume of 25 µl.

Sequencing and nucleotide blast

For nine strains that did not give expected results with the optimized triplex real-time PCR, a part of the genome, the 16S rRNA gene, was sequenced to ensure that the previous species identification was correct.

Amplification of the 16S rDNA was carried out in a reaction mixture containing 3 µl DNA, 2 µl each of 16SS and 16SR primer (5 pmol/µl, Table 2), and one illustra™ PuReTaq™ Ready-To-Go™ PCR bead (GE Healthcare, UK). Nuclease free water was added to a final volume of 25 µl. The PCR assay was initiated by denaturation at 94 °C for 30 sec, followed by annealing at 49 °C for 30 sec and extension at 72 °C for 2 min, repeated 29 times, and finally heated to 72 °C for 10 min (GeneAmp® PCR System 2700, Applied Biosystems).

Table 2. Primers for amplification of 16S rDNA

Primer	Primer sequence (5′ → 3′)
16SS	AGA GTT TGA TCC TGG CTC
16SR	CGG GAA CGT ATT CAC CG

The amplified PCR product was purified with QIAquick® PCR Purification kit (Qiagen), according to the manufacturer's instructions, and analysed, as described above, by gel electrophoresis and spectrophotometically. The purified PCR product was sequenced by the Rudbeck Laboratory, Uppsala. The sequences obtained were analysed by CLC Main workbench version 6.7.1 (CLC Bio). Sequences were trimmed before assembled, resulting in sequences with the length of 1200-1250 bp. The complete 16S rRNA gene is approximately 1500 bp. The obtained sequences were compared to known sequences using the NCBI blast collection (http://www.ncbi.nlm.nih.gov/), search inquiries were limited to bacterial DNA and only perfect matches were included.

3 Results

Phenotypic results

The phenotypical tests gave expected results, and are summarized in Table 3.

Table 3. Summary of results obtained by phenotypic tests, including three reference strains and nine strains giving non-expected results when analysed by triplex real-time PCR. Positive reaction is indicated by (+) while negative result is shown by (-)

	Strain											
Characteristic	*C. coli* SLV 271	*C. jejuni* SLV 540	*C. lari* SLV 559	C91	C96	CRL 117-08	CRL 127-08	CRL 143-08	CRL 263-08	CRL 337-08	Cb 7250-04	Cb 7252-04
Appearance in microscope	+	+	+	+	+	+	+	+	+	+	+	+
Hippurate hydrolysis	–	+	–	+	+	+	+	+	+	+	–	–
Indoxyl acetate hydrolysis	+	+	–	+	+	+	+	+	+	+	–	–
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+

DNA purification and extraction

Gel electrophoresis (Figure 4) and NanoDrop showed no RNA or other nucleotide residuals in the purified DNA, thus the DNA concentration determined spectrophotometically could be assumed to be correct.

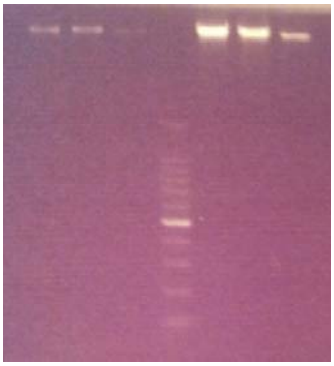

Figure 4. *From left to right the wells contained the following; well 1,* Campylobacter coli *SLV 271 1 µl; 2,* C. jejuni *SLV 540 1 µl; 3,* C. lari *SLV 559 1 µl; 4, ladder 100 bp; 5,* C. coli *SLV 271 5 µl; 6,* C. jejuni *SLV 540 5 µl; 7,* C. lari *SLV 559 5 µl.*

Two methods for template preparation were compared, a commercial kit (InstaGene™) and simple boiling. A last step was included in the method for the commercial kit, in which the upper part of the supernatant was transferred to a new eppendorf tube. This step turned out to be critical, in order to better avoid chelate beads in the PCR reaction. Chelate beads can inhibit the PCR reaction, giving false negative results. By including the last transfer step, this was avoided.

Boiling of colony material resuspended in water is an easy, fast, and cheap method, to get free DNA in solution. However, using an undiluted template could sometimes lead to unusual PCR results (amplification curves with unusual shapes) being obtained because of too high concentrations of DNA.

Optimizing of triplex real-time PCR

Unique primers and probes for *C. coli* and *C. jejuni* had previously been optimized by Toplak et al. (2003) and concentrations for these were therefore kept unchanged. Optimization focused instead on how to best include primers OT1559 and 18-1 and probe CT_16S_probe for thermotolerant *Campylobacter*.

Primers and probe

Figure 5 shows results from optimizing of primers OT1559 and 18-1. Samples with high amounts of DNA (10^5 genome copies/assay) give signals earlier, around cycle 14, while samples containing low amounts of DNA (10^2 genome copies/assay) give signals after cycle 24.

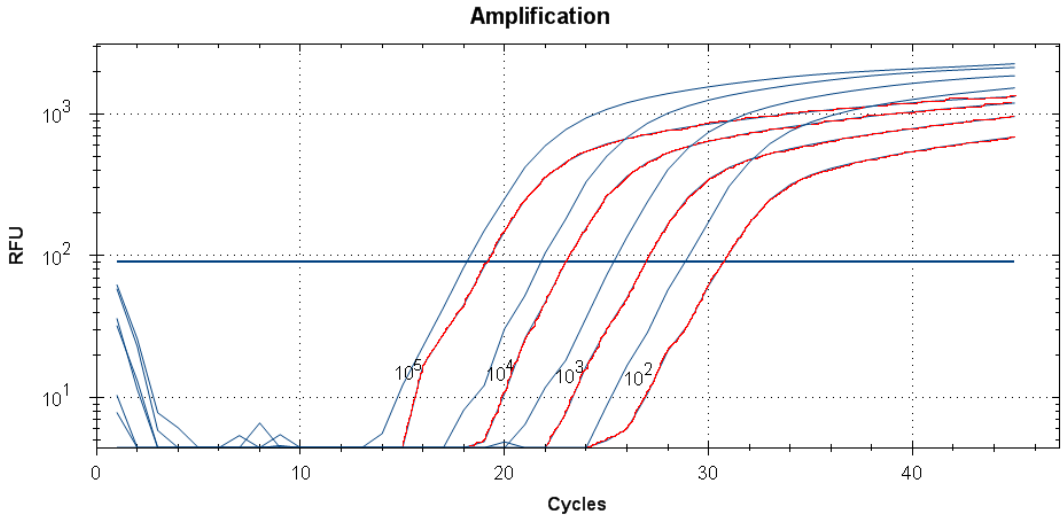

Figure 5. *Optimizing of primers OT1559 and 18-1. Primer concentrations varied between 0.1 µM (red lines) and 0.8 µM (blue lines) and template DNA was added in amounts of 10^2 to 10^5genome copies/reaction.*

Primer concentrations of 0.1 µM (red lines in Figure 5) gave an insufficient efficiency of 73.2 %, while 0.5 µM or higher gave efficiency above 90 % (data not shown). As primer concentration was lowered, it resulted in amplification occurring later shown as a lower relative fluorescence unit (RFU). The primer concentration had a, to some extent, higher impact on the results when template DNA was added in low amounts (10^2 genome copies/assay), shown in Figure 5 by a wider gap between the blue and red line, representing the same amount of template DNA, the gap is wider when the amount of DNA is low. Results showed that there were no major improvements of amplification if primer concentration 0.5 µM or higher was used. Since primers are relatively cheap, an optimal concentration was set to 0.6 µM.

Probe CT_16S_probe was added in concentrations ranging 0.1- 0.25 µM. The results were very similar to the ones shown in Figure 5, with the red lines representing probe concentration of 0.1 µM and blue lines 0.25 µM. Since probes are expensive, as low concentration as possible is to prefer, therefore 0.2 µM was set to optimum.

Temperature

After primers and probes targeting the 16S rDNA had been optimized, changes in temperature had only minor impact on the amplification of the 16S rRNA gene and *cadF*. The span of temperatures evaluated ranged 53-63 °C, in Figure 6 are parts of the results from the 55 and 60 °C experiments shown. There are no big differences in amplification and efficiency, unless very low amounts of template DNA is added which can give a slightly lower RFU if temperature is lowered (the curve to the very right in Figure 6). However, it turned out that a change in temperature had an effect on primers and probes targeting *hipO*.

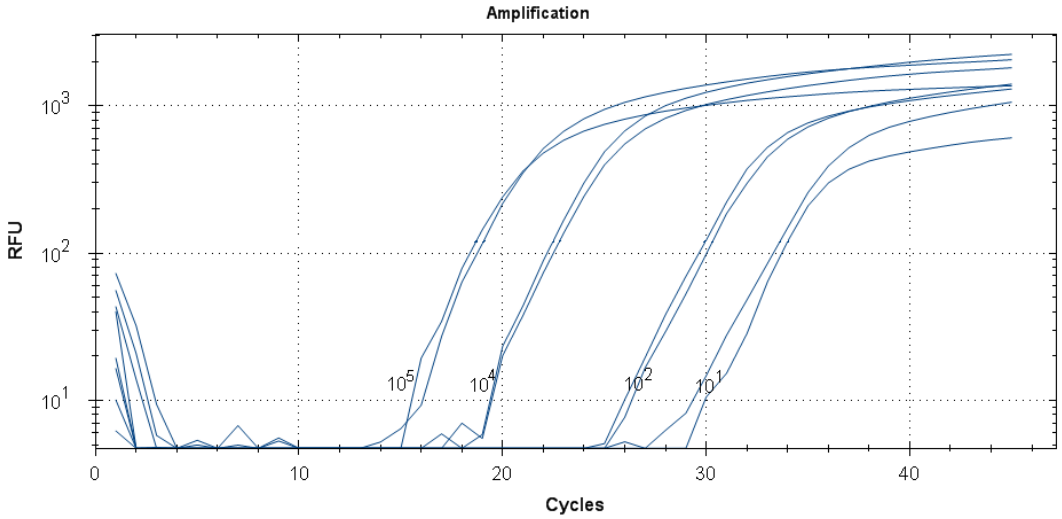

Figure 6. *Results from the 55 and 60 °C experiment, amplification of 16S rRNA gene. A change in temperature has little impact on the results, unless the sample contains very a low amount of template DNA.*

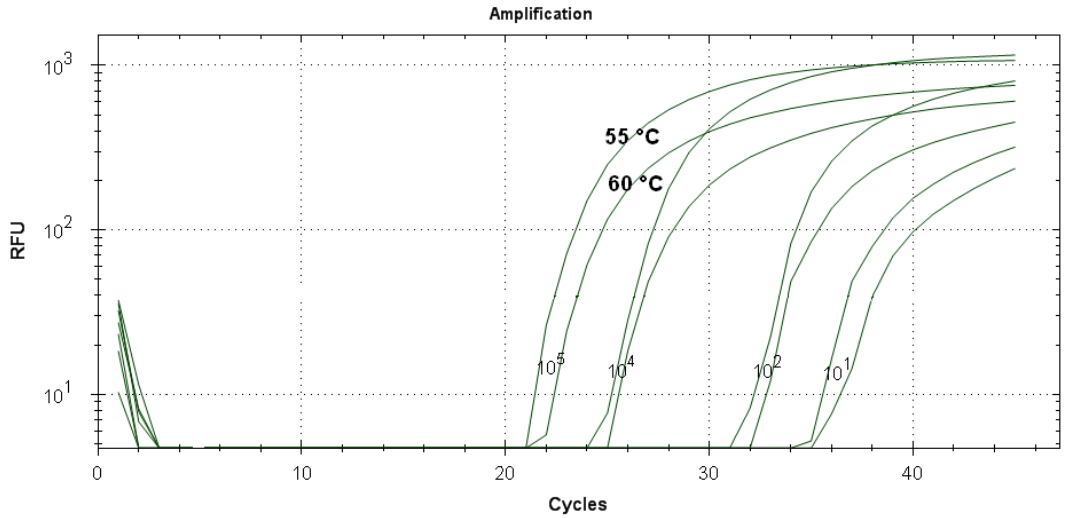

Figure 7. *Results from the 55 and 60 °C experiment. Amplification of* hipO *is favoured by a decrease in temperature.*

Results in Figure 7 are from the same experiment as in Figure 6, showing a different reporter dye, targeting *hipO*. The *hipO* primers and probe are unique for *C. jejuni*. Results show the effect of a change in temperature, amplification is favoured with a decrease in temperature. Optimal temperature, favouring all three sets of primers and probes, was 58 °C (data not shown).

Linearity and efficiency of triplex real-time PCR

Efficiency of the reaction should preferably be between 90-100 % (Tevfik Dorak, 2006), meaning doubling of the amplicon at each cycle. This corresponds to a slope of 3.1-3.6 when threshold cycles (C_t-value) are plotted against log quantity. Factors affecting the efficiency are for example length of amplicon, inhibitors, and primer design. Tevfik Dorak (2006) recommends that real-time PCRs with efficiency lower than 90 % should be further optimized or alternatively redesign of amplicon.

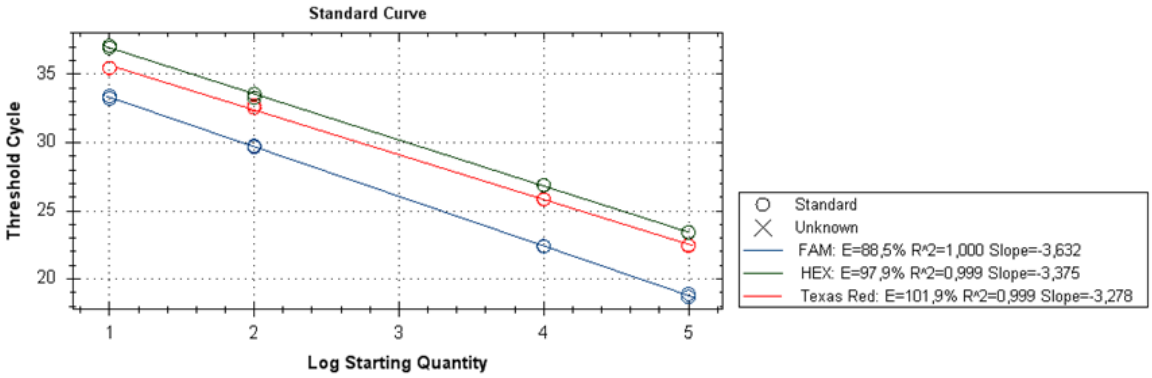

Figure 8. *A typical standard curve from the optimized triplex real-time PCR.*

The efficiency for the 16S rDNA was constant around 90 % (FAM, blue line in Figure 8). The somewhat low efficiency probably is due to that the amplified product is long (278 bp), compared to *hipO* (123 bp) and *cadF* (103 bp). The low efficiency was accepted since it was constant around 90 % and did not improve further as the triplex real-time PCR was optimized.

Inclusivity and exclusivity

The inclusivity and exclusivity was tested using 115 strains. Most isolates gave expected results when analysed the first time but some gave curves like presented in Figure 9. The results in Figure 9 could not be considered positive and needed to be reanalysed.

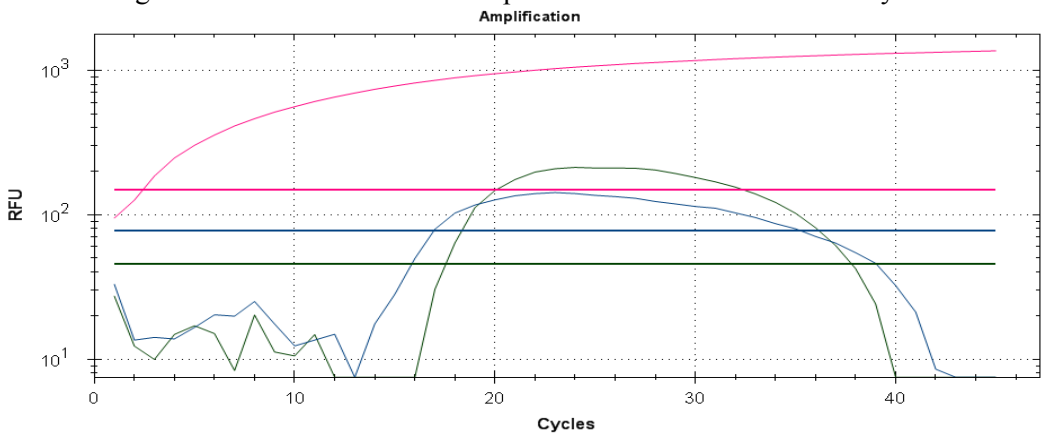

Figure 9. *Examples of unexpected results that occurred as the triplex real-time PCR was tested for inclusivity and exclusivity. None of these results were regarded as positive. Amplification of* cadF *(red) starts too early and has a somewhat linear amplification.* HipO *and* 16S rRNA *(green and blue) start to amplify when expected but at the end the probe seems to collapse, giving an ever weaker signal.*

Sometimes linear rather than exponential amplification of *hipO* occurred (Figure 10) giving doubts whether the results should be interpreted as positive (*C. jejuni*) or not. The results are possibly due to amplification of an atypical *C. jejuni* with one or more base pair mismatching within the binding region of the primers (one or both).

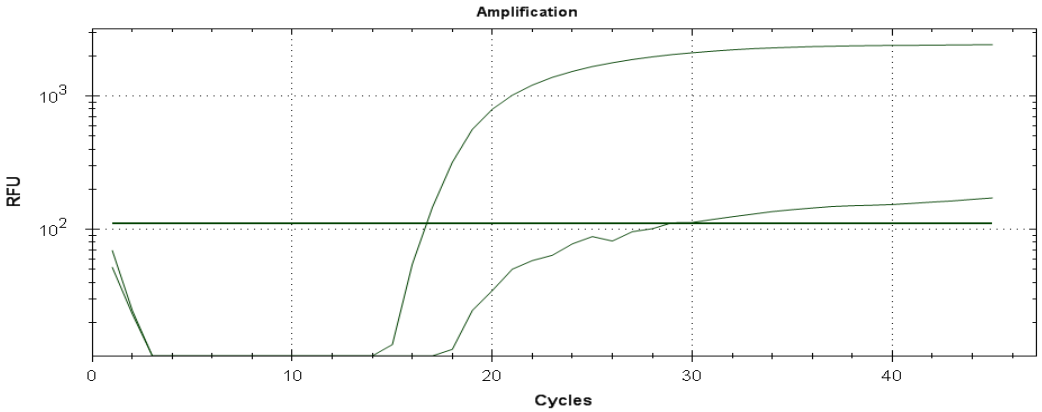

Figure 10. *Results from amplification of* hipO. *The left curve is an example of what an optimal amplification looks like. The curve to the right is rather linear than exponential, but was still interpreted as positive.*

Sequencing and nucleotide blast

For nine *Campylobacter* (7 *C. jejuni* and 2 *C. lari*), that at first did not give expected results when analysed by PCR, a part of the 16S rDNA was sequenced in order to conclude that previous identification was correct. The binding of the 16S rDNA primers to DNA were ruled out as a cause of not giving expected amplification by an alignment of the obtained 16S DNA sequences and the OT1559 and 18-1 primers. Results showed complete matches (no mismatches) of both primers to all our sequences, with starting binding in the region around base 836 of the PCR product obtained using primers 16SS and 16SR and ending around base 1122, giving as expected, an amplified product of 287 bp.

The BLAST search at NCBI gave unambiguous results. Seven strains (C91, C96, CRL 117-08, CRL 127-08, CRL143-08, CRL 263-08, and CRL 337-08) matched with *C. jejuni*, two strains (Cb 7250-04 and Cb 7252-04) matched with *C. lari*, and could thereby with certainty be said to have been correctly identified. The results corresponded to the one derived from the phenotypical tests. And when reanalysed with the optimized triplex real-time PCR all strains gave expected results.

The specificity and inclusivity/exclusivity of the six primers used in the triplex real-time PCR were confirmed by comparison to available sequences using the blast function at NCBI. As expected 100 % matches in *C. coli*, *C. jejuni* and *C. lari* were found. Perfect matches were also found in undefined bacterium ("uncultured bacterium clone"). Unexpectedly *Campylobacter insulaenigrae* (CCUG 48653) were amplified giving a clear signal in the triplex real-time PCR. Available at NCBI were two sequences of the 16S rRNA gene of *C. insulaenigrae* (NCTC 12927, EF433401) available. The partly sequenced genomes of *C. insulaenigrae* were compared with the OT1559 and 18-1 primers, giving a 87 % identity with the OT1559 primer, only differing in 3 out of 23 base pairs, and a complete match with 18-1 (Figure 11), resulting in an amplicon of 286 bp.

Primers from the study by Lübeck et al. (2003) were designed for thermotolerant *Campylobacter*, and should if designed correctly give positive results for all *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus*. However, Lübeck did not include *C. helveticus* in his definition of thermotolerant *Campylobacter*. According to the study by Lübeck et al. (2003) only one out of five analysed *C. upsaliensis* strains gave a positive result. In our study two of seven *C. upsaliensis* gave positive results when analysed by the triplex real-time PCR. The reason for the inconsistency in the results is to be found in the binding region of primer OT1559. As seen in Figure 11, the partly sequenced genome of *C. upsaliensis* matched with the OT1559 primer in 18 of 23 base pairs (78 %, 5 mismatches). *Campylobacter helveticus* (NCTC 12472) differs in the same region in 7 of 23 base pairs, giving too many mismatches for sufficient binding and amplification.

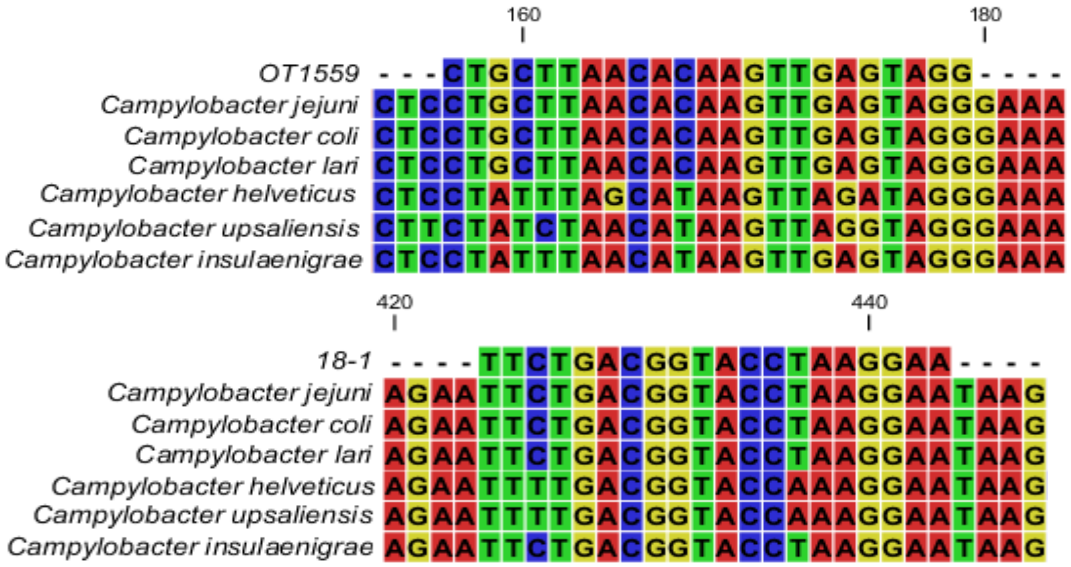

Figure 11. *Upper picture shows primer OT1559, and the lower 18-1, aligned to 16S DNA sequences of thermotolerant* Campylobacter *and* C. insulaenigrae. *Number above sequences indicates where, to what nucleotides, the primers bind.*

Inhibition

An indication of inhibition is when amplification curves appear later than the curves of NTC (Figure 12). The aim was to study whether the agar medium could have a negative effect on the PCR reaction, and if lysates prepared from colonies grown on BA or mCCDA were preferable. No differences in inhibition were observed between the two culturing media. However, inhibition was observed and the main problem turned out to be transfer of InstaGene™ chelate beads to the PCR assay. InstaGene™ beads are supposed to be spun down to the bottom of the eppendorf tube but can accidently be pipetted to the PCR reaction when loading the template DNA. The problem was solved with a minor modification of the template preparation method.

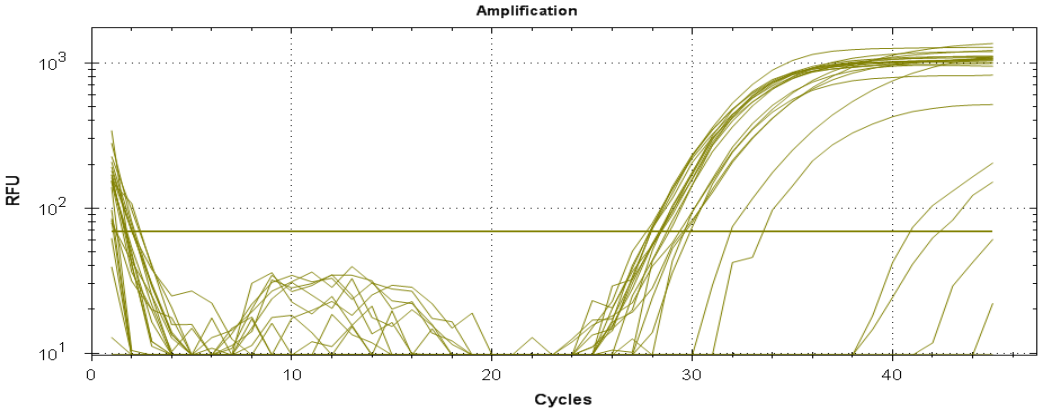

Figure 12. *Example of inhibition. NTC started amplify at cycle 25, all curves starting amplifying later are partly inhibited. Totally inhibited samples are not amplified and do not give a signal.*

4 Discussion

Template preparation

The triplex real-time PCR method developed in this study is intended to be a last step in a qualitative method for isolation and identification of thermotolerant *Campylobacter,* and specifically to identify *C. coli* and *C. jejuni* strains. The method will be used in situations where there is a need to identify the source of a foodborne outbreak of human gastroenteritis. In these cases, normally the microorganism causing the disease has been isolated from patients and identified. At the NFA, the food suspected of causing the outbreak will be analysed for the presence and isolation of this organism to enable further typing to strain level. Thus, the triplex real-time PCR will be preceded by selective isolation (atmosphere and media) of thermotolerant *Campylobacter*. Commonly, after isolation, single colonies are transferred from selective mCCDA to BA before further characterisation of the isolate as it is well known that selective media may interfere with e.g. phenotypic tests. However, this will delay the identification and therefore, the possibility of preparation of template DNA from both agar media without effect on the results is to prefer. We saw no effect of culturing media on the PCR results, DNA template can be prepared from both BA and mCCDA. However, the way template DNA was extracted could interfere with the PCR reaction. Both the commercial kit and boiling of colony material had advantages and disadvantages. Boiling of colony material is easy, cheap and fast, though some problems with inhibition were noticed and care has to be taken not to use too high DNA concentrations. The InstaGene™ kit on the other hand is also easy to use but requires some extra work, and extraction taking a little longer than boiling. There is also an additional cost associated with the commercial kit, but money may be well spent if a reanalyse of the triplex real-time PCR can be avoided. The problems first discovered with the InstaGene™ kit can be solved if methods described above are followed and that is recommended in future analysis.

Evaluation of optimized triplex real-time PCR

The developed triplex real-time PCR is fast and seems robust but has its drawbacks. The robustness has *per se* not been studied, but the fact that primers and probe concentrations

for 16S rDNA as well as annealing temperature can be varied without major effects on the results, indicate that the PCR is robust. Primers and probes specific for *C. jejuni* and *C. coli* worked very well, discriminating between the two species, with 100 % inclusivity and exclusivity. However, the results for primers and probe meant to be specific for thermotolerant *Campylobacter* lacked in both inclusivity and exclusivity. All *C. jejuni*, *C. coli*, and *C. lari* strains were included (amplified) by the 16S rDNA primers but only two of seven *C. upsaliensis* strains gave positive results. These results correspond to the original article (Lübeck et al., 2003), in which one of five *C. upsaliensis* gave positive results. One strain of *C. helveticus* was analyzed and was not included, but one strain of *C. insulaenigrae* was included even though it has not been recognized as thermotolerant. None of the non-Campylobacter species tested was amplified. That *C. helveticus* is not amplified is regarded as a minor problem since it has never been reported causing human gastroenteritis (Garrity et al., 2005). Since *C. upsaliensis* rarely causes campylobacteriosis, the fact that not all strains gave a positive result, is not regarded as a problem when the assay is used for the purpose described above. However, for assays when identification of *C. upsaliensis* is also required, other primers have to be used. *Campylobacter insulaenigrae* is closely related to *C. lari* (Figure 2) and was first isolated in 2004 (Foster et al., 2004). The recent discovery of the species explains why it is not mentioned in the latest publication of Bergey's Manual of Systematic Bacteriology published in 2005. So far there has been no report on *C. insulaenigrae* causing human gastroenteritis, and the strain cannot grow at 42 °C, thus amplification is regarded as a minor problem.

Further development of a multiplex real-time PCR

Examples of previously developed PCR methods, not all of them real-time PCR assays, are presented below, with no intention on being comprehensive but to rather inspire further development of a multiplex real-time PCR for thermotolerant *Campylobacter*. The first PCR for *Campylobacter* was developed in 1992, targeting the *flaA* gene of *C. jejuni* and *C. coli* (Moore et al., 2005). Since then a number of PCR methods for *C. jejuni* and *C. coli*, often in combination with addition of restriction enzymes, have been developed. In several previously developed PCR assays, the 16S rRNA gene has been used for the genus *Campylobacter*, but the gene cannot sufficiently distinguish between closely related species (Settanni & Corsetti, 2007).

Bonjoch et al. (2010) recently developed a multiplex real-time PCR assay for detection of *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*. The method targeted the gene *cje0832* encoding the ATP-binding protein CJE0832 to detect *C. coli* and *C. jejuni*, whereas the *bipA* gene, a member of the "GTP-binding elongation" family was targeted to detect *C. lari* and *C. upsaliensis*. Bonjoch et al. use the same fluorophore (6-FAM) for all probes and were thereby not able to discriminate between the species. A combination of the two methods, parts of Bonjoch's and Toplak's, could possibly result in a triplex real-time PCR fulfilling sufficient detection and discrimination between *C. coli*, *C. jejuni* and *C. lari*.

Another possibility is to combine the method developed by Toplak et al. with parts of the multiplex PCR method by Yamasaki-Matsune et al. (2007). Yamasaki-Matsune et al. developed a multiplex PCR consisting of seven pairs of primer, generating amplicons of varying sizes from 86 to 816 bp, giving both genus-specific and species-specific bands for six *Campylobacter* species. The amplicons of 86 bp (*lpxA*) and 251 bp (*glyA*) are specific for *C. upsaliensis* and *C. lari* respectively, and could due to their small size be suitable for a real-time PCR. In real-time PCR the amplicon should ideally have a maximal length of 250 bp (Tevfik Dorak, 2006). The combined methods will however not cover all thermotolerant *Campylobacter*, but the most frequent *Campylobacter* species associated with food- and waterborne outbreaks.

Numerous assays have been developed with the aim to distinguish between *C. coli* and *C. jejuni*, but no study has yet succeeded in discriminating all thermotolerant *Campylobacter* from non-thermotolerant ones. The one that came closest is Klena et al. (2004) were they developed a multiplex PCR for differentiation of thermotolerant *Campylobacter* (not including *C. helveticus*), amplifying parts of the housekeeping gene *lxpA*. Klena et al. have done a detailed study of the *lxpA* gene and developed novel primers for a multiplex PCR discriminating between *C. coli*, *C. jejuni*, *C. lari* and *C. upsaliensis*. The PCR method gave almost 100 % inclusivity and 97 % exclusivity. However the method is a traditional PCR, not a real-time PCR, and results are interpreted as bands on an agarose gel, with band varying in size between 206 -391 bp. The two smallest amplicons of 206 bp and 233 bp represents *C. upsaliensis* and *C. lari*, respectively. The two amplicons are of sizes that could suit a real-time PCR.

The future is probably to develop primers and probes for strains, rather than for a larger group of *Campylobacter*, especially since researchers do not fully agree on what strains should be included in the concept of thermotolerant *Campylobacter*.

5 Acknowledgments

I am grateful to my supervisor at the National Food Agency, Karin Jacobsson for her expertise, supportive personality and always being available whenever I had questions.

Stefan Roos, my supervisor at the Swedish University of Agricultural Sciences, for critical reading of the essay.

Eva Olsson Engvall and Boel Harbom, SVA, for providing this study with a large collection of reference strains, *Campylobacter* and *Arcobacter*.

Ronnie Eriksson for assistance in interpretation of PCR results.

Last but not least I give my warmest thanks to all the people working at the division of microbiology at the National Food Agency for making me look forward to come to work every day.

6 References

Adadayo, O. & Kirkpatrick, B.D. (2008). Campylobacter infections: update on presentation, diagnosis, and management. *Hospital Physician*, 44 (7) 9-15.

Amri, A.A., Senok, A.C., Ismael, A.Y., Al-Mahmeed, A.E. & Botta, G.A. (2007). Multiplex PCR for direct identification of campylobacter spp. In human and chicken stools. *Journal of Medical Microbiology*, 56, 1350-1355.

Anonymous (2012). *EURL-Campylobacter. Work programme for 1st of January 2012 to 31st of December 2012*. [online] Available at: http://ec.europa.eu/food/food/controls/reference_laboratories/docs/wp_2012 _eu_ reference_laboratory_for_campylobacter_en.pdf [Accessed 28 November 2012].

Bonjoch, X., Calvó, L., Soler, M., Ruiz-Rueda, O. & Garcia-Gil, L. (2010). A new multiplex real-time PCR assay to detect *Campylobacter jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*. *Food Analytical Methods*, 3, 40-46.

Botteldoorn, N., Van Coillie, E., Piessens, V., Rasschaert, L. Debruyne, L., Heyndrickx, M., Herman, L. & Messens, W. (2008). Quantification of *Campylobacter* spp. in chicken carcass rinse by real-time PCR. *Journal of Applied Microbiology*, 105, 1909-1918.

Campy-SET (2005). *Campylobacter: smittspårning, epidemiologi och typning*. Livsmedelsverket, Rapport 15/2005. Available at: http://www.slv.se/upload/dokument/rapporter/bakterier_virus_mogel/15_2005_%20 Livsmedelsverket.pdf [Accessed 3 January 2013].

Garrity, G.M., Bell, J.A. & Lilburn, T., (2005). Order I. Campylobacterales *ord. nov.*. In: G.M. Garrity (Ed.) 2005. *Bergey´s Manual of Systematic Bacteriology: Volume Two: the Proteobacteria*. 2 ed. New York: Springer.

Eberle, K.N. & Kiess, A.S. (2012). Phenotypic and genotypic methods for typing *Campylobacter jejuni* and *Campylobacter coli* in poultry. *Poultry Science Association Inc.*, 91, 255-264.

Foster, G., Holmes, B., Steigerwalt, A.G., Lawson, P.A., Thorne, P., Byrer, D.E., Ross, H.M., Xerry, J., Thomson, P.M. & Collins, M.D. (2004). *Campylobacter insulaenigrae* sp. nov., isolated from marine mammals. *International Journal of Systematic and Evolutionary Microbiology*, 54, 2369-2373.

Humphrey, T., O'Brien, S. & Madsen, M., (2007). Campylobacters as zoonotic pathogens: A food production perspective. *International Journal of Food Microbiology*, 117, 237-257.

Jordbruksverket (2008). *Jordbriuksverkets statisktikdatabas*. [online] Available at: http://statistik.sjv.se/Dialog/statfile2.asp [Accessed 22 November 2012].

Keramas, G., Bang, D.D., Lund, M., Madsen, M., Rasmussen, S.E., Bunkenborg, H., Tellerman, P. & Christensen, C.B.V. (2003). Development of sensitive DNA microarray suitable for rapid detection of *Campylobacter* spp.. *Molecular and Cellular Probes*, 17, 187-196.

Klena, J.D., Parker, C.T., Knibb, K., Ibbit, J.C., Devane, P.M.L., Horn, S.T., Miller, W.G. & Konkel, M.E. (2004). Differentiation of *Campylobacter coli*, *Campylobacter jejuni*, *Campylobacter lari*, and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid A gene *lpxA*. *Journal of Clinical Microbiology,* 42 (12) 5549-5557.

Lübeck, P.S., Wolffs, P., On, S.L.W., Ahrens, P., Rådström, P. & Hoorfar, J. (2003). Toward an international standard for PCR-based detection of food-borne thermotolerant campylobacters: Assay development and analytical validation. *Applied and Environmental Microbiology,* 69 (9) 5664-5669.

Moore, J.E., Corcoran, D., Dooley, J.S.G., Fanning, S., Lucey, B., Matsunda, M., McDowel, D.A., Mégraud, F., Millard, B.C., O'Mahony, R., O'Riordan, L., O'Rourke, M., Rao, J.R., Rooney, P.J., Sails, A. & Whyte, P. (2005). Campylobacter. *Veterinary Research*, 36, 351-382.

Popovic-Uroic, T., Patton, C.M., Nicholson, M.A. & Kiehlbauch, J.A. (1990). Evaluation of indoxyl acetate hydrolysis test for rapid differentiation of *Campylobacter*, *Helicobacter*, and *Wolinella* species. *Journal of Clinical Microbiology*, 28 (10) 2335-2339.

Settanni, L. & Corsetti, A. (2007). The use of multiplex PCR to detect and differentiate food- and beverage-associated microorganisms: a review. *Journal of Microbiological Methods*, 69, 1-22.

SMI (2008). *Vilka drabbas av Campylobacter-infektioner?* [online] Available at: http://www.smittskyddsinstitutet.se/temaar-2008-zoonoser-och-klimatforandringar/fokusomraden/livsmedel/campylobacter-infektioner--hur-vanliga-och-kan-man-vara-infekterad-av-flera-varianter-samtidigt/ [Accessed 8 November 2012].

SMI (2010). *Sjukdomsinformation om campylobacterinfektioner*.[online] Available at: http://www.smittskyddsinstitutet.se/sjukdomar/campylobacterinfektion/ [Accessed 8 November 2012].

SMI (2012). *Statistik för campylobacterinfektioner*. [online] Available at: http://www.smi.se/statistik/campylobacterinfektion/ [Accessed 13 November 2012].

Tevfik Dorak, M. ed. (2006). *Real-time PCR*. London: Taylor & Francis Ltd.

Toplak, N., Kovač, M., Piskernik, S., Smole Možina, S. & Jeršek, B. (2012). Detection and quantification of *Campylobacter jejuni* and *Campylobacter coli* using real-time multiplex PCR. *Journal of Applied Microbiology,* 112, 752-764.

VetBact (2009). *Biochemical tests*. [online] Available at: http://www.vetbact.org/vetbact/?biochemtest=1 [Accessed 22 November 2012].

WHO (2012). *Water-related diseases*. [online] Available at: http://www.who.int/water_sanitation_health/diseases/campylobacteriosis/en/ [Accessed 13 November 2012].

Yamazaki-Matsune, W., Taguchi, M., Seto, K., Kawahara, R., Kawatsu, K., Kumeda, Y., Kitazato, M., Nukina, M., Misawa, N. & Tsukamoto, T. (2007). Development of a multiplex PCR assay for identification of *Campylobacter fetus, Campylobacter hyointestinalis* subsp. *hyointestinalis*, *Campylobacter jejuni, Campylobacter lari* and *Campylobacter upsaliensis*. *Journal of Medical Microbiology*, 56, 1467-1473.

7 Appendix 1: List of bacterial strains

Table 4. List of *Campylobacter* and non-*Campylobacter* strains used to test inclusivity and exclusivity

Strain	**ID**	**Origin**
Arcobacter butzleri	CCUG 30485	Human feces, diarrhoea
Arcobacter butzleri	CCUG 32549	Human feces, 16-yr-old patient
Arcobacter skirrowii	CCUG 10374	Lamb feces, 5-month-old, persistent scour(diarrhoea)
Campylobacter coli	CCUG 11283	Porcine feces
Campylobacter coli	SLV 271 / CCUG 45147	Hen feces
Campylobacter coli	CRL 2-09	Carcass
Campylobacter coli	CRL 59-08	Carcass
Campylobacter coli	CRL 110-09	Caecum
Campylobacter coli	CRL 113-08	Caecum
Campylobacter coli	CRL 125-08	Caecum
Campylobacter coli	CRL 204-08	Carcass
Campylobacter coli	CRL 206-08	Caecum
Campylobacter coli	CRL 213-08	Carcass
Campylobacter coli	CRL 230-08	Caecum
Campylobacter coli	CRL 261-08	Carcass
Campylobacter coli	CRL 262-08	Caecum
Campylobacter coli	CRL 280-08	Caecum
Campylobacter coli	CRL 295-08	Caecum
Campylobacter coli	CRL 338-08	Carcass
Campylobacter coli	CRL 339-09	Caecum
Campylobacter coli	CRL 354-08	Caecum

Campylobacter coli	CRL 366-08	Caecum
Campylobacter coli	F926-2012	Unknown
Campylobacter coli	ZC 177	Unknown
Campylobacter concisus	CCUG 13144	Human gingival sulcus
Campylobacter curvus	CCUG 13146	Human alveolar (or jaw) abscess
Campylobacter fetus subsp. *fetus*	CCUG 6823	Sheep fetus brain
Campylobacter fetus subsp. *venerealis*	CCUG 538	Vaginal mucosa of heifer
Campylobacter gracilis	CCUG 27720	Human gingival sulcus
Campylobacter helveticus	CCUG 30682	Cat feces
Campylobacter hominis	CCUG 45161	Human feces
Campylobacter hyointestinalis subsp. *hyointestinalis*	CCUG 14169	Porcine intestine, proliferative ileitis
Campylobacter insulaenigrae	CCUG 48653	Marine mammal
Campylobacter jejuni	CCUG 11284	Bovine feces
Campylobacter jejuni	SLV 540	Chicken, Kristianstad, Sweden
Campylobacter jejuni	CRL 6-09	Carcass
Campylobacter jejuni	CRL 39-09	Caecum
Campylobacter jejuni	CRL 58-08	Carcass
Campylobacter jejuni	CRL 117-08	Carcass
Campylobacter jejuni	CRL 117-09	Caecum
Campylobacter jejuni	CRL 127-08	Caecum
Campylobacter jejuni	CRL 143-08	Caecum
Campylobacter jejuni	CRL 194-08	Caecum
Campylobacter jejuni	CRL 216-08	Caecum
Campylobacter jejuni	CRL 227-08	Caecum
Campylobacter jejuni	CRL 248-08	Caecum
Campylobacter jejuni	CRL 260-08	Caecum
Campylobacter jejuni	CRL 263-08	Carcass
Campylobacter jejuni	CRL 277-08	Caecum
Campylobacter jejuni	CRL 278-08	Carcass
Campylobacter jejuni	CRL 336-08	Carcass
Campylobacter jejuni	CRL 337-08	Caecum
Campylobacter jejuni	CRL 351-08	Caecum
Campylobacter jejuni	CRL 364-08	Carcass
Campylobacter jejuni	C12	Poultry product
Campylobacter jejuni	C13	Poultry product
Campylobacter jejuni	C17	Poultry product

Campylobacter jejuni	C20	Poultry product
Campylobacter jejuni	C26 green	Poultry product
Campylobacter jejuni	C26 red	Poultry product
Campylobacter jejuni	C30	Poultry product
Campylobacter jejuni	C33	Poultry product
Campylobacter jejuni	C36	Poultry product
Campylobacter jejuni	C40	Poultry product
Campylobacter jejuni	C48	Poultry product
Campylobacter jejuni	C54	Poultry product
Campylobacter jejuni	C62	Poultry product
Campylobacter jejuni	C67	Poultry product
Campylobacter jejuni	C70	Poultry product
Campylobacter jejuni	C71	Poultry product
Campylobacter jejuni	C72	Poultry product
Campylobacter jejuni	C82	Poultry product
Campylobacter jejuni	C87	Poultry product
Campylobacter jejuni	C91	Poultry product
Campylobacter jejuni	C96	Poultry product
Campylobacter jejuni	C102	Poultry product
Campylobacter jejuni	C108	Poultry product
Campylobacter jejuni	C110	Poultry product
Campylobacter jejuni	C114	Poultry product
Campylobacter jejuni	C118	Poultry product
Campylobacter jejuni	C119	Poultry product
Campylobacter lanienae	CCUG 44467	Human, asymptomatic worker in an abattoir
Campylobacter lari	CCUG 20707	Sea gull, UPTC
Campylobacter lari	CCUG 20708	Sea gull, UPTC
Campylobacter lari	CCUG 22396	Human appendicitis, UPTC
Campylobacter lari	CCUG 23947	Herring gull, *Larus argentatus*, cloacal swab
Campylobacter lari	CCUG 23948	Dog feces
Campylobacter lari	SLV 559 / Cb 227-99	Gull
Campylobacter lari	CRL 80-09	Caecum
Campylobacter lari	CRL 272-08	Caecum
Campylobacter lari	Cb 165-98	Gull
Campylobacter lari	Cb 192-87	Duck

Campylobacter lari	Cb 193-87	Duck
Campylobacter lari	Cb 221-99	Gull
Campylobacter lari	Cb 227-99	Gull
Campylobacter lari	Cb 7250-04	Chicken, cloaca
Campylobacter lari	Cb 7252-04	Chicken, cloaca
Campylobacter mucosalis	CCUG 6822	Porcine small intestine
Campylobacter rectus	CCUG 20446	Human periodontal pocket
Campylobacter showae	CCUG 30254	Human gingival crevice
Campylobacter upsaliensis	CCUG 14913	Canine feces
Campylobacter upsaliensis	CCUG 20818	Human stool
Campylobacter upsaliensis	CCUG 34019	Canine feces
Campylobacter upsaliensis	CCUG 34023	Canine feces
Campylobacter upsaliensis	C1002	Unknown
Campylobacter upsaliensis	C1004	Unknown
Campylobacter upsaliensis	C1005	Unknown
Escherisia coli	U226	Purified DNA
Escherisia coli O157	SLV 479	Purified DNA
Helicobacter cinaedi	CCUG 18818	Human rectal swab
Helicobacter pullorum	CCUG 33837	Broiler at slaughter
Listeria monocytogones	SLV 513	Purified DNA
Salmonella typhimurium	SLV 248	Purified DNA
Shigella dysenterieae	15/08	Purified DNA
Staphylococcus aureus	SLV 438	Purified DNA

8 Appendix 2: Popular summary in Swedish

Ny analysmetod utvecklad av Livsmedelsverket för att hitta *Campylobacter* i livsmedel

Om maten vi äter eller vattnet vi dricker inte har tillagats eller renats på rätt sätt kan det orsaka matförgiftning. Den bakterie som oftast orsakar matförgiftning kallas *Campylobacter* och varje år drabbas ca 8000 svenskar av *Campylobacter*. Bakterien finns över hela världen och det räcker att man får i sig ett fåtal bakterier för att man ska bli sjuk. Andra bakterier som ofta orsakar matförgiftning är salmonella, stafylokocker och kolera. Även virus, svampar (mögel) och parasiter kan orsaka matförgiftning. År 2010 drabbades till exempel dricksvattnet i Östersund av ett stort utbrott av parasiten *Cryptosporidium* och över 20 000 personer blev sjuka. Vad gäller *Campylobacter*, som vi har studerat närmar i denna studie, orsakar bakterien sällan stora utbrott. De vanligaste smittkällorna är rå kyckling, opastöriserad mjölk och vatten från åar och bäckar som inte har renats. Drabbas man av *Campylobacter* får man diarré, ont i magen, blir illamående och kräks. I sällsynta fall kan man även drabbas av en förlamningssjukdom som kallas Gullain-Barré syndrom.

Det finns olika arter av *Campylobacter*, de som oftast drabbar människor kallas *Campylobacter jejuni* som orsakar mellan 80 -85 % av fallen, följt av *C. coli* som är upphov till ca 10-15 % av alla Campylobacter-utbrott. När människor drabbas av matförgiftning och blir så sjuka att de måste uppsöka vård är det viktigt att ta reda på vilken mikroorganism (bakterie, virus, svamp eller parasit) de drabbats av för att kunna sätta in rätt behandling, t.ex. ge rätt antibiotika. Det är därmed nödvändigt att kunna skilja mellan olika arter av *Campylobacter*.

Livsmedelsverket har i denna studie utvecklat en ny analysmetod för att på ett snabbare och mer effektivt sätt ta reda på vilken art av *Campylobacter* som orsakat matförgiftning; *C. jejuni*, *C. coli* eller andra så kallad termotoleranta *Campylobacter*. Kännetecknade för termotoleranta *Campylobcater* är att de kan växa i temperaturer upp till 42 ºC och begreppet inkluderar *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. helveticus*. Metoden som har utvecklats kallas PCR (polymerase chain reaction) och har sin utgångspunkt i två tidigare publicerade metoder som man nu slagit samman till en. Mekanismerna för PCR bygger på att man letar efter DNA från *Campylobacter*. DNA är den genetiska koden för

allt levande, våra arvsanlag och gener. I traditionella PCR-metoder detekterar man en gen, men unikt för denna nya metod är att man kan detektera tre gener samtidigt i samma analys; en gen unik för *C. jejuni*, en för *C. coli* och en för termotoleranta *Campylobacter*. Att söka efter flera gener samtidigt sparar tid och pengar.

För att testa hur effektiv metoden är analyserades 115 prov representerande olika *Campylobacter* men även närbesläktade mikroorganismer och bakterier som ofta orsakar matförgiftning. Metoden hittade alla *C. jejuni* och *C. coli* och fungerade perfekt för dessa arter. Vad gäller den större spretiga gruppen termotoleranta Campylobacter hittade metoden alla *C. jejuni*, *C. coli* och *C. lari*, två av sju *C. upsaliensis* och inte *C. helveticus*. Att *C. helveticus* inte plockas upp ses inte som något större problem då den arten aldrig har isolerats från människa och är därmed mycket osannolik att finna i livsmedel. Resultaten vi fick för *C. upsaliensis* stämmer väl överens med de från tidigare studier och då bakterien ytterst sällan orsakar matförgiftning ses det inte som något större problem att man inte hittar alla *C. upsaliensis*. Viktigast att finna är *C. jejuni* och *C. coli* då de är de *Campylobacter* som oftast orsakar matförgiftning hos människor, vilket metoden klarar och syftet med studien anses därmed uppfyllt.

Genom att både ta prov från patienten och livsmedlet som man tror har orsakat matförgiftningen och analysera dessa med Livsmedelsverkets metod kan man se om provsvaren stämmer överens. Gör de det så vet man vilket livsmedel det var som orsakade matförgiftningen. Oftast tar man in flera livsmedel för analys och letar efter flera olika mikroorganismer. Hela analysmetoden tar dock ett par dagar och detta är något man kan titta vidare på, hur man kan förkorta analystiden ytterligare för att få ännu snabbare svar.