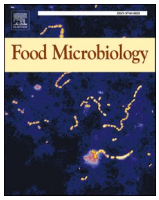
Food Microbiology 108 (2022) 104117

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Combining enrichment with multiplex real-time PCR leads to faster detection and identification of *Campylobacter* spp. in food compared to ISO 10272–1:2017

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ARTICLE INFO

Keywords:
EMRT-PCR
Rapid detection
Food samples
Enrichment
Molecular detection

ABSTRACT

Conventional protocols for the detection of *Campylobacter* from foods are laborious and time-consuming. This research describes an alternative procedure (EMRT-PCR) for the detection of *Campylobacter* from food by combining ISO 10272–1:2017 enrichment in Bolton broth (BB) with a multiplex real-time (MRT-) PCR assay. Species differentiation was done by targeting *C. jejuni* (*mapA*), *C. coli* (*ceuE*), and both species (*cje*). The detection limit of the MRT-PCR assay was 4.5 and 5.5 $\log_{10}$ cfu/ml in BB and BB containing chicken skin, respectively. A Monte Carlo simulation was conducted to predict the probability that campylobacters reach the MRT-PCR detection threshold throughout enrichment in BB, and results suggested that cold-stressed campylobacters could reach the detection limit after 40 h of enrichment ($p = 0.99$). As a proof of principle, 23 naturally contaminated meat products were enriched according to ISO 10272–1:2017 procedure A, and the EMRT-PCR in parallel. After 24 h, 12 and 11 samples already tested positive for *Campylobacter* with the ISO method and EMRT-PCR, respectively. After 40 h, the 24-h-negative sample was also positive with EMRT-PCR. The EMRT-PCR takes about 2 days to produce reliable results, while results using ISO 10272–1:2017 can take up to 8 days, which demonstrate the potential of the EMRT-PCR method.

1. Introduction

In 2020, the foodborne pathogen *Campylobacter* was the cause of the most reported zoonotic gastroenteritis cases in the European Union (EU) and has been doing so since 2005 (EFSA & ECDC, 2021). The latest EFSA report showed that illness was mostly associated with *Campylobacter jejuni* (88.1% of confirmed cases of which species information was provided) and *C. coli* (10.6%) followed by *C. fetus, C. upsaliensis* and *C. lari* (associated with less than 0.2%, each) (EFSA & ECDC, 2021). According to the latest EFSA report, *Campylobacter* was the fourth most frequently reported cause of foodborne outbreak in Europe but fortunately, hospitalization and death rates are rather low (7.1% and 0.04% of all reported illness cases, respectively) (EFSA & ECDC, 2021). In the United States of America, 9% of the foodborne illnesses is estimated to be caused by *Campylobacter* spp. annually (Scallan et al., 2011). *Campylobacter* is frequently found in foods such as raw milk, meat and meat products (broiler and turkey meat) (EFSA & ECDC, 2019; Rossler et al., 2019) but, to a lesser extent, was also isolated from fresh fruits and vegetables (Mohammadpour et al., 2018). Conventional methods for the qualitative detection of low levels of *Campylobacter* spp. make use of an enrichment followed by isolation and confirmation steps. Although theoretically, a single cell in 10 g of food product should be detectable, procedures are lengthy. In case of ISO 10272–1:2017 procedure A (ISO-A), a 48-h enrichment is followed by an isolation step of 48 h and confirmation steps of another 72–96 h, which means that negative results can be obtained after 4 days, while confirmed positive results can be obtained only after 7–8 days. Other culturing-based methods (e.g., direct plating on selective agar) can shorten detection times but have a higher detection limit. Furthermore, limited selectivity of the media has shown to complicate reliable detection due to overgrowth of competitors in culturing and plating media and subsequent masking of *Campylobacter* colonies (Chon et al., 2017; Hazeleger et al., 2016). Moreover, insufficient supply of microaerobic conditions during *Campylobacter* enrichment with common competitors resulted in decreased cell concentrations of *C. jejuni* after enrichment and possible detection failures (Lanzl et al., 2022).

Molecular detection approaches such as (quantitative) polymerase-chain reaction (qPCR) or immunological methods like enzyme-linked

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https://doi.org/10.1016/j.fm.2022.104117
Received 11 March 2022; Received in revised form 26 July 2022; Accepted 14 August 2022
Available online 19 August 2022

immunosorbent assays (ELISA) are generally less time-consuming and are often utilized in clinical settings (Granato et al., 2010; Shams et al., 2017; Valledor et al., 2020). However, it has been suggested that in samples with a complex matrix such as poultry, the utilization of quantitative PCR may be preferable over immunosorbent assays (Reis et al., 2018). Although molecular assays are much faster compared to culturing-based detection of pathogens, their detection limit is also considerably higher; generally, a minimal cell concentration between 10^3-10^5 cfu/ml is required for reliable results using PCR, and slightly lower concentrations are required for real-time PCR (Wang and Salazar, 2016). Furthermore, the sensitivity of qPCR is often affected negatively by the presence of interfering compounds or competing microbiota when food products are used as sample matrix (Jasson et al., 2010). Several approaches to molecularly detect campylobacters have been developed (e.g., Adekunle et al., 2019; De Boer et al., 2015; Melero et al., 2011; Nouri Gharajalar et al., 2020; Overesch et al., 2020; Seliwiorstow et al., 2015), however, in most applications, faecal material was used as sample matrix instead of food in order to identify and monitor highly colonized broiler batches. Often, *Campylobacter* concentrations are quite high in faecal samples (>6 $\log_{10}$ cfu/ml) (Seliwiorstow et al., 2015), while *Campylobacter* numbers are often much lower in foods (from less than 1 up to 3 $\log_{10}$ cfu/ml) (Sampers et al., 2010).

A considerable amount of research has been conducted to develop protocols utilizing qPCR for the detection of campylobacters from food products and some aimed at excluding the enrichment step completely (Josefsen et al., 2010; Liu et al., 2017; Papić et al., 2017; Schnider et al., 2010; Toplak et al., 2012; Ugarte-Ruiz et al., 2012; Vondrakova et al., 2014; Yang et al., 2003). Although cell concentrations can be already sufficiently high for detection through qPCR, Melero et al. (2011) showed that in 6% of naturally contaminated samples, *C. jejuni* could only be detected by qPCR after a 48-h enrichment, and Vondrakova et al. (2014) showed that food samples spiked with different concentrations of campylobacters could only be detected through qPCR when cell concentrations were above 10^2 cfu/ml. The initial contamination level therefore is a restricting factor for the utilization of direct molecular detection.

Therefore, a preceding enrichment step is often necessary to increase cell concentrations to reach the detection threshold of nucleic-acid-based procedures, and additionally, resuscitate sub-lethally injured campylobacters (He and Chen, 2010; Ivanova et al., 2014; Josefsen et al., 2004; Lanzl et al., 2020; Mayr et al., 2010; Rantsiou et al., 2010; Sails et al., 2003). Ivanova et al. (2014) demonstrated that *C. jejuni* was detected from slaughtering environment samples through real-time PCR from colonies grown on modified charcoal cefoperazone-deoxycholate agar (mCCDA) after enrichment in BB. Mayr et al. (2010) combined a 40–48 h enrichment in Preston broth (PB) with MRT-PCR to detect different *Campylobacter* species. Other studies applied modified versions of ISO 10272–1:2006, either utilizing blood-free Bolton broth (Rantsiou et al., 2010; Sails et al., 2003) or a modified sample preparation step (He and Chen, 2010) before enrichment in Bolton broth. The ISO 10272–1:2017 protocol states that an enrichment in Bolton broth should be applied when campylobacters are expected to be sub-lethally injured or stressed in examined food products (International Organization for Standardization, 2017), which can be expected for raw meat products which are often transported and stored at refrigeration- or freezing temperatures (Bhaduri and Cottrell, 2004; Lanzl et al., 2020; Maziero et al., 2010) and Bolton broth showed to be sufficiently nutritious for the recovery and growth of freeze-stressed campylobacters (Lanzl et al., 2022). Josefsen et al. (2004) followed the ISO 10272:2006 procedure and were able to detect campylobacters after conducting an enrichment in Bolton broth, however, differentiation between species within the group of thermotolerant campylobacters was not achieved. The aim of this study was to develop a rapid but reliable protocol for the detection and differentiation of *C. jejuni* and *C. coli* from food products combining enrichment in Bolton broth (based on ISO-A) with MRT-PCR, to, on the one hand allow repair of damaged *Campylobacter* and detect low levels of campylobacters, but on the other hand shorten the detection time compared to the ISO-A method, with identification included as an additional facet.

Table 1
Overview of sequences of primers and probes (cje, mapA, ceuE, IAC) used in this study. Fluorophores used (HEX, FAM, TexasRed and Cy5) are marked in bold while quenchers (Deep dark quencher 1 [DDQ 1], Black hole quenchers 2 and 3 [BHQ2 and BHQ3]) are marked in italics.

Target gene	Species	Primer/ probe	Sequence (5′-3′)
ATP-binding protein (*cje*)	*C. jejuni* & *C. coli*	Fw-primer	AGT GCC GAT AAA GGC TCA
		Rv-primer	ACT CGT CGA GCT TGA AGA ATA CG
		Probe	**HEX**-AAG CCA CTC TTT GCA TTT GTC CGC C-*DDQ1*
Membrane lipoprotein (*mapA*)	*C. jejuni*	Fw-primer	CTG GTG GTT TTG AAG CAA AGA TT
		Rv-primer	CAA TAC CAG TGT CTA AAG TGC GTT TAT
		Probe	**FAM**-TTG AAT TCC AAC ATC GCT AAT GTA TAA AAG CCC TTT-*DDQ1*
Periplasmic substrate binding protein (*ceuE*)	*C. coli*	Fw-primer	AAG CTC TTA TTG TTC TAA CCA ATT CTA ACA
		Rv-primer	TCA TCC ACA GCA TTG ATT CCT AA
		Probe	**TexasRed**-ATC ATG AAT GAT TCC AAA GCG AGA TTG AGG TCC A-*BHQ2*
Internal amplification control (*IAC*)	puC18	Fw-primer	CTG GCG TTT TTC CAT AGG CTC C
		Rv-primer	GGG GAA ACG CCT GGT ATC TTT A
		Probe	**Cy5**-CCT GAC GAG CAT CAC AAA AAT CGA CGC TCA A-*BHQ3*

2. Materials and methods

2.1. Bacterial strains and preparation of working cultures

17 *C. jejuni* and 12 *C. coli* isolates were used to test the probes- and primer specificity in this study (details of all *Campylobacter* strains used in this study can be found in Table S1 of the supplementary materials). For the preparation of working cultures, *C. jejuni* and *C. coli* were plated from the −80 °C vials onto Columbia agar base (CAB, Oxoid, supplemented with 5% (v/v) lysed horse blood (BioTrading Benelux B.V. Mijdrecht, Netherlands) and an additional 0.5% agar (Bacteriological agar No.1, Oxoid)) and grown for 24 h at 41.5 °C. Subsequently, a single colony was resuspended in Heart Infusion broth (HI; Bacto HI, Becton, Dickinson and Company) and cultured for 24 h at 41.5 °C to obtain stationary phase cultures. Afterward, a 1:500 dilution was made in unselective BB (Oxoid, supplemented with 5% (v/v) sterile lysed horse blood (BioTrading Benelux B.V., Mijdrecht, the Netherlands) without the addition of selective supplements) and cultured for 24 h at 41.5 °C to reach the stationary phase. Cell concentrations of the working cultures were determined by plating appropriate dilutions (made in peptone physiological salt solution (PPS, Tritium Microbiologie)) on CAB plates and incubation for 48 h at 41.5 °C. Throughout this study, *Campylobacter* was cultured under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) in flushed jars (Anoxomat WS9000, Mart Microbiology, Drachten, Netherlands).

2.2. MRT-PCR assay for detecting and differentiation C. jejuni and C. coli

For the preparation of the primer/probe mix for MRT-PCR (as further explained in section 2.5), primers and probes of four target genes were used, namely an ATP-binding protein (*cje*), a membrane lipoprotein

(*mapA*), a periplasmic substrate binding protein (*ceuE*) and an internal amplification control (IAC) (Table 1). Primer sequences for *cje* were obtained from a publication by Bonjoch et al. (2010), while primer sequences of *mapA* and *ceuE* were obtained from the Dutch Food Safety Authority (NVWA) and modified to ensure similar melting temperatures. All probes as well as the primer sequences of the IAC were developed using SnapGene Viewer (software version 4.2.11). Primers and probes were diluted in sterilized MilliQ to achieve a primer and probe concentration of 500 and 100 nM in the final assay, respectively. iQ Multiplex Powermix (BIORAD) was used to provide buffer, iTaq DNA polymerase and dNTPs. Since primer sequences for *cje*, *mapA* and *ceuE* were taken from recent publications or protocols and therefore, their individual specificity was not further tested.

The specificity of each primer/probe set was validated for four *C. jejuni* (NCTC 11168, 81–176, WDCM 00005 and ATCC 33560) and four *C. coli* isolates (ATCC 33559, WDCM 00004, Ca 2800 and Ca 1607) as well as purified pUC18 DNA in qPCR experiments using four different master mixes, each containing one primer/probe set (*cje, mapA, ceuE* and IAC) (data not shown). After specificity was confirmed in qPCR, the specificity of the multiplex primer/probe set (containing all four primer/probe sets (*cje, mapA, ceuE* and IAC)) was tested by performing MRT-PCR using working cultures of 17 *C. jejuni* and 12 *C. coli* isolates (as prepared in section 2.1). For DNA extraction, 1 ml of working culture of each strain was transferred to a 1.5 ml Eppendorf-tube and cells were lysed in a heat-block at 95 °C for 15 min, cooled down to room-temperature and 10 μl were used as template for MRT-PCR.

2.3. Determination of MRT-PCR detection limits

To determine the lower detection limit of MRT-PCR in different matrices and to extract DNA from enrichment samples, a cell lysis and DNA extraction step was performed using the GENE-UP Lysis kit (Biomérieux). The protocol provided by the supplier was followed with one modification, namely a sample volume of 10 μl instead of 20 μl (as stated in the GENE-UP Lysis kit protocol) was used.

The detection sensitivity of the primers and probes was tested with a subset of strains, namely four *C. jejuni* (NCTC 11168, 81–176, WDCM 00005 and WDCM 00156) and four *C. coli* (WDCM 00004, Ca 2800, Ca 1607 and ATCC 33559) isolates. Working cultures were prepared as stated in section 2.1 and 10-fold dilutions were made in three different matrices, namely HI, BB (BB base supplemented with 5% (v/v) sterile lysed horse blood and 0.5% (v/v) selective supplement SR0208) and the pooled liquids of three 48-h-BB-enrichments conducted with chicken skin that was tested *Campylobacter*-free. After DNA extraction, an MRT-PCR assay was conducted. Standard curves were generated for each primer-probe set and species tested by plotting the Ct from each dilution versus cell concentration and quantification cycles (Cq), correlation coefficients (R^2) and linear ranges were determined. The corresponding standard curves can be found in Fig. S1 of the supplementary materials. This way, thresholds were determined for each primer/probe set and matrix concerning the minimal relative fluorescent units (RFU) and range of Cq-values samples had to comply with, in order to be categorized as positive for *C. jejuni* or *C. coli*. As a result, it was determined that, for each target gene/fluorophore, an amplification curve had to reach a minimal RFU of 500 during the quantification cycles 15–35 in order to be viewed as positive. Furthermore, for each sample, a positive result was required for the IAC to rule out inhibition by matrix-components. Since this MRT-PCR protocol contained three primer/probe sets (*cje* for the detection of both *C. jejuni* and *C. coli*, and *mapA* and *ceuE* for the specific detection of *C. jejuni* and *C. coli,* respectively), an enrichment sample was only viewed as positive for *C. jejuni*, when both the amplification curves for *cje* and *mapA* complied with the set criteria. Likewise, a sample was only viewed positive for *C. coli*, when the amplification curves for *cje* and *ceuE* were compliant with the settings.

2.4. Determination of sampling times for enrichment samples

To determine the sample time points of interest during enrichment, a Monte Carlo simulation was conducted with 100,000 events to predict the probability that campylobacters reached the MRT-PCR detection limit throughout enrichment in BB at various initial cell concentrations varying from −2 to 2 $\log_{10}$ cfu/ml, to simulate contamination levels of 1 to 10^4 cfu/10 g of food that is diluted in 90 ml of BB at the start of the enrichment. For the simulation, the lag-duration (5.4 ± 1.3 h) and growth rate (0.42 ± 0.09 $\log_{10}$ h^{-1}) of freeze-stressed campylobacters during enrichment in BB was used (Lanzl et al., 2020). In the beforementioned study, the growth kinetics of 23 *Campylobacter* strains during enrichment in BB was tested and the variability in lag-duration and growth rate was assessed. For the strain selection, *C. jejuni* and *C. coli* strains isolated from different sources (food, human and environment) were chosen. The data was used for the simulation to predict the probability of severely stressed campylobacters to reach the detection limit of MRT-PCR.

2.5. Analysis of naturally contaminated food samples

As food samples, predominantly raw poultry products were used in this study since prevalence of *Campylobacter* is high compared to other foods (Zbrun et al., 2020). In total, 18 poultry and 5 beef products were examined. Samples were purchased at a local butcher and transferred to 4 °C for approximately 2 h before enrichment was conducted. Of each food product, 10 g was collected and used as sample for the enrichment procedure. For poultry products containing skin, as much of the skin was collected for the 10 g of sample required for the enrichment procedure. Subsequently, an enrichment was conducted according to ISO-A (using BB as enrichment medium). For that, 10 g of food product was transferred into a stomacher bag, diluted with 90 ml of BB and homogenized with a stomacher machine (Seward Stomacher Model 400) for 60 s at 230 rpm. To check whether samples were already highly contaminated at the start of the enrichment, 100 μl of the initial dilution was spread on Rapid *Campylobacter* agar (RCA; BIORAD) plates, which were incubated and subsequently counted to determine the cell concentration. In parallel, the enrichment bag was placed in an Anoxomat jar, which was flushed with a microaerobic gas mixture (5% O_2, 10% CO_2, 85% N_2) using an Anoxomat (WS9000, Mart Microbiology, Drachten, Netherlands) and incubated according to ISO-A (5 h at 37 °C followed with 43 h at 41.5 °C). After 24, 40 and 48 h samples were taken, and jars were flushed again and re-incubated for the remaining enrichment time. For the ISO detection, 10 μl were taken at each time point and cells were isolated according to the ISO-A protocol with the amendment that RCA was used as a selective isolation medium instead of mCCDA. Typical *Campylobacter* colonies were taken and confirmed through microscopy. Next to that, 1.1 ml of sample was used to determine cell concentrations using RCA-plates. Agar plates were incubated microaerobically for 48 h at 41.5 °C and cell concentrations (range: 1.0–5.5 $\log_{10}$ cfu/ml) were determined. For the EMRT-PCR procedure, 10 μl of sample were taken at each time point and cell lysis and DNA extraction were performed using the GENE-UP lysis kit protocol (as explained in section 2.3). Afterward, 10 μl of each sample was transferred into one well of a 96-well PCR-plate (Hard-Shell 96-well PCR plate HSL9001, BIORAD) filled with 15 μl of iQ Multiplex Powermix (BIORAD), 5 μl of primer/probe mix as prepared according to Table S3 of the supplementary materials and 1 μl of IAC sample DNA (250 ng/μl). After filling, the PCR-plate was sealed using the Microseal 'B' PCR Plate Sealing Film (MSB1001, BIORAD) and spun down at 3,800 rpm for 10 s. The plate was then transferred into the PCR-machine (CFX96, BIORAD) and run according to the following settings: Initial denaturation for 2:30 min at 95 °C followed by 40 PCR cycles consisting of 15 s at 95 °C and 60 s at 60 °C.

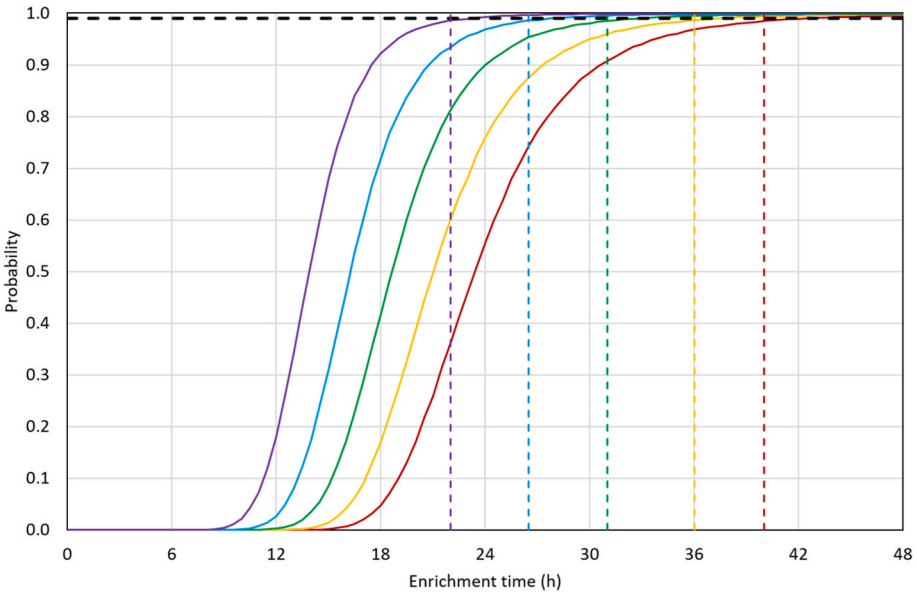

Fig. 1. Estimation of probability of campylobacters to reach the detection limit of MRT-PCR (5.5 log_{10} cfu/ml) throughout enrichment in BB at different cell concentrations at the start of enrichment. A Monte Carlo simulation was conducted utilizing the average lag-duration and growth rate of campylobacters during enrichment in BB after freeze-stress (5.4 ± 1.3 h and 0.42 ± 0.09 log_{10} h^{-1}, respectively). The Initial cell concentrations used for the simulation were 10^4, 10^3, 10^2, 10 and 1 cell in 10 g of food product, corresponding to 2, 1, 0, −1 and −2 log_{10} cfu/ml in enrichment, respectively. The respective line colours in the figure are depicted in purple, blue, green, orange and red). The detection probability of 0.99 is depicted in a striped, black line. For each initial cell concentration simulated, the enrichment time (h) was calculated, at which the probability of detection is 0.99 and depicted as vertical stripes lines in the respective colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results and discussion

3.1. Validation of primer/probe sets

All 17 *C. jejuni* and 12 *C. coli* strains were identified correctly using the primer/probe sets as depicted in Table 1. The primer/probe set for *cje* gave amplification curves for all *Campylobacter* strains, while the primer/probe sets for *mapA* and *ceuE* gave only amplification curves for *C. jejuni* and 12 *C. coli*, respectively (detailed results are depicted in Table S2 in the supplementary materials).

For this MRT-PCR assay, the primer/probe set *cje* (specific for *C. jejuni* and *C. coli*) was used in combination with two primer/probe sets specific for each of the two species. This served as double confirmation since food samples were only labelled positive for a species when amplification results were positive for *cje* as well as for one of the species-specific primer/probe sets (*mapA* or *ceuE*). The target species of the current assay were *C. jejuni* and *C. coli*, since these two species are most associated with campylobacteriosis (EFSA & ECDC, 2021). A further assay extension may also include less prominent species such as *C. lari* or even *C. upsaliensis* (Klena et al., 2004; Wang et al., 2002). Research suggests that primer/probe sets of *gyrA* or *peptT* could be used to reliably detect *C. lari* in rt-PCR assays (Chapela et al., 2015; He and Chen, 2010). However, when designing primers and probes the lengths of the newly designed primer pairs should be comparable to the other primer-sets in the assay (<100 bp differences between amplicons) (Ricke et al., 2019), to ensure that the time-temperature settings of the assay remain the same. Also, the choice of fluorophore and quencher is important to avoid fluorescence resonance energy transfer (FRET). After adaptation of the current setup, the assay should be revalidated to reconfirm species specificity and sensitivity of the assay (On et al., 2013).

The detection limits of the MRT-PCR in spiked BB were 4.5 log_{10} cfu/ml, for both *Campylobacter* species. However, when chicken skin was added as food sample in both media, the detection limited increased to 5.5 log_{10} cfu/ml. It has been recognized that food matrices can contain substances which can inhibit (q)PCR reactions (Schrader et al., 2012). A study by Schnider et al. (2010) found, that Cq values corresponding to *C. jejuni* and *C. coli* in a real time-PCR assay were more than five times higher in spiked buffered peptone water (BPW) containing broiler skin samples than in spiked BPW without the food matrix, indicating the presence of PCR inhibitors in the food matrix. Consequently, sample preparation protocols and DNA isolation methods have been developed to remove such inhibitors. In this study, different DNA extraction and isolation protocols were tested such as the application of thermal cell lysis (95 °C, 10 min), Chelex-100, the DNA isolation kits WIZARD Genomic DNA Purification Kit (Promega), GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich), DNeasy Blood &Tissue Kit (Qiagen) and the GENE-UP lysis protocol. In pure cultures, a thermal cell lysis step of 10 min was sufficient for subsequent detection of campylobacters through MRT-PCR, and longer (15, 20, 30 min) lysis steps did not enhance the MRT-PCR signal (no increase in RFU or lower Cq-values). In more complex matrices such as chicken meat products, the thermal lysis step was not sufficient to remove PCR inhibitors. The GENE-UP lysis protocol, however, proved to be fast and user-friendly to isolate bacterial DNA from an enriched food sample for subsequent MRT-QPCR analysis.

3.2. Determining enrichment sampling times for MRT-PCR detection

According to ISO-A, enrichment in BB is conducted for 48 h before the detection step takes place. To determine possible earlier detection time points, a Monte Carlo simulation was conducted to predict the probability that campylobacters reached the MRT-PCR detection limit throughout enrichment in BB at various initial cell concentrations. Data on the growth kinetics of freeze-stressed campylobacters was used to simulate enrichment of severely stressed cells, which need a longer recovery time resulting in a later reach of the required cell concentrations for the application of MRT-PCR. The probability of reaching the detection limit of 5.5 log_{10} cfu/ml throughout the course of enrichment for different initial cell concentrations is depicted in Fig. 1. The detection limit was reached already after 22 h of enrichment with a probability of 0.99 when the initial cell concentration was high (2 log_{10} cfu/ml), while for the lowest inoculum (−2 log_{10} cfu/ml, simulating 1 cell per 10 g of food sample) an enrichment duration of 40 h was needed. Apart from the 48-h sampling point, which is the initial duration of the ISO-A enrichment step, two additional sampling points were selected based on the simulation outcomes. In case a food product initially already contained relatively high amounts of *Campylobacter*, detection through MRT-PCR should be possible after 24 h (50% of the original enrichment time), while on the other hand, in a worst-case scenario (1 cell per 10 g of food), cell concentrations should reach the MRT-PCR detection limit within 40 h.

Table 2
Detection outcomes of food samples using the traditional ISO 10272–1:2017 procedure A protocol (ISO-A) and EMRT-PCR consisting of an enrichment step in BB followed by MRT-PCR. Cell concentrations were assessed at the beginning of enrichment (0 h) and samples were observed as negative (−) when cell concentrations were below the detection limit of 1 $\log_{10}$ cfu/ml BB. For ISO-A, detection outcomes were observed as negative (−) when cell concentrations were below 1 $\log_{10}$ cfu/ml BB, whereas outcomes were observed as positive (+) when cell concentrations were above 5.5 $\log_{10}$ cfu/ml BB. For food products 13 and 14, cell concentrations of enrichment samples after 24 h were positive, but did not reach the set detection limit of MRT-PCR. For the alternative procedure, EMRT-PCR assays were carried out for all samples and outcomes were observed as negative (−), if the MRT-PCR curves did not meet the set assay thresholds. Samples were labelled as positive (+), when the threshold settings were met and using species-specific primer/probe sets, distinctions could be made between *C. jejuni* (Cj) and *C. coli* (Cc).

Nr.	Food samples	0 h	24 h		40 h		48 h	
		Cell concentration ($\log_{10}$ cfu/ml BB)	ISO-A	EMRT-PCR	ISO -A	EMRT-PCR	ISO -A	EMRT-PCR
1	Chicken filet	-	-	-	-	-	-	-
2	Chicken drumstick	**1.8**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**
3	Chicken wing (marinated)	-	-	-	-	-	-	-
4	Chicken shawarma	-	-	-	-	-	-	-
5	Chicken liver	-	-	-	-	-	-	-
6	Minced beef	-	-	-	-	-	-	-
7	Chicken wing	-	+	**+ (Cj)**	+	**+ (Cj)**	+	**+ (Cj)**
8	Chicken hearts	-	-	-	-	-	-	-
9	Chicken thigh filet	-	-	-	-	-	-	-
10	Beef steak	-	-	-	-	-	-	-
11	Chicken shawarma	-	-	-	-	-	-	-
12	Minced beef	-	-	-	-	-	-	-
13	Chicken strips (marinated)	-	**+ (4.7 $\log_{10}$ cfu/ml BB)**	**+ (Cj)**	+	**+ (Cj)**	+	**+ (Cj)**
14	Chicken saté	-	**+ (2.7 $\log_{10}$ cfu/ml BB)**	-	+	**+ (Cj)**	+	**+ (Cj)**
15	Merguez sausage	**1.5**	+	**+ (Cj)**	+	**+ (Cj)**	+	**+ (Cj)**
16	Beef liver	-	+	**+ (Cj)**	+	**+ (Cj)**	+	**+ (Cj)**
17	Chicken liver	**1.9**	+	**+ (Cj)**	+	**+ (Cj)**	+	**+ (Cj)**
18	Chicken drumstick (marinated)	-	-	-	-	-	-	-
19	Chicken drumstick	**1.5**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**
20	Turkey leg	-	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**
21	Chicken leg	-	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**
22	Chicken wing	**1.3**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**
23	Chicken thigh filet	-	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**	+	**+ (Cj + Cc)**

3.3. Detection of campylobacters from food samples using ISO 10272–1:2017 procedure A and EMRT-PCR

As a proof of principle, 23 meat products were purchased, and 10 g of each sample were used to confirm the reliability of the EMRT-PCR by comparing it to the detection outcomes obtained by using the standard protocol, namely the ISO-A. After preparing the initial dilution in 90 ml of selective BB, the contamination level was assessed through spread-plating (100 μl). For 78% (18/23) of the samples, the initial cell concentration was below the detection limit of 1 $\log_{10}$ cfu/ml and therefore, cell concentrations in the food products itself were below 2 $\log_{10}$ cfu/g. The initial concentration in the other samples ranged from 1.3 to 1.9 $\log_{10}$ cfu/ml, yet still below the detection limit of the MRT-PCR detection limit. This highlights the necessity of enrichment since initial contamination levels are generally low. Samples were taken during the enrichment after 24, 40 and 48 h and as part of the ISO-A procedure a streak plate was done onto RCA. The ISO protocol states that the isolation step should be done on both modified charcoal cefoperazone-deoxycholate agar (mCCDA) and another solid medium with different selective principles (International Organization for Standardization, 2017). However, various studies have shown that Extended-spectrum beta-lactamase (ESBL-) producing *Enterobacteriaceae*, which are regularly present on especially broilers (Bortolaia et al., 2010; Depoorter et al., 2012) can also grow on mCCDA, thereby masking *Campylobacter* (Hazeleger et al., 2016; Jasson et al., 2009) and resulting in false-negative detection outcomes. Since RCA has proven to be sufficiently selective for the isolation of campylobacters (Lanzl et al., 2020, 2022; Seliwiorstow et al., 2016) only RCA was used for isolation. Since the detection limit of ISO-A is quite low (1 cfu per loop (~10 μl); 2 $\log_{10}$ cfu/ml), *Campylobacter* concentrations were also determined at each sampling point to assess whether cell numbers were high enough to reach the detection limit of 5.5 $\log_{10}$ cfu/ml of the MRT-PCR method. After 24 h of enrichment, 52% (12/23) and 48% (11/23) of the food samples tested positive and negative for *Campylobacter* spp., respectively, when ISO-A was applied. Samples which were positive after 24 h were also positive at the later sampling points (at 40 and 48 h). When EMRT-PCR was applied, only one of the samples (Nr. 14), which tested positive with ISO-A after 24 h, tested negative with MRT-PCR after 24 h of enrichment. However, the cell concentration in this sample determined with ISO-A was 2.7 $\log_{10}$ cfu/ml, which was indeed lower than the detection limit of MRT-PCR. For all other samples, detection outcomes with EMRT-PCR were the same as with ISO-A. Note that, for one sample (Nr. 13) the EMRT-PCR results were positive at the beginning of enrichment although cell concentration was below the MRT-PCR detection limit of 5.5 $\log_{10}$ cfu/ml. The outcomes of this comparison can be found in Table 2. Further shortening of the detection times may be obtained by lowering the detection limit of the MRT-PCR assay. The detection limit of the MRT-PCR was approximately one $\log_{10}$ higher in selective BB containing chicken than in selective BB. Further research to minimize PCR inhibitors and improve DNA isolation and purification may result in a lower detection limit which is inherent with shorter enrichment times. The experimental results obtained in this study showed that *Campylobacter* could be detected reliably from naturally contaminated food samples after 40 h of enrichment. This is in line with the predictions obtained through the Monte Carlo analysis, which was conducted based on experimental growth kinetics data of severely cold-stressed campylobacters during enrichment in BB. So, although the simulation results were experimentally confirmed, EMRT-PCR could be further tested for a higher number of food products also from different origins to strengthen the statistical power of the detection outcomes.

Application of EMRT-PCR allowed species differentiation, demonstrating that half of the *Campylobacter*-positive samples (6/12) were contaminated with *C. jejuni* only, while the other half was contaminated with both *C. jejuni* and *C. coli.* A study by Kramer et al. (2000) showed, that almost 30% of the meat samples examined in that study were contaminated by more than one strain of *Campylobacter*, and research conducted by Zhao et al. (2001) also revealed that meat sample were contaminated by more than one *Campylobacter* species. In a study by Schnider et al. (2010) a total 351 meat samples were examined for the presence of *C. jejuni* and *C. coli* in a qPCR assay utilizing primers for *hipO*

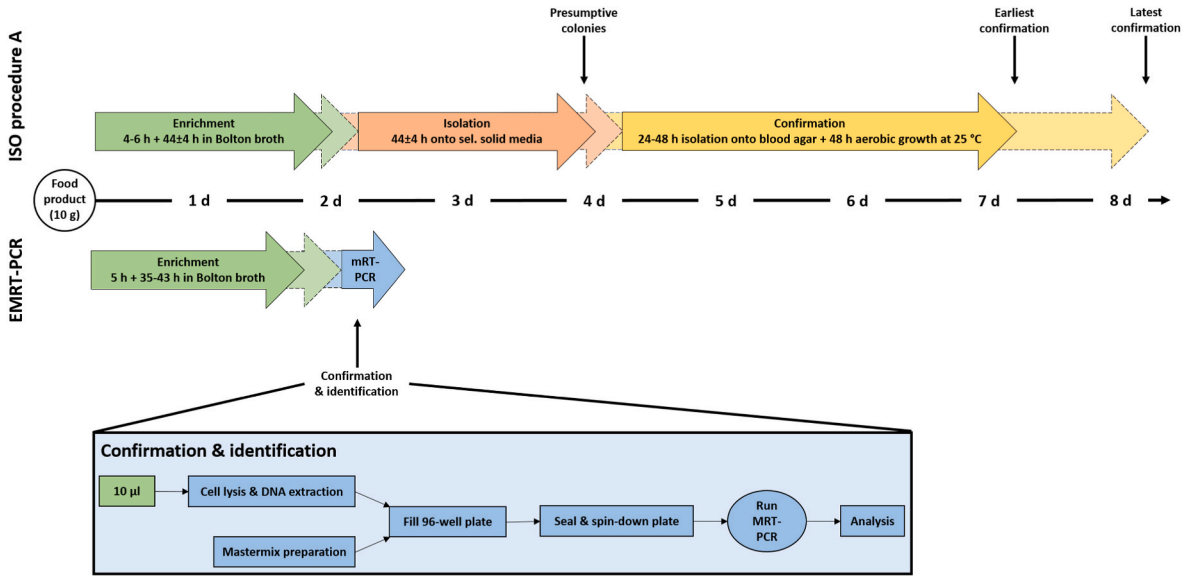

Fig. 2. Flowchart illustrating the detection procedure of *Campylobacter* spp. using ISO 10272–1:2017 (ISO procedure A) and the combination of enrichment with MRT-PCR (EMRT-PCR). Traditional detection using the ISO-A procedure takes between 7 and 8 days (±48 h of enrichment + ±48 h of isolation + 72–96 h of confirmation) while EMRT-PCR provides detection and species differentiation within ~2 days (40–48 h of enrichment + 3 h of confirmation and identification).

and *ceuE*. Results showed that approximately 36% and 18% of the samples were contaminated by either *C. jejuni* only or by both species, respectively, which is lower than results obtained in this research. However, samples for qPCR were taken without prior enrichment, and the choice of both liquid and solid media can have an impact on detection outcomes and strain diversity (Habib et al., 2011; Hayashi et al., 2013; Newell et al., 2001; Paulsen et al., 2005; Ugarte-Ruiz et al., 2013; Williams et al., 2012). Goossens et al. (1986) demonstrated that *C. coli* showed higher susceptibility to polymyxin B, a component of PB and was subsequently less successfully isolated from the medium compared to BB. Another study showed that campylobacters in naturally contaminated food samples grew significantly better in BB compared to PB (Baylis et al., 2000). Next to the enrichment medium, also the choice of isolation medium can affect the recovery of *Campylobacter* strains, as it has been found that the diversity of *Campylobacter* isolates was higher when isolated onto CampyFood agar compared to mCCDA, irrespective of a preceding enrichment step (Ugarte-Ruiz et al., 2013). Currently, BB is recommended by ISO for the recovery of sub-lethally injured campylobacters, while PB is advised when the presence of ESBL-producing *Enterobacteriaceae* is expected. Both media utilize a cocktail of different antibiotics to suppress the growth of competing microbiota, however, these compounds can also cause a selection bias between *Campylobacter* species. Studies by Ugarte-Ruiz et al. (2013) and Newell et al. (2001) showed that the enrichment step (using different selective enrichment media) reduced the diversity of *Campylobacter* isolates and that some strain types were recoverable by direct plating from the samples but could not be recovered after enrichment.

3.4. Comparison of steps required for detection of Campylobacter spp. using ISO and EMRT-PCR

The detection of *Campylobacter* spp. using ISO-A contains an enrichment step in BB for 4–6 h at 37 °C + 44±4 h at 41.5 °C for cell repair and growth of campylobacters, followed by an isolation step on selective solid media (mCCDA and another selective plate) of 44 ± 4 h. Presumptive colonies are streaked onto non-selective blood agar plates and incubated for 24–48 h, dependent on the size of the colonies. Then, presumptive *Campylobacter* colonies are confirmed by examination of morphology and motility, the presence of oxidase activity and absence of aerobic growth at 25 °C. To test the latter, colonies grown on non-selective blood agar are streaked onto non-selective blood agar again and incubated aerobically for 44 ± 4 h. All these steps combined, a negative detection outcome can be determined after 4 days (2 days of enrichment and 2 days of isolation), while a confirmed positive detection outcome takes 7–8 days (2 days of enrichment and 2 days of isolation and 3–4 days of confirmation [1–2 days for growth on non-selective blood agar and 2 days to test for absence of aerobic growth at 25 °C). EMRT-PCR, consisting of an enrichment in BB for 40–48 h, followed by detection and species differentiation through a 1 h sample preparation and 2 h MRT-PCR run provides results in about 2 days. In comparison to ISO-A, the application of EMRT-PCR shortens the detection of *Campylobacter* spp. from food samples by approximately two days for *Campylobacter*-negative and 5–6 days for *Campylobacter*-positive results. The flowchart in Fig. 2 gives an overview concerning the timeline of the two procedures.

4. Conclusion

This study aimed to develop a rapid but reliable protocol for the detection and differentiation of *C. jejuni* and *C. coli* from food products and combines an enrichment with MRT-PCR (EMRT-PCR). The study demonstrated through predictive modelling and experimental results that 40 h of enrichment according to ISO-A followed by MRT-PCR allowed the detection of, and differentiation between *C. jejuni* and *C. coli* in naturally contaminated meat samples. EMRT-PCR takes approximately 2 days, while confirmed positive detection outcomes using traditional ISO protocols can take up to 8 days.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank Dr. Greetje Castelijn and Dr. Menno van der Voort from Wageningen Food Safety Research for providing strains used in this

research and Marcel Tempelaars from the Laboratory of Food Microbiology at Wageningen University and Research for his help and advice in setting up the MRT-PCR. Furthermore, the authors thank all project partners (Biomérieux, DSM, Innosieve Diagnostics, Merck Darmstadt, NWO, Dutch Food Safety Authority and Wageningen Food Safety Research) for their financial and in-kind contributions. This work is part of the research program "Rapid and reliable enrichment-based detection of foodborne pathogens" with project number 15002, which is partly financed by the Dutch Research Council (NWO).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2022.104117.

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