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RAPID IN-HOUSE DETECTION METHOD OF *CAMPYLOBACTER* SPP. FROM FOOD BY REDOX POTENTIAL MONITORING COMBINED WITH REAL-TIME PCR

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The rapid detection of *Campylobacter* spp. is of utmost importance for the reduction of infections in humans by contaminated food products. The standard culturing method (ISO 10272-1:2006) involves a high time and labour demand. In this paper, we present a method that reduces the detection time of *Campylobacter* spp. to or below one third as compared to the ISO method, at a reduced cost per test. We used redox potential change of enrichment cultures (Bolton broth with Bolton selective supplement) for reliably selecting *Campylobacter*-contaminated raw milk and broiler meat samples. Identification of *Campylobacter* spp. in the contaminated samples was done by real-time PCR method. Culturing time to conclusive redox monitoring varied between 6 and 24 h for positive samples, depending on the contamination rate, in contrast to 136 h with the standard culturing process. However, now the *Campylobacter*-negative majority of food samples will not need to be tested by real-time PCR because redox potential monitoring can identify them in the selective enrichment phase. This method could be potentially used as a faster alternative to the current standard ISO 10272-1:2006, for non-regulatory monitoring purposes.

Key words: Rapid detection method, *Campylobacter* spp., redox potential, food safety, raw milk, broiler meat

Campylobacter spp. have been the most commonly reported gastrointestinal bacterial pathogens in humans in the European Union (EU) since 2005. The most important pathogenic strains belong to the group of thermotolerant campylobacters, notably *Campylobacter (C.) jejuni*, *C. coli,* and, to a lesser extent, *C. lari* (Griffiths and Park, 1990).

According to the European Food Safety Authority (EFSA), there were 229,213 confirmed cases of human campylobacteriosis in the European Union in

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2015 (EFSA, 2016). Since most patients recover without consulting their physicians, the actual number of cases is assumed to be much higher. The infection can also trigger complications such as reactive arthritis and Guillain-Barré syndrome. A small number of patients with pre-existing health conditions may die (Kemmeren et al., 2005). Considering the role of *Campylobacter* spp. bacteria in public health care, the availability of fast and accurate identification methods is very desirable.

Broiler meat was the most commonly identified source of *Campylobacter* outbreaks in the EU in 2015. However, there was also an outbreak involving 28 people hospitalised after raw milk consumption (EFSA, 2016).

Conventional methods for the detection of *Campylobacter* spp. in food are sensitive and are being continuously improved, but they are rather time consuming. The identification of a suspected *Campylobacter* colony takes 4 to 6 days, and phenotypic identification schemes for *Campylobacter* spp. are often difficult to interpret (Churruca et al., 2007).

Molecular methods such as the polymerase chain reaction (PCR) can rapidly detect and identify foodborne pathogenic microbes (Rijpens and Herman, 2002). Among the different PCR methods, real-time PCR has a sensitivity similar to that of culture methods (Navas et al., 2006). However, the use of PCR is limited by its cost (Rodríguez-Lázaro et al., 2004).

Current research methods for the detection of *Campylobacter* suggest the need for an enrichment step to increase the target pathogen concentration and to revitalise stressed and injured cells of *Salmonella, Listeria* and *Campylobacter* (Moran et al., 2009; Lynch et al., 2011; Chon et al., 2013). The enriched pathogens then become more accurately identifiable by real-time PCR (Garrido et al., 2013).

Redox potential monitoring has been applied for the rapid assessment of viable counts of several microorganisms (*E. coli*, *Salmonella, Enterococcus,* etc.) in water, milk, foods, and surface samples without an influence of the food matrix (Reichart et al., 2007; Erdősi et al., 2012; Erdősi et al., 2014). The method monitors the downward trend of redox potential caused by typical oxidation-reduction reactions performed by microorganisms in biological systems. The redox potential decreases quite early. The shape of the redox potential curve is characteristic of the type of microorganism (Reichart et al., 2007). This makes the monitoring of redox potential a useful tool for the qualitative and quantitative determination of microbial contamination as well.

The aim of the present work was, on the one hand, to develop a rapid method for detecting *Campylobacter* spp. in food by redox potential monitoring as early as during the enrichment phase. On the other hand, we attempted to demonstrate the practical applicability of this rapid method using retail raw milk and broiler meat samples. Real-time PCR was used for the identification of *Campylobacter* spp. in positive samples.

Materials and methods

Bacterial strains

Since several bacterial species can co-exist and therefore be detected as 'background' microflora in food samples, the following bacterial strains were used for investigating the specificity and reliability of the combined method: *Campylobacter jejuni* (ATCC 33560), *C. lari* (ATCC 35222), *C. coli* (ATCC 43478), *Listeria monocytogenes* (ATCC 19111), *Staphylococcus aureus* (ATCC 12600), *Escherichia coli* (ATCC 10536), *Bacillus cereus* (ATCC 9634), *B. subtilis* (NCTC 3610), *Pseudomonas aeruginosa* (ATCC10145), *Enterococcus faecalis* (ATCC 19433), *Klebsiella oxytoca* (ATCC 700324), and *Enterobacter cloacae* (ATCC 13047).

Culture media

Bolton Broth (Merck 100068) with Bolton Selective Supplement (Merck 100079) was used as enrichment and culture medium.

Determination of calibration curves of Campylobacter *spp.*

The redox potential measurements were performed in Bolton Broth with Bolton Selective Supplement. For the determination of the calibration curve a tenfold dilution series was prepared from a single pure culture of *C. jejuni*, *C. coli* and *C. lari* with peptone water to the 6th dilution level. From each dilution 1 ml was pipetted into a redox measuring cell containing 250 ml Bolton Broth with Bolton Selective Supplement. While incubating the measuring cells, the redox potential of each cell was continuously monitored. The equipment automatically determined the detection times belonging to the different dilution levels. After inputting the viable count of the undiluted inoculum (determined by plate counting), the software computed the calibration curve. The equations of the calibration curves were calculated by linear regression from the logarithm of the initial viable cell numbers in the measuring cells (logN) and the time to detection (TTD) values. The theoretical time requirement of detection of the target microorganism in the measuring cell (logN = 0) can be determined from the intercept TTD(0) of the calibration curve. If no TTD could be obtained in that period, the measuring cell was considered free of the target microorganism.

The redox potential measuring system consisted of 250-ml measuring cells equipped with Schott Blue Line 31RX redox electrodes and a MicroTester device (manufactured by MicroTest Ltd.) supplied with the Windows-based software MicroTester Redox v.2.5.16 for data collection and evaluation (url:1).

Validation of the redox potential method for the detection of Campylobacter *spp.*

The ISO 10272-1:2006 standard method was applied as a reference of detection efficiency. A simplified flow diagram of the standard method is shown in Fig. 1.

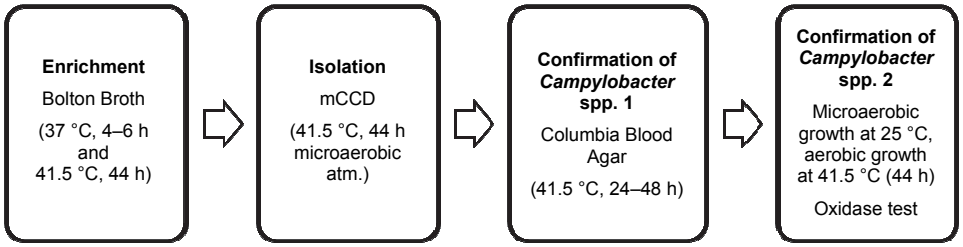

Fig. 1. Conventional method of detection of *Campylobacter* spp. The enrichment culture Bolton Broth is standardised in ISO 10272-1.2006

Selectivity

Selectivity of the redox potential method was tested using pure cultures of non-target bacterial strains (*L. monocytogenes*, *Staph. aureus*, *E. coli*, *B. cereus*, *B. subtilis*, *P. aeruginosa*, *E. faecalis*, *K. oxytoca*, and *E. cloacae*; Table 1). The growth medium was inoculated with the appropriate dilution of the pure culture of each test strain.

Table 1

Selectivity of Bolton Broth with added Bolton Supplement as compared to Tryptic Soy Broth (TSB) at respective incubation temperatures

Bacterium	TTD (h) Bolton Broth T = 41.5 °C	TTD (h) TSB T = 37 °C	Initial bacterial count (cfu/ml)
Escherichia coli	–	1.83	2.2×10^6
Listeria monocytogenes	–	1.17	4.0×10^7
Bacillus cereus	–	4.50	2.5×10^6
Bacillus subtilis	–	3.33	8.2×10^6
Staphylococcus aureus	–	1.00	5.8×10^7
Enterococcus faecalis	–	3.33	1.5×10^7
Klebsiella oxytoca	–	4.17	3.5×10^6
Enterobacter cloacae	–	2.67	1.9×10^6
Pseudomonas aeruginosa	31.17	5.00	4.8×10^7
Campylobacter jejuni	8.17	–	1.7×10^3
Campylobacter lari	4.17	–	4.8×10^3
Campylobacter coli	3.83	–	1.1×10^6

TTD: time to detection (in hours)

Determination of the relative detection level of C. jejuni *in contaminated raw milk and broiler meat samples*

The relative detection level was determined after contamination of food samples with the target microorganism *C. jejuni,* at three concentrations: negative controls (0 cfu/25 g), samples spiked at low level (2 cfu /25 g), and samples spiked at high level (2×10^3 cfu/25 g). Raw milk and broiler chicken meat samples were processed in this phase. Only samples tested negative by the ISO method for *C. jejuni*, *C. coli* and *C. lari* were used. Each combination was replicated six times, totalling 18 raw milk and 18 broiler meat samples.

The samples were subjected to redox potential monitoring in parallel with processing according to the ISO reference method.

Testing raw milk and broiler meat from retail outlets by the method of redox potential monitoring combined with real-time PCR

A total of 95 raw milk and 145 broiler meat samples obtained from local retail shops were examined by redox potential monitoring. Twenty-five ml or 25-g samples were homogenised in 225 ml Bolton Broth (Merck 100068) with Bolton Selective Supplement (Merck 100079) for selective enrichment at 41.5 °C. Enrichment culture incubation of 250-ml homogenates was done in redox potential measuring cells. Samples showing the characteristic redox potential change during monitoring were presumed to be *Campylobacter*-positive samples.

To verify the presence or absence of *Campylobacter* spp. in each enriched culture, real-time PCR technique was used. Genomic DNA was isolated from 1 ml aliquots of enriched food samples. The *Mericon* DNA Bacteria Kit (Qiagen) was used according to the manufacturer's instructions. Real-time PCR amplification was performed on SLAN® Real-Time PCR System (Hongshi) using the *Mericon Campylobacter* spp. Kit (*Mericon*® Pathogen Detection Handbook, Qiagen, 2012).

In parallel with the redox potential monitoring and real-time PCR, each sample was processed by the conventional ISO 10272-1:2006 method.

Mathematical–statistical evaluation

Detailed mathematical–statistical evaluation was performed using MS EXCEL 2016 software package (Analysis ToolPak for Excel).

Results

Selectivity of Bolton Broth complemented with Bolton Selective Supplement for Campylobacter *spp.*

The time to detection (TTD) values of the examined bacteria are shown in Table 1. In Bolton Broth complemented with Bolton Selective Supplement, only

the *Campylobacter* spp. and *Pseudomonas aeruginosa* multiplied. However, the *Campylobacter* spp. and *P. aeruginosa* can be differentiated from each other based on the shape of the redox curve (Fig. 2).

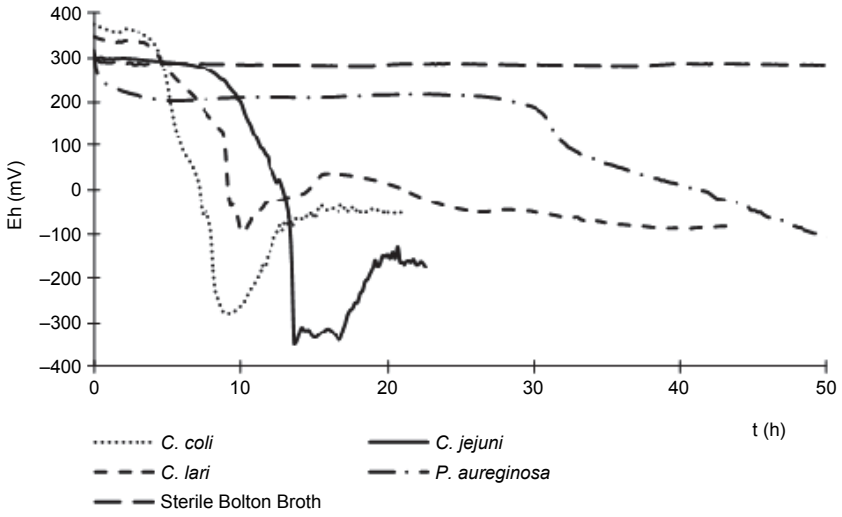

Fig. 2. Characteristic redox potential curves obtained by monitoring the growth of pure bacterial cultures of three *Campylobacter* ssp. and *Pseudomonas aureginosa* in selective Bolton broth. Eh: redox potential related to normal hydrogen electrode

Determination of calibration curves of Campylobacter *spp.*

It could be established that the growth rate of *Campylobacter jejuni* is lower and the TTD belonging to the same logN is higher than that of *C. coli* and *C. lari*.

Equations of calibration curves:

Campylobacter jejuni: TTD (h) = 34.313–8.100·logN $R^2 = 0.9965$

Campylobacter coli: TTD (h) = 22.707–3.085·logN $R^2 = 0.9753$

Campylobacter lari: TTD (h) = 31.971–7.560·logN $R^2 = 0.9954$

The highest TTD (h) value for *C. jejuni* coincides with the slowest growth of the bacterium.

The main results of the detailed mathematical–statistical evaluation of the *Campylobacter jejuni* regression are as follow.

Intercept, TTD(0) = 34.31 h 95% confidence interval: 33.14 – 37.49

Slope = –8.100 h/1 log unit 95% confidence interval: –9.552 – –6.648

If we do not obtain TTD within 38 h, the inoculum of the measuring cell is free from *Campylobacter*.

Validation characteristics

Linearity: valid in the total range of detection
Sensitivity (the slope of the calibration curve): 8,100 h/log unit
Detection limit: theoretically 1 living microbe in the test cell
Range of detection: 10^0–10^7cfu/ml

The calibration curve could be applied for the calculation of *C. jejuni* count in those cases when the redox curves (and the TTD values) represented exclusively the growth of *C. jejuni.* Taking into account the upper limit of the intercept, if we reach TTD within 38 h, the sample of the measuring cell could contain *Campylobacter*.

Results obtained from meat and milk samples inoculated with C. jejuni *at three different concentrations*

Limit of detection by the system is considered to be 1 viable cell/sample, represented by the logN = 0 value of the calibration curve. The time requirement to determine 1 viable cell/sample for a bacterial agent can be read or calculated as the intercept value on the *y* axis with the projection of the calibration curve of the agent. The result of the calculation is, from the practical point of view, the 'time to detection' (TTD value) belonging to the logN = 0, which means 1 viable cell/measuring cell.

The average time requirements for detection (TTD) for the six samples of raw milk and six samples of broiler meat at three concentration levels of *C. jejuni* were as follow:

Negative: 0 cfu/25g (ml) TTD = –
Low: 2 cfu/25g (ml) TTD = 32.9 h
High: 2×10^3 cfu/25g (ml) TTD = 8.6 h

Campylobacter-positive as well as negative samples detected by redox potential monitoring were identical with those detected by the culturing process according to the ISO standard.

Testing of raw milk and broiler meat by the method of redox potential monitoring in combination with real-time PCR

Campylobacter detection results obtained by the combination of redox potential monitoring and real-time PCR were identical with those of the standard method of culture. However, the time requirement of the instrumental approach was significantly shorter compared to the conventional method (Table 2).

In case of negative samples, the results could be read in 38 h by the redox potential monitoring, without PCR investigation. At low contamination level (e.g. N = 10 cfu/g) the detection of *Campylobacter* required 26 h, while at 5×10^2 cfu/g concentration, which is the infective dose of *Campylobacter* (Robinson, 1981), only 12.5 h was needed. Compared to the standard method of culture

which requires 138 h for the detection of *Campylobacter* spp., we achieved detection in no more than 38 h. The results of PCR verification indicated that there were neither false positive nor false negative test results.

Table 2

Time requirement of detecting *Campylobacter* spp. in broiler meat and raw milk

Food sample		Redox potential measurement alone		Redox + real-time PCR		Conventional method time (h)
		TTD (h) minimum	TTD (h) maximum	Minimum (h)	Maximum (h)	
Broiler meat n = 145	positive n = 78	3.17	21.17	6.17	24.17	138
	negative n = 67	38	38	negative	negative	94
Raw milk n = 95	positive n = 0	negative	negative	negative	negative	–
	negative n = 95	38	38	negative	negative	94

TTD: time to detection (in hours)

Discussion

Investigation of foodborne infectious disease outbreaks requires rapid isolation and identification of an implicated pathogen. However, *Campylobacter* spp. are slow-growing bacteria that are, additionally, sensitive to environmental conditions. Enrichment culture using Bolton Broth has been widely used for the detection of *Campylobacter* from meat samples (Baylis et al., 2000) and the procedure is standardised in ISO 10272-1:2006. The major disadvantage is the time length and labour demand of the procedure. For these reasons, the use of some alternative method that can rapidly identify *Campylobacter* spp. in a human foodborne disease outbreak has been recommended (de Boer et al., 2015).

In order to detect a low number, even a single cell, of *Campylobacter* spp. with the highest efficiency, an enrichment phase of the initial sample is a crucial step in culturing (Cocolin et al., 2002). Several methods have been developed to combine improved enrichment methods with other new methods such as real-time PCR or a commercially available immunochromatographic assay (Kawatsu et al., 2010; Chon et al., 2013; Suh et al., 2014). However, the total time requirements of these detection methods were 48 h or more. Further development of real-time PCR, microarray PCR, miniaturised biosensors, chromatographic techniques and DNA sequencing may improve the monitoring capacity at a lower cost in the future (Josefsen et al., 2015).

In this study, the possibility of rapid and reliable detection of *Campylobacter* spp. in food was demonstrated by the application of redox potential monitoring using milk and meat samples. Standard Bolton Broth with Bolton Selective Supplement was used for enrichment and redox monitoring was done in this early phase. The theoretical limit of detection was 1 cfu/sample. According to our results, redox potential monitoring is suitable already during the enrichment process for selecting samples positive for *Campylobacter* spp.

Our results show that detection of *Campylobacter* at a low contamination level of $N \leq 10$ cfu/g required 26 h in enrichment medium, using redox potential monitoring. However, at the clinically significant 5×10^2 cfu/g concentration, which is the infective dose of *C. jejuni* (Robinson, 1981), only a time to detection as short as 12.5 h was needed. Compared to the 138 h, that is 5.75 days, required by the ISO standard method of culture, our method provides positive detection results in just 0.5 day to 1.6 days (up to 38 h for negative samples). Then, only positive samples would need to be processed for species identification with a PCR test. In the case of negative samples, the results can be obtained within 38 h by redox potential monitoring alone without any further verification by PCR.

Although *P. aeruginosa* was able to grow under the conditions favouring the growth of *Campylobacter* spp., its multiplication was significantly slower. Considering the TTD values found, the initial contaminating bacterial density of *P. aeruginosa* would have to be unrealistically high (10^7 cfu/ml or g), otherwise it would not be able to interfere with the redox potential curve of *Campylobacter* in the case of mixed contamination.

We believe that the application of this redox potential monitoring method would significantly accelerate verification of the absence or presence of *Campylobacter* spp. in food samples before a product is released for consumption. For food processing establishments, this is a significant time reduction. The method also allows to reliably satisfy non-regulatory monitoring requirements on *Campylobacter* spp. at greatly diminished laboratory costs of a faster testing for this pathogen.

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Disclosures

The authors declare that they have no financial/commercial interests in the subject matter or materials and equipment discussed in the manuscript, with the exception of Katalin Szakmár who has an involvement with the company manufacturing the measuring equipment applied.

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