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OPTIMISATION OF SPECIFIC PCR DETECTION OF *CAMPYLOBACTER COLI* IN ENRICHMENT BROTH*

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Campylobacter jejuni and *C. coli* are among the most important causes of acute diarrhoea in humans throughout the world. Poultry meat is a major source of *Campylobacter* infections. Sensitive detection methods are necessary to identify contaminated samples. Detection of campylobacters by culturing is slow and tedious, whereas PCR technology offers the potential for rapid and sensitive detection, however, it may be inhibited when used directly for food or pre-enriched food samples. Different methods for sample and/or DNA preparation were studied to find an optimal combination for sensitive PCR detection of *C. coli* in enrichment broth. Buoyant density centrifugation (BDC) prior to cell lysis improved PCR detection of *C. coli* by 100–1000-fold. Preston enrichment broth spiked with 10^1–10^2 CFU ml^{-1} was detected positive after 18 h of enrichment. Specific *flaA* PCR detection of *C. coli* in enrichment broth following BDC and simple heat lysis of the cells can be conducted within two working days. This study is a part of the undergoing development of a rapid and sensitive molecular procedure for specific detection of *C. coli* in foods.

Keywords: *Campylobacter, C. coli*, detection, PCR*, flaA* gene, Preston enrichment broth, buoyant density centrifugation, BDC

Campylobacter jejuni and *C. coli,* the causative agents of acute human enterocolitis, are the most common cause of food-borne diarrhoea in many industrialised countries (ALTEKRUSE et al., 1999). The incidence of *Campylobacter* infections continues to rise and already exceeds the number of salmonellosis in many European countries (WHO, 2000; ANON., 2000).

Handling and consumption of contaminated, undercooked poultry meat is suspected to be an important source of *Campylobacter* infections (CORRY & ATABAY, 2001). High amount of poultry meat from Slovenian retail market was found contaminated with campylobacters (ZORMAN & SMOLE MOŽINA, 2002). To implement effective control measures in food, particularly poultry meat production, rapid and sensitive detection methods are needed. Conventional cultural detection methods of campylobacters are laborious, time-consuming and affected by their fastidious growth requirements and relatively inert biochemical characteristics (WANG et al., 1999). Standardised procedure (ISO, 1995) for thermotolerant *Campylobacter* detection in

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food requires 5–7 days and is affected by subjective interpretation of test results and by bacterial inoculum size (WANG et al., 1999; 2001). More, *Campylobacter* cells can be injured by intrinsic food parameters or processing conditions and transformed into non-culturable coccoid forms that could not be detected by culturing methods (MOORE, 2001; PARK, 2002). PCR technology is an extensively used genetic approach for rapid and sensitive detection of a number of food-borne pathogens (OLSEN et al., 1995; SCHEU et al., 1998; OLSEN, 2000; HILL & JINNEMAN, 2000). However, substances that act as DNA polymerase inhibitors lower PCR detection capacity. The enrichment step, although it lengthens the time of analysis, dilutes inhibitors in the food and increases the number of viable target cells. However, the appropriate sample preparation is crucial for successful amplification. Several procedures for cell concentration and PCR inhibitors removal are used such as centrifugation, washing and filtering (LAMPEL et al., 2000), immobilization with metal hydroxides (LUCORE et al., 2000), immunomagnetic separation (YU et al., 2001) and others (HILL & JINNEMAN, 2000). A simple and convenient sample treatment method based on buoyant density centrifugation (BDC) was applied for PCR detection of pathogenic bacteria in foods (LINDQVIST, 1997; LANTZ et al., 1998). It was used before NASBA-ELGA detection of *C. jejuni* (UYTTENDAELE et al., 1999), improved PCR detection of *C. jejuni* in enriched chicken rinses (WANG et al., 1999) and gave comparable results with cultural and commercial automated ELISA after a 48-h enrichment assay (LILJA & HÄNNINEN, 2001).

In the present study we report on the application of BDC in improving PCR detection of *C. coli* in artificially spiked selective Preston medium after a shorter time of enrichment.

1. Materials and methods

1.1. Bacterial strain

The strain *C. coli* ŽM 140, poultry meat isolate, previously identified phenotypically and genotypically (ZORMAN & SMOLE MOŽINA, 2002), was used in all experiments. It was cultured on 5% horse blood agar plates in gas-tight containers under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42 °C for 24 h.

1.2. Preparation of Preston enrichment broth

Preston enrichment broth composed of Nutrient Broth No. 2, 5% defibrillated horse blood and *Campylobacter* Growth Supplement (Oxoid) was used as enrichment medium for *C. coli* growth as recommended by different international committees (ANON., 1990, 2001; ISO, 1995; JOSEFSEN et al., 2002). Preston broth (50 ml) was inoculated with cell dilutions (from 1 to 10^4 cells) of an overnight culture of *C. coli* ŽM 140 in Ringer solution and incubated microaerobically at 42 °C for 12–24 h. The cell concentrations were calculated following plating on Columbia blood agar plates (Oxoid) for 48 h.

1.3. Sample preparation by buoyant density centrifugation (BDC)

A standard isotonic medium (SIM) was made by adding 0.1% (w/v) peptone (Oxoid) and 0.85% (w/v) NaCl into Percoll (Sigma). The bacterial cells were separated from the broth by layering 0.9 ml of sample over the top of 0.6 ml of 40% SIM (in peptone water) and centrifuging at 14 500 r.p.m. for 1 min. The supernatant was carefully removed down to 0.1 ml, which was resuspended in 100 µl of PCR reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) and centrifuged at 10 000 r.p.m. for 5 min. The supernatant was removed down to a final volume of 10–20 µl. The cells were lysed by heating at 90 °C for 17 min (LINDQVIST, 1997; WANG et al., 1999).

1.4. DNA preparation

– with heat lysis

One ml aliquot of enriched broth was centrifuged for 5 min at 13 000 r.p.m. Precipitated cells were resuspended in 100 µl of sterile HPLC grade water, washed again, lysed by heating (90 °C, 17 min) and stored at –20 °C (NACHAMKIN et al., 1993).

– with alkaline lysis

One ml aliquot of enriched broth was primarily treated as described above. The washed pellet was then resuspended in 100 µl of 0.05 M NaOH and 100 µl of 0.125% SDS (HERMAN et al., 1995) lysed by heating (90 °C, 17 min) and stored at –20 °C.

– with CTAB method

After centrifuging the enriched broth for 5 min at 13 000 r.p.m., precipitated cells were resuspended in 500 µl of TE and further treated as previously described (WILSON, 1987). Isolated DNA was stored at –20 °C.

1.5. PCR conditions

Consensus pair of primers (WASSENAAR & NEWELL, 2000) was used to amplify a 1.7 kb *C. coli* specific DNA fragment as previously described (NACHAMKIN et al., 1993). Tween (0.5%) was included in the reaction mixture to prevent PCR inhibition. Twenty-five µl PCR reactions were performed using Promega reagents and GeneAmp PCR System 2400 (Perkin Elmer) and 1 µl aliquots of the bacterial DNA were used as templates. In all PCR experiments, negative controls for PCR reactions and sample preparations were included. The amplified products (3 µl) were analysed by agarose gel electrophoresis using 1% (w/v) gel in 0.5×TAE. A 100 bp DNA ladder (Gibco) was used for molecular size determinations. The fragments were stained with ethidium bromide and photographed on an UV transilluminator.

1.6. The effect of BDC and DNA preparation on PCR sensitivity

Different cell treatments before PCR amplification were compared: (a) heat lysis, (b) alkaline lysis with NaOH and SDS, (c) BDC prior to heat lysis, (d) BDC prior to alkaline lysis and (e) BDC prior to DNA isolation with CTAB.

The experiment was done in two steps. After 24-h enrichment of spiked broths, three aliquots (1 ml) of each broth were sampled. The first was heat lysed, the second was treated by alkaline lysis and the third with BDC prior to heat lysis. One µl aliquots were used in *flaA* PCR amplifications. PCR sensitivity was estimated based on the lowest initial cell concentration in the enrichment broths where the amplicons were produced. It was the best when BDC was used, so it was included in all further tests. In the second step, three aliquots were taken again from each of the enrichment broths. They were all treated with BDC and then heat lysis, alkaline lysis or CTAB method was used. After *flaA* PCR, detection levels were compared as described above.

1.7. The time needed for PCR detection of C. coli *in enrichment broth*

As we wanted to shorten the enrichment without limitations on detection level the broths were incubated for 12, 15, 18 and 24 h and then detection levels were compared.

2. Results and discussion

All experiments were performed at least twice and the average values are presented. The concentration of *C. coli* 140 ŽM was roughly estimated by measuring the absorbance at 650 nm. Serial culture dilutions were made and the concentration calculated after plating on Columbia blood agar plates for 48 h. The absorbance (650 nm) A=0.1 corresponded to approximately 10^5 CFU ml^{-1}. When the concentration of the cells was 10^6 or more, the absorbance was always higher than 0.2.

The enrichment broth was spiked with dilutions of *C. coli* cells, and different sample/DNA preparation methods were used prior to *flaA* PCR. The best sensitivity was obtained when the samples were prepared by buoyant density centrifugation (BDC) prior to PCR. After 24 h of enrichment, the detection limit was 10^1–10^2 CFU ml^{-1} of the initial enrichment broth. In the case of alkaline lysis without BDC, detection limit was 10^3–10^4 CFU ml^{-1}, but in case of DNA isolation by heat lysis without BDC PCR detected only 10^4–10^5 CFU ml^{-1} of initial enrichment broth. These results showed that BDC prior to lysis increased detection sensitivity by 100–1000 fold (data not shown). Similar improvement was obtained when BDC was applied for detection of *C. jejuni* in chicken rinse (WANG et al., 1999).

When incubation was shortened from 24 h to 12 h only BDC treated sample was PCR positive, all other procedures failed to detect *C. coli* (data not shown). While better PCR detection sensitivity was achieved with BDC treated samples we tried to select the most efficient DNA preparation procedure to be used with BDC. The combination of BDC and CTAB method of DNA isolation was expected to remove more PCR

inhibitors and consequently allow the best sensitivity but that was not the case. After 18 h of enrichment, sensitivity was 100-fold lower than with the other two methods, where PCR detection limit was in the range of 10^1–10^2 CFU ml^{-1} of the initial enrichment broth (Table 1). Several unsuccessful repetitions of PCR detection after BDC/CTAB sample preparation even at high cell concentrations could be explained by the fact that intensive DNA cleaning means loss of DNA and consequently lower PCR sensitivity.

Table 1. Detection of *C. coli* in Preston enrichment broth using BDC and different DNA preparation methods prior to *flaA* PCR

Initial concentration (CFU ml^{-1})	Incubation time (h)	Concentration after incubation (CFU ml^{-1})	DNA preparation after BDC	*flaA* PCR
10^4–10^5	18	10^4–10^8	heat lysis	+
10^3–10^4	18	10^4–10^8	heat lysis	+
10^2–10^3	18	10^4–10^6	heat lysis	+
10–100	18	10^4–10^6	heat lysis	+
1–10	18	10^2–10^4	heat lysis	–
10^4–10^5	18	10^4–10^8	alkaline lysis	+
10^3–10^4	18	10^4–10^8	alkaline lysis	+
10^2–10^3	18	10^4–10^6	alkaline lysis	–
10–100	18	10^4–10^6	alkaline lysis	–
1–10	18	10^2–10^4	alkaline lysis	–
10^4–10^5	18	10^4–10^8	CTAB	+
10^3–10^4	18	10^4–10^8	CTAB	+
10^2–10^3	18	10^4–10^6	CTAB	–
10–100	18	10^4–10^6	CTAB	–
1–10	18	10^2–10^4	CTAB	–
10^4–10^5	24	10^7–10^9	heat lysis	+
10^3–10^4	24	10^7–10^9	heat lysis	+
10^2–10^3	24	10^6–10^7	heat lysis	+
10–100	24	10^4–10^6	heat lysis	+
1–10	24	10^4–10^5	heat lysis	–
10^4–10^5	24	10^7–10^9	alkaline lysis	+
10^3–10^4	24	10^7–10^9	alkaline lysis	+
10^2–10^3	24	10^6–10^7	alkaline lysis	+
10–100	24	10^4–10^5	alkaline lysis	–
1–10	24	10^4–10^5	alkaline lysis	–
10^4–10^5	24	10^7–10^9	CTAB	–
10^3–10^4	24	10^7–10^9	CTAB	–
10^2–10^3	24	10^6–10^7	CTAB	–
10–100	24	10^4–10^6	CTAB	–
1–10	24	10^4–10^5	CTAB	–

BDC: Buoyant density centrifugation followed by heat lysis of cells
+: amplicon detected
–: amplicon not detected

Buoyant density centrifugation of the sample followed by simple heat lysis of cells was found to be the most efficient and the easiest of all tested methods for sample preparation prior to PCR amplification.

Finally, we tried to shorten the time of enrichment and of the whole procedure without additional limitation on detection level. After 18 h of enrichment, the initial concentration 10^1–10^2 CFU ml^{-1} of *C. coli* was detected, while shorter time of enrichment did not allow PCR detection. When incubated for less than 18 h, the cell concentrations in enrichment broth were found to be too low for detection (data not shown). The cells were still in exponential phase, where variations in cell concentrations are much higher than in the stationary phase. We assumed that the Preston broth enrichment time could be shortened to 18 h but not further more. In this way, specific *flaA* PCR detection of *C. coli* in enrichment broth including BDC and simple heat lysis of the cells can be conducted within two working days.

3. Conclusions

A sample treatment, based on buoyant density centrifugation (BDC) that separates and concentrates bacteria from food or enrichment medium, improved *flaA* PCR detection of *C. coli* in Preston enrichment broth by 100–1000 fold compared to methods without BDC. The enrichment time could be shortened to 18 h at maximum. At shorter incubation times campylobacters could not reach sufficient concentration and consequently PCR detection would not be reliable. The described method allows specific PCR detection of *C. coli* in enrichment broth within two working days. This study is a part of an undergoing development of a rapid and sensitive molecular procedure for specific detection of *C. coli* in foods.

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