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Comparison of Immunomagnetic Separation and Multiplex PCR Assay for Detection of *Campylobacter jejuni* and *Campylobacter coli* in Chicken Meat

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ABSTRACT

Campylobacter is one of the most common food-borne pathogens in the developed and developing countries. Poultry and poultry products are important vehicles for this bacterium to infect humans. Due to only a short time required for analysis of food products, more rapid, sensitive and specific methods have been developed for *Campylobacter* detection. Two alternative methods, multiplex PCR (mPCR) and immunomagnetic separation (IMS) followed by plating to charcoal cefoperazone-deoxycholate agar (CCDA), were compared for their suitability to detect *Campylobacter jejuni* and *C. coli* in chicken meat. IMS followed by plating could detect *C. jejuni* and *C. coli* inoculated at 10^0 cfu/g in meat after 12 h of incubation. The mPCR method could detect both species at the same inoculation level after 16 h of incubation. However, the total analytical time to identify *C. jejuni* and *C. coli* in chicken meat using IMS followed by plating was 72-96 h while the time used by mPCR was only 22 h. A single cell of *C. jejuni* or *C. coli* in 1 g of chicken meat was detected by mPCR after 16 h of incubation in Preston broth.

Key words: *Campylobacter*, chicken meat, immunomagnetic separation, multiplex PCR

INTRODUCTION

Campylobacter is one of the most common food-borne pathogens in the developed and developing countries, causing gastroenteritis characterized by watery and/or bloody diarrhea. It is associated with Guillain-Barré (GBS), Reiter's and haemolytic uremic (HUS) syndromes and reactive arthritis (Tauxe, 2000; FSAI, 2002; Lake *et al*., 2003). During the last 20 years, the infection rate of *Campylobacter* has continued to increase in many developed countries, part of this increase may be due to the improvement of detection and reporting (Tauxe, 2000). The infective dose of *Campylobacter* is considered to be low, ranging from 500 to 10,000 cells (FSAI, 2002). Only three

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Campylobacter species, *C. jejuni*, *C. coli* and *C. lari*, represent about 90% of all human campylobacteriosis cases (Stern and Line, 2000). In the European Union, approximately 0.2 million human cases of campylobacteriosis are reported annually (EFSA, 2004). Wild birds and domestic animals are well-known as reservoirs for *Campylobacter*, and poultry products are important vehicles for this bacterium to infect humans. Epidemiological studies show that consumption or handling of poultry meat should be considered as a major risk factor for human infection with *C. jejuni* or *C. coli* (Coker, 2000; FSAI, 2002).

Traditional analytical methods for determination of *Campylobacter* are based on phenotyping which requires four to five days. Due to the perishable nature of food products, this time-consuming method is unacceptable. Advanced analytical assays, *i.e.* rapid, sensitive and specific methods, are urgently needed to support veterinary intervention programs. For this reason, immunomagnetic separation (IMS) using magnetic beads coated with immunoglobulins is exploited to isolate and concentrate *C. jejuni* from sample tested leaving cells viable, and reduces the time of the pre-enrichment step. A short pre-enrichment step is needed to improve the sensitivity and overcome the problems of inhibitors from the food sources (Yu *et al*., 2001). In addition, IMS can be used to concentrate bacteria from a volume of 225 ml of pre-enrichment broth mixed with 25 g of suspected food, which is the standard amount for microbiological investigations.

Polymerase chain reaction (PCR) assay is a relatively rapid, sensitive and specific method to detect *Campylobacter* in food samples. Multiplex PCR (mPCR) may not only identify *Campylobacter*, but also discriminate simultaneously between different species of *Campylobacter*, including *C. jejuni* and *C. coli* (Denis *et al*., 1999). However, one disadvantage of PCR assay is the presence of inhibitors of amplification in food that require effective clean-up methods to overcome possible interference (Denis *et al*., 2001). The aim of this study was, therefore, to evaluate the suitability of the detection of *C. jejuni* and *C. coli* in pure culture and chicken meat samples following different incubation times (0-21 h) using IMS followed by plating to charcoal cefoperazone-deoxycholate agar (CCDA) or mPCR.

MATERIALS AND METHODS

Bacterial strains and culture conditions

C. jejuni strain ATCC 33291 and *C. coli* strain ATCC 33559 were bought from Microbiologics (St. Cloud, MN, USA) and were used as positive control. *Campylobacter* were grown under a microaerophilic atmosphere, which was generated by using a gas package (BBL, Becton Dickinson, Sparks, USA). Following multiplication in tryptone soya broth (TSB; Oxoid, Hampshire, England) for 24 h at 42°C, *Campylobacter* were plated onto CCDA and incubated for 48 h at 42°C. The agar was composed of *Campylobacter* blood-free selective agar base (Oxoid) and CCDA selective supplement (Oxoid), which contained 32 μg/ml cefoperazone and 10 μg/ml amphotericin B. One colony was transferred from the CCDA plate to a tryptone soya agar-containing tube (TSA; Oxoid) incubated for 24 h at 42°C to acquire pure *Campylobacter*. The identity of bacteria was confirmed using API Campy strips (BioMériuex, Lyon, France) developed according to the protocol of the manufacturer. Pure *Campylobacter* were stored at 4°C until used.

Sample preparation

Pure *Campylobacter* was grown in TSB as described above, and 1 ml of the suspension was decimally diluted with peptone physiological salt solution (Biotrading, Mijdrecht, The Netherlands) containing 5 g/l peptone and 8.5 g/l

sodium chloride, to give cell concentrations ranging from 10^1 to10^6 cfu/ml. *Campylobacter* pure culture samples, containing 1 ml diluted *Campylobacter* suspension, were added to 225 ml Preston broth in a stomacher bag. For artificial inoculate chicken meat samples collected from the local market in Utrecht, The Netherlands, aliquots of 25 g minced chicken fillet were fortified with 1 ml diluted *Campylobacter* suspension followed by the addition of 225 ml Preston broth in the stomacher bags. A negative control was prepared for each chicken meat sample without adding *Campylobacter* inoculation. The Preston broth was prepared according to the manufacturer's instructions and consisted of nutrient broth No.2 (Oxoid), which contained 5% (v/v) lysed horse blood (Oxoid), *Campylobacter* growth supplement (Oxoid) and modified Preston *Campylobacter* selective supplement (Oxoid). Both pure culture and chicken meat samples were homogenized for 90 s in a stomacher (Interscience, St.Nom, France), and the suspension was incubated under microaerophilic atmosphere at 42°C for incubation times indicated in the text.

Immunomagnetic separation

Following enrichment in Preston broth, each sample-containing stomacher bag was placed into an incubation pot of the IMS machine operating at 37°C (Pathatrix™, Microscience, Cambridgeshire, UK). *Campylobacter* were then captured according to the protocol of the manufacturer. In brief, 50 µl anti-*Campylobacter* magnetic beads (Pathatrix PC50, Microscience) were added to the sample and the bead-containing suspension was circulated for 30 min. The magnetically immobilized beads were released, washed with 100 ml of pre-warmed buffered peptone water, which was compose of 10 mg/ml peptone (Becton Dickinson), 5 mg/ml sodium chloride (Merck Darmstadt, Germany), 4.5 mg/ml disodium hydrogen phosphate dehydrate (Merck) and 1.5 mg/ml potassium dihydrogen phosphate (Merck) adjusted to pH 7.2. After washing, the beads were drawn to the magnet again, and the wash solution was removed to leave 0.2 ml bead-containing suspension for analysis.

Microbial test

The number of *Campylobacter* cells in pure culture samples and chicken meat samples was determined by transferring 100 µl of a sample on a CCDA plate. The number of *Campylobacter* colonies with smooth, gray, moist, flat and effuse was counted following incubation at 42°C for 48 h, and the result was expressed as colony forming units per volume or weight of the original sample. Microbial tests were conducted before and after IMS.

mPCR analysis

The identification of *Campylobacter* by its genus and species level was based on PCR amplification of *16S* rRNA (*Campylobacter* genus), *mapA* (*jejuni* species) and *ceuE* (*coli* species) genes (Denis *et al*., 1999). In short, *16S* rRNA gene-based MD16S1 (5′-ATC TAA TGG CTT AAC CAT TAA AC-3′) and MD16S2 (5′-GGA CGG TAA CTA GTT TAG TAT T-3′) oligonucleotides were used as genus-specific primers. The *mapA* gene-based oligonucleotide primers were MDmapA1 (5′-CTA TTT TAT TTT TGA GTG CTT GTG-3′) and MDmapA2 (5′-GCT TTA TTT GCC ATT TGT TTT ATT A-3′), whereas COL3 (5′-AAT TGA AAA TTG CTC CAA CTA TG-3′) and MDCOL2 (5′-TGA TTT TAT TAT TTG TAG CAG CG-3′) reflected the *ceuE* gene. All oligonucleotide primers were synthesized commercially by Isogen Bioscience (Maarsen, The Netherlands).

After enrichment in a chicken meat sample, bacterial DNA was extracted from 1 ml of Preston broth suspension using a QIAamp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The aimed genes were amplified using 30 µl extracted DNA in an end

volume of 50 µl containing 200 µM dNTPs (Amersham, Buckingshamshire, UK), 1.5 mM $MgCl_2$, 1X PCR reaction buffer, 2 U *Taq* DNA polymerase (Promega, Madison, WI, USA), 0.5 µM of each MD16S1 and MD16S2 primers, 0.42 µM of each MDmapA1, MDmapA2, COL3 and MDCOL2 primers. The amplification reactions were carried out in a Mycycler™ thermal cycler (Biorad, Hercules, CA, USA). In brief, the sample was heated at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 1 min 30 s and extension at 72°C for 1 min. The PCR reaction was completed by the final extension step at 72°C for 10 min. This amplification step generated 857 bp, 589 bp and 462 bp DNA fragments for *Campylobacter* spp., *C. jejuni* and *C. coli*, respectively. These products were assessed by electrophoresis using 5 µl PCR product loaded onto a 1% (w/v) agarose gel (Invitrogen, Palsley, UK) containing 0.05 µg/ml ethidium bromide (Sigma-Aldrich, St. Louis, MD, USA) run at 100 V for 30 min and photographed under UV light.

RESULTS AND DISCUSSION

In order to assess the efficacy of IMS, Preston broth was spiked at various levels of *C. jejuni* and after IMS, beads were grafted on CCDA plates in order to count the number of captured viable cells after selective growth. It can be deduced from Table 1 that the recovery of added cells is only 0.5% to 9%, which may impair favorable sensitivity. Furthermore, despite the increasing spiking level, the number of colonies of retrieved cells did not show an upward trend revealing an unsatisfactory precision as well. From the ratio of the number of spiked and retrieved colonies, a detection limit could be calculated for each spiking level. This value ranged broadly and users applying IMS to detect *Campylobacter* might, therefore, reckon with a detection limit at least 2.1×10^2 cfu/ml instead of 11 cfu/ml.

In a similar way, 25 g minced chicken meat was inoculated at different levels of *C. jejuni*, mixed with 225 ml Preston broth, processed using

Table 1 Detection limit of *C. jejuni* pure culture and inoculated with chicken meat detected by the IMS followed by plating method.

Pure culture samples[a]			Chicken meat samples		
Inoculum level (cfu/ml)	After IMS (cfu)[b]	Detection limit[c] (cfu/ml)	Inoculum level (cfu/g)	After IMS (cfu)[b]	Detection limit (cfu/g)
2×10^2	1	2×10^2	2×10^3	1	2×10^3
3×10^2	13	23	4×10^3	4	1×10^3
7×10^2	38	18	1.5×10^4	3	5×10^3
2×10^3	1.8×10^2	11	4×10^4	14	2.9×10^3
5×10^3	1.1×10^2	45	8×10^4	28	2.9×10^3
6×10^3	29	2.1×10^2	1.5×10^5	91	1.7×10^3
7×10^3	77	91	3×10^5	81	3.7×10^3
8×10^3	3×10^2	27	1×10^6	4.2×10^2	2.4×10^3
9×10^3	75	1.2×10^2	2×10^6	9.2×10^2	2.2×10^3
1×10^4	4.1×10^2	24			

[a] Preston broth used without modified Preston *Campylobacter* selective supplement;

[b] originally present in 0.2 ml bead suspension;

[c] ratio of the number of spiked colonies and the number of IMS-retrieved colonies.

IMS and the number of retrieved colonies determined using CCDA plates (Table 1). In this case, the recovery ranged between 0.02 and 0.1% of the original amount of added cells. The corresponding calculated detection limits were higher as well, and at least 5×10^3 cfu/g meat could be detected.

This sensitivity was reflected in the number of *Campylobacter* colonies on CCDA plates following different incubation time intervals at different inoculation levels of medium and meat (Table 2). At any starting level, *C. coli* was detected faster giving rise to more colonies on the CCDA plate than *C. jejuni*. For example, at an inoculum level of 10^{-2} cfu/ml, *C. coli* was detected in Preston broth after an incubation time of 12 h whereas *C. jejuni* was only visualized after 19 h of incubation. Likewise, *C. coli* was detected earlier in meat than *C. jejuni*. Compared to broth, detection of *Campylobacter* in meat was strongly delayed, as expected from the results in Table 1. To detect 1 to 9 *Campylobacter* cells of both species in 1 g chicken meat, an incubation time of at least 12 h was necessary. However, at this inoculum level in pure culture, *C. jejuni* and *C. coli* were detected after only a 4 h incubation period (Table 2).

Using PCR, it is probable that not only reproductive cells but also the damaged and dead cells are detected. In addition, PCR offers a possibility to confirm the presence of *Campylobacter* and the identification of the involved species in a so-called multiplex mode. To assess this sensitivity, mPCR analysis was performed before using IMS on the same chicken meat samples as well (Table 3). At the genus level, mPCR analysis was able to detect the bacterium following a 14 h incubation period in meat inoculated at 1 cfu/g, while species were determined after 16 h of incubation. Without pre-enrichment, at least 10^5 *C. jejuni* and *C. coli* in 1 g chicken meat were detected with mPCR

The efficacy of IMS followed by plating could detect at least 5×10^3 cfu/g of *C. jejuni* in chicken meat, which was not different from the other IMS methods, such as a combination of IMS with atomic force and fluorescence microscopy to enable the detection of 10^4 cfu/g in ground poultry meat (Yu *et al*., 2001) or IMS coupled with a tyrosinase modified enzyme electrode had a detection limit of 2.1×10^4 cfu/g in the chicken carcass samples (Che *et al*., 2001). To compare the efficacy of IMS followed by plating between pure culture samples and chicken meat samples, we found that in pure culture samples the efficacy of IMS followed by plating gave better detection than in chicken samples. At the same inoculum level, such as 10^0 cfu/ml in Preston broth and 10^0 cfu/g in chicken meat, this method detected *C. jejuni* and *C. coli* in pure culture with less pre-enrichment time than when contaminated in chicken meat for 8 h.

This study used mPCR assay conditions the same as Denis *et al*. (2001), who reported the detection of artificial contamination of *C. jejuni* and *C. coli* of chicken samples and faecal samples after 24 h enrichment. The sensitivity was 1.5×10^3 cfu/ml and initially inoculated with 5 cfu/g of samples before the enrichment step. Our results showed that *C. jejuni* and *C. coli* contaminated in chicken meat samples were identified by mPCR at an inoculum level 10^0 cfu/g (1-9 cfu/g) after only 16 h enrichment. It meant that we could reduce the enrichment to 8 h in this mPCR assay condition. Mateo *et al*. (2005) determined that detection limit of the mPCR method to detect 1 ml *C. jejuni* pure culture without pre-enrichment was 50 cfu/ml. However, the study showed that the mPCR method could detect chicken meat without pre-enrichment inoculated by 10^5 cfu/g of *C. jejuni* and *C. coli*. The difference in detection limits of both studies was caused by inhibitors of amplification reactions of PCR contained within chicken meat. From Tables 2 and 3, the results showed the pre-enrichment time of chicken samples or pure culture samples detected by mPCR

Table 2 Number of counted colonies following CCDA plating of IMS beads used to capture *C. coli* and *C. jejuni* from either pure culture samples or chicken meat samples which were inoculated at indicated levels and incubated for indicated time intervals. The counted number of *C. jejuni* is given between brackets.

Incubation time (h)	Inoculum levels (cfu/ml or cfu/g)											
	10^{-2}	10^{-1}		10^{0}		10^{1}		10^{2}		10^{3}	
	Pure[a]	Pure	Meat[b]	Pure	Meat	Pure	Meat	Pure	Meat	Pure	Meat
1								12			2
2				nd[c]		3 (2)		184			
4		nd (1)		2 (1)		14 (14)	(nd)	379 (111)	1 (4)	(49)	23
6	(nd)	4 (2)		20		(45)	(nd)		5		
8	(nd)	17 (5)			(nd)	(184)	2 (nd)		118		
10		95									
12	19	438			9 (3)		13 (18)				
14	112	737			28						
16	10^{2}		84		385						
18	10^{3}		96								
19	(55)		(nd)								
20			830								
21			(332)								

[a] *Campylobacter* pure culture ; [b] *Campylobacter* inoculated with chicken meat; [c] nd, not detected.

Table 3 mPCR analysis to detect chicken meat inoculated with *C. coli* or *C. jejuni*, which were inoculated at indicated levels and incubated for indicated time intervals. The result of *C. jejuni* is given between brackets.

Incubation time (h)	Inoculum levels (cfu/g)						
	10^{-1}	10^{0}	10^{1}	10^{2}	10^{3}	10^{4}	10^{5}
0					nd[c]	nd (nd)	G[a],S[b] (G,S)
2				nd	nd	nd (nd)	G,S (nd)
4				nd	G,S (nd)	G,S (nd)	(G,S)
6			nd	nd (nd)	G,S (G,S)		
8			nd	nd (nd)	G,S (nd)		
10		nd (nd)	G,S (nd)	G,S			
12		nd (G)	G (nd)				
14	G (G)	G (G)					
16	G,S (G,S)	G,S (G,S)	(G,S)				
17			G,S (G,S)	G,S (G,S)			
18	G,S	G,S	G,S	(G,S)	(G,S)		
20		G,S	(G,S)				

[a] G, PCR product detected in genus; [b] S, PCR product detected in species; [c] nd, not detected.

or IMS followed by plating, to depend on the inoculum levels of *C. jejuni* and *C. coli*. If the inoculum levels were high, the pre-enrichment time would be short.

CONCLUSION

IMS followed by plating could detect *C. jejuni* and *C. coli* inoculated at 10^0 cfu/g in meat after at least 12 h of pre-enrichment time. The mPCR method could detect both species at the same inoculation level with at least 16 h of incubation. However, the total analytical time of IMS followed by plating to identify at the species level was 72-96 h while the time used by mPCR was only 22 h. IMS followed by plating and mPCR could detect *Campylobacter* in chicken but required different times for the total analysis. In conclusion, the mPCR method for the detection of *C. jejuni* and *C. coli* in chicken meat could be performed with less total analytical time than IMS followed by plating. A single cell of *C. jejuni* or *C. coli* in 1 g of chicken meat was detected by mPCR after 16 h of incubation in Preston broth.

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