Isolation and identification of *Campylobacter* spp. and *Campylobacter coli* from poultry carcasses by conventional culture method and multiplex PCR in Mashhad, Iran

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(Received 14 Mar 2007; revised version 23 Jul 2007; accepted 11 Sept 2007)

Summary

The genus *Campylobacter* is of great importance to public health because it includes several species that may cause diarrhoea. Poultry and poultry products are known as important sources of human campylobacteriosis. In this study, during the autumn months of 2005, a total of 100 samples from poultry carcasses, representing 20 broiler flocks were obtained by rinse test, after the chilling stage of processing. The samples were enriched in Preston broth, followed by streaking on selective media. Then, the suspected colonies were isolated on sheep blood agar and tested for morphology, motility and Gram-staining. Biochemical tests and hippurate hydrolysis activity were also performed. Concurrently, a multiplex PCR assay (m-PCR) with two sets of primers was employed for identification of *Campylobacter* genus and *Campylobacter coli*. The m-PCR assay was applied on bacterial cultures harvested from selective media plates. By conventional culture method, including hippurate hydrolysis test from suspected colonies, 76% of samples were positive for *Campylobacter* spp. and 2% for *C. coli*. In m-PCR assay 28% of the harvested cultures, were positive for *Campylobacter* genus but *C. coli* were not detected in any of the samples. According to this preliminary study, it seems that the contamination rate of poultry carcasses with other species of *Campylobacter* genus is higher than contamination with *C. coli.*

Key words: *Campylobacter coli*, *Campylobacter* spp., m-PCR, Poultry carcass

Introduction

Campylobacter spp. are Gram-negative, microaerophilic and/or anaerobic, mainly spiral-shaped bacteria, which most of them are recognized or suspected as human gastrointestinal pathogens (Skirrow, 1994). *Campylobacter jejuni* and *Campylobacter coli* are frequently associated with human campylobacteriosis (Skirrow, 1990). In many western countries, the incidence of campylobacteriosis is higher than diseases caused by *Salmonella* (Rohner *et al*., 1997). Poultry and poultry products are known as important sources of human campylobacteriosis and play important roles in disease transmission (Deming *et al*., 1987; Evans, 1992). However, in developing countries due to inappropriate detection method, a number of cases might have been undetected (Trachoo, 2003). The main transmission route of infection is the ingestion of food of animal origin (Butzler and Oosterom, 1991). The fastidious growth requirements, complex taxonomy and unreliable biochemical tests, present significant challenges in the identification of *Campylobacter* spp. (On, 2001). Furthermore, *C. coli* and *C. jejuni* are closely related by phylogenetic and genetic criteria (Dedieu *et al*., 2004), so identification of *Campylobacter* species is difficult (Morris *et al*., 1985). For the treatment of human campylobacteriosis, differentiation of *C. jejuni* and *C. coli* is necessary (Cloak and Fratamico, 2002). Thus, development of simple methods for detection and reliable differentiation of the

thermophilic *Campylobacter* species are necessary. Molecular tests due to their relative ease of use, low cost, and potential application in large-scale screening programs, by means of automated technologies, appears to be attractive candidates (Kricka, 1998). The polymerase chain reaction (PCR) assay allows not only detection of viable but also non-cultivable forms of *Campylobacter* (Wegmuller *et al*., 1993; Hazeleger *et al*., 1994). Indeed, a number of genetically based detection and typing methods have been developed for identification of *Campylobacter* species in food, and in clinical and environmental samples (Eyers *et al*., 1993; Wegmuller *et al*., 1993; Stucki *et al*., 1995; Jackson *et al*., 1996; Gonzalez *et al*., 1997; Linton *et al*., 1997; Van Doorn *et al*., 1997; Metherell *et al*., 1999). However, the earlier methods suffer from drawbacks, like the need for multiple PCRs, restriction enzyme digestion or the requirement of a hybridization step with species-specific probes after the completion of the PCR.

The objective of this preliminary study was to determine the contamination rate of poultry broiler carcasses with *Campylobacter* spp. and *C. coli*, using conventional culture method and multiplex PCR (m-PCR) assay.

Materials and Methods

Bacterial reference strain

In this study *C. coli* CCUG11283 kindly provided by Department of Microbiology, University of Tehran Medical Science, was used for PCR optimization and also employed as positive control in the m-PCR assay.

Rinse test sampling

During the autumn months of 2005, a total of 100 samples were randomly collected from broiler chickens, representing 20 broiler flocks, after the chilling stage in the processing line at a commercial broiler slaughtering facility in Mashhad city. Chicken carcasses were rinsed in 250 ml of 0.1% (w/v) peptone water supplemented with 0.15% sodium thioglycolate by shaking for 1 min in sterile plastic containers, followed by filtration through two layers of sterilized cheesecloth. The samples were brought to the laboratory on crashed ice.

Isolation and identification of *Campylobacter* spp. by conventional culture method

In the laboratory, filtrated chickens rinse water were centrifuged at 16000 × g for 10 min at 4°C. The supernatant was discarded and the pellet suspended in 5 ml of enrichment Preston broth. After resuspention of the pellet, the samples were incubated in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2) at 37°C for 24 h, followed by further incubation at 42°C for another 24 h. Enrichment broth was consisted of nutrient broth, supplemented with trimethoprim 10 mg/L, rifampicin 5 mg/L, polymyxin B 2500 IU/L, cefoperazone 15 mg/L and amphotericin 2 mg/L. Then the enriched cultures were plated onto a selective media which was consisted of blood agar base supplemented with 7% lysed horse blood and antibiotics, including vancomycin 10 mg/L, polymyxin B 2500 IU/L and trimethoprim 5 mg/L. The plates were incubated under a microaerobic atmosphere condition at 42°C for 48 h.

Suspected colonies on selective media were examined for morphology and motility by phase-contrast microscope and Gram-staining. In the next step, the suspected colonies were isolated on blood agar plates, containing 5% sheep blood, and incubated under microaerophilic conditions at 42°C for 72 h, followed by biochemical tests, including catalase, oxidase, and hippurate hydrolysis. *Campylobacter* colonies with negative hippurate hydrolysis result were tested for susceptibility to 30 µg discs of nalidixic acid on blood agar plates.

For hippurate hydrolysis test, a loopful of the suspected colonies isolated on sheep blood agar was added to 0.5 ml of a 1% sodium hippurate solution and mixed by shaking, followed by 2 h incubation at 37°C in a water bath. Then, 0.2 ml of 3.5% ninhydrin solution in a mixture of acetone and butanol (1:1) was added in each tube on the top of the hippurate solution. For colour development, further incubation was carried out at 37°C for 10 min. A deep purple

colour, crystal violet-like, was recorded as a positive result, indicating the presence of glycine which was resulted from the hydrolysis of the hippurate. Remaining colourless or a pale purple in colour showed a negative result in hippurate hydrolysis. The test was performed twice on each suspected colony.

DNA extraction

Bacterial cultures on selective media plates were collected and suspended in sterile, deionized distilled water and heated in a boiling water bath for 10 min. The samples were cooled immediately on ice for 5-10 min and centrifuged at 13000 × g for 5 min. The supernatants were used as DNA templates for PCR.

Multiplex PCR assay

The reaction mixture consisted of 2.5 µl of bacterial lysate, 2.5 µl of 10 × BSA buffer (1 ml of 10 X contained 500 µl of 1 M Tris-HCl, pH = 8.5, 200 µl of 1 M KCl, 30 µl of 1 M $MgCl_2$, 5 mg of BSA and 270 µl of deionized water), 2.4 µl of 10 × dNTP mixture (2.5 mM of each dNTP), 0.7 µl of each *cadF* and the *ceuE* gene primer mixture (25 µM stock concentration), 0.2 µl of Taq polymerase (5 U/µl) and deionized water to a final volume of 25 µl. The oligonucleotide primers used are shown in Table 1. The reaction mixture was amplified in a thermocycler (Bio-Rad). The following PCR conditions were used: heat denaturation at 94°C for 4 min, 33 cycles with denaturation at 94°C for 1 min, annealing at 45°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR product was separated by electrophoresis in 1.5% agarose gel at 100 V for 40 min in Tris-acetate buffer, visualized by ethidium bromide staining, illuminated by UV-transilluminator and documented by a gel documentation apparatus. A 100 bp DNA ladder was used as a size reference for PCR assay. Genomic DNA extracted from *C. coli* CCUG11283 was used as positive controls in all PCR reactions.

Results

In conventional culture method, 76 out of 100 samples studied (76%), identified as *Campylobacter* genus. In hippurate hydrolysis test, 74 samples evaluated as positive and two samples (2%) as negative.

The results of m-PCR assay on cultures collected from selective agar plates are shown in Fig. 1. The m-PCR generated two PCR products with a length of 400 bp and 894 bp, for the *Campylobacter* spp. and *C coli*, respectively. Out of 100 samples that were analysed by m-PCR assay, 28 (28%) samples were positive for *Campylobacter* genus, but none of them identified as *C. coli* (Fig. 1).

Two samples that were negative in hippurate hydrolysis test and sensitive to nalidixic acid, identified as *C. coli*. These

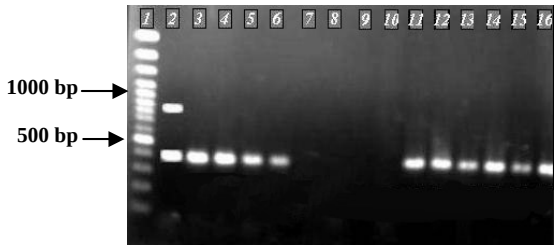

Fig. 1: Detection of *Campylobacter* genus and *C. coli* in chicken carcass samples by multiplex PCR assay amplifying 400 bp segment of *cadF* gene, specific for *Campylobacter* genus and 894 bp segment of *ceuE* gene, specific for *C. coli*: Lane 10: negative control, Lane 2: *C. coli* positive control, Lane 1: 100 bp markers, Lanes 3-6 and 11-16: positive for *Campylobacter* genus

Table 1: PCR primers used for detection of *Campylobacter* genus and *C. coli*

Target gene	Sequence (5′→3′)	Gene location	PCR product (bp)	Reference
cadF—outer membrane protein (*Campylobacter*)	(F) TTG AAG GTA ATT TAG ATA TG	101→120	400	Nayak *et al*. (2005)
	(R) CTA ATA CCY[1] AAA GTT GAA AC	497→478		
ceuE—lipoprotein component of enterochelin (*C. coli*)	(F) ATG AAA AAA TAT TTA GTT TTT GCA	2689→3675	894	Nayak *et al*. (2005)
	(R) ATT TTA TTA TTT GTA GCA GCG	3578→3558		

Y[1]: C or T

two samples were negative in m-PCR assay.

Discussion

Detection of *Campylobacter* in poultry carcasses is important for the identification of the source of outbreaks associated with the consumption of improperly cooked poultry meat (Pearson and Healing, 1992).

Conventional culture method for isolation of *Campylobacter* generally requires 4 days to give a negative result and 6-7 days to confirm a positive result. In this method discrimination between the closely related species, *C. jejuni* and *C. coli* is only based on the hippurate hydrolysis test (Barrett *et al*., 1988), but this phenotypic distinction is not always accurate (Totten *et al*., 1987; Nicholson and Patton, 1993).

Polymerase chain reaction is an excellent and more rapid genetic assay for identification and differentiation of *C. jejuni* and *C. coli* (Lawson *et al*., 1998). Earlier PCR-based methods required two or three individual DNA amplifications and agarose gel electrophoresis analyses per sample.

Several m-PCR assays have also been used to detect *Campylobacter* spp., *C. coli* and *C. jejuni*. In these assays a variety of species-specific gene targets such as *omp50*, 16S rRNA, 23S rRNA, *hipO*, *mapA*, or putative aspartokinase have been employed for identification of *C. coli* and *C. jejuni* (Giesendorf and Quint, 1995; Denis *et al*., 1999; Burnett *et al*., 2002; Wang *et al*., 2002; On and Jordan, 2003; Dedieu *et al*., 2004).

In our study for m-PCR assay, the first set of primers was specific for *Campylobacter* genus and the other one was specific for *C. coli*. The sets of primers have also been used by Cloak and Fratamico (2002) and Nayak *et al*. (2005). After a BLAST search it was revealed that a degeneracy is necessary in one of the primers (*cadF*-R) (Table 1).

The 894 bp *ceuE* gene from *C. coli* encodes a 34.5 to 36.2 kDa lipoprotein component of the binding-protein-dependent transport system for the siderophore enterochelin (Richardson and Park, 1995; Gonzalez *et al*., 1997).

The *cadF* gene from *Campylobacter* genus encodes a 37-kDa outer membrane protein that promotes the binding of these pathogens to intestinal epithelial cells (Konkel *et al*., 1999). In our experiment a 894 bp of *ceuE* gene and a 400 bp fragment of *cadF* gene were selected for amplification.

A previous study conducted by Nayak *et al*. (2005) showed that *cadF* gene was also amplified in three non-*Campylobacter* strains including: *Enterococcus casseliflavus* ATCC 25788, *E. coli* ATCC 43889 and *Pasteurella aerogenes* ATCC 29554 and the *ceuE* gene offered cross-reactivity only with *Enterococcus faecalis* ATCC 51299 (Nayak *et al*., 2005). Therefore, the 400 bp and 894 bp bands can be observed concurrently in *C. coli*. The use of selective enrichment broth and selective plating agar containing different antibiotics, along with the requirement of microaerophilic atmosphere for the growth and the necessity of specific incubation temperature (42°C) for the optimum growth of *Campylobacter*, will make conditions unfavorable for growth of the four mentioned non-*Campylobacter* strains.

In the four previous experiments performed by different researchers, the annealing temperatures used for PCR amplification of *cadF* and *ceuE* gene (for *C. coli*) were 45, 57, 56 and 52°C (Winters and Slavik, 1995; Gonzalez *et al*., 1997; Konkel *et al*., 1999; Nayak *et al*., 2005). We found that annealing temperature of 45°C produces the expected bands without any non-specific PCR product.

In our experiment the analysis of data resulted from conventional and molecular methods revealed huge differences. From 76 out of 100 samples which were positive by conventional culture method, only 28 samples were detected as *Campylobacter* genus by m-PCR. Our results in this study are in agreement with the findings of Nayak *et al*. (2005) which reported that 67% of total *Campylobacter* isolates gave false results with conventional culture method (Nayak *et al*., 2005).

While the hippurate hydrolysis test is rapid, it appears that the positive results are not reliable, because other amino acids or peptides which are transported from the culture media or produced during the incubation can give false-positive results

(Megraud, 1987).

Positive results from hippurate hydrolysis test are based on the observation of the deep purple colour. Pale purple colour is considered as negative. The judgement based on qualitative criteria is not reliable and may lead to misinterpretation.

In our study two samples were negative in hippurate hydrolysis test and susceptible to nalidixic acid and identified as *C. coli*, but these two samples were negative in m-PCR assay. Both *C. jejuni* and *C. coli* are susceptible to nalidixic acid and this result is not surprising, as several strains of *C. jejuni* have been reported to be hippurate-negative (Totten *et al*., 1987; On and Jordan, 2003). The specificity of this PCR assay for detection of the *C. coli* has been shown to be 97%, which was obtained by testing against 11 Gram-positive and 25 Gram-negative isolates (Nayak *et al*., 2005).

The high specificity level of the m-PCR assay which was employed in our experiment indicates that the results of this method could be more reliable than that of the conventional culture method. It is probable that the prevalence of *C. coli* in poultry flocks, is more than what we obtained in this preliminary study.

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