Isolation and identification of *Campylobacter jejuni* from poultry carcasses using conventional culture methods and multiplex PCR assay

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Abstract

Campylobacter jejuni is a major cause of food-borne diarrhea in many countries. Poultry and poultry products are known as important sources of human campylobacteriosis. In this study, conventional culture and multiplex PCR methods were compared for the detection of *C. jejuni* isolated from poultry carcasses. A total of 100 samples, representing 20 broiler flocks, were collected from poultry carcasses after the evisceration stage in the processing line at a commercial broiler slaughtering facility in Mashhad, Iran. In the conventional culture method, samples were processed by enrichment followed by selective plating, and then suspected colonies were isolated on sheep blood agar and tested for morphology, motility, Gram staining, biochemical properties and hippurate hydrolysis activity. For the identification of the *Campylobacter* genus and its *jejuni* serovar by molecular methods, a multiplex PCR assay (m-PCR) with two sets of specific primers was used. In the hippurate hydrolysis test of suspected colonies, 76% of the samples were determined as positive, while in the m-PCR assay 28% of cultures harvested were identified as *C. jejuni*. Two percent of hippurate hydrolyze negative colonies were found positive in the m-PCR test.

It appears that the conventional method, based on the hippurate hydrolysis test for detection of *C. jejuni*, is a less reliable test. The use of the m-PCR method, based on amplification from conserved genes, allows reliable detection and identification of *C. jejuni*.

Introduction

Different species of *Campylobacter* genus are recognized or suspected as human gastrointestinal pathogens (Skirrow, 1994). *C. jejuni* and *C. coli* are frequently associated with human campylobacteriosis, of which more than 80-90% are caused by *C. jejuni* (Skirrow, 1990). In addition to gastroenteritis, *C. jejuni* has also been associated with the development of Guillain-Barré syndrome (GBS), a disorder of the peripheral nervous system, which is characterized by temporary paralysis due to acute autoimmune inflammatory polyradiculoneuropathy (Nachamkin *et al.,* 1998; Hadden & Greson, 2001). Nearly 40% of *C. jejuni* infections have shown to precede GBS (Dingle *et al,.* 2001). In many western countries, the incidence of Campylobacteriosis is higher than diseases caused by *Salmonella spp.* (Rohner *et al.*, 1997). However, due to inappropriate detection methods and lack of training, a number of cases might have been undetected in developing countries (Trachoo, 2003).

The main transmission route of infection is the ingestion of food of animal origin (Butzler & Oosterom, 1991). Poultry and poultry products are known as important sources of human campylobacteriosis and play an important role in disease transmission (Deming *et al.,* 1987; Evans, 1992; Genigeorgis & Hassuney, 1986; Harris *et al.,* 1986). The fastidious growth requirements, complex taxonomy and unreliable biochemical tests present significant challenges in the identification of *Campylobacter spp.* (On, 1996, 2001). Furthermore, *C. coli* and *C. jejuni* are closely related by phylogenetic and genetic criteria (Dedieu *et al.,* 2006), thereby making identification of *Campylobacter* at species level is difficult. Although the hippurate hydrolysis test is widely used to differentiate *C. jejuni* from other species of *Campylobacter* (Morris *et al.,* 1985), hippurate-negative *C. jejuni* strains have been isolated (Denis *et al.,* 1999; On & Jordan, 2003). 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 absolutely necessary. Molecular tests

appear to be attractive candidates due to their relative ease of use, low cost, and potential application in large-scale screening programs, by means of automated technologies (Kricka, 1998). The PCR assay also allows not only detection of viable but also non-cultivable forms of *Campylobacter* (Hazeleger *et al.,* 1994; Wegmuller *et al.,* 1993). Several multiplex PCR assays have 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*, putative aspartokinase, *cad F* and oxidoreductase subunit have been employed for identification of *C. coli* and *C. jejuni* (Dedieu *et al.,* 2004; Denis *et al.,* 1999; On & Jordan, 2003; Giesendorf & Quint, 1995; Burnett *et al.,* 2002; Wang *et al.,* 2002; *Nayak et al.,* 2005).

The objective of this preliminary study was to determine the contamination rate of broiler carcasses with *C. jejuni* by using a conventional culture method and compare its results with a multiplex PCR assay.

Materials and Methods

Bacterial reference strain

C. jejuni ATCC 33291, purchased from Mast International Inc. (UK), was used for PCR optimization and also employed as positive control in the multiplex PCR assay.

Rinse test sampling

A total of 100 samples were randomly collected from broiler chickens, representing 20 broiler flocks, after the evisceration stage in the processing line at a commercial broiler slaughtering facility in Mashhad, Iran. 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 kept on crushed ice until further processing.

Conventional culture method

Filtrated chicken rinses were centrifuged at 16,000 × g for 10 min at 4°C. The supernatant was discarded and the pellet was suspended in 5 ml of enrichment broth. After resuspension of the pellet, the samples were incubated in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) at 42°C for 48 h. The enrichment broth consisted of nutrient broth (Merck), supplemented with trimethoprim 10mg/l, rifampicin 5mg/l, polymyxin B 2500 IU/l, cefoprazone15mg/l, amphotricin 2mg/l, using selectatab (SV59 series, Mast Diagnostics). The enriched cultures were plated onto a selective medium, consisting of blood agar base supplemented with 7% lysed horse blood and antibiotics, including vancomycin 10mg/l, polymyxin B 2500 IU/l, trimethoprim 5mg/l, using selectavial (SV3 series, Mast Diagnostics). The plates were incubated under microaerobic atmosphere conditions 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, five colonies from each plate were isolated on blood agar plates containing 5% sheep blood, under micro-aerophilic conditions, at 42°C for 72 h, followed by biochemical tests, including catalase, oxidase, and hippurate hydrolysis (Denis *et al.*, 1999, Vanderzant & Splittstoesse, 1992, Nayak *et al.,* 2005).

Hippurate hydrolysis test

A loopful of suspected colonies isolated on sheep blood agar was transferred 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 (Merck) solution in a 1:1 mixture of acetone and butanol was added to each tube on the top of the hippurate solution. For color development, further incubation was carried out at 37°C for 10 min. A deep purple color, crystal violet-like, was recorded as a positive result. It indicated the presence of glycine, resulting from the hydrolysis of hippurate. A pale purple color or colorless results were considered as negative results for hippurate hydrolysis. The test was performed twice on each suspected colony (Denis *et al.,* 1999).

DNA extraction

The colonies from selective media plates described above 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 13,000 × g for 5 min. The supernatants were used as DNA templates for PCR (Nayak *et al.,* 2005).

Multiplex PCR assay

The reaction mixture consisted of 2.5 µl of bacterial lysate, 2.5 µl of 10 BSA buffer (1 ml 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 primer (100 pmol/µl), 0.2 µl of Taq DNA polymerase (5 U/µl) and deionized water to a final volume of 25 µl. The oligonucleotide primers used are shown in Table 1. After a BLAST search, it was revealed that two degenerated sites were necessary in cad F (R) and oxidoreductase subunit (F) primers (Table 1). The reaction mixture was amplified in a thermocyler (BioRad iCycler) and 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 sec, extension at 72°C for 1 min, and 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 (Fermentas) was used as a size reference for PCR assay. Genomic DNA extracted from *C. jejuni* ATCC 33291 was used as positive control and deionized distilled water was used as template for negative control in all PCR reactions.

Results

A total number of 100 samples from poultry carcasses, representing 20 broiler flocks, were analyzed for the presence of *C. jejuni* by a conventional culture method. This included enrichment, selective plating, biochemical tests from suspected colonies and examining them for morphology, motility and gram staining. The results showed that 76 samples (76%) were contaminated with *C. jejuni*.

Results of the multiplex-PCR are shown in Fig. 1. The m-PCR assay generated two PCR products with a length of 400 bp and 160 bp, indicating the presence of *Campylobacter* genus and *C. jejuni*, respectively. Out of 100 suspected colonies that were analyzed by m-PCR assay, 28 (28%) were determined as positive for both *Campylobacter* genus and *C. jejuni* serovar (Figure 1). Two samples that were negative in hippurate hydrolysis test were found to be positive in the 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 & Healing, 1992). The conventional culture method for the 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 (Barret *et al*., 1988), but this phenotypic distinction is not always accurate (Totten *et al*., 1987; Nicholson & Patton 1993). Alternative methods have been investigated for the detection of *Campylobacter spp*. in poultry meat and poultry products. Polymerase chain reaction (PCR) is an excellent and more rapid genetic assay for

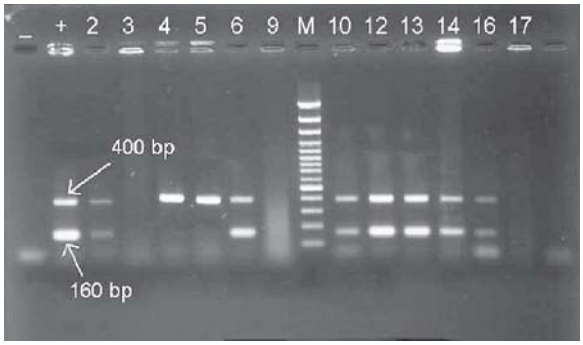

Figure 1: Detection of *C. jejuni* in chicken carcass rinses by multiplex PCR assay, amplifying a 400 bp segment of *cadF* gene, specific for *Campylobacter* genus and 160 bp segment of *oxidoreductase subunit*, specific for *C. jejuni*. Lane (-): negative control (DW), Lane (+): positive control (*C. jejuni* ATCC 33291), Lane (M): 100-bp markers. Lanes 4, 5: positive samples for *Campylobacter* genus, lanes 2, 6, 10, 12, 13, 14 and 16: positive samples for *C. jejuni*.

identification and differentiation of *C. jejuni* and *C. coli* (Gonzalez *et al*., 1997; Linton *et al*., 1997; Lawson *et al*., 1998). The first set of primers used in this study was specific for the *Campylobacter* genus, while the second pair was specific for *C. jejuni*. These sets of primers have also been used previously by Cloak & Fratamico (2002) and Nayak *et al*. (2005). A previous study conducted by Nayak *et al*. (2005) showed that the *cadF* gene was also amplified in three non-*Campylobacter* species, including: *Enterococcus casseliflavus* ATCC 25788, *Escherichia coli* ATCC 43889 and *Pasteurella aerogenes* ATCC 29554, but the 400 bp and 160 bp bands were observed concurrently only in *P. aerogenes* (Nayak *et al*., 2005). The use of selective conditions (such as enrichment broth and selective plating agar containing different antibiotics), along with the requirement of a microaerophilic atmosphere and a specific incubation temperature (42°C) for the optimum growth of *Campylobacter*, make conditions unfavorable for the growth of the three mentioned non-*Campylobacter* strains, although the m-PCR method employed in this study cannot detect non-cultivable forms of *C. jejuni*.

In four previous experiments performed by different researchers, the annealing temperatures used for PCR amplification of *cadF* and the undefined gene (for *C. jejuni*) were 45°C, 57°C, 56°C and 52°C, respectively (Konkel *et al.,* 1999; Gonzalez *et al.,* 1997; Winter & Slavik, 1995; Nayak *et al.,* 2005). It was found that an annealing temperature of 45°C

Table 1: PCR primers used for *C. jejuni* detection.

Target gene	Sequence (5'→3')	Gene location	PCR product (bp)
cadF—outer membrane protein (*Campylobacter* genus)	(F) TTG AAG GTA ATT TAG ATA TG (R) CTA ATA CCY[1] AAA GTT GAA AC	101→120 497→478	400
Oxidoreductase subunit (*Campylobacter jejuni*)	(F) CAA ATA AAR[2] TTA GAG GTA GAA TGT (R) GGA TAA GCA CTA GCT AGC TGA T	66983→67007 67141→67120	160

Y[1]: C or T R[2]: A or G

produced the expected bands without any non-specific PCR products. Analysis of the data obtained from conventional and molecular methods revealed huge differences. From 76 out of 100 samples that were positive according to the conventional culture method, only 26 samples were confirmed to be positive by m-PCR. Our results support earlier research conducted by Nayak *et al.*, which reported that 67% of chicken isolates of *C. jejuni* gave false results with the hippurate hydrolysis test (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 that 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 observing a deep purple color. A pale purple color is considered as negative, so the judgment based on qualitative criteria could not be reliable, and may lead to misinterpretations. In our study, two samples were negative in hippurate hydrolysis test, but positive in the m-PCR assay. This result is not surprising, as several strains of *C. jejuni* have been reported to be hippurate-negative (On & Jordan 2003; Totten *et al.,* 1987). The specificity of this PCR assay for detection of the *C. jejuni* has been shown to be 97%, which were determined by testing against 11 Gram positive and 25 Gram negative isolates (Nayak *et al.,* 2005).

The high specificity level of the m-PCR assay that was employed in our experiment indicates that the obtained results by this method could be more reliable than the results of the conventional method. The sensitivity of this m-PCR assay in detecting both *Campylobacter* genus and *C. jejuni* at low levels in different foods deserves further investigations. Further application of DNA-based detection methods will improve our understanding of the true prevalence of *Campylobacter* species in different reservoirs and will improve our understanding of the role of these reservoirs in the epidemiology of human infections.

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