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Isolation and Identification of *Campylobacter jejuni* from Bulk Tank Milk in Mashhad-Iran

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Abstract: *Campylobacter jejuni* is a major cause of food-borne diarrhea in many countries. In this study a total number of 200 bulk tank milk samples which were delivered to Pegah pasteurization factory in Mashhad were randomly collected, in consecutive days during summer. The presence of *Campylobacter* genus and serovar *Campylobacter jejuni* in collected samples were assessed by performing the enrichment followed by streaking on selective media. The suspected colonies were isolated on sheep blood agar and tested for morphology, motility, gram staining, biochemical properties and hippurate hydrolysis activity. For comparison, a multiplex PCR assay (m-PCR) from suspected colonies with two sets of primers was employed for concurrent identification of *Campylobacter* genus and its *jejuni* serovar. By conventional culture method including hippurate hydrolysis test from suspected colonies, 12.5% of samples were determined as positive, but in m-PCR assay 8% of cultures harvest, identified as *C. jejuni*. It seems that conventional method, based on hippurate hydrolysis for detection of *C. jejuni*, could not be a reliable test. The use of m-PCR method based on amplification from conserved genes, allows reliable detection and identification of *C. jejuni*.

Key words: *Campylobacter jejuni* · Culture method · m-PCR · Bulk tank milk

INTRODUCTION

Different species of *Campylobacter* genus are recognized or suspected as human gastrointestinal pathogens [1]. *C. jejuni* and *C. coli* are frequently associated with human campylobacteriosis. More than 80-90% of Campylobacteriosis are caused by *C. jejuni* [2]. *C. jejuni* is more prevalent and frequently has been isolated from bulk tank milk [3-5]. In addition to gastroenteritis, *C. jejuni* has also been associated with the development of Guillain-Barre syndrome (GBS), which is a serious neurological disease with symptoms that include flaccid paralysis [6]. Nearly 40% of *C. jejuni* infections have shown to precede GBS [7]. *Campylobacter* was also reported as a causative agent of diarrhea in travelers staying for different times in developing areas including the Middle East, North Africa, Southeast Asia and Latin America [8]. In many developed countries, the incidence of campylobacteriosis is higher than diseases caused by *Salmonella* [9]. However, in developing countries due to inappropriate detection method and ignorance, a number of cases might have been undetected [10]. The main transmission route of infection is ingestion of food of animal origin [11]. Consumption of raw milk, inadequately pasteurized milk and cheese contaminated with *Campylobacter* was shown to be responsible for enteric infection outbreaks [12-14]. The prevalence rates of pathogens including *Campylobacter* spp. in bulk tank milk vary considerably among surveys and could be influenced by several factors such as geographical area, season, farm size, number of animals on farm, hygiene and farm management practices [4].

The fastidious growth requirements, complex taxonomy and unreliable biochemical tests present significant challenges in the identification of *Campylobacter* spp. [15,16]. Furthermore, *C. coli* and *C. jejuni* are closely related by phylogenetic and genetic criteria [17], so identification of *Campylobacter* at species level is difficult. For the treatment of human campylobacteriosis, differentiation of *C. jejuni* and *C. coli* is necessary [18]. Although the hippurate hydrolysis test is widely used to differentiate *C. jejuni* from other species of *Campylobacter* [19], but *C. jejuni* hippurate-negative

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strains have been isolated [20, 21]. Thus, development of simple methods for detection and reliable differentiation of the thermophilic *Campylobacter* species are absolutely 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, appear to be attractive candidates [22]. 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 [17, 20, 21, 23-26].

The objective of this study was to determine the contamination rate of bulk tank milk with *C. jejuni*, using conventional culture method and compare it with a multiplex PCR assay.

MATERIALS AND METHODS

Bacterial Reference Strain: In this study *Campylobacter jejuni* (ATCC 33291) purchased from Mast International Inc, was used for PCR optimization and also employed as positive control in the multiplex PCR assay.

Sample Collection: A total numbers of 200 samples, from bulk tanks milk which was collected from different dairy herds, located in Mashhad suburb and were delivering to Pegah pasteurization factory in Mashhad (Iran) and were randomly obtained. The samples were brought to the laboratory on crashed ice.

Conventional Culture Method: In the laboratory 10 ml of raw milk was centrifuged at 14,000 rpm for 20 min at 4°C and the resulting pellet was resuspended in 45 ml of *Campylobacter* enrichment broth [27]. The tubes were incubated in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) at 42°C for 48 h. Enrichment broth was consisted of nutrient broth (Merck), supplemented with trimethoprim 10mg/L, rifampicin 5mg/L, polymyxinB 2500 IU/L, cefoprazone15mg/L, amphotricin 2mg/L, using selectatab (SV59 series-Mast Diagnostics). Then the enriched cultures were plated onto a selective media, consisted of blood agar base supplemented with 7% lysed horse blood and antibiotics including, vancomycin 10mg/L, polymyxinB 2500 IU/L, trimethoprim 5mg/L, using selectavial (SV3 series-Mast Diagnostics). The plates were incubated under 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, utmost five suspected colonies from each plate were isolated on blood agar plates containing 5% sheep blood, under microaerophilic conditions, at 42°C for 72 h, followed by biochemical tests, including catalase, oxidase and hippurate hydrolysis.

Hippurate Hydrolysis Test: A loopful of the suspected colonies isolated on sheep blood agar was transferred to0.5 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 in 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 positive result, indicating the presence of glycine, resulted from the hydrolysis of the hippurate. A pale purple color or colorless results were considered as negative for hippurate hydrolysis. The test was performed twice on each suspected colony.

DNA Extraction: The same suspected colonies 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 13,000 rpm for 5 min. The supernatants were used as DNA templates for PCR assay.

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× 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, 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. After a BLAST search it was revealed that two degeneracies are necessary in cad F (R) and oxidoreductase subunit (F) primers (Table 1). The reaction mixture was amplified in a thermocycler (Bio-Rad 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 and 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 100V for 40 min in

Table 1: PCR primers used for *Campylobacter jejuni* detection

Target gene	Sequence (5'→3')	Gene location	PCR product (bp)	Reference
CadF—outer membrane protein				
(*Campylobacter genus*)	(F) TTG AAG GTA ATT TAG ATA TG	101→120		
	(R) CTA ATA CCY[1] AAA GTT GAA AC	497→478	400	[18-26]
Oxidoreductase subunit (*C. jejuni*)	(F) CAA ATA AAR[2] TTA GAG GTA GAA TGT	66983→67007		
	(R) GGA TAA GCA CTA GCT AGC TGA T	67141→67120	160	[18-26]

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

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. jejuni* (ATCC 33291) was used as positive controls in all PCR reactions.

RESULTS

Conventional Method: In conventional culture method, numbers of 200 samples from bulk tank milk were analyzed by enrichment, selective plating and biochemical tests from suspected colonies and examining them for morphology, motility, gram staining and hippurate hydrolysis test. Total of 25 samples (12.5%) were determined as contaminated with *C. jejuni*.

PCR Method: Results of the m-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* spp and C. *jejuni,* respectively. Out of 200 samples that were analyzed by m-PCR assay, 31 (15.5%) were determined as positive for *Campylobacter* genus and number of 16 (8%) determined as *C. jejuni*.

DISCUSSION

Transmission of *Campylobacter* infections to humans via the consumption of raw milk has been reported in numerous outbreaks [14, 28]. The infective dose of *C. jejuni* cells is very small and it has been estimated that as few as 500 cells could cause human illness [29]. This means that even a very small number of *C. jejuni* cells, present a potential health hazard. Thus, sensitive methods are needed to detect *C. jejuni* in different foods. The outcome of studies examining the presence of bacterial pathogens in foods largely depends on the efficiency of the method employed in pathogen detection. As with other pathogens, there is no single method that could be successfully applied for the detection of *Campylobacter* in all different food samples [30].

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 [31], but this phenotypic distinction is not always accurate [32, 33].

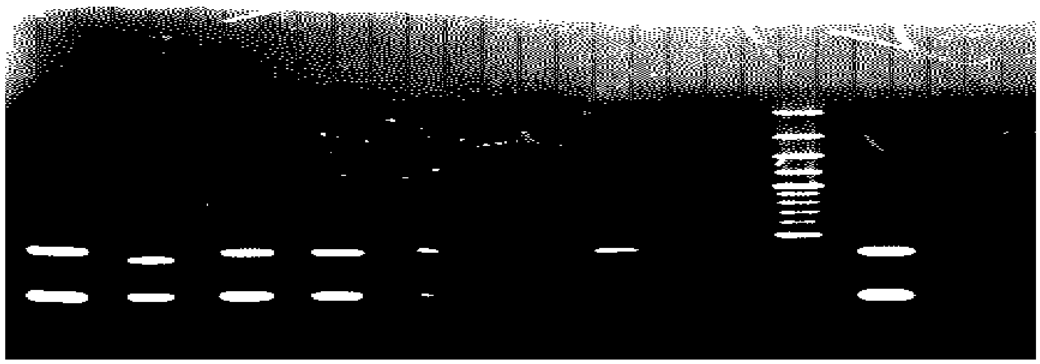

Fig. 1: Detection of *Campylobacter jejuni* in bulk tank milk samples by multiplex PCR assay, amplifying 400 bp segment of *cadF* gene, specific for *campylobacter* genus and 160 bp segment of *oxidoreductase subunit* gene, specific for *C. jejuni*: Lane: (11) negative control (DW), Lane(10) positive control (*C. jejuni* ATCC 33291), Lane(9) 100bp markers. Lanes 6, 7, 8 positive samples for *Campylobacter* genus, Lanes 1, 2, 3, 4, 5 positive samples for *C. jejuni*.

Alternative methods have been investigated for detection of *campylobacter* spp in foods. Polymerase chain reaction (PCR) is an excellent and more rapid genetic assay for identification and differentiation of *C. jejuni* and *C. coli* [34-36]. 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 .jejuni*. The sets of primers have also been used by Cloak and Fratamico [18] and Nayak [26].

A previous study conducted by Nayak *et al.* [26] showed *cadF* gene was also amplified in three non *Campylobacter* strains 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*. In this study the use of selective enrichment broth and selective plating agar containing different antibiotics, along with the requirement of microaerophilic atmosphere for the growth and necessity of specific incubation temperature (42°C) for the optimum growth of campylobacter, will make conditions unfavorable for growth of *P. aerogenes*.

Although the m-PCR method employed in this study can not detect noncultivable forms of *C. jejuni*, but it should be noted that an acceptable specimen should be free of PCR inhibitory substances that could produce a false-negative result. Samples may contain substances which are not always removed by the extraction process and which may inhibit the PCR amplification, so because of the presence of calcium ions as a PCR inhibitor in raw milk [37], it seems that direct PCR is not a recommendable procedure.

In four previous experiments performed by different researchers, the annealing temperatures used for PCR amplification of *cadF* and the *oxidoreductase subunit* for *C. jejuni* were 45, 57, 56 and 52°C [26, 34, 38, 39]. We found that annealing temperature of 45°C produces the expected bands without any non-specific PCR product.

In this study by using m-PCR assay, *C. jejuni* was found in 16 (8%) of the BTM samples, but in conventional culture method, number of 25 (12.5%) samples determined as contaminated whit *C. jejuni*. Our results are in agreement by the research conducted by Nayak *et al.* [21, 32] which reported that 67% of total isolates of *C. jejuni,* gave false results with the hippurate hydrolysis test. Besides, it has been reported that several strains of *C. jejuni* are hippurate-negative. Previous studies have reported the prevalence of *C. jejuni* in raw milk samples as ranging from <1 to 12% [4, 40].

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 [41]. Positive results from hippurate hydrolysis test are based on observing a deep purple color. Pale purple color is considered as negative. The judgment based on qualitative criteria is not reliable and may lead to miss interpretations.

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 [26].

The high specificity level of the m-PCR assay which was employed in our experiment indicates that the obtained results by this method could be more reliable than what resulted from conventional method. The sensitivity of this m-PCR assay in detecting *campylobacter* genus and *C. jejuni* at low levels in different food matrices needs further investigation.

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