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Comparative analysis of cultural isolation and PCR based assay for detection of *Campylobacter jejuni* and *Campylobacter coli* in foods of animal origin

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ABSTRACT

The present study was designed to determine the occurrence of *C. jejuni* and *C. coli* in chicken meat, chicken offal, chevon and milk samples from Bareilly, Uttar Pradesh (India) to elucidate the role of these products in transmission of *Campylobacter* to humans. The samples were examined using both cultural as well as polymerase chain reaction based detection method. Out of 280 samples examined, 29 (10.36%) samples were found positive by cultural method, where as 68 (24.29%) samples showed presence of *Campylobacter* species by polymerase chain reaction. All the samples depicted positive by culture were also found positive by PCR. The highest occurrence rate was observed in chicken meat (46%), followed by chicken offal (20%), chevon (18%) and milk (3.75%). The results depicted the superior efficacy of PCR for rapid screening of samples owing to its high rapidity, specificity and automation potential. The study suggested the potential of transmission of *Campylobacter* species to humans due to the consumption of raw or contaminated meat or milk.

Keywords: *Campylobacter jejuni, Campylobacter coli,* Culture, Polymerase chain reaction, Chicken meat

Thermophilic *Campylobacter* species, particularly *C. jejuni* and *C. coli* are well known food-borne pathogens and recognized as most common agents of acute gastroenteritis in humans (Scallan *et al.,* 2011). Nowadays, campylobacteriosis is a disease of great public health concern in underdeveloped, developing and developed countries (Humphrey *et al.,* 2007; Prasad *et al.,* 2001). Amongst all *Campylobacter* species, approximately 90 - 95% of enteric infections in the industrialized nations are incriminated to *C. jejuni* subspecies *jejuni*, usually referred to as *C. jejuni* and only 3 - 4% cases are reported due to *C. coli* (Vandamme, 2000), but the epidemiology and the outcomes of these disease conditions are less targeted and least understood in developing countries (Prasad *et al.,* 2001).

A low dose containing 500 – 800 organisms of *C. jejuni* has proved to be completely infectious for humans (Nachamkin *et al.,* 2008). Poultry as well as non-poultry food animals such as beef cattle, sheep, goat, pig and their products are significant source of human campylobacteriosis (Allos 2001; McCrea *et al.,* 2006; Humphrey *et al.,* 2007; Suzuki and Yamamoto, 2009). Conventional methods for isolation and identification of *Campylobacter* spp. from foods are arduous and extremely time consuming as the entire process goes on for 5 days (Corry *et al.,* 1995). The limitations of cultural methods have prompted researchers to develop more rapid, sensitive and reliable alternatives for detection of *Campylobacter* species. Molecular techniques such as polymerase chain reaction have been found to be reliable and rapid methods for detection of *Campylobacter* species (Lubeck *et al.,* 2003; Vanniasinkam *et al.,* 1999). Thus, this study was conducted to adjudge the efficiency of classical cultural and polymerase chain reaction based diagnostic method for the detection of *C. jejuni* and *C. coli*, keeping in view the duration for processing and quality of results.

Table 1. Sequence of primers used (Klena *et al*., 2004) for the detection of *Campylobacter jejuni* and *Campylobacter coli*

Primers	Sequences (5`- 3`)	Product size
mP - Cj Forward	ACAACTTGGTGACGATGTTGTA	331 bp
mP - Cc Forward	AGACAAATAAGAGAGAATCAG	391 bp
mP - R Reverse	CAATCATGDGCDATATGASAATAHGCCAT	

MATERIALS AND METHODS

Sample Collection

A total of 280 food samples comprising 100 chicken meat (breast and leg muscles), 50 chicken offal, 50 chevon (leg muscles) and 80 raw milk samples from Bareilly, Uttar Pradesh (India) were targeted in the study. The samples were taken aseptically and transported to the laboratory in Carry Blair transport media.

Selective enrichment

The commercially available Preston enrichment broth supplemented with Preston selective supplement (HiMedia Laboratories, India) was used for enrichment of the samples. Approximately 25 g of chicken meat, chicken offal and chevon was enriched in 100 ml Preston enrichment broth with Preston selective supplement and incubated at 42°C for 24-36 hours under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) using CampyPak (BD, Oxoid) gas generating packs. In case of milk, 50 ml of each sample was centrifuged at 20,000 × g for 40 min. The supernatant was discarded and pellet was redissolved in 100 ml of Preston enrichment broth and incubated under microaerophilic conditions at 42°C for 24 - 36 hours (OIE, 2008).

Selective plating

The modified charcoal cefoperazone deoxycholate agar (mCCDA) supplemented with CCDA supplement (HiMedia Laboratories, India) was used as selective plating medium. A loopful of the enriched culture was streaked onto mCCDA and incubated under microaerophilic conditions at 42°C for 48 hours. The suspected colonies exhibiting the typical characteristics (greyish, flat and moistened with a tendency to spread and with or without metal sheen) were picked up and subcultured again onto mCCDA to isolate pure colonies for further processing (Figure 1). The isolates were identified to genus level by a typical Gram stain appearance, characteristic corkscrew-like motility, oxidase test, catalase test and nitrate reduction test. The identification to species level was done by indoxyl acetate hydrolysis test, H_2S production in triple sugar iron agar, growth in 1% glycine, resistance to cephalothin and sensitivity to nalidixic acid. Hippurate hydrolysis test was performed to differentiate *C. jejuni* and *C. coli* isolates (OIE, 2008).

PCR based detection

A PCR assay targeting the lipid gene *lpx*A (Klena *et al.,* 2004) was used for the detection and differentiation of *C. jejuni* and *C. coli* from enriched broths. However, the primers developed by Klena *et al.,* (2004) could differentiate four species of Campylobacters; *C. jejuni*, *C. coli*, *C. lari* and C. *upsaliensis*. As human and pigs are major reservoirs for *C. lari* and C. *upsaliensis* and the present study was targeting chicken meat, chevon and milk only, so primers for these two species were not considered. The genomic DNA from enriched broths was extracted using DNeasy Blood and Tissue Kit (QIAGEN, USA) following the manufacturer's instructions.

The details of primer set used, along with the product size are listed in Table 1. The reaction mixture (25 µl) comprised 2.5 µl of 10X Dream *Taq* Buffer (MBI fermentas, USA), 2.5 µl of 2 mM of dNTP mix (MBI fermentas, USA), 15 pmol of each primer, 1 U *Taq* Polymerase (MBI fermentas, USA), 4 µl of bacterial DNA and nuclease-free water to make volume upto 25 µl. The amplification was carried out in a thermal cycler (Corbett Research, Australia). The cyclic conditions followed were: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were analysed on 1.5% agarose in gel electrophoresis unit (Merck Biosciences, India) and photographed using gel documentation system (Ultra Violet Product Ltd. U.K.).

Table 2. Comparison of culture and PCR for detection of *Campylobacter jejuni* and *Campylobacter coli*

Type of sample	Number	Culture (%)			Polymerase chain reaction (%)			
		C. jejuni	C. coli	Total	C. jejuni	C. coli	Mixed	Total
Chicken meat	100	18(18)	4(4)	22(22)	28(28)	10(10)	8(8)	46(46)
Chicken offal	50	3(6)	1(2)	4(8)	5(10)	3(6)	2(4)	10(20)
Chevon	50	2(4)	1(2)	3(6)	7(14)	2(4)	0(0)	9(18)
Milk	80	0(0)	0(0)	0(0)	1(1.25)	2(2.5)	0(0)	3(3.75)
Total	280	23(8.21)	6(2.14)	29(10.36)	41(14.64)	17(6.07)	10(0.03)	68(24.29)

To test the specificity of primers, the PCR assay was also applied on *E. coli, Salmonella* and *Arcobacter*. PCR reaction mixture and cyclic conditions were kept same as described above.

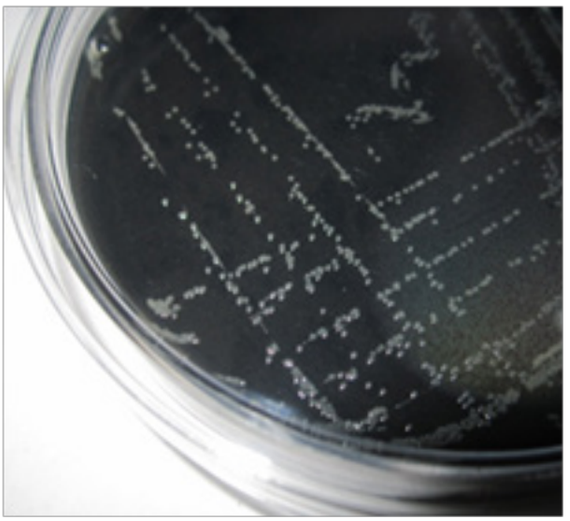

Figure 1. *Campylobacter* **colonies on Modified charcoal cefoperazone deoxycholate agar**

Diagnostic specificity

The level of agreement according to precision was expressed as the kappa statistic, defined as the proportion of potential agreement beyond chance exhibited by two tests. The diagnostic specificity was calculated as $d/(b+d)$, where d is the number of samples negative by both PCR and culture and b is the number of samples positive by PCR but negative by culture. The level of agreement between two tests was calculated as $(a+d)/n$, where a is the number of samples positive by both PCR and culture, d is the number of samples negative by both methods, and n is the total number of samples under examination (Martin *et al*., 1997).

Table 3. Diagnostic specificity of PCR assay for detection of *Campylobacter jejuni* and *Campylobacter coli*

PCR results	Cultural method results			Diagnostic specificity	Level of agree-ment
	Positive	Negative	Total		
Positive	29	39	68		
Negative	0	212	212	0.85	0.86
Total	29	251	280		

RESULTS AND DISCUSSION

A total of 29 (10.36%) samples were found positive by selective culture, out of which 79.31% (23/29) were identified as *C. jejuni* and 20.69% (6/29) as *C. coli*. Biochemically, the isolates were positive for oxidase, catalase, nitrate reduction tests as well as species specific tests like indoxyl acetate hydrolysis test, growth in 1% glycine and were sensitive to nalidixic acid. The isolates found positive by hippurate hydrolysis test were considered as *C. jejuni* and rest as *C. coli*. The highest isolation rate was observed in chicken meat (22%) followed by chicken offal (8%), chevon (6%) and milk (0%). *C. lari* which also prefers to grow at 42ºC was not considered in the present study because the isolates proved sensitive to nalidixic acid whereas, *C. lari* is resistant to nalidixic acid and does

not hydrolyse hippuric acid. Also, 95% of the foods borne gastroenteritis cases are caused by the targeted species and *C. lari* is not of much public health importance.

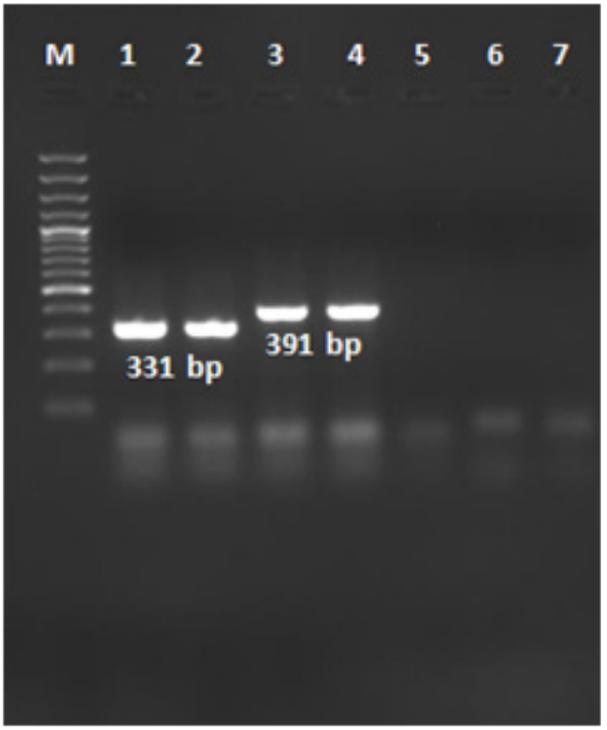

Figure 2. Specificity of PCR assay for the detection of *Campylobacter jejuni* **and** *Campylobacter coli***: (M) 100 bp plus ladder; (1&2)** *C. jejuni* **(331 bp); (3&4)** *C. coli* **(391 bp); (5)** *Salmonella***; (6)** *E. coli***; (7)** *Arcobacter*

A total of 68 (24.29%) samples were found positive by PCR out of which 60.29% (41/68) were identified as *C. jejuni* and 25% (17/68) as *C. coli* and 14.71% (10/68) as mixed comprising both *C. jejuni* and *C. coli. C. jejuni* amplicons produced 331 bp product where as *C. coli* amplicons produced 391 bp product (Figure 2). All the samples depicted positive by culture were also found positive by PCR. The highest occurrence rate was observed in chicken meat (46%), followed by chicken offal (20%), chevon (18%) and milk (3.75%). The samples positive by culture were also found positive by PCR. *C. jejuni* was detected at a higher rate (8.21% by culture and 14.64% by PCR) than *C. coli* (2.14% by culture and 6.07% by PCR). The comparison between two techniques is shown in Table 2. The results were in concordance with the findings of Vandamme (2000), who reported higher prevalence of *C. jejuni* (90-95%) as compared to *C. coli* (3-4%) in developed nations.

No amplification was observed in PCR using DNA extracted from *E. coli, Salmonella* and *Arcobacter* organisms (Figure 2). The absence of desired amplicons from these organisms confirmed the specificity of the primers.

In this study, *C. jejuni* was isolated in higher numbers as compared to *C. coli* and maximum isolation was recovered from chicken meat samples. Rahimi and Esfahani (2010) also reported that *C. jejuni* is most frequently isolated species from chicken meat than *C. coli* and our findings are in agreement with their results, where most of the isolates (93%) were of *C. jejuni.*

Chicken offal and chevon showed comparatively lesser occurrence rate as compared to chicken meat. In the present study, occurrence rate of 18% was reported from chevon samples using PCR. The studies conducted in other parts of the world revealed comparatively lower occurrence rate; 10.5% from Ethiopia (Dadi and Asrat 2008) and 11.8% from Ireland (Whyte *et al.,* 2004). In this investigation, no *Campylobacter* isolate was recovered from raw milk samples and the findings are comparable to observations of Wegmuller *et al.* (1993) and Singh *et al.* (2011) who reported 0% isolation rate from raw milk samples. The difficulty in isolation from milk might be due to low concentrations of the organisms in milk. However, 3 raw milk samples were found positive by PCR with overall occurrence rate of 3.75% and findings are in accordance with the observations of various workers, who reported contamination rates of 0 to 8.1% in raw milk with *Campylobacter* species using PCR (Rosef *et al.,* 1983; Orr *et al.,* 1995; Stanley and Jones, 2003).

A total of 280 samples were tested for the occurrence of *C. jejuni* and *C. coli* both by culture and PCR. A total of 68 samples were positive by PCR and 29 samples were positive by culture. Thirty nine samples positive by PCR were found to be negative by culture. The difference between the performance of PCR and culture was also calculated (Table 3) and the diagnostic specificity for the comparison of PCR to culture was 0.85, with an agreement of 0.86.

The isolation and identification of the campylobacters based on selective culture and biochemical differentiation

up to species level is tedious and non reliable. It generally requires 4 days to give a negative result and 5-6 days to confirm a positive result and also, there are limited biochemical tests used for identification as well as differentiation of *Campylobacter* species due to their inability to ferment or oxidize carbohydrates. But, culture can be considered for the detection of live organisms as it causes only their multiplication leaving the dead ones. Whereas, the limitation with PCR based method is that the DNA of the dead organisms may also get amplified giving false results. Hence, on the basis of our study, we can say that PCR based methods are more rapid and reliable, particularly while processing a large number of samples.

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