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, whereas 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.
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% O₂, 10% CO₂ and 85% N₂) 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 ceferazone 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, H₂S 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 lpxA (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>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’- 3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>mP - Cj Forward</td>
<td>ACAACTTGTTAGCTTTTGTA</td>
<td>331 bp</td>
</tr>
<tr>
<td>mP - Cc Forward</td>
<td>AGACAAATAAGAGAGAATCAG</td>
<td>391 bp</td>
</tr>
<tr>
<td>mP - R Reverse</td>
<td>CAATCATGDCDATATGASAATAHGCCAT</td>
<td></td>
</tr>
</tbody>
</table>

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

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](image)

### 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>
<thead>
<tr>
<th>Type of sample</th>
<th>Number</th>
<th>Culture (%)</th>
<th>Polymerase chain reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C. jejuni</td>
<td>C. coli</td>
</tr>
<tr>
<td>Chicken meat</td>
<td>100</td>
<td>18(18)</td>
<td>4(4)</td>
</tr>
<tr>
<td>Chicken offal</td>
<td>50</td>
<td>3(6)</td>
<td>1(2)</td>
</tr>
<tr>
<td>Chevon</td>
<td>50</td>
<td>2(4)</td>
<td>1(2)</td>
</tr>
<tr>
<td>Milk</td>
<td>80</td>
<td>0(0)</td>
<td>0(0)</td>
</tr>
<tr>
<td>Total</td>
<td>280</td>
<td>23(8.21)</td>
<td>6(2.14)</td>
</tr>
</tbody>
</table>

#### 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 food borne gastroenteritis cases are caused by the targeted species and C. lari is not of much public health importance.

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
Comparative detection of Campylobacter spp. in food and

...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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REFERENCES