

Original Research Article

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Prevalence of *Campylobacter jejuni* in Chicken Meat from Chennai, Tamil Nadu, India

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A study was conducted to assess the prevalence of thermophilic *Campylobacter jejuni* in retail chicken meat sold in meat stalls at Chennai Corporation. For this, samples collected from randomly selected five different zones of Chennai were screened for presence of *Campylobacter jejuni* by conventional culture method and further confirmed by Polymerase Chain Reaction. The results of prevalence study showed that 38. 67% (58/150) of the chicken carcasses examined were positive for *Campylobacter jejuni*. Chicken meat samples from Ambattur zone had highest percentage (63. 33%) of positive samples closely followed by Anna Nagar (56. 67%). Royapuram zone had lowest percentage (13. 33%) of positive samples. Percentage of positive samples from Thiruvottiyur and Adyar zones were 43. 33% and 16. 67%, respectively.

Introduction

Raw chicken have been a significant sources of the bacterial pathogens like *Salmonella* and *Campylobacter* spp. (Deming *et al.*, 1987; Madden *et al.*, 2011; Drinceanu *et al.*, 2013). *Campylobacter* infections have been linked to poultry in many outbreaks (Tauxe *et al.*, 1988; Edwards *et al.*, 2014; Hope *et al.*, 2014; Lahti *et al.*, 2016), primarily due to the consumption of raw or undercooked chicken. Thermophilic *Campylobacter* spp. particularly

Campylobacter jejuni and *Campylobacter coli* have emerged as major cause of diarrhoeal illness globally and are also responsible for the mortality and morbidity among children. In developed countries, *Campylobacter* spp. have been found to be second leading cause of diarrhea/gastrointestinal illness after *Salmonella* spp. Among human beings, *Campylobacters* have also been reported to cause severe conditions like Guillain Barre Syndrome, Chinese paralytic syndrome, hepatitis, meningitis and reactive arthritis

(Allos, 1997; Korman *et al.*, 1997; Tenkate and Stafford, 2001). In India, several studies have revealed moderate to high prevalence of *Campylobacter* spp. in humans and livestock including poultry and their products (Raja, 2012; Rajendran *et al.*, 2012; Geetha, 2013; Mukherjee *et al.*, 2013; Dhanalakshmi *et al.*, 2014; Rajagunalan *et al.*, 2014; Begum *et al.*, 2015; Vaishnavi *et al.*, 2015). However, very limited studies have been conducted to assess the prevalence of *Campylobacter jejuni* in retail chicken carcasses sold in Tamil Nadu, which is one of the leading poultry producing and consuming state in the country. Keeping this in view, the present study was proposed to determine the prevalence of *Campylobacter jejuni* in commercially available chicken meat in Chennai, Tamil Nadu.

Materials and Methods

Collection of chicken meat samples

For this study, a total of 150 chicken meat samples were randomly collected on separate days from different retail chicken meat stalls located at five different zones of Chennai Corporation, namely, Thiruvottiyur, Royapuram, Ambattur, Anna Nagar and Adyar. The zones were randomly chosen to represent northern, southern, eastern, western and central parts of Chennai. Thirty samples were collected from meat stalls at each zone. The samples were collected within 3 to 4h of slaughter, packed individually in clean, sterile polyethylene bags and transported to the Food and Industrial Microbiology Laboratory at College of Food and Dairy Technology, Koduvalli, Chennai, in an insulated container maintained at $4\pm1^{\circ}\text{C}$ under hygienic condition.

Chemicals, Media, Buffers and Reagents

All the chemicals used in the study were of analytical grade purchased from reputed national and international firms. Media,

supplements, laboratory aids and DNA extraction kits used in the study were procured from HiMedia, Oxoid and Qiagen.

Prevalence of *Campylobacter jejuni* in retail chicken meat samples

Isolation and characterization as well as confirmation of *Campylobacter jejuni* was done by conventional culture procedure and Polymerase Chain Reaction (PCR).

Isolation and Characterization of *Campylobacter jejuni* from retail chicken meat samples by conventional culture procedure

Enrichment

Twenty five grams of chicken meat samples were homogenized with 225ml of buffered peptone water (BPW). Then, 1ml of homogenate was taken for enrichment in 9ml of Park and Sander's broth base added with 5% sterile defibrinated lysed sheep blood as well as reconstituted contents of Park and Sander's selective supplement I and II. Then the homogenate were incubated for 24h at 42-43°C under micro-aerophilic conditions.

Selective plating

After incubation, the enriched inoculum was streaked onto selective media (Park and Sander's broth base) along with 2% agar, 5% sterile defibrinated lysed sheep blood, reconstituted contents of Park and Sander's selective supplement I and II. Then, the streaked petri plates were incubated for 48h at 42-43°C under micro-aerophilic conditions.

Selection of suspected *Campylobacter* spp. Colonies and identification

Characteristic *Campylobacter* colonies *i. e.* small, mucoid, non-hemolytic, gray

(sometimes tan and watery) and discrete or spreading swarming types were picked up and subjected to presumptive identification tests like Gram staining, motility, oxidase, catalase tests. These isolates were further subjected to other biochemical tests for confirmation and species differentiation. These tests includes growth at 25°C and 42°C, growth in 3. 5% sodium chloride, H₂S production as detected by reactions on triple sugar iron (TSI) agar, hippurate hydrolysis test, sensitivity to nalidixic acid and resistance to cephalothin.

Biochemical characterization of presumptive *Campylobacter jejuni* colonies

Biochemical tests for identification and speciation as described by Skirrow and Benjamin (1980) and Gracia *et al.*, (1985) were followed with modifications. A brief description employed in various biochemical tests is described below.

Catalase activity

A loopful of test culture was mixed with 2-3 drops of hydrogen peroxide on a clean glass slide and examined for the release of nascent oxygen in the form of gas bubbles. A positive reaction was indicated by the appearance of gas bubbles within 1-2min.

Oxidase reaction

Surface growth of the test culture on Park and Sander's agar plates was smeared on filter paper strips impregnated with oxidase test reagent using a platinum loop. A positive reaction consisted of appearance of a dark purple colour on paper within 30sec.

Growth at 25°C and 42°C

Duplicated Park and Sander's agar plates inoculated with test cultures were incubated under micro-aerophilic conditions at 25°C and

42°C. A positive reaction was indicated by the development of surface colonies within 48h of incubation.

Growth in 1% glycine

The test culture was inoculated into 1% glycine broth and incubated at 42°C for 48h under micro-aerophilic conditions. A loopful of this broth was streaked on the Park and Sander's agar plates and incubated to study the development of clear growth.

Growth in 3. 5% Sodium chloride

The test culture was inoculated into the 3. 5% sodium chloride broth and incubated at 42°C for 48h under micro-aerophilic conditions. Growth was detected by streaking a loopful from this broth onto the Park and Sanders' agar plates followed by incubation at 42 °C for 48h under micro-aerophilic conditions.

Hydrogen sulphide (H₂S) production in Triple Sugar Iron (TSI) agar medium

In TSI agar, with butt stabbed and the slant streaked with the culture under test, was incubated at 42°C in a micro-aerophilic conditions and observed daily for 7 days. A positive reaction was indicated by blackening at the butt due to hydrogen sulphide production.

Sensitivity to Nalidixic acid and resistance to Cephalothin

Disc diffusion test was employed in determining the susceptibility of organism to nalidixic acid. Sterile normal saline suspension or overnight growth cultures from Park and Sander's broth was spread on Mueller-Hinton agar plate. After drying the plate, antibiotic discs of nalidixic acid (30 µg) and cephalothin (30 µg) was placed on the medium. The plates were incubated at 42°C

under micro-aerophilic conditions for 48h. The presence of clear zone of inhibition of growth was taken as sensitive.

Hippurate hydrolysis test

A loopful of 48h old culture growth was inoculated into 1ml of sodium hippurate solution. The inoculated tubes were incubated at 42°C for 4h under micro-aerophilic condition. Then, 0.5 ml of ninhydrin indicator was added and re-incubated at 42°C for 10min. A positive reaction was indicated by the appearance of a deep blue colour.

Confirmation of *Campylobacter jejuni* isolates from retail chicken meat samples by Polymerase Chain Reaction

DNA extraction

DNA extraction was carried out using DNeasy blood and tissue kit (QIAGEN) following the procedure described by the manufacturer. A loopful of 48h grown culture from Park and Sander's agar plates was suspended in 200µl of phosphate buffer saline & mixed by gentle vortexing. Then, 20µl of proteinase K followed by 200µl of AL buffer was added with intermittent mixing by gentle vortexing and incubated at 56°C for 10min in water bath.

Further, 200µl of 99. 9% ethanol was added and again mixed by gentle vortexing. The mix was pipetted into a new spin column tube and centrifuged for 1min at 8000rpm. The flow through and collection tube was discarded and the spin column was placed in a new collection tube. Then, 500µl of buffer AW1 was added and centrifuged for 1min at 8000rpm. The flow through and collection tube was discarded again and the spin column was placed in a new collection tube. Now, 500µl of buffer AW2 was added and centrifuged for 3min at 14000rpm. Finally, the

flow-through and collection tube was discarded and the spin column was placed into a 2ml micro centrifuge tube. Then, 40µl of buffer AE added to the centre of the spin column membrane and incubated at room temperature for 1min. After incubation, the mixture was centrifuged at 8000rpm for 1min and the extracted DNA was collected in 2ml centrifuge tube and stored at -18 to -20°C.

Oligonucleotide primer

A set of primers described by Dhanalakshmi (2011) were used in this study. The details of the primers are as shown in the Table 1.

Standardization of PCR Protocol

The PCR amplification of flaA gene fragment was set up in 25µl reactions. The PCR protocol was initially standardized by using standard culture of *Campylobacter jejuni* procured from Division of Veterinary Public Health, Indian Veterinary Research Institute, Bareilly, Uttar Pradesh by optimizing the concentration of components of reaction mixture in PCR assay and by varying the annealing temperature and cyclic condition as shown in Table 2.

A 25µl of reaction volume which contains 12. 5µl of PCR mastermix, 1µl (10pmol) of each forward and reverse primers, 3µl of template DNA and 7. 5µl of nuclease free water. The reaction was performed in a thermocycler (Master Cycler, Gradient, and Eppendorf, Germany).

The cyclic conditions comprises of following steps:

Agarose Gel Electrophoresis

PCR amplified products were electrophoresed on agarose gel (1. 5%). Agarose gel (1. 5%) was prepared by boiling agarose in an

appropriate volume of 1 x SBB buffer. After cooling for about 5min, 3 μ l of Ethidium Bromide was added to the agarose solution to a final concentration of 0.5 μ g/ml.

Then, molten agarose was poured into the tray. The comb was fitted into the slots on the tray. The tray was kept undisturbed till the gel had solidified.

The comb was then taken-out carefully and the tray containing gel was then placed in a submarine horizontal electrophoresis unit filled with 1 x SBB buffer up to a level of 1mm above the gel surface.

About 8 μ l of each PCR product was loaded into each well. Electrophoresis was performed at 5V/cm and the mobility was monitored by the migration of the dye. After sufficient migration, the gels were observed under UV transilluminator to visualize the bands. The PCR product size was determined by comparing with a standard molecular weight marker and was photographed by the gel documentation system.

Results and Discussion

Colony, morphology and motility of the *Campylobacter* spp. Isolates

Out of 150 chicken carcasses samples examined, 145 showed the presence of watery and dew drop like colonies. Then, all the 145 colonies were subjected to Gram staining and motility test, of these 73 colonies were found to be Gram negative, short spiral or "S" shaped or coccoid shaped bacteria and while incubating their hanging drop preparations for 48h all showed typical cork screw darting type motility. Then, these isolates were further characterized by biochemical tests.

Biochemical characterization

Various biochemical tests were performed on suspected isolates to confirm *Campylobacter* spp.

Biochemical characterization of suspected *Campylobacter* spp. isolates

Suspected colonies of organisms which gave Gram negative stain with spiral or vibrioid morphology and showing cork screw or darting motility were taken as tentative *Campylobacter* spp. and streaked on selective media (Park and Sander's broth base) as per 2. 3. 1 (b).

After the necessary incubation period colonies were tested to a combination of following biochemical tests for characterization of the isolates.

- i. Catalase - Positive
- ii. Oxidase - Positive
- iii. Growth at 25°C – Negative
- iv. Growth at 42°C - Positive
- v. Growth in 1% Glycine – Positive
- vi. Growth in 3. 5% sodium chloride – Negative
- vii. H₂S production (TSI agar method) – Negative
- viii. Sensitivity to nalidixic acid – Sensitive
- ix. Resistance to cephalothin – Resistant
- x. Hippurate hydrolysis - Positive

Table.1 Details of Primer used in PCR

PCR Target Spices	Primer	Sequence(5' To 3')	Size (bp)	Reference
<i>C. jejuni</i>	flaA-Cj F	TCTGCTAAGGCTCCAAGT	367bp	Dhanalakshmi (2011)
	flaA-Cj R	CTCAAGCGGCTCAAGATG		

Table.2 Different temperature and cycling conditions for the PCR assay

S. No.	Steps	Temperature	Time
1	Initial Denaturation	94°C	5 min
2	Denaturation	94°C	1 min
3	Primer annealing	54°C	45 sec
4	Extension	72°C	1 min
Repeat for 34 cycles (Step 2-Step 4)			
5	Final extension	72°C	10 min

Table.3 Zone-wise prevalence of *Campylobacter jejuni* in chicken meat samples

S. No.	Zone	Name of the Zone	No. of samples screened	No. of positive samples
1	Zone I	Thiruvottiyur	30	13
2	Zone V	Royapuram	30	04
3	Zone VII	Ambattur	30	19
4	Zone VIII	Anna Nagar	30	17
5	Zone XIII	Adyar	30	05

Fig.1 Characteristic *Campylobacter* colonies in park and Sanders agar after 48h of Incubation at $42\pm1^{\circ}\text{C}$



Fig.3 Oxidase reaction



Fig.2 Catalase activity



Fig.4 Growth at 42°C



Fig.5 Growth at 25°C

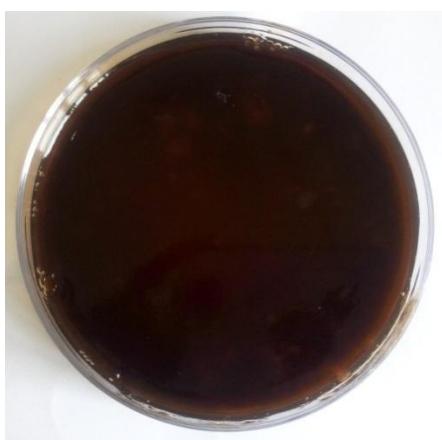


Fig.6 Growth in 1% Glycine



Fig.7 Growth in 3.5% NaCl



Fig.8 Test For H₂S in TSI agar



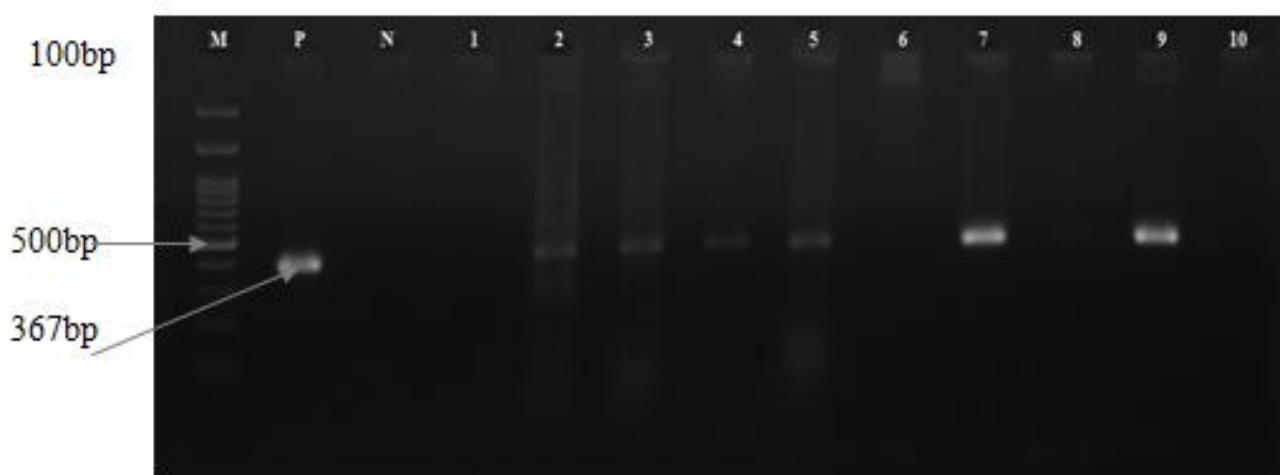
Fig.9 Sensitivity to Nalidixic Acid and Resistance to Cephalothin



Fig.10 Hippurate Hydrolysis Test



Fig.11 Screening of *Campylobacter jejuni* targeting flaA gene



M-Marker, P-positive control, N-Negative control, Samples -1-10, Positive samples-2, 3, 4, 5, 7 and 9. Negative samples-1, 6, 8 and 10.

Based on this a total of 73 suspected samples, which showed the typical morphology and motility were tested for oxidase and catalase reaction. Out of these, 58 isolates were positive for both oxidase and catalase tests which were further subjected to the rest of the biochemical tests. All the 58 isolates were considered presumptive for the *Campylobacter* spp. and then these isolates were further subjected to the biochemical tests like hippurate hydrolysis, rapid H₂S test, Growth at 25°C, Growth at 42°C, Growth in 1% Glycine, Growth in 3. 5% NaCl, Nalidixic acid sensitivity and Cephalothin resistance. The results revealed that all the isolates were belonging to *Campylobacter jejuni*.

Confirmation of *Campylobacter jejuni* by Polymerase Chain Reaction

For early detection of *Campylobacter jejuni*, Polymerase Chain Reaction (PCR) assay was standardized employing a set of primers targeting species specific flaA gene. A total of 150 chicken samples obtained from meat stalls in different zones of Chennai were enriched in enrichment medium as per 2. 3. 1(a) for 24h. The Genomic DNA was obtained from the grown culture following the procedure described in '2. 3. 2(a) DNA extraction', and was used as template. The details of the primer set used for PCR assay and standardization of conditions for PCR reaction are discussed in 2. 3. 2(b) and 2. 3. 2(c), respectively. On completion, amplified products were subjected to electrophoresis on agarose gel (1. 5%) as per 2. 3. 2(d). After sufficient migration, the gels were observed under UV transilluminator to visualize the bands. The PCR product size was determined by comparing with a standard molecular weight marker and was photographed by the gel documentation system (Fig. 11). All the 58 (38. 67%) isolates that were found to be positive for *Campylobacter jejuni* by conventional culture method have also shown

positive results in PCR method.

Overall, a total of 58 (38. 67%) *Campylobacter jejuni* isolates have been found from 150 retail broiler chicken carcasses. The number of positive samples from each zone has been shown in Table 3. In general, the prevalence varied between 13. 33% and 63. 33%. The results showed that the chicken samples from Ambattur zone had highest percentage (63. 33%) of positive samples closely followed by Anna Nagar (56. 67%). Royapuram zone had lowest percentage (13. 33%) of positive cases. Percentage of positive samples from Thiruvottiyur and Adyar zones were 43. 33% and 16. 67%, respectively.

Several authors studied the prevalence of *Campylobacter jejuni* in various food animals including their products in India (Rajan and Mathan, 1982; Chattopadhyay *et al.*, 2001; Bandekar *et al.*, 2005; Elango *et al.*, 2012; Mukherjee *et al.*, 2013; Dhanalakshmi *et al.*, 2014; Kapadnis *et al.*, 2014; Malik *et al.*, 2014; Modi *et al.*, 2015) and also in abroad (Kim *et al.*, 2010; Ilida and Faridah, 2012; Williams and Oyarzabal, 2012; Hosseinzadeh *et al.*, 2015; Belmar *et al.*, 2015; Hald *et al.*, 2016). A study conducted by Alagic *et al.*, 2016 revealed that 27. 4% of chicken carcasses were positive for the presence of *Campylobacter jejuni* out of 84 samples examined.

Further, the author stated that among the *Campylobacter* isolates obtained, *Campylobacter jejuni* (91. 9%) was predominant. Similarly, Singh *et al.*, 2009 also observed lower prevalence (12. 79%) of *Campylobacter* Spp. in poultry meat and carcasses. Comparatively, overall higher prevalence (38. 67%) resulted in the present study might be attributed either to the fecal contamination of feathers and skin during transport to the slaughter facility, leakage of

fecal content from the cloaca, intestinal breakage and contact with contaminated equipment, water and other carcasses or to the unhygienic processing as well as pooling of carcasses during dressing and slaughtering operations.

Similarly, Garbyal (2000) reported 37. 41% prevalence of thermophilic *Campylobacters* in chicken samples from Barielly, Uttar Pradesh region which is almost identical to our finding (38. 67%). Further, highly variable prevalence of *Campylobacter jejuni* in meat stalls at different zones of Chennai clearly indicates that slaughter and post-slaughter factors would be the major risk factors that influence the prevalence rate rather than pre-slaughter factors.

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