



## Research Article

# PRELIMINARY STUDY TO DETERMINE THE PREVALENCE OF *Campylobacter* SPECIES IN RETAIL POULTRY IN MUMBAI, INDIA

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**Abstract-** *Campylobacter* is a leading foodborne zoonosis, and is frequently associated with the handling and consumption of poultry meat. Various studies have indicated that *Campylobacter* causes a substantial human disease burden in low to middle income countries. With the rapid growth of urban conglomerates, such as India's commercial capital Mumbai, changes in diets, food production and retailing dynamics, it is likely that exposure to this pathogen will impact a significant role. It was thought worthwhile to conduct a preliminary study of the prevalence of *Campylobacter* species in retail poultry locales from Mumbai. In this study, 74% of the 120 retail poultry meat samples were found to be contaminated with *Campylobacter* which on speciation using multiplex PCR were differentiated to *C. jejuni* (57%) and *C. coli* (29.8%). The high incidence of *Campylobacter* in poultry meat in the city is indicative of its emergence as a potential risk to the consumers.

**Keywords-** *Campylobacter*, Poultry Meat, *C. jejuni*, *C. coli*, Food Safety

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## Introduction

*Campylobacter* is one of the leading causes of gastrointestinal infections ranking 4<sup>th</sup> after rotavirus, cryptosporidiosis and *Salmonella*. According to the Center for Disease Control (CDC), *Campylobacter* infections were reported to have an incidence rate of 19.1 per 100,000 population which is higher than that resulting from *Salmonella*. i.e., 16 per 100,000 population [1]. In the USA, *Campylobacter* infections affect nearly 1.3 million people every year while the European Union has a higher incidence rate as reported by European Food Safety Authority (EFSA) with more than 200,000 cases of campylobacteriosis costing around 2.4 billion euros being the cost of productivity lost and hospitalization [2]. Although there is a focus on surveying and tracking *Campylobacter* spp. in developed countries, there is limited data available on prevalence of this pathogen in developing countries. In India, the high burden of many pathogens with higher mortality rate have restricted the inclusion of *Campylobacter* in national surveillance program [3]. The prevalence data on the incidence rate of *Campylobacter* infections in India is mainly generated through center-based surveillance studies. These reports have confirmed the presence of *Campylobacter* species from diarrheal patients as well as in animal products such as poultry meat, dogs, pork etc. with incidence rate of approximately 13% [3-5]. There are several food items that serve as the source of *Campylobacter* such as milk (9%) [6], beef (3%) [7], chevon meat (64%) [8], fish (2%) [9] and pork (9%) [10]. Amongst the various sources of *Campylobacter*, poultry is the primary vehicle of transmission, with consumption of undercooked poultry meat and mishandling of raw poultry products being the main risk factors for campylobacteriosis [11]. This claim has been corroborated by multiple studies one of which was conducted by Hoffmann *et al.*, 2017, in which 11 foodborne diseases across 14 world subregions were assessed to trace their food sources and poultry meat was found to be responsible for 50-70% of reported campylobacteriosis cases [12]. The poultry market in this country is mainly composed of 'live' bird retail shops that account for 90% of total poultry sales and are preferred over frozen meat [13]. These 'live' bird markets consist of small shops with barely one workstation

without segregation of areas for animal slaughter, de-feathering and evisceration. The lack of sanitation measures for the workers or the dressing floors that results in increased chances of cross contamination of the poultry meat with the caecal content of the contaminated bird. The birds are known to get contaminated with *Campylobacter* spp. as early as 7 days post-hatching becoming a part of their intestinal normal flora [14, 15]. Thus, these contaminated birds further contaminate the poultry carcass during the slaughtering process. With the infective dose for *Campylobacter* infection being as low as 500 cells, the contaminated carcass presents an increased risk to the consumers [16, 17]. The present study was designed to study the prevalence of *Campylobacter* species in retail poultry meat samples from live bird markets in the city of Mumbai, India to assess and generate preliminary surveillance data highlighting the prominent presence of this pathogen in Mumbai which would enable subsequent remedial solution impacting public safety.

## Materials and Methods

### Poultry Sampling

A total of 120 poultry samples were collected from 'live' bird retail shops from various locations in Mumbai, India. Samples were collected from different representative regions of Mumbai spanning over a period of one year. The poultry samples i.e., boneless thigh, breast and wing pieces were collected from retail poultry markets in sterile sampling bottles (Himedia Pvt. Ltd) and transported to the lab for further processing within 2 hours.

### Microbiological Analysis

The poultry samples were analyzed using the modified ISO 10272:2006 method [18, 19]. In this method, 10g of poultry meat was rinsed with 10ml of buffered peptone water (BPW) and the surface of the meat sample was hand massaged for at least 2 min to ensure the dissolution of the bacteria attached. This chicken rinsate (5ml) was then added to Bolton broth base containing antibiotic

Table-1 Oligonucleotide primers and mPCR cycle details for identification and differentiation of *Campylobacter* spp.

Species & Genes	Genes	Sequences (5' to 3')	PCR Product size	PCR cycle Details -30
<i>Campylobacter</i> genus specific 16S rRNA	16S rRNA	F-ATC TAA TGG CTT AAC CAT TAA AC R- GGA CGG TAA CTA GTT TAG TAT T	857 bp	Initial Denaturation - 95°C/5mins Denaturation- 95°C/30 sec Annealing-59°C/1min Extension- 72°C/1min Final Extension- 72°C/5min
<i>C. jejuni</i>	mapA	F- CTA TTT TAT TTT TGA GTG CTT GTG R- GCT TTA TTT GCC ATT TGT TTT ATT A	589 bp	
<i>C. coli</i>	ceuE	F- AAT TGA AAA TTG CTC CAA CTA TG R- TGA TTT TAT TAT TTG TAG CAG CG	462 bp	

supplements (HiMedia Pvt. Ltd.) with 5% of lysed horse blood (Haffkine Pvt. Ltd) which was then incubated in an anaerobic gas jar for 24 hrs. at 37°C with Campy Gen gas pack (Oxoid Pvt. Ltd.) to generate a microaerophilic environment (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>) needed for *Campylobacter* spp. growth [17, 20]. In accordance with the method suggested by De *et al.*, isolation of *Campylobacter* from enriched samples was achieved using nitrocellulose membrane filters of 0.45 µm pore size. The filters were placed on the surface of the modified charcoal Cefoperazone deoxycholate agar (mCCDA) plates and 100 µl of the enriched broth divided into 5 to 7 droplets was added onto the filter. The filter was removed after 30 ± 5 mins, allowing motile *Campylobacter* spp. to pass through the filter onto the surface of the solid media. The mCCDA plates were incubated for 24 hrs. at 37°C with Campy Gen gas pack (Oxoid Pvt. Ltd.) in an anaerobic gas jar. The plates were checked for growth after 24 hrs. and the plates with no growth were further incubated for another 5 days to confirm the absence of *Campylobacter* spp. [21–23]. Typical *Campylobacter* colonies were transferred onto St. Columbia blood agar (CBA) with 5% lysed horse blood and mCCDA. The presumptive cultures were Gram stained to microscopically confirm the presence of typical *Campylobacter* morphology. The pure cultures were also tested for the presence of the three important enzymes *i.e.*, oxidase, catalase and hippuricase.

#### DNA extraction

All oxidase and catalase positive cultures further identified using multiplex PCR designed to identify *C. jejuni* and *C. coli*. DNA was extracted from the presumptive *Campylobacter* isolates and standard strains of *C. jejuni* and *C. coli* were used as positive control while *E. coli* were used as negative control. The culture suspensions prepared in molecular biology grade water were kept in boiling water bath maintained at (95°C) for 10 mins and immediately transferred to ice before centrifuging at 10,000 rpm for 5 mins. The supernatant was stored at 4°C to be used as template DNA for mPCR amplification.

#### mPCR for species identification

*Campylobacter* species were confirmed using mPCR targeting the three genes *i.e.*, 16s rRNA to confirm the *Campylobacter* genera, mapA and ceuE to differentiate *C. jejuni* and *C. coli*. The primers and the mPCR protocol used in this study was published by Denis *et al.*, in 1999 and the details are mentioned in Table 1 [21, 24, 25]. The PCR products were visualized by gel electrophoresis with 1% agarose gel stained with ethidium bromide.

#### Results

In this study, typical colony, *i.e.*, grey colored pinpoint colonies on mCCDA were selected for further preliminary identification of *Campylobacter* species. A total of 114 strains of *Campylobacter* were obtained from positive samples that were tested biochemically for presence of oxidase, catalase and hippuricase enzyme. The morphological characteristics of the growth on the mCCDA plates was studied and tested for the presence of 2 enzymes *i.e.*, oxidase and catalase. All oxidase and catalase positive cultures revealed the typical curved rod morphology however, the older cultures were observed to have coccoid morphology that is believed to be a result of environmental stress [26]. Further to differentiate *C. jejuni* from other species of *Campylobacter*, hippurate hydrolysis test was performed. Distinct purple coloration as a result of reaction between ninhydrin and glycine, a by-product of the hippuricase enzyme is indicative of the presence of the hipO gene known to be present in *C. jejuni* and consequently used as a differentiation test for the species. Faint or light coloration was taken as negative reaction and only deep purple coloration was considered as positive reaction for *C. jejuni* [27]. All the hippuricase positive cultures were considered to be *C. jejuni*

differentiating them from the other species of *Campylobacter*. The identification of *Campylobacter* spp. cannot be based merely on the biochemical reactions as this species neither ferments nor oxidizes sugars and the other tests such as nitrate, IMViC or lysine are not conclusive [28]. Thus, mPCR was used to identify and speciate the isolates obtained through mass screening of poultry samples. The presumptively positive cultures identified by conventional culture method were confirmed using multiplex PCR targeting the 16S rRNA, mapA and ceuE gene for *Campylobacter* species, *C. jejuni* and *C. coli* respectively. The distinctive band pattern by the amplicon of the PCR products of varying sizes such as 857bp, 589 bp and 462 bp were used to speciate *Campylobacter* isolates as seen in the Fig 1. Among the 114 putative *Campylobacter* samples, 89 of the 120 poultry meat samples collected from the live bird markets in Mumbai city were found to be positive for *Campylobacter* species. On the basis of the number and the size of the bands observed, the prevalence of *C. jejuni*, *C. coli* and other species of *Campylobacter* was calculated to be 57%, 29.8% and 13.2% respectively (Fig. 2).

#### Discussion

Sporadically occurring *Campylobacter* infections are often underestimated because of its self-limiting and rarely fatal nature. However, even though the implications of *campylobacteriosis* are similar to other gastroenteric infections such as vomiting, fever, watery or bloody diarrhea, *Campylobacter* infections are known for their post-infection complications like Guillain Barré Syndrome (GBS), Miller Fischer syndrome, reactive arthritis and irritable bowel syndrome. Studies have reported that approximately 25-40% of GBS patients have a history of *Campylobacter* infections [29, 30]. Additionally, the low infective dose of *Campylobacter* to establish an infection emphasizes on the importance of this pathogen. Multiple studies in India have isolated *Campylobacter* species from stool of diarrheal patients [31], poultry meat samples [9], poultry fecal samples [32] and pets such as dogs [33]. The absence of *Campylobacter* from the list of high priority diseases in India does not mean the absence of this pathogen in the country. The under-reporting of *Campylobacter* species can be attributed to its fastidious nature with stringent growth requirement. In the present study to ensure maximum recovery from poultry sampling, the isolation efficiency was improved by combining the ISO 10272:2006 enrichment method with the use of membrane filters. The addition of membrane filters for the isolation of *Campylobacter* spp. relies on the pathogen size allowing the motile *Campylobacter* strains to pass through the filter while restricting other background bacteria. Additionally, it also acts as a selective barrier after enrichment of poultry rinsate samples in Bolton broth with antibiotics and horse blood [21]. The use of membrane filters for isolation of the other species of *Campylobacter* *i.e.*, *C. upsaliensis*, *C. fetus* and *C. concisus* was reported by De *et al.*, with improved isolation rate from 14 to 16% [34]. In another study by Jokinen *et al.*, the combination of the enrichment step and membrane filters reduced the false positive rate from 30.7% to 1.6% as compared to the conventional cultural method. Hence, membrane filters in conjunction with pre-enrichment were used in this study to achieve higher efficiency in isolation of *C. jejuni* and *C. coli* from poultry samples. Two different pore sizes are commonly used for isolation of *Campylobacter* spp. are 0.65µm [35] and 0.45µm [22]. In a comparative study published by Nachamkin *et al.*, 0.65µm polycarbonate membrane had a higher isolation rate in comparison to 0.65µm and 0.45µm nitrocellulose filters [36]. Likewise, Speegle *et al.* stated that 0.45µm retained 90% of the bacteria and had a comparatively lower efficiency as compared to that of 0.65µm filter [37]. However, a pre-enrichment step prior to the use of nitrocellulose membrane filters of 0.45µm pore size improves its efficiency in isolation of *Campylobacter* spp. considering the economics and ease of availability

of 0.45 µm pore sized nitrocellulose membrane filters, these were used in this study to isolate *C. jejuni* and *C. coli*. Fig 3 shows the colonies that were obtained at the spots where the enriched Bolton broth was dropped on the 0.45 µm filters. The transmission of *Campylobacter* is primarily through consumption of contaminated poultry meat which is either undercooked or raw. The high prevalence of *Campylobacter* spp. in poultry is expected as it is part of the normal flora of the chicken intestines which gets inhabited as early as 7 days after chicken hatches. Once infected, the pathogen persists in the intestines of the bird throughout their life till their slaughter age and is continuously shed aiding in horizontal transmission of the pathogen. It has been established by earlier reports that the higher prevalence rate of *Campylobacter* spp. in the caecal content of the infected flock is directly associated with higher prevalence rate in chicken carcass and its poultry products [38, 39]. Multiple studies in India have reported high incidence rate of *Campylobacter* species in poultry caecal samples ranging from 11 to 17% [33, 40]. In addition to the consumption of contaminated poultry meat, of contaminated poultry, water, close proximity to poultry farms and its employees, a case-control study by Ravel et al., 2017, reported that, the other factors that caused *Campylobacter* infections included attending barbecue and fast food from restaurants [41–43]. Another study by Khan et al., 2018, reported the presence of *Campylobacter* on chopping boards and knives thereby highlighting the importance of role of cross contamination from poultry meat to other vegetables often consumed raw [44].

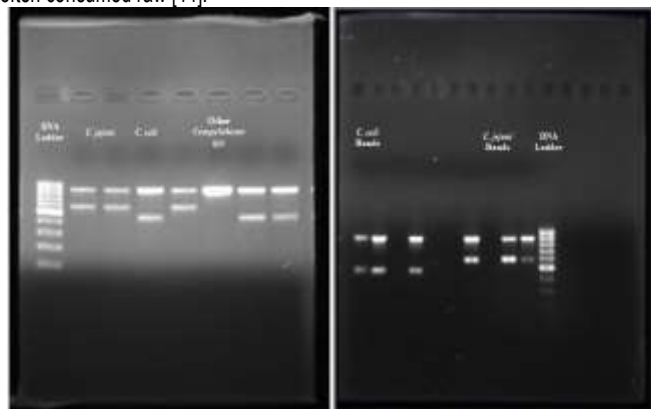


Fig 1- Gel electrophoresis images of PCR products for DNA bands observed for *C. jejuni* (589bp), *C. coli* (462bp) and other *Campylobacter* species (857bp) result of mPCR.

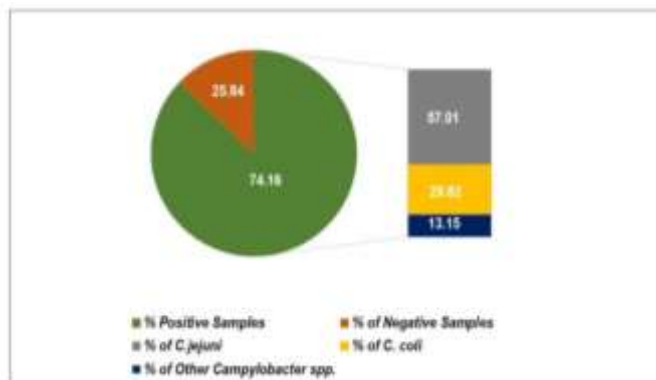


Fig 2- Percentage distribution of *Campylobacter* species in poultry meat samples collected from various markets in Mumbai

Of 25 species of *Campylobacter*, *C. jejuni* is predominantly present in poultry responsible 90% of Campylobacteriosis followed by *C. coli*. The results of this study validate this claim where the prevalence of *C. jejuni* was found to be 57% as compared to that of 29.8% of *C. coli*. Even though the incidence rates of *C. jejuni* in this city was found to be comparatively lower than that reported by the neighboring city of Pune with 76.9% [17]. However, Furukawa et al. reported the incidence rate of *C. jejuni* to be 64% in Japan [45]. Numerous studies conducted across the Indian subcontinent have reported a higher prevalence rate of *C. jejuni* with 71% in Chandigarh, 81% in Izatnagar [31, 33]. However, some areas such as

Barielly (93.75%) and Uttarakhand (60.40%) had higher prevalence rates of *C. coli* [46, 47]. Similar reports have been reported by Pedonese et al., with a prevalence rate of *C. coli* (58.1%) exceeding that of *C. jejuni* (41.9%) [48]. The exact reason for the difference in prevalence rates is unknown but several theories have been proposed such as geographic location, poultry breeding conditions or pathogen source [46, 49].

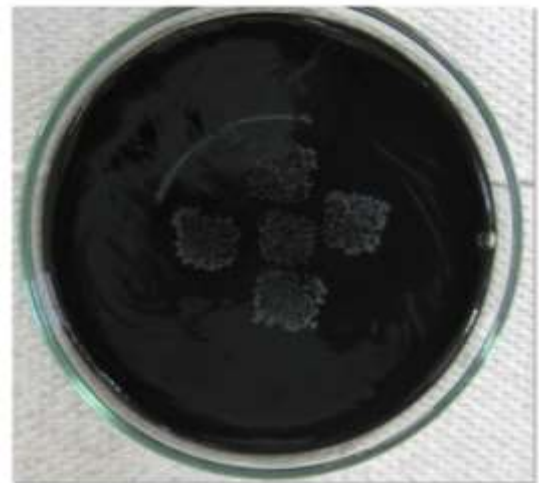


Fig 3- *C. jejuni* colonies on mCCDA plates

### Conclusion

Our study revealed a high prevalence of *Campylobacter* species in retail poultry sampled from Mumbai outlets thereby indicating that the raw retail poultry products may be vehicles for transmission of the pathogen in the city. It is critical that risk reduction strategies are employed throughout the food chain from farm to fork. These include healthy monitored on farm practices to reduce pathogen carriage, increased hygiene at slaughter and processing plants, continued implementation of HACCP systems and increased consumer education efforts. Additionally, consumption of undercooked poultry products and cross contamination during handling and preparation must be avoided at the domestic and food service industry levels. Further research focusing on effective prevention via identification of various risk factors is essential for developing intervention and mitigation strategies to reduce the presence of *Campylobacter* at the retail level. Also, it is important to include the pathogen in standards for poultry quality assessment.

**Application of research:** The current study presents pilot data of *Campylobacter* levels in the city of Mumbai that can help in designing the necessary intervention plans to control the spread and rise of antimicrobial resistant strains of *Campylobacter*.

**Research Category:** Veterinary Microbiology, Food Microbiology

### Abbreviations:

CDC- Center for Disease Control  
EFSA- European Food Safety Authority  
HACCP- Hazard Analysis and Critical Control Points  
mCCDA- Modified Charcoal Cefoperazone Deoxycholate Agar  
GBS- Guillain Barre Syndrome

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Research project name or number: PhD Thesis

**Author Contributions:** All authors equally contributed



**Author statement:** All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

**Study area / Sample Collection:** Retail shops from various locations in Mumbai

**Animal name:** Chicken - *Gallus gallus domesticus*

**Conflict of Interest:** None declared

**Ethical approval:** Ethical approval taken from Department of Microbiology, Bhavan's College, University of Mumbai, Mumbai, 400058, India.  
Ethical Committee Approval Number: Nil

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