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Research Article

Phenotypic & Molecular characterization of *Campylobacter* species from broilers in and around Kolkata Wet Markets

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ABSTRACT

Thermophilic Campylobacter spp. are one of the primary causes of bacterial human diarrhoea. The consumption of poultry meat is suspected to be a major cause of human campylobacteriosis. The major purpose of this study was isolation, identification and characterization of thermophilic Campylobacter spp. in fresh poultry samples by conventional culture methods and to confirm Campylobacter jejuniand Campylobacter coli isolates by using the multiplex PCR assay. 100 broiler intestines were collected from meat shops (n=6). The samples were analyzed based on morphological and cultural characteristics in accordance with the international standard ISO 10272:1995 for Campylobacter spp. Concurrently, a multiplex PCR was used for the identification of C. jejuniand C. coli. of the 100 samples of intestines, i.e., caecal contents, 15% were contaminated with Campylobacter spp. of the 15 thermophilic Campylobacter isolates, 8 possessed the cdtB gene and thus were confirmed as Campylobacter jejuni (53.3%), and Seven (46.7%) were identified as Campylobacter coli. The study concluded that there was a high proportion of contamination of poultry meat marketed in and around Kolkata by Campylobacter spp. Therefore, we need to implement effective control measures to address these public health issues. Furthermore, poultry meat must be properly cooked before consuming to ensure food safety.

Keywords: *Campylobacter jejuni, Campylobacter coli, Poultry, Isolation, Multiplex PCR.*

INTRODUCTION

Foodborne illnesses are usually caused by bacteria, viruses, parasites, or chemical substances that enter the body through contaminated water or food. Foodborne pathogens can cause severe diarrhoea, which may lead to long-lasting disability and death**.**

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Campylobacteriosis is one of the most frequently reported foodborne diseases worldwide. Foodborne illnesses due to *Campylobacter* are mainly transmitted by raw milk, raw or undercooked chicken, and drinking water (www.who.int). *Campylobacter* arenon-spore-forming, pleomorphic bacteria and are shaped (0.2 to 0.8 by 0.5 to 5 μm), may be spiral, "S", curved or rodlike. Presently, there are 17 species and 6 subspecies belonging to the genus *Campylobacter*, of which *C. jejuni* (subspecies *jejuni*) and *C. coli* have beenfrequently reported in human diseases. Most *Campylobacter* species grow under microaerophilic conditions and have a respiratory type of metabolism (Kaakoush et al., 2015). As enteric organisms, *Campylobacter* spp. are carried in the intestinal tracts of food animals, especially poultry, and they are often present in food of animal origin through faecal contamination during processing (Luangtongkum et al., 2007). Poultry are reservoirs of thermophilic *Campylobacter* species, especially *Campylobacter jejuni* and *Campylobacter coli* (Varga et al., 2019). Although diarrhoea is the most frequent clinical manifestation of *Campylobacter* species infection, it is also associated with Guillain-Barré syndrome, a severe immunoreactive complication (Ruiz-Palacios et al., 2007). In light of their importance, FAO and WHO have already undertaken risk assessments on *Campylobacter* in broiler chickens (WHO, 2009).

Agars that are more selective and differential are needed to facilitate isolation and enumeration (Line, 2001). Endtz et al. later confirmed there is a higher isolation rate for *Campylobacter* while using the charcoal selective medium (Aryal, 2022) by streaking on selective media consisting of blood agar base supplemented with 7 per cent lysed horse blood and antibiotics (Jamshidi et al., 2008). Suspected colonies were examined for morphology and motility tests, followed by biochemical tests. Use of selective antibiotics to eliminate non-*Campylobacter* contaminants is necessary for isolation of the organism. On existing agar plates, *Campylobacter* colonies are frequently small and colourless, or they may appear similar to a water droplet on the agar surface (Line, 2001)**.** Lauwers et al. developed a selective medium for *Campylobacter* isolation containing cycloheximide, cefazolin, bacitracin, colistin sulfate, and novobiocin to eliminate non-*Campylobacter* contaminants, which is necessary for isolation (Lauwers et al., 1978). PCR is used in the detection and confirmation of *Campylobacter*. *Campylobacter* produces cytolethal distending toxin (CDT), which causes progressive cellular distension, ultimately leading to cell death (Ge et al., 2008). *Cdt*B has been found as the active component of holotoxin localized in the host nucleus. It cleaves dsDNA molecules during the G1 and G2 phases. *Cdt*B also cause cell distention and irreversible cell-cycle arrest (Al-Mahmeed et al., 2006). The *cdt*A, *cdt*B or *cdt*C gene of *C. jejuni*, *C. coli* or *C. fetus*, respectively, could be successfully amplified using the corresponding set of primers in a highly species-specific manner. Hence, it indicates that the *cdt* gene-based multiplex PCR assay may be useful for rapid and accurate detection as well as identification of *Campylobacter* strains in a species-specific manner (Asakura et al., 2008). So, isolation of *Campylobacter* species is often tedious**.** The present study was undertaken with the aim of isolating and identifying *Campylobacter* spp. from poultry (chicken) samples sold at retail markets in Salt Lake areas of North 24 Parganas district in West Bengal, India, and to confirm the isolates using molecular tools.

MATERIALS AND METHODS

Collection of Samples:

Sampling was carried out in retail outlets located in and around Salt Lake, North 24 Parganas (West Bengal), during October 2022 to February 2023. The study encompassed isolation of *Campylobacter* spp. from caecal contents of chicken and to confirm the isolatesusing molecular tools. Likewise, a total of 100 chicken intestines were collected in

sterile plastic bags & placed in a thermocol box with ice and brought to the laboratory for isolation of *Campylobacter*.

Isolation and identification:

Isolation and identification of *Campylobacter* based on morphological and cultural characteristics in accordance with the international standard ISO 10272:1995. For selective isolation of *Campylobacter*by direct plating, the selective medium was prepared by adding Brain Heart Infusion agar (BHIA; BD Difco, USA), which was used as base media supplemented with 0.03% Charcoal powder (M/s. SRL),7% sterile Horse serum (Himedia, India) (Jamshidi et al., 2008) and antibiotics (Cycloheximide-25mg, Bacitracin-12,500 IU, Cefazolin sodium-7.5 mg, Colistin sulphate-5,000 IU, Novobiocin sodium-2.5 mg) as mentioned by Butzler (Lauwers et al., 1978). The plates were incubated at 37°C for 48 hours under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) in a McIntosh anaerobic gas jar created by using commercially available Anaerogas Campylo pack (BD GasPak^{TM} EZ Campy Container System and BD BBL^{TM} Bio-Bag^{TM}Environmental Chamber Type (Cfj). Further the colonies were subjected to Gram's staining, motility, oxidase, catalase and Hippurate hydrolysis. All the isolates were stored by dispensing a lawn of coloniesfrom the serum plates to 15% BHIB-Glycerol stock and stored at -70°C till further use.

Molecular Characterization:

DNA extraction:

Total DNA was extracted from the cultured isolates as follows. The lawn of suspected bacteria was collected and transferred to 200 µl of nuclease-free water, vortexed and boiled in a water bath at 100°C for 10 minutes. It was immediately chilled on ice for cell lysis for 10 minutes. Then, they were centrifuged at 4,000 rpm for 10 minutes to collect the supernatant. Supernatant containing target template DNA was used for PCR amplification. PCR was performed in a Biorad T100 thermocycler (Biorad, USA).

Genotypic Identification of *Campylobacter*:

Multiplex PCR was performed to detect *Campylobacter* specific cytolethal distending toxinB (*cdt*B) gene following the protocol described by Asakura et al (Asakura M et al., 2008) to detect *C. jejuni* and *C. coli*. The details of primers were depicted in Table 1.

Table 1: Oligonucleotides for *cdtB*multiplex PCR:

Primers	Sequence (5´-3´)	Annealing temperature (°C)	Product (bp)	Reference
Cj-CdtBU5	F: ATC TTT TAA CCT TGC TTT TGC	56	714	(Asakura M et al.,2008)
Cj-CdtBR6	R: GCA AGC ATT AAA ATC GCA GC			
Cc-CdtBU5	F: TTT AAT GTA TTA TTT GCC GC		413	
Cc-CdtBR5	R: TCA TTG CCT ATG CGT ATG			

All reactions contained two primer sets **(***Cj* and *Cc*), each of volume 1.00 µL**,** DNase-RNase free water of 8.00 µL, PCR master mix of 5.00 µL and DNA Template of 3.00 µLin a 20µL reaction volume. PCR was performed on a Bio-Rad T100 thermocycler (Bio-Rad, USA). PCR protocols and cycling conditions used for PCR amplification of the *cdt*B gene are described below (Table 2). The PCR products were analyzed by 1.5% agarose gel electrophoresis, then bands were visualized with UV light with the help ofethidium bromide added to the gel before, by placing over a gel documentation system (UVP, UK: DNR Bio imaging system and BioGene) to understand amplification and the images were captured.

Table 2: PCR protocol for amplification of the *cdt* Bgene

Sl No.	Step		Temperature	Duration	Cycle
1.	Initial denaturation		94°C	90 sec	1
2.	CYCLING	Denaturation	94°C	30 sec	35
		Annealing	56°C	30 sec	
		Extension	72°C	45 sec	
3.	Final extension		72°C	5 min	1

RESULTS

A total of fifteen (n:15) *Campylobacter* isolates were recovered from 100 poultrycaecal contents (n:100) by direct plating of a loopful of stool specimen onto Brain Heart Infusion Agar (supplemented with charcoal, antibiotics and 7% Horse serum) platesto get single colonies, which were incubated at 37°C for 48 hours under microaerophilic conditions. Greyish, small, flat and glistening colonies growing along the streak were subjected to Gram's staining. Suspected colonies were further inoculated on BHI agar with 5% Horse serum plates(subculture) for pure cultures (Fig 1). The colonies were picked up and on Gram staining observed Gram negative, spiral-shaped rods, observed darting motility, biochemical tests positive for Oxidase and PCR confirmation for species-level identification. All the *Campylobacter* isolates were subjected to PCR based species confirmation. Eight possessed *the cdt*B gene (714bp) and thus were confirmed as *Campylobacter jejuni* (53.3%). Seven were identified as *Campylobacter coli* (46.7%) by detection of *the cdt*B gene (413bp) (Fig 2).

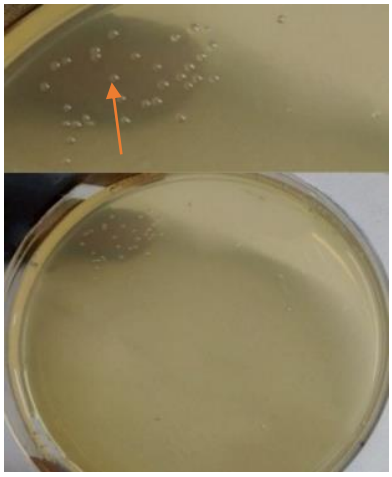

Fig 1: Small dewdrop-like or spreading watery colonies on 5% Horse serum plate after subculture

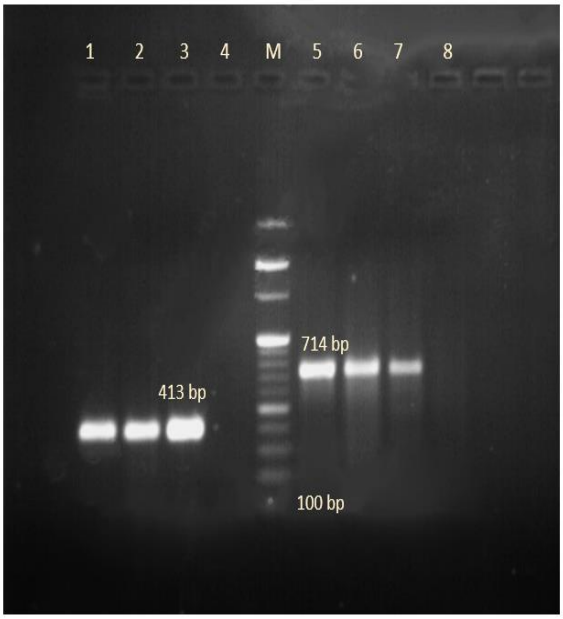

Fig 2: Agarose gel electrophoresis of PCR amplicon of *the cdt*B gene of *Campylobacter* isolates

DISCUSSION

Foodborne illness is one of the greatest public health concerns across the globe. This is mainly due to the consumption of intoxicated or contaminated food and water. According to the WHO, annually, there are about 600 million cases of foodborne illnesses and 420000 deaths due to the consumption of unsafe food (www.who.int). Diarrhoeagenic bacteria are often responsible for such foodborne illness, and it is predominantly seen in LMIC when compared with developed countries. Among bacterial agents, *Campylobacter* is one of the most common foodborne pathogens infecting millions of people around the world. Foodborne illnesses due to *Campylobacter* infection are mainly caused by consumption of raw milk, raw or undercooked chicken and drinking water (www.who.int). Again, chickens are implicated as the reservoirs for *Campylobacter* (Kazwala et al., 1990). They are well adopted in the chicken gut microbiome. *Campylobacter* spp., in particular *Campylobacter jejuni* and *Campylobacter coli*, are the most common causes of bacterial enteritis in humans (Post et al., 2017).

In order to understand the isolation rate of *Campylobacter* from chickens from various retail poultry meat shops in and around Kolkata, the study was conducted employing molecular tools. About 15% of the chickens were found to harbour thermophilic campylobacters in this study area, and of them, 8 were positive for *C. jejuni* and 7 for *C. coli*. Chickens are well-known reservoirs of Campylobacter and thus can serve as a potential source of this pathogen to humans. Unprocessed or poorly cooked chicken posed a significant public health threat. Similar results were obtained in several studies conducted in different parts of India (Sharma et al., 2016; Singh et al., 2009; Prince Milton et al., 2017), wherein occurrence was found to vary from 3% to as high as 22%. In another study, Rajagunalan et al. (2014) also reported this pathogen in 16% of the caecal and faecal samples of chicken, of which the majority belonged to *C. coli* (Rajagunalan et al., 2009). Likewise, another study in the Bareilly region also reported a higher isolation rate of *C. coli* (93.75%) (Malik H et al., 2014). The variation in isolation rate of these two campylobacters in different studies may be attributed to different geographical locations, environmental factors, farm management practices and adopted laboratory methods for isolation of this capnophilic organism.

CONCLUSION

In conclusion, this study reports on the occurrence of *Campylobacter* (15%) in broilers in and around Kolkata. All the *Campylobacter* isolates (n: 15), which encompassed 8 *C. jejuni* and 7 *C. coli*, carried virulence associated toxin i.e., cytolethal distending toxin B (*cdt*B) gene. Thus, the presence of foodborne pathogens like *Campylobacter* in poultry meat, as revealed in this study, is a great cause of concern. Therefore, there is an immediate need to implement an effective control programme to reduce the contamination of poultry meat by the pathogen. Bringing awareness among the poultry farm workers in the poultry industry may be promoted to avoid contamination during the poultry processing chain to tackle the issue. Further, continued surveillance for understanding the transmission dynamics of foodborne pathogens is essential to formulate and implement successful containment measures to solve these public health issues.

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Conflict of Interest:
The authors declare that they have no conflict of interest.

Author Contribution
All authors have participated in critically revising the entire manuscript and approving the final manuscript.

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