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Brief Communication

Prevalence and antibiotic susceptibility of thermophilic *Campylobacters* from sources implicated in horizontal transmission of flock colonisation

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Abstract

Thermophilic *Campylobacter* are commonly associated with poultry as commensals of the avian gut and are the causative agent responsible for human Campylobacteriosis. This study aimed to establish the prevalence of *Campylobacter* spp. from environmental sources that have previously been implicated as sources of horizontal transmission. The highest prevalence of thermophilic *Campylobacter* was found in water samples (87.5%) and lowest from flies (7.2%). Only *C. jejuni* was isolated from all sources. A secondary aim was to provide a baseline of resistance profiles of *Campylobacter* spp. isolates obtained. Alarmingly all the *C. jejuni* isolates from environmental sources as well as humans were multi-drug resistant.

Key words: *Antibiotic resistance, campylobacter, horizontal transmission*

Introduction

Campylobacter jejuni and *C. coli* are the most commonly reported bacterial causes of acute gastroenteritis in humans in both developing and developed countries.[1] *Campylobacters* may cause a spectrum of illness in humans. The signs manifested by the patients include abdominal cramping and diarrhoea. Other extra intestinal diseases may result from *Campylobacter* infections including bacteraemia, endocarditis, meningitis, urinary tract infection (UTI), and Guillain-Barré syndrome, which are the acute paralytic diseases of the peripheral nervous system.

The most important sources of *Campylobacter* infections for humans are associated with poultry.[1] Various studies have demonstrated high levels of *Campylobacter* on broiler chickens from poultry farms[2] and on retail chickens.[3] The presence of *Campylobacter* in the agricultural context has widely been documented in scientific literature; however, there are few reports for the same from India.[4]

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The treatment and clinical management of Campylobacteriosis in man is adversely affected due to the escalating rates of antibiotic resistance.[5] This is because antimicrobial resistance prolongs the infection and causes further complications in patients with bacteraemia. Infections due to antimicrobial resistant enteric bacteria are highest in the developing world, where the use of antimicrobial drugs in humans and animals is relatively unrestricted.[6] The unregulated use of antimicrobial agents in food animal production has led to the emergence and spread of antibiotic resistance among *Campylobacter* spp. After the approval and thereafter the subsequent use of fluoroquinolones in poultry in Europe and USA, there was an increase in fluoroquinolone resistance in *Campylobacter* spp. from animals and human patients.[7]

The epidemiology of *Campylobacter* in broiler production is not wholly understood to date. There is a difference of opinion over, which are the main sources for flock colonisation.[8] The transmission of *Campylobacter* occurs in the flock, after original introduction of the organism into that group and as intra-intestinal colonisation of its members commences.[9] Two types of transmission have been pursued by researchers. The first is vertical transmission from parent chicken to progeny. The second being horizontal transmission from external sources such as feed, water, domestic animals, insects, rodents and wild birds to chicken. Carryover from previous flocks and horizontal transmission via contaminated water, domestic and wild animals, personnel working in the broiler house, and the external environment has been implicated.[10,11] Vertical transmission from *Campylobacter*-positive breeder flocks via the egg to their progeny has not been found to be very likely.[12] Therefore, based on preceding substantiation, and because, investigations on bacteriological, pathological, clinical, and epidemiological aspects of *Campylobacter*s

in India are moderately recent, the present study was undertaken to determine frequency of occurrence of pathogenic *Campylobacters* in the farm environment and to determine antimicrobial susceptibility patterns of the isolates.

Materials and Methods

During the study, 233 samples were collected from poultry farms and from several retail establishments located in Pune, India [Table 1]. Samples were collected from different wild birds and animals, poultry chickens, rodents, humans, water, soil, and flies. These were examined for presence of *Campylobacter spp.* All the samples were transported to the laboratory under refrigerated conditions, and microbiological analyses were carried out immediately. The poultry birds were sampled non-invasively in that there was no entry into the body cavity of the birds. All scientific ethical practices were respected. In this study, fresh faecal, cloacal swabs and feather swabs each were collected from the poultry chickens. Faecal samples were collected from wild birds in and around the poultry farm using a reported protocol.[13] The rectal contents were collected from rodents and analysed by reported protocol.[14] Flies and treated drinking water in the farm area were analysed using the protocol by Khalil *et al*., (1994).[15] Litter samples were obtained from poultry broiler house.

For analysis of samples from humans and rodents about 0.5 g of rectal contents/faeces were directly plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) plates.[14] This was followed by incubation at 42 ± 1°C for 48 h under the microaerobic conditions.

The swabs were transported in sterile Preston enrichment broth (PEB). *Campylobacter* was isolated from collected flies by dissecting them vertically and horizontally into four pieces followed by addition of each fly to 5 ml sterile PEB. Water was filtered through 0.45 μm millipore filters. The filter was removed and added to 50 ml sterile PEB. Preston *Campylobacter* selective enrichment broth (PEB) (HiMedia, Mumbai India) was supplemented with selective supplement (HiMedia, Mumbai India) containing polymyxin B (5 IU/ml), rifampicin (10 μg/ml), trimethoprim 10 (μg/ml) and cycloheximide (100 μg/ml) along with 10% horse blood (Haffkine Biopharmaceutical Ltd, Pune, India) for all isolations. Hundred microlitres were immediately cultured onto mCCDA with antibiotics. This was followed by incubation at 42 ± 1°C for 48 h under the microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) in an anaerobic jar (Anaerobic System Mark VI, HiMedia, Mumbai India).

Presumptive thermotolerant *Campylobacter* colonies were picked based on their morphological characteristics. Biochemical tests, which consisted of hippurate hydrolysis, catalase test, indoxyl acetate hydrolysis and H_2S test, were performed on colonies isolated from the blood agar plates. The Hi*Campylobacter*™ Latex Test Kit (Hi-Media, India), a rapid latex agglutination test was used for confirmation of the isolates as thermophilic *Campylobacter*s.

Templates for polymerase chain reaction (PCR) were obtained from single bacterial colony, which was selected for every isolate and suspended in sterile saline. Cells were centrifuged to obtain pellet and washed thrice with sterile water to remove media components. Purified DNA was prepared using commercial DNA isolation kits (Chromous Biotech (P) Ltd., India) according to manufacturer's instructions.

The presumptive *Campylobacter* were authenticated by the presence of a 450-bp amplicon obtained using the primers designed to specifically amplify coding regions from the flagellin gene by previously reported method[16] using.

Forward primer, Pg50 5'-ATGGGATTTCGTATTAAC-3' and Reverse primer, Pg3 5'-GAACTTTGAACCGATTTG-3'. Tubes were subjected to 25 cycles of 94°C for 1 min, 37°C for 1 min, and 72°C for 1 min, followed by a 5 min extension at 72°C. Primers were synthesised by BioResource Biotech (P) Ltd.

The 16S rRNA sequencing was carried out and sequenced data were subjected to Basic Local Alignment Search Tool (BLAST) analysis.

The minimum inhibitory concentration of *Campylobacter* was determined using the agar dilution method towards ampicillin, azithromycin, chloramphenicol, ciprofloxacin, doxycycline, gentamycin, nalidixic acid, norfloxacin, and tetracycline. There is a dearth of internationally validated criteria for breakpoints of susceptible or resistant isolates for *Campylobacter*. Consequently, where breakpoints from the Clinical and

Table 1: *Campylobacter jejuni* isolated from farm sources

	Wild birds and animals	Poultry chickens	Rodents	Flies	Human	Water	Soil
Total samples*	64	45	10	83	15	8	8
Campylobacter Positive samples	10	20	0	6	12	7	4
Prevalence (%)	15.62	44.44	0	7.2	80	87.5	50

*The total number of samples examined was 233

Laboratory Standards Institute (CLSI) were not available, established breakpoints were used.[17]

Results

In this study, 64 samples were collected from different wild birds and animals. Faecal samples were collected from 45 poultry chickens from the farm environments and neighboring retail markets, 10 rodents, and 15 humans. Alongside, water, soil, and flies were also examined for presence of *Campylobacter spp.* In total, 46 *Campylobacter* isolates were obtained from poultry farms as well as retail poultry. The highest prevalence was found in water samples (87.5%) followed by human faecal samples (80%), soil (50%) and poultry chickens (44.44%), wild birds (15.62%) and flies (7.2%). All rodent samples were negative for *Campylobacter* spp.

The isolates were positively identified using the PCR-based assays for identification of *Campylobacter* spp. The PCR yielded the expected amplicon of product size of 450 bp, with the primers specific for flagellin gene as shown in Figure 1.

Partial 16S rRNA sequences of the 46 different isolates were obtained and subjected to BLAST analysis using the NCBI Blast software. The isolates were identified as *C. jejuni* according to the BLAST results as well as phenotyping.

The nucleotide sequences of 30 isolates were deposited in NCBI GenBank (accession numbers JQ972883-912). Figure 1 represents the 450-bp amplicon obtained after PCR of these isolates. These results positively confirm the presence of *Campylobacter* spp. in the Indian poultry environment.

The proportion of isolates resistant to each antimicrobial agent for *C. jejuni* isolates [Table 2] from poultry farm was as follows: 100% for ciprofloxacin, norfloxacin and nalidixic acid, 97.82% for erythromycin and tetracycline, and 43.4% for gentamycin. All the isolates in this set were sensitive to ampicillin, azithromycin, chloramphenicol, clindamycin, doxycycline and streptomycin. The majority of the isolates were grouped into similar categories. In Table 3 the multidrug resistance phenotypes of the isolates are elucidated. The isolates could be divided into 4 multidrug resistance profiles. Almost 52.2% and 43.5% of the isolates belonged to one of the two multidrug resistance phenotypes.

Discussion

Previous studies in India have shown that 39.3% of the poultry, tested positive for *Campylobacter* in Calcutta, 64% in Vellore, 57% in Maharashtra and 17.14% in the

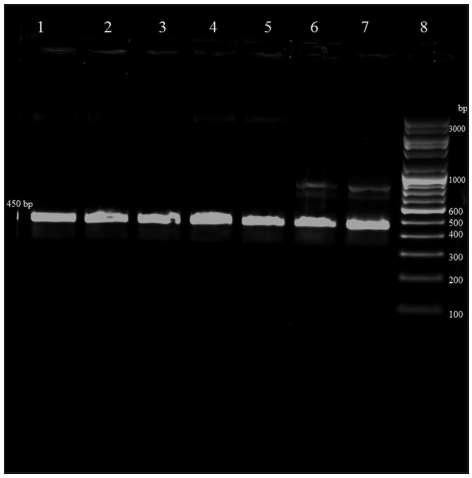

Figure 1: Gel image depicting 450-bp amplicons obtained after PCR assay of genomic DNA of Campylobacter isolates, using the flagellin gene primer Lane1, *C. coli* MTCC 131; Lane 2, JQ972908; Lane3, JQ972909; Lane4, JQ972910; Lane5, JQ972911; Lane6, JQ972912; Lane7, JQ972901; Lane8, Thermo Scientific GeneRuler 100-bp Plus DNA Ladder (100-3000 bp)

Table 2: Grouping of *Campylobacter jejuni* isolates[a] based on MIC[b] values of antibiotics

Antibiotic	Number of isolates with MIC (µg/ml) of													MIC_{50}	MIC_{90}	Resistant isolates	
	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256			(No.)	(%)
Ampicillin						2	33	11		I				4	8	0	0
Azithromycin	46					I								0.0625	0.0625	0	0
Chloramphenicol					23	23				I				1	2	0	0
Ciprofloxacin							I					46		128	128	46	100
Clindamycin			13	33			I							0.5	0.5	0	0
Doxycycline			45			1			I					0.25	0.25	0	0
Erythromycin					1			I3	25	17				16	32	45	97.8
Gentamycin				26					10I	10				0.5	32	10	21.7
Nalidixic Acid										I			46	128	128	46	100
Norfloxacin									I	3	25	6	12	64	256	46	100
Streptomycin							46				I			4	4	0	0
Tetracycline								1	1I	43	1			32	32	45	97.8

[a]Isolates from environmental/poultry farm environments, [b]MIC was determined by agar dilution method in MHA at 42°C for 48 h in microaerobic atmosphere, I: Indicate breakpoints for resistance

Table 3: Antibiotic resistance profile of *Campylobacter jejuni* isolates

Profile*	No. of *C. jejuni* isolates	
	(No.)	(%)
M, Q	1	2.17
Q, T	1	2.17
M, Q, T	24	52.17
G, M, Q, T	20	43.47

*Q: Fluoroquinolones (nalidixic acid, ciprofloxacin and norfloxacin), M: Macrolides (erythromycin and azithromycin), C: Clindamycin, P: Phenicols (chloramphenicol), T: Tetracyclines (tetracycline and doxycycline); A: Ampicillin, G: gentamicin and streptomycin

Meghalaya-Assam region.[17] Our results from Pune, Maharashtra are in agreement with these reported values. However, only *C. jejuni* was isolated from the poultry farm environments. We do not conclude on this, because of biased detection in favour of this species. It must be noted that the prevalence of *Campylobacters* in poultry, depends on not only the isolation method but also flock size and type, geography, season, animal age, and number of animals investigated. We have no explanation as to why no other species were isolated. Nevertheless, this occurrence was reported in a similar study from New Zealand as well.[13]

The presence of *Campylobacter* in the agricultural context has widely been documented in scientific literature; however, there are few similar reports from India.[4] The highest prevalence was found in water samples (87.5%) followed by human faecal samples (80%), soil (50%) and poultry chickens (44.44%), wild birds (15.62%) and flies (7.2%). Though the presence of *Campylobacter* in 11% of rodents was detected in a similar study, we could not detect any *Campylobacters* from rodents.[18] Wild animals, wild birds, humans and even flies could be potential reservoirs of *C. jejuni* in poultry farms. It is thus postulated that either or all these reservoirs may be responsible for horizontal transmission of *Campylobacter* in the Indian poultry farm environment. Moreover, a large number of *Campylobacter* isolates obtained in this study showed the same multidrug resistance phenotype despite being isolated from different environmental sources. However, further molecular studies using *flaA*- RFLP or PFGE will be able to elucidate the route of transmission.

An important observation was that 80% of humans associated with poultry were found to be positive for *Campylobacter*. *Campylobacter* enteritis are usually treated using fluoroquinolones and macrolides.[1] Recent studies have reported the appearance of fluoroquinolones resistant *Campylobacter* spp. among poultry flocks,[7] necessitating the survey of prevalence of *Campylobacter* spp. in poultry and their antimicrobial resistances. Earlier reports from India show 30.6% and 35.83% of strains from retail poultry were multidrug resistant.[17,19] However, we noted that alarmingly all the *C. jejuni* isolates from environmental sources as well as humans were multidrug resistant.

We wish to highlight these results due to a scarcity of *Campylobacter* research in India. Further, the results paint a dismal picture because there are no national surveillance studies and hardly any projects taken up to study the problem of *Campylobacter*. With a population of 1.2 billion people, the increasing rates of *Campylobacter* carriage in poultry, the alarmingly low infective dose of *Campylobacter* (only 500 bacterial cells) and the battery of complications following enteric infections, stringent measures must be taken to improve our understanding of *Campylobacter* in India.

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