Characterization of isolated thermophilic campylobacters and associated risk factors in poultry farms of Uttarakhand, India

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Abstract

Background

Campylobacters are the common commensals of poultry responsible for several cases of gastroenteritis in humans. The illness, if severe can result into complications causing a nervous disorder named Guillian Barre syndrome. Owing to its serious health implications, the study aimed to screen eight organized poultry farms and their environment (water, litter, manure, and feed) of Uttarakhand state, India for the presence of thermophilic Campylobacter species and their virulence and antibiotic resistance profile. It also undertook identification of risk factors associated with the occurrence of campylobacters in each farm using a questionnaire survey comprising eleven potential risk factors (other animals on farm, reuse of litters, use of foot bath, in house or branded feed, chlorination of water, distance of manure heap, housing system, flock size, floor type, shoe use by farm personnels, moist or dry litter and number of broiler floor).

Results

Of eight, six farms showed varying occurrence of C.jejuni and C.coli with an overall prevalence of 12.29%. Not a single isolate of C.lari and C.upsaliensis was recorded. Poultry faecal, water and litter samples observed 18.2%, 6% and 1.9% presence, respectively. Feed and manure samples did not appear positive. In 48 revived Campylobacter isolates, 100% presence of cadF and flaA virulence genes were detected followed by cdtB (97.9%), cgtB (22.9%) and ciaB (12.5%), respectively. Ten isolates 23.80%) were multidrug resistant (MDR) exhibiting resistance to at least 3 or more antimicrobial classes. The most common MDR patterns were AMP CX CIP TE (n = 2) and AMP CX CIP (n = 2). Feeding of branded feed was found to have significant association with Campylobacter presence in the examined broiler flocks (p-value 0.0047).

Conclusions

The study highlights the occurrence of food pathogens, Campylobacter jejuni and C.coli in the poultry farms and their environment of the state. The organisms possessed significant virulence genes capable of developing critical human illness. Overall, the presence of MDR thermophilic campylobacters appears to be a severe public threat.

Background

The incidences of food-borne illnesses are observed in developing as well as developed nations. These illnesses are mainly caused by pathogenic bacteria present in food (1, 2). As per Center for Disease Control and Prevention (CDC), campylobacters stand as 4th major cause of food-borne illness (9%), 3rd major cause of hospitalization (15%) and 5th main cause of human deaths (6%) due to food-borne infections annually in the United States (3). India, a developing country, lacks a decent data on foodborne diseases as many cases go unreported. The Integrated Disease Surveillance Programme (IDSP) network, launched
in India in 2004, highlights that food-borne outbreaks together with acute diarrhoeal diseases constitute nearly half of all reported outbreaks based on data collected from 2011-15(4). Among the well known food-borne pathogens, thermophilic campylobacters namely *Campylobacter jejuni* (*C.jeuni*) and *Campylobacter coli* (*C.coli*) contribute approximately 95% of human infection (3). Two others, *C. lari* and *C. upsaliensis* also account for many diarrhoeal cases in humans (5, 6). These microorganisms constitute the normal gastrointestinal microflora in many animals, especially birds (7). Poultry birds can be infected with the bacteria at a very high level without showing any visible clinical symptoms. Campylobacters, *C. jejuni* and *C. coli* are well adapted to birds because of their ability to grow at 41–42 (the approximate body temperature of a bird). These organisms have been frequently isolated from the caecal microflora (8). Intestinal content is thus one of the primary suspected source of meat contamination during slaughter. Hence, managing *Campylobacter* spp. in the poultry reservoir is a crucial step in prevention and control of food-borne campylobacteriosis in humans. Other possible sources like contaminated drinking water, consumption of unpasteurized milk and ready to eat food products, faecal run-off of birds and domestic animals contaminating surface water and direct contact with animals are significant in transmitting illness to humans (9).

Campylobacter illness in humans occur worldwide with estimated 500 million infections annually (10). Although it is a self limiting disease, the emergence of antimicrobial resistance in campylobacters has become a concern for food safety. Development of antimicrobial resistant (AMR) campylobacters has been linked to the indiscriminate use of antimicrobials in food animal production system (poultry and swine) for disease prevention and growth promotion (11, 12). Sub-therapeutic use of antimicrobials in food production systems is believed to create selection pressure and force microorganisms to develop resistance in order to survive (13). A rapid increase in the proportion of *Campylobacter* strains resistant to antimicrobial agents, particularly fluoroquinolones and macrolides, has been reported in many countries (14, 15, 16, 17). Nevertheless, there still exists paucity of data on the presence of antimicrobial resistant campylobacters and various risk factors responsible for the prevalence of these organisms in poultry production systems in India. Very few researchers have reported campylobacters in poultry (18, 19, 20, 21) thus, more future research awaits in this direction.

Uttarakhand, an Indian state with high tourist footfall of around 34.36 million with foreign tourist visits over 0.13 million in 2017(22) finds limited data on campylobacter presence in farms. To fill this knowledge gap, the present study was designed to estimate the occurrence of thermophilic campylobacters, virulence, antibiotic resistance and risk factor associated with campylobacters in eight commercial poultry farms located in Kumaon region of Uttarakhand.

**Results**

*Prevalence of thermophilic campylobacters*

Out of 545 samples comprising 346 poultry faecal and 199 environmental samples viz; feed (n=52), water (n=50), litter (n=51) and manure (n=46) samples collected from eight poultry farms, 67 samples tested positive for *Campylobacter* yielding a total prevalence of 12.29% (Table 2). *Campylobacter* genus-specific
amplicon of 816 bp (16SrRNA) was present in all the positive isolates. Faecal prevalence of *Campylobacter* was 18.2% (63/346) while environmental sources showed a prevalence of 2.01% (4/199), which included 3 (6%) isolates from water and 1 isolate (1.96%) from litter sample. None of the feed and manure samples yielded *Campylobacter* spp. Of the 67 isolates obtained, multiplex PCR targeting *lpxA* gene for species differentiation identified 16 (23.88%) as *C. jejuni* (331bp) and 51 (76.11%) as *C. coli* (391bp). None of the isolates produced an amplicon size of 233 bp (*C. lari*) and 206 bp (*C. upsaliensis*).

Varying prevalence was observed among the farms studied. Highest prevalence was detected in Bazpur farm (31.4%) followed by Pantnagar farm 2 (25.0%), Pantnagar farm 1 (24.4%), Haldwani farm (16.3%), Bindukhatta farm (7.5%) and Jawaharnagar farm (5.6%). No *Campylobacter* isolate was recovered from Kiccha and Ramnagar farms.

The species distribution of *Campylobacter* across farms revealed highest prevalence of *C. coli* in Bazpur farm (90.9%) followed by Pantnagar farm 1 (81.81%), Pantnagar farm 2 (66.67%), Jawaharnagar (60%), Haldwani farm (37.5%) and Bindukhatta (33.33%). However, the highest prevalence of *C. jejuni* was observed in Bindukhatta farm (66%) followed by Haldwani farm (62.5%), Jawaharnagar farm (40%) Pantnagar farm 2 (33.33%), Pantnagar farm 1 (18.18%) and Bazpur farm (9.09%)(Table 3).

**Prevalence of virulence genes**

All the 48 revived *Campylobacter* isolates (39 C. coli and 9 C. jejuni) showed 100% presence of *cadF* and *flaA* virulence genes followed by *cdtB* (97.9%), *cgtB* (22.9%) and *ciaB* (12.5%), respectively. None of the *Campylobacter* isolate harboured *wlaN* gene. The *ciaB* gene was detected only in *C. jejuni* isolates (66.66%, 6/9). None of the C. coli isolates harboured *ciaB* gene. Gene *cdtB* was detected in all C. coli (100%, 39/39) and (88.88%, 8/9) C. jejuni isolates. Virulence gene *cgtB* was identified in (33.33%, 3/9) C. jejuni and (20.51%, 8/39) C. coli isolates(Table 4).

Virulence genes *cadF* and *flaA* were detected in all isolates (100%) recovered from all four farms. Highest frequency of virulence gene *ciaB* was detected from Bindukhatta farm (33.3%) followed by Jawaharnagar farm (20%), Bazpur farm (9.09%) and Pantnagar farm 2 (6.66%). Virulence gene *cdtB* was detected from Pantnagar farm 2 (100%), Jawaharnagar farm (100%), Bindukhatta farm (100%) and Bazpur farm (95.4%). Highest frequency of *cgtB* gene was detected from Bazpur farm (36.3%) followed by Jawaharnagar farm (20%), Bindukhatta farm (16.6%) and Pantnagar farm 2 (6.6%). Virulence gene *wlaN* was not detected in any of the farms(Table 5).

**Phenotypic Antimicrobial Susceptibility**

On subjecting 42 revived isolates to disc diffusion test, forty one isolates (n=41, 97.6%) exhibited resistance to at least one antimicrobial on the disc diffusion assay and one isolate (ID.C4) was pan-susceptible. Ten isolates (n=10, 23.80%) were multidrug resistant (MDR) exhibiting resistance to at least 3 or more antimicrobial classes. Three *Campylobacter* isolates were found resistant to four classes of antimicrobials while seven isolates showed phenotypic resistance to three classes of antimicrobials. However, twenty two isolates were found to be resistant to two classes of antimicrobials.
β-lactam antimicrobials (cefoxitin, ceftriaxone and ampicillin) observed higher resistance than other classes studied. Highest frequency of resistance was found against cefoxitin (97.61%) followed by ciprofloxacin (64.28 %), nalidixic acid (33.33 %), ampicillin (28.5%) and ceftriaxone (14.28%). Two isolates (4.76%) were resistant to tetracycline. However, only one isolate showed resistance to clindamycin, sulfafurazole and erythromycin. All isolates (n=42) were susceptible to levofloxacin and gentamicin.

Variable resistance was seen in the two thermophilic campylobacters (C. jejuni and C. coli). Out of 42 isolates, 41 (97.61%) showed resistance to second generation cephalosporin, cefoxitin. Of these 41, 11 (27.5%) were C. jejuni and 30 (73.17%) were C. coli. Only 12 (28.5%,12/42) isolates showed resistant to ampicillin, of which, 4 (33.33%) were C. jejuni and 8 (66.66%) were C. coli. Six isolates (14.28%, 6/42) showed resistance to ceftriaxone of which 4 (66.66%) were C. jejuni and 2 (33.33%) were C. coli. However, resistance against ciprofloxacin, nalidixic acid and tetracycline was shown by 27 (64.28%,27/42), 14 (33.33%,14/42) and 2 (4.76%,2/42) isolates, respectively. Of which 7 (25.92%), 6 (42.85%) and 2 (100%) were C. jejuni and 20 (74.07%), 8 (57.14%) and 0 (0%) C. coli respectively(Table 6).

A total of 16 different AMR combinations were detected of which, 8 resistance patterns were MDR represented by 10 isolates. The most common MDR patterns were AMP CX CIP TE (n=2) and AMP CX CIP (n=2). Distribution of antimicrobial resistance patterns across sample types and farm location is detailed in Table7.

**Genotypic Characterization of AMR Determinants**

Presence of four antibiotic resistance genes (ARGs) namely \(bla\)OXA-61, \(tet\)(O), \(cme\)B and \(erm\)B conferring resistance to different classes of antibiotics were detected by specific Antibiotic Resistance Genes-PCRs. Out of 41 isolates showing phenotypic resistance, 29 isolates showed presence of at least one resistance genes targeted (\(bla\)OXA-61, \(tet\)(O), \(cme\)B and \(erm\)B. However, 12 resistant isolates did not harbour any of the four resistance genes. β-lactam resistance gene \(bla\)OXA-61 was detected in 18 (58.06%) out of 31 isolates showing phenotypic resistance. Resistance gene \(cme\)B was detected in 19 fluoroquinolone resistant isolates (79.16%) out of 24 tested. One lincosamide (Clindamycin) resistant isolate harboured \(cme\)B gene. Tetracycline resistant \(tet\)(O) gene was detected in all isolates showing phenotypic tetracycline resistance (n=2). Macrolide resistance gene \(erm\)B was absent in a single erythromycin resistant isolate. Most prevalent resistance gene combination was \(bla\)OXA-61+\(cme\)B, which was detected in 11 isolates(Table 8).

**Risk factor analysis**

Of the 11 parameters studied as risk factors using a questionnaire distributed to farm owners, only one risk parameter i.e., feeding of branded feed was found to have significant association with Campylobacter presence in the examined broiler flocks (p-value 0.0047).

**Discussion**
The present study was designed to determine the prevalence of thermophilic campylobacters in poultry raised at farms and their living environment.

**Prevalence of thermophilic campylobacters**

The overall *Campylobacter* comprising poultry faeces (n=346) and environmental samples(n=199) was recorded as 12.29% (67/545). Other findings reported from the studies conducted in broiler flocks have also reported almost similar overall prevalence. Chokboonmongkol et al. (23) reported 11.2% *Campylobacter* spp. prevalence in broiler flocks from Thailand while another study from Ecuador reported 12.4% prevalence of *Campylobacter* in broiler flocks (24). In India, limited studies have been done on *Campylobacter* prevalence in poultry. These studies have revealed prevalence ranging from 13.54-21.8% (13.54%,21; 15.89%,19; 21.8%,25 14.28%,26 and 20%, 27). However, a much higher prevalence of *Campylobacter* as high as 72.2% from cloacal swab samples has also been documented from poultry by Vaz et al. (28). Another study by Ingresa et al. (29) reported 71.4% prevalence of *Campylobacter* in poultry caecal samples and 69.1% for poultry faecal samples.

Faecal prevalence of *Campylobacter* was 18.2% (63/346).Detection of campylobacters in poultry faeces poses a significant risk for contamination of chicken meat. The organisms frequently colonize the bird’s intestine and shed in large numbers through faeces. Faecal shedding of *Campylobacter* spp. is a source of infection to other birds in the flock. Bacteria present in faeces can contaminate feed and water supply of the same flock. Moreover, there is a risk of *Campylobacter* transmission to their flocks by means of frequent human movement.

Only 4 isolates could be recovered from 199 environmental samples with a prevalence of 2.01%, which included 3 isolates from water and 1 isolate from litter sample However, Vaz et al. (28) recorded much higher 63.8% *Campylobacter* prevalence in litter samples from Brazilian broiler flocks. Similarly, Lisa et al. (30) reported 64.3%, 64.3% and 45.7% *Campylobacter* prevalence in soil, compost, and processed waste water respectively. Presence of campylobacters in environment is significant as campylobacters are able to form biofilms as a survival mechanism outside the host (31). Detection of *Campylobacter* spp. from water samples is important because all the birds in a flock drink water from the same waterer which aid in further spread within a flock. Also, capability to form a biofilm poses the risk of its presence in cold water inspite of chemical treatment (32).

Feed and manure samples of our study did not reveal any presence of *Campylobacter* spp. However, these sources cannot be neglected as a source of infection. Zero prevalence of *Campylobacter* in manure and feed samples could be due to less number of samples processed.

Interestingly, in this study majority of the isolates were identified as *C. coli* (76.11%) and only 23.88% isolates were *C. jejuni*. (Table4). *C. jejuni* is considered to be the predominant species colonizing poultry (33,34). Many studies (35,36,37) report the dominance of *C.jejuni* over *C.coli* in poultry. In India, Chattopadhyay et al. (38) and Rajendran et al. (39) also showed that *C. jejuni* were more frequent than *C. coli* in poultry faecal samples. However, in accordance to our study, many other authors have reported
C. coli dominance. Pergola et al. (40) reported 70.71% prevalence of C. coli and 17.14% C. jejuni from cloacal swab samples. Monika (21 and 19) also reported higher C. coli presence of 75% and 67.44%, respectively of the total isolates recovered from poultry faeces of Uttarakhand. Also, Wieczorek et al (41) in their retrospective study of five-years on prevalence and antimicrobial resistance of Campylobacter from poultry carcasses in Poland also found C. coli as a dominant species over C. jejuni. In our opinion, the initial dominance of a species and further spread due to improper control measures can decide the higher presence of a species. Better colonization ability of either of the two species in poultry intestine and persistence in outside environment may decide the dominance.

No C. lari and C. upsaliensis were detected in this study. However, C. lari isolation from poultry is reported by some authors. Very few studies support the presence of C. lari in poultry isolates. Pillai (42) and 25 isolated 2 and a single isolate of C. lari from poultry samples in Bangalore and Bareililly respectively. Oyarzabal and Hussain (43) are of the opinion that, with the development of DNA based methods for the identification of isolates, C. lari has not been reported for more than 10 years in the United States, which suggests that previous reports may have been misidentifications from the traditional biochemical tests which were used for species confirmation. Further studies on poultry using molecular diagnostic techniques would answer the same. Acke et al (44) reported that dogs are the main reservoirs for C. upsaliensis which could probably be the reason for non-isolation of this organism in our study.

No previous data on Campylobacter prevalence in poultry farms is available for selected locations except for Pantnagar and Haldwani. Probably isolation of Campylobacter from the locations except the two (Pantnagar and Haldwani) has not been reported so far. Poultry farms at Pantnagar screened before have reported the prevalence rates of 16 % (45), 11.66 % (46) and 13.54 % (21). However, a lower prevalence of 6.9 % (19) and 5.34 % (47) also has been reported from Pantnagar. Rawat et al (20) reported 4.17 % Campylobacter prevalence in faecal samples of broilers collected from an organized farm of Pantnagar.

Prevalence of virulence genes

Total 48 Campylobacter isolates including 39 C. coli and 9 C. jejuni were included for virulence gene detection using PCR. Nineteen isolates (n=19) could not be revived and thus were not included in the virulence gene analysis. The genes associated with bacterial motility (flaA) and adhesion to epithelial cells (ca df), were present in all (100%) the isolates. These genes are known to be conserved in Campylobacter spp. (48,49) and play a key role in the development of Campylobacter infection. The cdtB (97.9%) was second most prevalent gene. This gene along with cdtA and cdtC cytoxin gene has the ability to interfere with the division and differentiation of the intestinal crypt cells, thus has an important role in diarrhoea. This combination has been recorded with a prevalence of 96.6–97.6% in positive strains (50) which is in accordance with our study. It also suggests that the three genes (cdtA, cdtB and cdtC) should be included together in future studies for assessing toxic property.

The cgb gene was found in 22.9% of the positive Campylobacter spp. isolates. Not much data is available on the presence of this gene in the campylobacters though this gene, as wlaN, also codes for a β-1,3-
galactosyltransferase enzyme that is required for the production of sialylated lipooligosaccharide responsible for Guillain-Barré syndrome (GBS)(51)

Other gene ciaB exhibited in 12.5% isolates. This gene is important for Campylobacter survival in the intestinal tract. The product of the ciaB marker, which play a role both in the intestinal invasiveness and in colonization of the epithelial cells (52), was identified in campylobacters by other authors also in a lower percentag (53,54). The presence of this gene is significant as it helps the organisms to overcome the stress conditions presented by the intestine and cause disease. Additionally, expression of ciaB has been observed to reduce under nutritional stress (55).

None of the Campylobacter isolate harboured wlaN gene. Many studies conducted on C. jejuni and C.coli have reported total absence of this gene (48,56,57). However, Kim et al.(58) identified the wlaN gene among 100% of 63 human and in 78.6% of 42 animal C. jejuni isolated tested in Korea. The product of the wlaN gene is also thought to be involved in development of of Guillain–Barre’ syndrome after C. jejuni infection (49,58,59).

In our study, C.jeunii (cadF(100%), flaA(100%), ciaB(66.66%), cdtB(88.88%) and cgtB(33.33%) ) possessed more number of virulent genes than C.coli (cadF and flaA(100%), cdtB(100%) and cgtB(20.51%)). Moreover, ciaB gene presence (responsible for both epithelial and intestinal mucosal invasion) only in C.jeunii isolates may suggest this species dominance over C.coli in being more pathogenic (60) and a cause for regulars diarrhoeal cases in humans (7). The virulent profile of C. jejuni (59, 61) showed that the greatest potential of this species over the other in causing clinical cases in humans (81.1%) (62) is due to the properties of invasion, colonization and toxin production which are essential to elicit its pathogenesis. In contrast, C. Coli shows its priority is to ensure the survival through mechanisms (63).

Either of the virulence genes except wlaN were found in both faecal and environmental (water(n=3) and litter(n=1)) samples. This indicates potential risk to consumers.

Virulence genes cadF and flaA were detected in all isolates (100%) recovered from all four farms. Pant (45) recorded 100% prevalence of flaA and cadF genes in the isolates recovered from diverse sources collected from Udham Singh Nagar district. The presence of virulence genes such as cdtA and cdtB have been reported (46,64) who screened the sources from Pantnagar and nearby areas. Campylobacter isolates of the same region were also shown to express wlaN, iam, ciaB and dnaJ virulent genes (47).

High frequency of detection of virulence genes cadF (100%), flaA (100%) and cdtB (97.9%) in Campylobacter species in farms is a matter of concern. Casabonne et al. (65) studied the prevalence of seven virulence and toxin genes, i.e. flaA, cadF, ciaB, cdtB, cgtB, docC and wlaN from the diarrhic patients. He found all the isolates were positive for flaA, cadF and cdtB genes (100%) and 40.0%, 23.3%, 20.0% and 6.7% were positive for ciaB, docC, wlaN and cgtC, respectively. Wieczorek and Osek (66) showed the presence of cadF and flaA gene in 100% of the isolates obtained from poultry and human. Talukder et al. (57) studied pathogenic genes namely flaA, cadF, pldA, ciaB, cdtA, cdtB, cdtC and wlaN in 40 C. jejuni and 5 C. coli strains isolated from diarrheal patients in Bangladesh and found 100% prevalence of flaA,
cadF and pldA genes. The detection rates of ciaB, cdtA, cdtB, cdtC and wlaN genes were reported as 95%, 97.5%, 97.5%, 97.5% and 7.5% respectively.

**Phenotypic Antimicrobial Susceptibility**

Forty two isolates (11 *C. jejuni* and 31 *C. coli*) were revived for the phenotypic antimicrobial susceptibility. *Campylobacter* isolates exhibited highest frequency of resistance to cefoxitin (97.61%) followed by ciprofloxacin (64.28%), nalidixic acid (33.33%), ampicillin (28.5%) and ceftriaxone (14.28%) (Fig. 19). Two isolates (4.76%) were resistant to tetracycline. However, only one isolate showed resistance to clindamycin, sulfafurazole and erythromycin. All isolates (n=42) were susceptible to levofloxacin and gentamicin (Table 14).

The antibiotic resistance profile in this study was almost identical to the findings of Rajagunalan (19) who observed *C. jejuni* to be 100% sensitive to gentamicin, ampicillin and erythromycin and 100% resistant to cephalothin and co-trimoxazole. Narvaez *et al.* (67) reported that 71.4% of *Campylobacter* isolates had sensitivity against nalidixic acid followed by tetracycline (48.1%), ciprofloxacin (5.5%), azithromycin (1.78%) and erythromycin (1.78%). All isolates were susceptible to clindamycin, florfenicol, gentamicin and telithromycin and tetracycline resistance was attributable to the presence of the tet(O) gene. Kashoma *et al.* (68) reported *Campylobacter* isolates with resistance to ampicillin (63%), ciprofloxacin (9.3%), erythromycin (53.7%), gentamicin (0%), streptomycin (35.2%), and tetracycline (18.5%), azithromycin (42.6%), nalidixic acid (64.8%), chloramphenicol (13%) and tylosin (90.2%) respectively. The variation in the antimicrobial sensitivity pattern of the *Campylobacter* isolates has been reported earlier.

Ten isolates of 41 (n=10, 23.80%) were multidrug resistant (MDR) exhibiting resistance to at least 3 or more antimicrobial classes. Only one isolate (ID.C4) was pan-susceptible. Higher resistance to β-lactam antimicrobials was detected in our study such as cefoxitin, ceftriaxone and ampicillin. Resistance to ampicillin (28.5%), a “critically important antimicrobial”, crucial in human medicine is alarming, since it limits our options to treat critical human infections. Resistance was also detected against tetracycline (n=2) and clindamycin (n=1); antibiotics classified as “highly important” in human medicine according to WHO. Clinical management of *Campylobacter* infection becomes more difficult because of increasing development of resistance against antibiotics.

Of 16 different AMR combinations, 8 resistance patterns were MDR represented by 10 isolates. The most common MDR patterns were AMP CX CIP TE (n=2) and AMP CX CIP (n=2). Resistance pattern AMP CX CIP TE (n=2) had faecal origin and was identified from two separate locations, viz Haldwani and Pantnagar farm 2. Another MDR pattern AMP CX CIP (n=2) also had faecal origin. However, this pattern was identified from Bazpur and Bindukhatta farms. Distribution of antimicrobial resistance patterns across sample types and farm location is detailed in (Table 17). Most number of AMR patterns were detected from Bazpur farm (n=7), followed by Pantnagar farm 2 (n=6) and Bindukhatta farm (n=5). Four AMR patterns per farm were detected from Haldwani, Pantnagar farm 1 and Jawaharnagar farm. Significant diversity in the AMR patterns was detected across different farms and sample types. This may conclude the presence of genotypic diversity among the isolates circulating across locations and within a single location.
Genotypic Characterization of AMR Determinants

Out of 41 isolates showing phenotypic resistance, 29 isolates showed presence of at least one resistance genes targeted (blaOXA-61, tet(0), cmeB and ermB). Most prevalent resistance gene combination was blaOXA-61+ cmeB, which was detected in 11 isolates. A variety of antimicrobial resistance genes (ARGs) conferring resistance to various classes of antibiotics detected in this study is a matter of concern because these antibiotics are frequently used in human medicine and also these resistant determinants can be transferred to susceptible bacterial population by horizontal gene transfer (HGT). Nesme and Simonet (69) reported that soil is prone to genetic exchange by means of horizontal gene transfer between ecologically distinct lineages present in other ecosystems. Kashoma et al. (68) reported antimicrobial resistance genes blaOXA-61 (52.6%), cmeB (26.3%), tet(0) (26.3%) and aph-3-1 (5.3%) in Campylobacter isolates.

Risk factor analysis

Out of 11 risk parameters tested, only feeding of branded feed was found to be significantly associated with Campylobacter colonization of the examined broiler flocks (p-value 0.0047). In a similar study, Hald et al. (70) reported that 35% Campylobacter positive flocks used purchased wheat. Authors further reported that farmers who purchased wheat from a feedstuff dealer (p value 0.026) had a higher risk of Campylobacter infections in their broiler flocks compared to farmers who fed home-grown wheat. Various studies have been conducted to determine potential risk factors for Campylobacter infection in poultry farms (70,71,72,73,74). Cardinale (75) reported that an elevated risk of Campylobacter infection at poultry farms was associated with several factors namely presence of other animals (mainly laying hens, cattle and sheep) in the farm, farm staff not wearing proper work clothing while working in poultry houses, un-cemented poultry-house floors and the use of cartons that transport chicks from the hatchery to the farm as feed plates (rather than specifically designed feed plates). However, thorough cleaning and disinfection of poultry-house surroundings and manure disposal outside the farm were associated with decreased flock risk. In our study, the strength of association of risk factors with the prevalence of Campylobacter organism could be better identified with more number of samples screened at much larger number of farm locations.

Conclusion

The study highlights the occurrence of food pathogens, Campylobacter jejuni and C.coli in the poultry farms and their environment of the state. The organisms possessed significant virulence genes capable of developing critical human illness. Moreover, their resistance for frequently used antibiotics and attaining multi drug resistance is a point of concern. Eight different multi drug resistant patterns point towards reinforcing strict regulations against frequent misuse of antibiotics in farms for commercial gains. Majority of isolates possessing blaOXA-61+ cmeB gene combination may increase the peril by further possible horizontal spread in the surrounding microflora. Evaluation of potential risk factors in colonization of campylobacters suggests a thorough examination of feed before use, though this finding needs a more detailed study with more number of samples. To conclude, improved biosecurity in farms is
of paramount importance. Also, pre-harvest and post harvest interventions are valuable in reducing the risks linked with consumption /contamination of poultry meat.

Materials And Methods

Study Design and Sample collection

The present study was conducted in the Uttarakhand state of India. Samples were collected from eight poultry farms (n=8) farms located at Haldwani, Panthagar, Kiccha, Ramnagar, Bazpur, Jawaharnagar and Bindukhatta regions of the state, India from September 2016 to May 2017. A total of 545 samples collected comprised poultry faeces (n=346) and environment samples (n=199). The environmental samples represented water (n=50), poultry feed (52), litter (51) and manure (46). Sterile 100 ml whirlPak bags (Nasco, Fort Atkinson, WI) were used to collect poultry faeces, poultry feed, litter and manure. The water samples were collected in 100ml sterile sample container (Abdos India). The samples were collected aseptically and immediately brought to the laboratory for processing as per previously published protocols (76, 77, 78).

Isolation and Molecular Confirmation

Poultry faecal samples were streaked directly onto the modified Charcoal-Cefoperazone-deoxycholate agar (mCCDA, Hi media) plates and incubated at 42°C with 5% CO₂ in a CO₂ incubator for 48 hrs (OIE terrestrial manual 2008). The poultry feed and the environmental samples however were initially enriched in 9 ml Bolton broth (Oxoid, UK) supplemented with 5% sheep blood. Thereafter, a loopful of the enriched broth suspension was streaked onto mCCDA plates and were incubated at same time-temperature combination. The characteristic campylobacter colonies (1-2 mm size, circular, flat to slightly raised, sticky, spreading and shiny grey) were selected from each plate and tested biochemically. All the presumptive Campylobacter isolates that were catalase and oxidase positive while urease and TSI negative were subjected to DNA isolation using heat-shock method. A simplex PCR and a multiplex PCR assay targeting the 16SrRNA (79) and lipid gene lpxA (80) respectively were used for the Campylobacter genus and species identification. The primer sequence and the cyclic conditions used were as per references (79, 80 for Campylobacter genus and species, respectively). All PCR confirmed Campylobacter isolates were stored as 20% glycerol stock at -80°C.

Antibiotic Susceptibility Testing (AST)

The antimicrobial resistance (AMR) profile of Campylobacter isolates was determined using standard Kirby-Bauer disc diffusion method. A total of 42 isolates out of 67 isolates could be recovered for antimicrobial sensitivity testing. A panel of eleven antibiotics representing 5 classes of antimicrobials included Ampicillin (AMP,10µg), Gentamicin (GEN,10µg), Erythromycin(E,15 µg) ciprofloxacin(CIP,5µg) , levofoxacin (LE,5µg), nalidixic acid(NA,30µg), ceftriaxone(CTR,30 µg), cefoxitin(CX, 30µg), sulfafurazole(SF,300 µg), tetracycline(TE, 30µg)and clindamycin(CD, 30µg). The isolates were revived on mCCDA plates supplemented with (FD009) supplement. The growth suspension prepared in
PBS (0.5 McFarland) was spread on Mueller-Hinton agar (MHA) plates and incubated at 37 °C for 24h. Zone diameter was measured and break points were interpreted based on the recommendations of Clinical and Laboratory Standards Institute standards for disk-diffusion assay (81). The isolates showing resistance to three or more classes of antimicrobials were classified as Multidrug Resistant (MDR) (82). The isolates with intermediate level of resistance were categorized as susceptible to avoid overestimation of resistance.

**Detection of antimicrobial resistance genes (ARGs)**

*Campylobacter* isolates were screened for the presence of five antimicrobial resistance genes coding resistance to the antimicrobials used. PCR was performed to detect the presence of β-lactam resistance coding *bla*OXA-61 gene (83), gentamicin resistance coding *aph*A-3-1 gene (84), tetracycline resistance coding *tet*(O) gene (84), macrolide resistance coding *erm*B gene (85)) and a multidrug resistance gene *cme*B coding for fluoroquinolone and lincosamide antibiotics (83). PCR reaction and cycling conditions were used as described in respective references.

**Detection of virulence genes**

All *Campylobacter* isolates were screened for the presence of various virulence genes by PCR. Virulence genes screened were *Cad*F (86), *fla*A and *Cia*B (56), *cdt*B (87), *wla*N (88) and *cgt*B (89). PCR reaction and cycling conditions were used as described earlier in respective references.

**Risk factor analysis**

A questionnaire was prepared to study various risk factors associated with the *Campylobacter* prevalence in the poultry farms. All farm owners were requested to respond to the questionnaire (Table 1). However, no records were taken if an owner showed unwillingness to answer the questionnaire.

**Statistical analysis**

Univariate analysis was used to analyze differences in the proportion of *Campylobacter* in various poultry farms. The statistical significance level was defined as a two-tailed $p \leq 0.05$. All data analysis was carried out using Statistix7 software (Tallahassee, Florida, US).

**Declarations**

**Ethics approval**

The study was performed under the project on Zoonotic diseases. The project involves routine commection of probable samples for isolation and identification of food pathogens. The samples were analyzed using referred analytical methods. Therefore no informed consent was obtained.

**Consent for publication**
Not applicable.

**Availability of data and materials**

All data generated or analysed during this study are included in this published article and its additional information files. Strains are available from the corresponding author on request.

**Competing interests**

The authors declare that they have no competing interests.

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**Author’s contribution**

GG collected the samples and analyzed the samples. M designed the study. DK performed analysis of the data and AKU provided help as and when required and edited the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 1: Questionnaire for risk factor analysis for *Campylobacter* prevalence in poultry farms.

| Sl. No. | Questions                                      | Response        |
|---------|------------------------------------------------|-----------------|
| 1.      | Other animals on farm                          | Yes/No          |
| 1.      | How many times litter is reused?               | Once/twice/more |
| 1.      | Use of foot bath at entrance site              | Yes/No          |
| 1.      | Feed                                           | In house/Branded|
| 1.      | Chlorination for drinking water                | Yes/No          |
| 1.      | Distance of manure heap from farm              | <200m/>200m     |
| 1.      | Housing System                                 | Free moving/Cage system |
| 1.      | Flock size                                     | As informed     |
| 1.      | Floor type                                     | Cemented/Earthen|
| 1.      | Use of shoes by farm personnel                 | Yes/No          |
| 1.      | Litter                                         | Moist/Dry       |
| 1.      | No. of broiler flocks                          | As informed     |

Table 2: Prevalence of *Campylobacter* from different source

| Sample               | Total samples | Isolates found | Prevalence (%) |
|----------------------|---------------|----------------|----------------|
| Poultry faeces       | 346           | 63             | 18.2%          |
| Feed                 | 52            | 0              | 0.0%           |
| Litter               | 51            | 1              | 1.96%          |
| Water                | 50            | 3              | 6.0%           |
| Manure               | 46            | 0              | 0.0%           |
| Total                | 545           | 67             | 12.29%         |

Table 3: *Campylobacter* species distribution at different poultry farms
Table 4: Species-wise distribution of various virulence genes

| Species     | cadF          | flaA          | ciaB         | cdtB          | wlaN         | cgtB          |
|-------------|---------------|---------------|--------------|---------------|--------------|---------------|
| C. jejuni(9)| 9 (100)       | 9 (100)       | 6 (66.66)    | 8 (88.88)     | 0 (0)        | 3 (33.33)     |
| C. coli(39) | 39 (100)      | 39 (100)      | 0 (0)        | 39 (100)      | 0 (0)        | 8 (20.51)     |
| Total       | 48(100)       | 48(100)       | 6(12.50)     | 47(97.9)      | 0(0)         | 11(22.9)      |

Figure in parentheses indicates prevalence.

Table 5: Distribution of virulence genes in Campylobacter isolates (n=48) at different farms

| Farm (no. of isolates) | Number of positive isolates (%) | cadF    | flaA    | ciaB   | cdtB      | wlaN   | cgtB   |
|------------------------|---------------------------------|---------|---------|--------|-----------|--------|--------|
| Pantnagar farm 2 (15)  |                                 | 15(100) | 15(100) | 1(6.66)| 15(100)   | 0(0)   | 1(6.6) |
| Bazpur farm (22)       |                                 | 22(100) | 22(100) | 2(9.09)| 21(95.4)  | 0(0)   | 8(36.3)|
| Jawaharnagar farm (5)  |                                 | 5(100)  | 5(100)  | 1(20)  | 5(100)    | 0(0)   | 1(20)  |
| Bindukhatta farm (6)   |                                 | 6(100)  | 6(100)  | 2(33.3)| 6(100)    | 0(0)   | 1(16.6)|
| Total (48)             |                                 | 48(100) | 48(100) | 6(12.5)| 47(97.9)  | 0(0)   | 11(22.9)|

Table 6: Distribution of resistant Campylobacter isolates in C. jejuni and C. coli
| Antibiotics          | Resistant isolates | *C. jejuni* (%) | *C. coli* (%) |
|----------------------|--------------------|----------------|--------------|
| Ampicillin (AMP)     | 12/42              | 4(33.33)       | 8(66.66)     |
| Clindamycin (CD)     | 1/42               | 0(0)           | 1(100)       |
| Ceftriaxone (CTR)    | 6/42               | 4(66.66)       | 2(33.33)     |
| Cefoxitin (CX)       | 41/42              | 11(27.5)       | 30(73.17)    |
| Levofloxacin (LE)    | 0/42               | 0(0)           | 0(0)         |
| Ciprofloxacin (CIP)  | 27/42              | 7(25.92),      | 20(74.07)    |
| Nalidixic acid (NA)  | 14/42              | 6(42.85)       | 8(57.14)     |
| Erythromycin (E)     | 1/42               | 1(100)         | 0(0)         |
| Tetracycline (TE)    | 2/42               | 2(100)         | 0(0)         |
| Gentamicin (G)       | 0/42               | 0(0)           | 0(0)         |
| Sulphafurazole (SF)  | 1/42               | 0(0)           | 1(100)       |

**Table 7: Distribution of antimicrobial resistance patterns as per type of sample and farm location**
| Resistance Pattern (N)\(^a\) | Samples | Farm/Location\(^b\) |
|-------------------------------|---------|-------------------|
|                              | Poultry faeces | Water | Litter | H | P1 | P2 | BA | JW | BI |
| CX (6)                        | 6        | 0     | 0      | 0 | 3  | 2  | 0  | 1  | 0  |
| CX NA (2)                     | 2        | 0     | 0      | 0 | 0  | 2  | 0  | 0  | 0  |
| AMP CX (3)                    | 3        | 0     | 0      | 1 | 0  | 0  | 0  | 1  | 1  |
| CX CIP (11)                   | 9        | 2     | 0      | 0 | 2  | 1  | 6  | 1  | 1  |
| AMP SF NA (1)                 | 1        | 0     | 0      | 0 | 0  | 1  | 0  | 0  | 0  |
| CX CIP CD (1)                 | 1        | 0     | 0      | 0 | 0  | 0  | 1  | 0  | 0  |
| CX CTR NA (1)                 | 1        | 0     | 0      | 0 | 1  | 0  | 0  | 0  | 0  |
| CX CIP CTR (1)                | 1        | 0     | 0      | 0 | 0  | 0  | 0  | 0  | 1  |
| AMP CX CIP (2)                | 2        | 0     | 0      | 0 | 0  | 0  | 1  | 0  | 1  |
| CX CIP NA (6)                 | 5        | 0     | 1      | 1 | 1  | 2  | 1  | 1  | 0  |
| CX CIP CTR NA (1)             | 1        | 0     | 0      | 0 | 0  | 0  | 0  | 0  | 1  |
| AMP CX CTR NA (1)             | 0        | 1     | 0      | 0 | 0  | 0  | 1  | 0  | 0  |
| AMP CX CIP TE (2)             | 2        | 0     | 0      | 1 | 0  | 1  | 0  | 0  | 0  |
| AMP CX CIP NA(1)              | 1        | 0     | 0      | 0 | 0  | 0  | 1  | 0  | 0  |
| AMP CX E CIP CTR (1)          | 1        | 0     | 0      | 1 | 0  | 0  | 0  | 0  | 0  |
| AMP CX CIP CTR NA (1)         | 1        | 0     | 0      | 0 | 0  | 0  | 1  | 0  | 0  |

\(^a\)Resistance pattern (Number of isolates)

\(^b\)H (Haldwani farm), P1(Pantnagar farm 1), P2 (Pantnagar farm 2), BA (Bazpur farm), JW (Jawaharnagar farm), BI (Bindukhatta farm).
Table 8: Antimicrobial resistance (AMR) as per phenotype and genotype of *Campylobacter* isolates
| ID  | Resistance Pattern | Species | No. of antibiotics | No. of classes | Resistance genotype | Farm           | Source       |
|-----|--------------------|---------|--------------------|----------------|---------------------|----------------|--------------|
| C1  | CX                 | C. coli | 1                  | 1              | -                   | Pantnagar 1    | Poultry faeces |
| C2  | CX CIP             | C. coli | 2                  | 2              | cmeB                | Bazpur         | Poultry faeces |
| C3  | AMP CX CIP TE      | C. jejuni | 4              | 4              | tet(O)              | Haldwani      | Poultry faeces |
| C5  | AMP CX E CIP CTR   | C. coli  | 5                  | 4              | blaOXA-61, cmeB     | Haldwani      | Poultry faeces |
| C6  | CX CIP NA          | C. jejuni | 3              | 2              | -                   | Haldwani      | Poultry faeces |
| C7  | AMP CX CIP TE      | C. coli  | 4                  | 4              | cmeB, tet(O)        | Pantnagar 2    | Poultry faeces |
| C8  | CX CIP CTR NA     | C. jejuni | 4              | 2              | blaOXA-61, cmeB     | Bindukhatta   | Poultry faeces |
| C9  | CX CIP             | C. coli  | 2                  | 2              | -                   | Pantnagar 2    | Poultry faeces |
| C10 | AMP CX CIP CTR NA | C. coli  | 5                  | 3              | cmeB                | Bazpur         | Poultry faeces |
| C11 | CX CIP NA          | C. coli  | 3                  | 2              | cmeB                | Pantnagar 1    | Poultry faeces |
| C12 | CX CIP             | C. coli  | 2                  | 2              | -                   | Pantnagar 1    | Water         |
| C13 | AMP CX             | C. coli  | 2                  | 2              | -                   | Jawaharnagar   | Poultry faeces |
| C14 | CX CIP NA          | C. coli  | 3                  | 2              | -                   | Pantnagar 2    | Poultry faeces |
| C15 | CX CIP NA          | C. coli  | 3                  | 2              | cmeB                | Pantnagar 2    | Poultry faeces |
| C16 | CX CIP NA          | C. coli  | 3                  | 2              | cmeB                | Bazpur         | Litter        |
| C17 | CX CIP             | C. coli  | 2                  | 2              | blaOXA-61, cmeB     | Bazpur         | Poultry faeces |
| C18 | CX CIP             | C. coli  | 2                  | 2              | blaOXA-61, cmeB     | Bazpur         | Poultry faeces |
| C19 | CX CIP             | C. coli  | 2                  | 2              | -                   | Pantnagar 1    | Poultry faeces |
| C20 | CX CIP             | C. coli  | 2                  | 2              | cmeB                | Bazpur         | Water         |
| C21 | AMP SF             | C. coli  | 3                  | 3              | blaOXA-              | Pantnagar     | Poultry faeces |
|   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|
| C22 | CX NA | C. coli | 2 | 2 | blaOXA-61, cme B | Pantnaga r 2 | Poultry faeces |
| C23 | AMP CX CIP | C. coli | 3 | 3 | blaOXA-61, cme B | Bazpur | Poultry faeces |
| C24 | CX | C. coli | 1 | 1 | blaOXA-61 | Pantnaga r 1 | Poultry faeces |
| C25 | AMP CX CIP | C. coli | 3 | 3 | blaOXA-61 | Bindukhatta | Poultry faeces |
| C26 | CX CIP CD | C. coli | 3 | 3 | blaOXA-61, cme B | Bazpur | Poultry faeces |
| C27 | CX CTR NA | C. jejuni | 3 | 2 | blaOXA-61 | Pantnaga r 1 | Poultry faeces |
| C28 | CX | C. jejuni | 1 | 1 | - | Pantnaga r 1 | Poultry faeces |
| C29 | CX CIP | C. jejuni | 2 | 2 | - | Bazpur | Poultry faeces |
| C30 | CX CIP NA | C. jejuni | 3 | 2 | blaOXA-61, cme B | Jawaharnagar | Poultry faeces |
| C31 | CX | C. coli | 1 | 1 | - | Pantnaga r 1 | Poultry faeces |
| C32 | CX CIP | C. coli | 2 | 2 | blaOXA-61, cme B | Bazpur | Poultry faeces |
| C33 | CX | C. jejuni | 1 | 1 | - | Pantnaga r 1 | Poultry faeces |
| C34 | AMP CX CTR NA | C. jejuni | 4 | 3 | cmeB | Bazpur | Water |
| C35 | AMP CX CIP NA | C. coli | 4 | 3 | blaOXA-61, cmeB | Bazpur | Poultry faeces |
| C36 | CX NA | C. coli | 2 | 2 | cmeB | Pantnaga r 2 | Poultry faeces |
| C37 | CX CIP | C. coli | 2 | 2 | blaOXA-61, cme B | Jawaharnagar | Poultry faeces |
| C38 | CX | C. coli | 1 | 1 | blaOXA-61 | Jawaharnagar | Poultry faeces |
| C39 | CX CIP CTR | C. jejuni | 3 | 2 | - | Bindukhatta | Poultry faeces |
| C40 | CX CIP | C. coli | 2 | 2 | blaOXA-61 | Bindukhatta | Poultry faeces |
| C41 | AMP CX | C. coli | 2 | 2 | blaOXA-61 | Bindukhatta | Poultry faeces |
| C42 | AMP CX | C. coli | 2 | 2 | - | Haldwani | Poultry faeces |