Isolation, molecular identification and antimicrobial resistance patterns of Campylobacter species of dairy origin: First report from Bangladesh

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Abstract
This study was aimed for isolation, identification and characterization of Campylobacter species from Bangladesh Agricultural University dairy farm during the period of January to May, 2016. A total of 80 samples (fecal samples of calves, heifers and cows; milk samples of cows) were collected from Bangladesh Agricultural University dairy farm for isolation and identification of Campylobacter species by using cultural, biochemical and molecular methods. Moreover, the isolated Campylobacter species were subjected for antimicrobial susceptibility test. Campylobacter like organisms were presumptively identified in 20 samples. Isolates were biochemically positive to catalase and oxidase tests and in hippurate hydrolysis test some of the isolates (n=6) shown negative that indicated the isolates were C. coli and some of the test isolates (n=14) shown positive that indicated the isolates were C. jejuni. Campylobacter specific 16S rRNA genes were amplified from the isolates. Out of 20 isolated Campylobacter 14 (17.5%) were detected as C. jejuni and the rest 6 (7.5%) were detected as C. coli by cdC gene based multiplex PCR assay. C. jejuni were resistant to amoxicillin, erythromycin, azithromycin and susceptible to gentamicin, ciprofloxacin, norfloxacin and streptomycin. Furthermore, C. coli were resistant to amoxicillin and erythromycin and susceptible to gentamycin, ciprofloxacin. Out of 20 Campylobacter isolates, 57.14% C. jejuni and 33.33% C. coli were identified as multidrug resistant. To the best of our knowledge, this study has brought the first report on the occurrence of Campylobacter species with their antibiogram profiles in any dairy farm of Bangladesh.

Introduction

Campylobacter spp. are Gram negative, microaerophilic bacteria with slightly curved or spiral rods shaped under the family of Campylobacteriaceae.1 At least a dozen of Campylobacter spp. has been associated with human disease and the most common are C. jejuni and C. coli.2 Development of human infection may occur by direct contact with infected animals or by consumption of contaminated unpasteurized milk or milk products, contaminated water and raw meat and domestic birds are considered as important reservoirs of food-borne infection for humans.3 The importance of milk for the development of human gastroenteritis due to Campylobacter spp. was confirmed by the summary report of European Union on food-borne disease outbreaks.4 It is assumed that contamination of raw milk by Campylobacter spp. derived from secondary fecal contamination during the milking process.5 The infection has been developed due to consumption of raw milk that is the most important source of Campylobacter.6 Longer life span of dairy cattle than beef cattle can serve as a long-term reservoir of Campylobacter spp. in dairy cattle.5,7 The development of environmental contamination through indirect exposure of cattle feces is considered a high risk to human infections.7 The ideal environment for optimal growth of Campylobacter spp. requires an atmosphere containing approximately 5% O2, 10% CO2, and 85% N2 at 37°C to 42°C.8 The selective blood-containing agar are recommended medium that are used for culture of Campylobacter spp. although alternative media may be used. The viability of C. jejuni in faces and milk may remain for up to 9 days and 3 days respectively.9 Contamination of raw milk with Campylobacter spp. mainly associated with fecal contamination.10

The concerns for human health are the inappropriate use of antibiotics in cattle production and the development of antimicrobial resistant strains of bacteria. The increasing rate of human infections caused by antimicrobial-resistant Campylobacter spp. makes more difficult to clinical management of campylobacteriosis.11 Campylobacter spp. with resistant to antimicrobial agent has been reported worldwide.12-14

In Bangladesh several studies such as occurrence, molecular detection and antibiotic sensitivity test of Campylobacter spp. in poultry farms have been performed.14,15 However, there are no documented reports exist yet on the occurrence and antibiogram profiles of Campylobacter spp. in dairy farm where milk is widely consumed in Bangladesh. Therefore, this study was aimed to isolate and identify Campylobacter spp. inhabiting feces and milk originating from Bangladesh Agricultural University dairy farm and to assess antibiogram profiles of the isolated Campylobacter spp.

Materials and Methods

Collection, transportation and processing of samples
A total of 80 samples (60 fecal samples...
and 20 milk samples) were collected from Bangladesh Agricultural University (BAU) dairy farm during the period of January to May, 2016. Then the collected samples were transferred to Molecular Bacteriology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh through thermos flask. Then the samples were processed immediately for the isolation and identification of Campylobacter species.

**Isolation of Campylobacter species**

Isolation of Campylobacter species were carried out by filtration method (0.45 µm filter; Biotech, Germany) as described by Shiramatu et al.16

**Identification of Campylobacter spp. by biochemical tests**

Differentiation of isolated Campylobacter spp. were performed by various biochemical tests such as catalase, oxidase and hippurate hydrolysis test according to the methods described by Foster et al.17

**Preparation of DNA templates**

DNA templates were prepared by boiling method according to the procedures mentioned by Hoshimo et al.18

16S rRNA-gene-based PCR for identification of the genus Campylobacter

The 16S rRNA gene was selected for the identification of the genus Campylobacter. Primers (Invitrogen, USA) used for the amplification of 16S rRNA gene are shown in Table 1. The reaction mixture (20 µL) was prepared by mixing 10 µL master mixes (Promega, USA), 1 µL forward primer (10 pmol), 1 µL reverse primer (10 pmol), 3 µL DNA template and 5 µL deionized water. The PCR reactions were carried out using a thermocycler (Astec, Japan) with the following program: initial denaturation with 1 cycle of 5 min at 94°C, 30 cycles each consisting of denaturation with 30 s at 94°C, annealing with 30 s at 47°C, extension with 1 min 30 s at 72°C and a final extension step of 10 min at 72°C. PCR products were analyzed by 1.5% agarose (Invitrogen, USA) gel electrophoresis and the bands were visualized with UV light after staining with ethidium bromide (0.5 µg/mL) for 10 minutes in a dark place. Bands were visualized and images were captured on a UV transilluminator (Biometra, Germany).

**Table 1. Primers used for the various PCR.**

| Primer         | Sequence (5’-3’)                        | Target                   | Amplicon size (bp) | Reference |
|----------------|----------------------------------------|--------------------------|--------------------|-----------|
| 16S9F          | GAGTTTGATGCTGGGCTC                     | 16S rRNA gene            | 1530               | [22]      |
| 16S1540R       | GGGGTAGCAGCTGTTAA                      | C. coli                  | 524                | [19]      |
| Cj-cdtCU1      | TTAGCCTTTGCACCTCATA                    | Cj-cdtC                 | 313                | [19]      |
| Cj-cdtCR2      | AAGGGTAGCAGCTGTTAA                     | Cj-cdtC                 | 313                | [19]      |
| Cc-CdtCU1      | TAGGGAATAGCGCCCGAGG                    | Cc-cdtC                 | 397                | [19]      |
| Cc-CdtCR1      | GCTTAATACAGTTACGATAG                   | Cc-cdtC                 | 397                | [19]      |

**Table 2. Percentages (%) of Campylobacter species available in fecal and milk samples.**

| Types of sample | Species | No. of sample | No. (%) of Campylobacter isolates | Overall no. (%) of Campylobacter species |
|-----------------|---------|---------------|----------------------------------|----------------------------------------|
|                 |         |               | C. jejuni                        | C. coli                                |
| Faecal          | Calves  | 20            | 2 (10)                           | 0 (0)                                  | 20 (25)                                |
|                 | Heifers | 20            | 4 (20)                           | 2 (10)                                | 20 (25)                                |
|                 | Cows    | 20            | 5 (25)                           | 3 (15)                                | 20 (25)                                |
| Milk            | Cows    | 20            | 2 (10)                           | 1 (5)                                 | 20 (25)                                |
| **Total**       |         | **80**        | **14 (17)**                      | **6 (7.5)**                           | **20 (25)**                            |

**Antimicrobial susceptibility test**

All Campylobacter spp. were tested against eight commonly used antibiotics (HiMedia, India) by the method of disk diffusion as described by Luangtongkum et al.20 The zones of growth inhibition were compared with the zone size interpretative standards as described by Clinical and Laboratory Standard Institute.21 E. coli ATCC 25922 was kept as a quality control bacterium in this study. At least two separate experiments were performed for confirmation of all susceptibility data.

**Results**

**Isolation and identification of Campylobacter species using conventional methods**

The occurrences of Campylobacter species available in fecal and milk samples are shown in Table 2. A total of 80 samples [fecal (60) and milk (20)] were subjected for isolation of Campylobacter strains by filtration method. Campylobacter spp. produced grey color spreading colonies on Blood agar base no. 2 media after 48 hrs of incubation at 37°C using microaerophilic condition (5% O2, 10% CO2 and 85% N2). In Gram’s staining examination, the organism shown Gram negative, pink color, small.
curved shape arranged as single or in pair under microscope (100X). Out of 60 fecal samples only 16 (26.66%) were positive for *Campylobacter* spp. and out of 20 milk samples, 4 (20%) were positive for *Campylobacter* spp. *Campylobacter* like organisms were then subjected for biochemical tests. All the isolates of *Campylobacter* spp. (n=20) produced bubbles were found positive in catalase test. All the isolates of *Campylobacter* spp. (n=20) produced deep blue color within 10 seconds were found positive in oxidase test. In hippurate hydrolysis test some of the isolates (n=6) did not develop any purple color that indicated the isolates were *C. coli* and some of the test isolates (n=14) developed purple color that indicated the isolates were *C. jejuni*.

**Molecular identification of *Campylobacter* spp.**

Genus specific PCR with the gene of 16S rRNA was performed. 1530 bp fragment of targeted gene was amplified successfully (Figure 1). The multiplex PCR assay targeting the *cdtC* gene was used and 14 *C. jejuni* gave specific amplification (524 bp) (Figure 2). Similarly, 6 *C. coli* gave specific amplification (313 bp) by multiplex PCR assay targeting *cdtC* gene (Figure 3). None of the tested strains produced a specific band corresponding to the gene of *cdtC* of *C. fetus* (data not shown).

**Antibiogram profiles of isolated *Campylobacter* spp.**

14 isolates of *C. jejuni* were subjected to antimicrobial susceptibility testing against 8 selected antibiotics (Table 3). Among all the isolates 10 (71.42%) were susceptible to gentamicin, 4 (28.57%) were susceptible to norfloxacin, 4 (28.57%) were susceptible to ciprofloxacin, 5 (35.71%) were susceptible to streptomycin and 1 (7.14%) was susceptible to azithromycin. All data of antibiogram profile shown that the isolates were resistant to amoxicillin 12 (85.71%), tetracycline 12 (85.71%), azithromycin 12 (85.71%), erythromycin 14 (100%). Furthermore, 8 (57.14%) were resistant to streptomycin, 7 (50%) were resistant to norfloxacin, 2 (14.28%) were resistant to gentamicin and 4 (28.57%) were resistant to ciprofloxacin. 6 (42.85%) isolates were intermediate resistant to ciprofloxacin, 3 (21.42%) were intermediate resistant to norfloxacin, 1 (7.14) was intermediate resistant to streptomycin, 2 (14.28) were intermediate resistant to amoxicillin. Six isolates of *C. coli* were subjected to antimicrobial susceptibility testing against 8 selected antibiotics (Table 4). Among all the isolates 4 (66.67%) were susceptible to gentamicin, 3 (50%) were susceptible to ciprofloxacin, 1 (16.67) was susceptible to norfloxacin, 1 (16.67) was susceptible to azithromycin, 2 (33.33%) were susceptible to streptomycin. Antibiogram profiles revealed that the isolates were resistant to amoxicillin 4

![Figure 1. Detection of *Campylobacter* spp. by 16S rRNA gene based PCR. Lanes: 1, 100 bp DNA ladder (Promega, USA); 7, negative control; 2-6, tested positive samples.](image1)

![Figure 2. Detection of *Campylobacter jejuni* by *cdtC* gene based multiplex PCR assay. Lanes: 1-5, tested positive samples; 6, negative control; 7, 100 bp DNA ladder (Promega, USA).](image2)

![Figure 3. Detection of *Campylobacter coli* by *cdtC* gene based multiplex PCR assay. Lanes: 1-5, tested positive samples; 6, negative control; 7, 100 bp DNA ladder (Promega, USA).](image3)

**Table 3. Antimicrobial susceptibility pattern of *C. jejuni* (n=14) identified by the disk diffusion method.**

| Antimicrobial agents | S (%) | Number (%) of *C. jejuni* | I (%) | R (%) |
|----------------------|-------|---------------------------|-------|-------|
| Amoxicillin          | 0 (0) | 2 (14.28)                 | 12 (85.71) |       |
| Tetracycline         | 0 (0) | 2 (14.28)                 | 12 (85.71) |       |
| Gentamicin           | 10 (71.42%) | 2 (14.28) | 2 (14.28) |       |
| Erythromycin         | 0 (0) | 0 (0)                     | 14 (100) |       |
| Azithromycin         | 1 (7.14) | 1 (7.14) | 12 (85.71) |       |
| Ciprofloxacin        | 4 (28.57%) | 6 (42.85) | 4 (28.57) |       |
| Norfloxacin          | 4 (28.57%) | 3 (21.42) | 7 (50) |       |
| Streptomycin         | 5 (35.71) | 1 (7.14) | 8 (57.14) |       |

Note: AMX, Amoxicillin (30 μg); ADM, Amphotericin (30 μg); CIP, Ciprofloxacin (5 μg); E, Erythromycin (30 μg); GEN, gentamicin (10 μg); NOR, Norfloxacin (10 μg); S, Streptomycin (10 μg); TE, Tetracycline (30 μg); b: S = Susceptible; I = Intermediate resistance; R = Resistance.

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were identified by considering resistant to 2 or more drugs as described in Table 5. A total of 14 C. jejuni were isolated and 8 (57.14%) were identified as multidrug resistant. Out of 6 C. coli 2 (33.33%) were identified as multidrug resistant.

### Discussion

To our knowledge, this study has brought the first report on investigating the prevalence of *Campylobacter* spp. in dairy farms of Bangladesh. Cultural examination, staining characteristics, biochemical tests and finally PCR were performed for the characterization of the *Campylobacter* spp. and the colony characteristics were exhibited grey color which was supported by several researchers.15,23 The routine isolation and identification of *Campylobacter* spp. in laboratories were conducted on the basis of cultural and biochemical methods which was supported by several researchers.24 Hippurate hydrolysis test was used for discriminating between *C. jejuni* and *C. coli* which was also used by several researchers.13,15,16 The current study recorded 16 (26.66%) and 4 (20%) *Campylobacter* spp. from 60 fecal and 20 milk samples respectively during the study period. Out of 80 samples, 14 (17%) isolates were *C. jejuni* and the remaining 6 (7.5%) isolates were *C. coli*. Ramonaite et al.24 also recorded *Campylobacter jejuni* and *Campylobacter coli* from dairy farm in Lithuania. PCR primers targeting 16S rRNA gene of *Campylobacter* spp. were amplified 1530 bp fragments of DNA confirmed the identity of *Campylobacter* spp. (Figure 1). All *Campylobacter* isolates were positive to 16S rRNA gene based PCR which is supported by Kabir et al.13,14 The *cdtC* gene was amplified for detecting and discriminating between Cj-cdtC and Cc-cdtC (Figures 2 and 3) likewise several researchers.13 Despite of the fact that *Campylobacter* spp. is common in dairy cattle, our study revealed a moderate rate of prevalence (20%) in BAU dairy farm, Mymensingh, Bangladesh as shown in Table 2. Other researchers reported prevalence between 5% and 78.5%.25,26 Since sampling design, cultural methods and conditions were varied among these studies, a direct comparison of the results is troublesome. However, our data contribute to previous conversation that dairy cattle are significant assortment for *Campylobacter* spp. and could be a source of infections. The present study recorded that *Campylobacter* spp.

### Table 4. Antimicrobial susceptibility pattern of *C. coli* (n=6) identified by the disk diffusion method.

| Antimicrobial agents | S (%) | Number of *C. coli* isolates | I (%) | R (%) |
|----------------------|-------|-----------------------------|-------|-------|
| Amoxicillin          | 0 (0) | 2 (33.33)                   | 0 (0) | 2 (33.33) |
| Tetracycline         | 0 (0) | 2 (33.33)                   | 0 (0) | 2 (33.33) |
| Gentamicin           | 4 (66.67) | 1 (16.67)             | 1 (16.67) |
| Erythromycin         | 0 (0) | 2 (33.33)                   | 0 (0) | 2 (33.33) |
| Azithromycin         | 1 (16.67) | 2 (33.33)                | 3 (50) |
| Ciprofloxacin        | 3 (50) | 2 (33.33)                   | 1 (16.67) |
| Norfloxacin          | 1 (16.67) | 2 (33.33)                | 3 (50) |
| Streptomycin         | 2 (33.33) | 1 (16.67)                | 3 (50) |

### Table 5. Antimicrobial resistance pattern of *C. jejuni* and *C. coli*.

| Species | Resistance patterns | No. of resistant isolates (%) | No. of multidrug resistant isolates (%) |
|---------|---------------------|-------------------------------|---------------------------------------|
| *C. jejuni* (n=14) | No resistance demonstrated | - | 8 (57.14) |
|         | Resistant to 1 agent (AMX) | 3 (21.42) | |
|         | Resistant to 1 agent (E) | 2 (14.29) | |
|         | Resistant to 1 agent (AZM) | 1 (7.14) | |
|         | Resistant to 2 agents (AMX-TE) | 1 (7.14) | |
|         | Resistant to 3 agents (AMX-S-TE) | 1 (7.14) | |
|         | Resistant to 3 agents (E-S-CIP) | 2 (14.29) | |
|         | Resistant to 2 agents (AMX-NOR-AZM-TE) | 1 (7.14) | |
|         | Resistant to 5 agents (AMX-S-E-AZM-TE) | 1 (7.14) | |
|         | Total resistant isolates | 14 (100) | |

| *C. coli* (n=6) | Resistant to 1 agent (AMX) | 4 (66.67) | 2 (33.33) |
|                | Resistant to 2 agents (AMX-E) | 1 (16.67) | |
|                | Resistant to 3 agents (AMX-S-TE) | 1 (16.67) | |
|                | Total resistant isolates | 6 (100) | |

[Veterinary Science Development 2018; 8:7838]
species may be found more frequently in fecal samples than milk samples. In the antimicrobial susceptibility testing most of the isolates were susceptible to ciprofloxacin, gentamicin and all the isolates were resistant to amoxicillin, erythromycin. These findings are close to the findings of several researchers. The current study also recorded some multidrug resistant spp. in collected samples of BAU dairy farm. Out of 20 isolates, 57.14% Campylobacter jejuni and 33.33% Campylobacter coli were detected as multidrug resistant. Resistant profiles of multidrug resistant Campylobacter spp. were close to the result of some researchers. Findings of this study suggested that multidrug resistant Campylobacter spp. isolated from dairy farm might be an important concern for veterinary practitioners.

Conclusions

The findings of this study demonstrated the presence of multidrug resistant Campylobacter jejuni and Campylobacter coli in feces and milk samples that are not only harmful for cattle itself but also are harmful for consumers on milk consumption. Nevertheless, more studies are needed to clearly understand the genomic diversity in Campylobacter jejuni and Campylobacter coli as well as molecular mechanisms for the development of antimicrobial resistance.

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