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 (60 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 hirupate 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.8 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 Shiramaru 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 Hoshino 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 mixtures (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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Target</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S9F</td>
<td>GAGTTTGATCCTGGCTC</td>
<td>16S rRNA gene</td>
<td>1530</td>
<td>[22]</td>
</tr>
<tr>
<td>16S1540R</td>
<td>AAGGAGGTAGTCCAGGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cj-cdtCU1</td>
<td>TTTAGCCTTGGCACTCTTA</td>
<td>Cj-cdtC</td>
<td>524</td>
<td>[19]</td>
</tr>
<tr>
<td>Cj-cdtCR2</td>
<td>AAGGGGATAGCCAAGTGGTA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cc-CdtCU1</td>
<td>TAGGGATATGCACGCAAAG</td>
<td>Cc-cdtC</td>
<td>313</td>
<td>[19]</td>
</tr>
<tr>
<td>Cc-CdtCR1</td>
<td>GCTTAATACAGTTACGATAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CfspCU2</td>
<td>AAGCATAGTTTTGCCAGAGC</td>
<td>CfspCU2</td>
<td>397</td>
<td>[19]</td>
</tr>
<tr>
<td>CfspCR1</td>
<td>GTTTGAGTTTTCAAGTTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Types of sample</th>
<th>Species</th>
<th>No. of sample</th>
<th>No. (%) of Campylobacter isolates</th>
<th>Overall no. (%) of Campylobacter species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C. jejuni</td>
<td>C. coli</td>
</tr>
<tr>
<td>Faecal</td>
<td>Calves</td>
<td>20</td>
<td>2 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Heifers</td>
<td>20</td>
<td>4 (20)</td>
<td>2 (10)</td>
</tr>
<tr>
<td></td>
<td>Cows</td>
<td>20</td>
<td>5 (25)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Milk</td>
<td>Cows</td>
<td>20</td>
<td>3 (15)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>80</td>
<td>14 (17)</td>
<td>6 (7.5)</td>
</tr>
</tbody>
</table>
16S rRNA was performed. 1530 bp fragments were amplified from 20 milk samples, 4 (20%) were positive for Campylobacter spp. and out of 20 milk samples, 4 (20%) were positive for Campylobacter jejuni. 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 cdIC 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 cdIC gene (Figure 3). None of the tested strains produced a specific band corresponding to the gene of cdIC of C. fetus (data not shown).

Antibiogram profiles of isolated Campylobacter jejuni

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 anti-biogram 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 streptomycin, 2 (33.33%) were susceptible to azithromycin. Antibiogram profiles revealed that the isolates were resistant to amoxicillin 4

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

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

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

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

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>S (%)</th>
<th>Number (%) of C. jejuni</th>
<th>R (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>0 (0)</td>
<td>2 (14.28)</td>
<td>12 (85.71)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0 (0)</td>
<td>2 (14.28)</td>
<td>12 (85.71)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 (71.42)</td>
<td>2 (14.28)</td>
<td>2 (14.28)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>14 (100)</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>1 (7.14)</td>
<td>1 (7.14)</td>
<td>12 (85.71)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>4 (28.57)</td>
<td>6 (42.85)</td>
<td>4 (28.57)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>4 (28.57)</td>
<td>3 (21.42)</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>5 (35.71)</td>
<td>1 (7.14)</td>
<td>8 (57.14)</td>
</tr>
</tbody>
</table>

Note: AMX, Amoxicillin (30 μg); ADM, Amoxicillin (50 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.
Campylobacter jejuni and C. coli are shown in Table 5. Out of 14 C. jejuni isolates, 3 (21.42%) were resistant to 1 agent (AMX), 2 (14.29) were resistant to 2 agents (E-S-CIP), 1 (7.14%) was resistant to 4 agent (AMX-NOR-AZM-TE) and 1 (7.14%) was resistant to 5 agents (AMX-NOR-AZM-TE) respectively. Out of 6 Campylobacter coli isolates, 4 (66.67%) were resistant to 1 agent (AMX), 2 (33.33) were resistant to 2 agents (AMX-S-TE), 1 (16.67) were resistant to 3 agents (AMX-S-TE) respectively. Moreover, 1 (7.14%) was resistant to 3 agents (AMX-S-TE), 2 (14.29) were resistant to 3 agents (E-S-CIP), 1 (7.14%) was resistant to 4 agent (AMX-NOR-AZM-TE) and 1 (7.14%) was resistant to 5 agents (AMX-S-E-AZM-TE) respectively. Out of 6 Campylobacter coli isolates, 4 (66.67%) were resistant to 1 agent (AMX), 2 (33.33) were resistant to 2 agents (AMX-S-CIP), 1 (16.67) were resistant to 3 agents (AMX-S-CIP) respectively. In this study, multidrug resistant Campylobacter spp. were identified by considering resistant to 2 or more drugs as described in Table 5. A total of 14 Campylobacter jejuni and 8 Campylobacter coli were identified as multidrug resistant. Out of 6 Campylobacter coli (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. 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. The Hippurate hydrolysis test was used for discriminating between C. jejuni and C. coli which was also used by several researchers. 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. Ramonaitė et al. 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. The cdT gene was amplified for detecting and discriminating between Cj-cdTC and Cc-cdTC (Figures 2 and 3) likewise several researchers. 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%. 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 jejuni and C. coli.
species may be found more frequently in fecal samples than milk samples.

In the antimicrobial susceptibility testing of 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% C. jejuni and 33.33% C. 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 C. jejuni and C. 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 C. jejuni and C. coli as well as molecular mechanisms for the development of antimicrobial resistance.

References