Vol. 12(32), pp. 771-778, 28 August, 2018
DOI: 10.5897/AJMR2018.8946
Article Number: 863B6F758401
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Screening of human diarrhoeal samples in Mymensingh city of Bangladesh for the isolation, identification and antimicrobial resistance profiles of *Campylobacter* spp.

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Received 23 July, 2018; Accepted 13 August, 2018

***Campylobacter* spp. (*Campylobacter jejuni* and *Campylobacter coli*) are one of the major cause of food-borne bacterial diarrhoea in human worldwide. This study was conducted for the isolation, identification and antimicrobial resistance profiling of *Campylobacter* spp. from diarrhoeal samples of human collected from Surya Kanta (SK) hospital, Mymensingh Medical College, Mymensingh during the period of August 2016 to October 2017. Using cultural and biochemical techniques, a total number of 150 samples were subjected to *Campylobacter* isolation and identification. The isolated *Campylobacter* species (*C. jejuni* and *C. coli*) were characterized by antimicrobial susceptibility testing. Among 40 positive *Campylobacter* isolates, 23 (57.50%) were *C. jejuni* and the rest 17 (42.50%) isolates were *C. coli*. Furthermore, out of 40 *Campylobacter* like organisms, 22 *Campylobacter* isolates were found in male patient and 18 *Campylobacter* isolates were found in female. 13 (16.04%) C. *jejuni* and 9 (11.11%) C. *coli* were found in male and 10 (14.49%) C. *jejuni* and 8 (11.59%) C. *coli* were found in female. Considering the different age groups, 5 (33.33%), 12 (27.91%), 19 (29.68%) and 4 (14.28%) *Campylobacter* isolates were found in 1 to 15, 16 to 30, 31 to 50 and above 50 years respectively during the period of August 2016 to October 2017. Majority of the *Campylobacter jejuni* were resistant to ampicillin, nalidixic acid, tetracycline and norfloxacin. However, majority of the *Campylobacter jejuni* were susceptible to gentamycin, chloramphenicol, ciprofloxacin, azithromycin and streptomycin. Furthermore, *C. coli* were resistant to ampicillin, tetracycline, erythromycin, nalidixic acid, norfloxacin and susceptible to streptomycin, chloramphenicol, gentamycin and ciprofloxacin. Out of 40 *Campylobacter* isolates 65.21% *C. jejuni* and 52.94% *Campylobacter coli* were detected as multidrug resistant. The findings of the study revealed the presence of multidrug resistant *Campylobacter* species in human diarrhoeal samples in Mymensingh.**

Key words: Human diarrhoeal samples, *Campylobacter* spp., isolation, identification, antimicrobial resistance profiles.

INTRODUCTION

Campylobacter is one of the most commonly identified bacterial infections and is considered as the vital bacterial cause of acute human gastroenteritis worldwide (Allos and Taylor, 1998). Campylobacteriosis is the major public health hazard and a common cause of gastro-enteritis of human in the industrialized world (Friedman

et al., 2000). FoodNet surveillance identified 13.4 diagnosed *Campylobacter* infections per 100,000 persons (2002). Approximately 95% of diagnosed Campylobacter infections are due to *C. jejuni* (Altekruse et al., 1999). *Campylobacter* species are commonly inhibited in the intestinal tracts of poultry, livestock and food products of animal origin which are mostly associated with diarrhoea (de Boer et al., 2000). *Campylobacter* spp. is highly pathogenic causing acute diarrhoea and sometimes reactive arthritis and Guillain –Barre syndrome (Zia et al., 2003). *Campylobacter* spp. has a zoonotic impact (Anonymous, 2008) and causes food-borne infection resulting to human gastro-enteritis (Butzler et al., 1991; White et al., 1997; Kabir, 2011).

The primary sources of infection are considered to be the poultry and poultry products. Therefore, food animals such as cattle, sheep and pigs may be considered as asymptomatic carriers of *Campylobacter* species and during slaughter and carcass dressing, these animal food products can be contaminated by this pathogen (Berndtson et al., 1996). It is also reported that illness was associated with the consumption of raw milk and participation in hand milking of cows (Black et al., 1988).

Consumption of contaminated food stuffs are the major source of Campylobacteriosis in human which is predominantly acquired through it (Humphrey et al., 2007). About 2.5 million cases of Campylobacteriosis. The Centers for Disease Control and Prevention (CDC) estimates that over 200 deaths occur annually in the United States. Although, *Campylobacter* out-breaks are relatively rare, waterborne out-breaks have occurred worldwide in many developed countries (Karagiannis et al., 2010).

In campylobacterial diarrhoea, most of the patients had diarrhoea with vomiting and fever and the onset was mostly sudden which usually lasted for less than a week (Goossens et al., 1990). In human and veterinary practices, microbial resistance to antibiotic agents is a matter of concern all over the world (Sarker et al., 2018). Resistance of *Campylobacter* species to antimicrobial agents have been reported worldwide (Isenbarger et al., 2002). This condition seems to exaggerate rapidly in developing countries where widespread and uncontrolled use of antibiotics is practiced (Englen et al., 2003). Increasing level of antimicrobial resistant *Campylobacter* species is due to the excessive use of antimicrobial agents in food animals resulting to the emergence and dissemination of antimicrobial resistant bacteria (Hassan et al., 2014; Engberg et al., 2001), which has a serious impact on both Veterinary and Human health with regard to food safety. In veterinary industry antibiotics are commonly used as a therapeutic, prophylactic and growth promoting agents for livestock and poultry production and its causes accumulation in antibiotic residue in animal food products and development of antibiotic resistance (Donoghue, 2003).

As a microaerophilic bacteria, *Campylobacter* can be detrimental to their survival within certain environmental stresses like exposure to air, drying, low pH and prolonged storage (Oh et al., 2015). *Campylobacter* is frequently isolated from stools of infants with diarrhoea in developing countries which is resulted from the consumption of contaminated food or water (Coker et al., 2002). Only few studies in Bangladesh have been reported for the isolation of *Campylobacter* species from patients having diarrhoea (Blaser et al., 1997; Alam et al., 2006). Furthermore, no documented reports exist yet on the prevalence and antimicrobial resistance of *Campylobacter* species in diarrhoeal patients in Mymensingh where patients are available in that case.

Therefore, the aim of the present study was to isolate, identify and analyze the antimicrobial resistance patterns of *Campylobacter* species from diarrhoeal patients in Surya Kanta (SK) hospital, Mymensingh Medical College, Mymensingh, Bangladesh.

MATERIALS AND METHODS

Collection and transportation of samples

A total of 150 diarrhoeal samples were collected and immediately taken to the Bacteriology Laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh through cool chain maintaining. Thereafter, the samples were processed immediately for the isolation and identification of *Campylobacter* spp.

Isolation and identification of bacteria

Isolation of *Campylobacter* spp. were carried out by filtration method (0.45 µm filter) as described by Shiramaru et al. (2012). Each of the stool samples was suspended in 500 µl of sterile saline. 100 µl of the samples was spread on the filters that were placed on the surface of Blood agar base no. 2 and allowed to stand for 30 min at room temperature. After 30 min, the filter was removed from the BBA and then the plates were incubated at 37°C for 48 h in microaerophilic condition (5% O_2, 10% CO_2 and 85% N_2). After 48 h, the incubated media were then examined for growth of bacteria. Grey, flat and irregularly spreading colonies were observed on BBA. The colony was then subjected to Gram's Method of staining and observed under microscope for Gram negative curve. The organisms from the agar media were then sub-cultured into Blood agar base no. 2 with the help of an inoculating loop in the case of Gram negative curve in the smears. In the case of Blood agar grey, flat and irregularly spreading colony were observed. Thus, single pure colony was obtained. These pure isolates obtained were used for further study.

For differentiation of isolation of *Campylobacter,* isolated organisms with supporting growth characteristics of *Campylobacter*

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Table 1. List of primer used for *Campylobacter* spp.

Primer	Sequence (5'-3')	Target	Amplicon size (bp)	Reference
16S9F 16S1540R	GAGTTTGATCCTGGCTC AAGGAGGTGATCCAGCC	*Campylobacter* spp.16S rRNA gene	1530	Samosornsuk et al.(2007)

Table 2. Isolation of *Campylobacter* spp. according to sex and age of patients.

Parameter		Number of patients	Number (%) of *Campylobacter* positive case	Number (%) of *Campylobacter* spp.	
				C. jejuni (%)	*C. coli* (%)
Sex	Male	81	22(27.16)	13 (16.04)	9 (11.11)
	Female	69	18(26.09)	10 (14.49)	8 (11.59)
	Total	**150**	**40 (26.67)**	**23 (15.33)**	**17 (11.33)**
Age (years)	1-15	15	5 (33.33)	3 (20.00)	2 (13.33)
	16-30	43	12 (27.91)	7 (16.27)	5 (11.62)
	31-50	64	19 (29.68)	11 (17.18)	8 (12.50)
	>50	28	4 (14.28)	2 (7.14)	2 (7.14)
	Total	**150**	**40 (26.67)**	**23 (15.33)**	**17 (11.33)**

were subjected to various tests according to the procedures previously described by Nachamkin (2003) and Foster et al. (2004).

Molecular identification by PCR

DNA templates were prepared with the boiling method as described by Hoshino et al. (1998). All the samples were examined by two pairs of primers (Table 1) to detect the 16S rRNA gene of *Campylobacter* spp.. The PCR reactions were carried out using a thermocycler (ASTEC, Japan) with the following programme: initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 47°C for 30 sand extension at 72°C for 1 min and 30 s. The final extension was conducted at 72°C for 10 min. The holding temperature was 4°C until the thermo cycler was removed. 1.5% agarose (Invitrogen, USA) gel was used for electrophoresis of the PCR products.

Antimicrobial sensitivity test

All *Campylobacter* strains were tested against ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), streptomycin (10 µg), gentamicin (10 µg), erythromycin (15 µg), azithromycin (15 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg) and norfloxacin (10 µg) by disk diffusion method as described by Luangtongkum et al. (2007) with few modifications. All antimicrobial disks were obtained from Hi Media Laboratories Pvt. Ltd, India. Briefly, within 15 min after adjusting the turbidity of the inoculum suspension (equivalent to 0.5 McFarland turbidity), a sterile cotton swab was dipped into the adjusted suspension and then, the swab was rotated several times followed by pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. Thereafter, the dried surface of a Muller-Hinton agar supplemented with 5% defibrinated sheep blood was inoculated by streaking the swab over the entire sterile agar surface and this procedure was repeated two more times, and the plate was rotated at 60° each time to ensure a confluent lawn of bacterial growth. After the inoculates were dry, five antimicrobial disks were applied per plate and incubated in the inverted position at 37°C for 48 h under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). The zone diameter breakpoints of each antimicrobial agent were determined according to the breakpoints used by the National Antimicrobial Resistance Monitoring System (NARMS) and the CLSI-established guideline for bacteria isolated from animals (CDC, 2003; National Committee for Clinical Laboratory Standards 2002a, 2002b).

RESULTS

Cultural examination and biochemical tests

Campylobacter spp. produced grey color spreading colonies on blood agar base no. 2 media after 48 h of incubation at 37°C. In Gram's staining under microscope, the organism revealed Gram negative, pink color, small curved shape arranged as single or pair. The organisms were checked and confirmed by their purity using special blood agar media (Blood agar base no. 2). All the isolates of *Campylobacter* spp. were found positive for catalase test and oxidase test. Only *C. jejuni* were found positive in hippurate hydrolysis test but *C. coli* were found negative for hippurate hydrolysis test.

Isolation of *Campylobacter* spp. from patients according to sex and age

A total of 150 diarrhoeal samples (male=81 and female=69) were subjected to isolation of *Campylobacter* strains by filtration method. A total of 40 (26.67%) *Campylobacter* organisms were found in the studied samples where 22 (27.16%) were male and 18 (26.09%) were female as shown in Table 2.

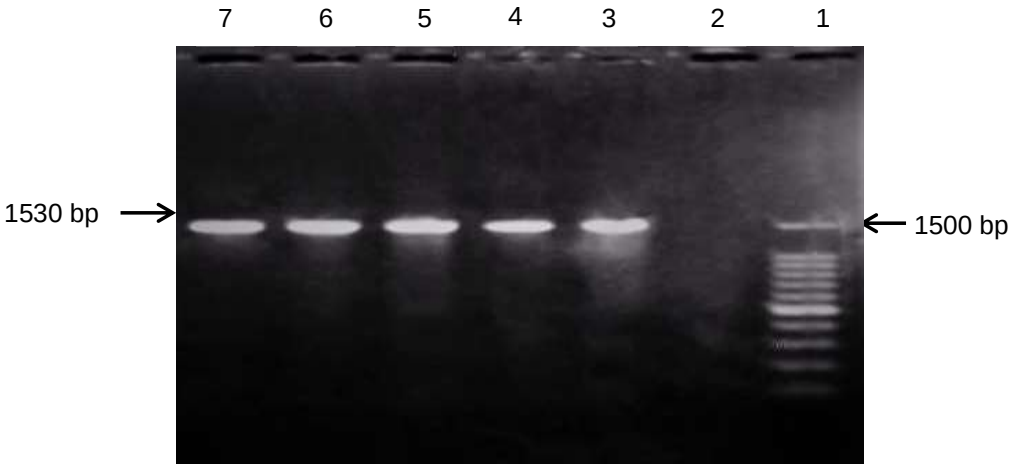

Figure 1. PCR product of 16S rRNA gene. A 1530 bp gene product in the picture confirmed the 16S rRNA gene of *Campylobacter* spp. Lane, 1 was 100 bp DNA ladder (Promega, USA); lane 2 was negative control and lanes 3 to 7 were positive samples.

The age groups of experimented samples were divided as; 1 to 15 years (n=15), 16 to 30 years (n=43), 31 to 50 years (n=64) and above 50 years (n=28) and were subjected to isolation of *Campylobacter* strains. Out of 40 *Campylobacter* organisms; 5 (33.33%) were in 1 to 15 years, 12 (27.91%) were in 16 to 30 years, 19 (29.68%) were in 31 to 50 years and above 50 years, 4 (14.28%) were found (Table 2).

Molecular detection by PCR

Genus specific (16S rRNA gene) polymerase chain reaction (PCR) was performed. 1530 bp fragment of targeted gene was amplified successfully. The results of PCR are shown in Figure 1.

Antibiogram study for *Campylobacter* spp.

Antimicrobial susceptibility of C. jejuni and C. coli

Details results of antimicrobial susceptibility testing by disc diffusion method with 10 chosen antimicrobial agents are presented in Table 3. Out of 23 *C. jejuni* isolates, 23 (100%) were resistant to ampicillin, 7 (30.43%) were intermediately resistant to streptomycin, and 19 (82.61%) were susceptible to gentamicin.

Out of 17 *Campylobacter coli* isolates, 17 (100%) were resistant to ampicillin, 7 (41.17%) were intermediately resistant to azithromycin and 12 (70.59%) were susceptible to ciprofloxacin

Antimicrobial resistant pattern of C. jejuni and C. coli

The results of antimicrobial resistance patterns of *C. jejuni* and *C. coli* are summarized in Table 4. Out of 23 *C. jejuni* isolates, 5 (21.73%) and 3 (13.04%) were resistant to each of 1 antibiotic (AMP) and (E) respectively. Moreover, 6 (26.09%) and 2 (8.69%) were resistant to each of 2 antibiotic agents (AMP-TET) and (AMP-ST) respectively. Furthermore, 2 (8.69%), 1(4.34) and 2 (8.69%) were resistant to each of 3 antibiotic agents: (AMP-ST-TET), (E-ST-CIP) and (AMP-TET-ER) respectively and 2 (8.69%) were resistant to 4 antibiotic agents (AMP-TET-ER-NOR).

Out of 17 C. coli isolates, 3 (17.64%) and 5 (29.41%) were resistant to 1 antibiotic (AMP) and (E) respectively. Furthermore, 2 (11.76%) and 2 (11.76%) were resistant to 2 antibiotics (AMP-TET) and (AMP-ST) respectively. 1 (5.88%), 2 (11.76%) and 1 (5.88%) were resistant to each of 3 antibiotic agents: (AMP-ST-TET), (E-ST-CIP) and (AMP-TET-ER) respectively. 1 (5.88%) were resistant to 4 antibiotic agents (AMP-TET-ER-NOR).

Multidrug resistant *Campylobacter* spp. was identified by considering resistant to 2 or more drugs. A total of 15 (65.21%) C. jejuni (out of 23) and 9 (52.94%) C. coli (out of 17) were identified as multidrug resistant.

DISCUSSION

For the characterization of the *Campylobacter* spp. isolation, cultural characteristics based examination, staining characteristics, biochemical testing and finally PCR were very crucial which were performed from the isolated *Campylobacter* spp. Blood agar base no. 2 as a selective agar media was used to culture the organism (*Campylobacter* spp.) under required microaerophilic environment (5% O_2, 10 % CO_2 and 85% N_2) as the experiment was conducted by several researchers (Haseena, 2017; Kabir et al., 2014a, 2014b) and

Table 3. Antimicrobial susceptibility pattern of *Campylobacter* spp. (n=40) identified by the disk diffusion method.

Name of isolates		Number (%)									
		AMP	**TET**	**CHL**	**ST**	**GEN**	**E**	**AZM**	**NAL**	**CIP**	**NOR**
C. jejuni	S (%)	0(0)	5(21.74)	17(73.91)	13(56.52)	19(82.61)	8(34.78)	15(65.21)	2(8.69)	16(69.56)	7(30.43)
	I (%)	0(0)	2(8.69)	6(26.08)	7(30.43)	3(13.04)	7(30.43)	5(21.73)	0(0.00)	2(8.69)	4(17.39)
	R (%)	23(100)	16(69.56)	0(0)	3(13.04)	1(4.34)	8(34.78)	3(13.04)	21(91.31)	5(21.73)	12(52.17)
C. coli	S (%)	0(0)	6(35.29)	10(58.82)	11(64.70)	9(52.94)	3(17.64)	4(23.52)	5(29.41)	12(70.59)	1(5.88)
	I (%)	0(0)	2(11.76)	5(29.41)	5(29.41)	3(17.64)	3(17.64)	7(41.17)	4(23.52)	1(5.88)	3(17.64)
	R (%)	17(100)	9(52.94)	2(11.76)	1(5.88)	5(29.41)	11(64.71)	6(35.29)	8(47.05)	4(23.52)	13(76.47)

Susceptible: I = Intermediate; R = Resistance. AMP, Ampicillin; TET, tetracycline; E, erythromycin; AZM, Azithromycin; CIP, Ciprofloxacin; ST, Streptomycin; NOR, Norfloxacin; GEN, Gentamicin; NAL, Nalidixic acid; CHL, Chloramphenicol.

Table 4. Antimicrobial resistance pattern of *Campylobacter* spp.

Isolate	Resistance profiles	Number of isolates (%)
C. jejuni (n=23)	a. No resistance demonstrated	-
	b. Resistant to 1 agent (AMP)	5(21.73)
	c. Resistant to 1 agent (E)	3(13.04)
	d. Resistant to 2 agents (AMP-TET)	6(26.09)
	e. Resistant to 2 agents (AMP-ST)	2(8.69)
	f. Resistant to 3 agents (AMP-ST-TET)	2(8.69)
	g. Resistant to 3 agents (E-ST-CIP)	1(4.34)
	h. Resistant to 3 agent (AMP-TET-ER)	2(8.69)
	i. resistant to 4 agents (AMP-TET-ER-NOR)	2(8.69)
	Total resistant isolates	**23(100)**
C. coli (n=17)	a. No resistance demonstrated	-
	b. Resistant to 1 agent (AMP)	3(17.64)
	c. Resistant to 1 agent (E)	5(29.41)
	d. Resistant to 2 agents (AMP-TET)	2(11.76)
	e. Resistant to 2 agents (AMP-ST)	2(11.76)
	f. Resistant to 3 agents (AMP-ST-TET)	1(5.88)
	g. Resistant to 3 agents (E-ST-CIP)	2(11.76)
	h. Resistant to 3 agent (AMP-TET-ER)	1(5.88)
	i. Resistant to 4 agents (AMP-TET-ER-NOR)	1(5.88)
	Total resistant isolates	**17(100)**

filtration method (0.45 µm filter paper) was used for selection of the *Campylobacter* spp. (Shiramaru et al., 2012). The colony characteristics of *Campylobacter* spp. exhibited pink or light pink color, gram negative and slightly curved shaped that is similar to the results of several researchers (Doyle, 1990; Rowe and Madden, 2000; Jamshidi et al*.,* 2008; Kabir, 2011).

This study recorded 40 *Campylobacter* spp. by biochemical tests. Out of 40 isolates, 23 (57.50%) *C. jejuni* and 17 (42.50%) *C. coli* were identified. In this study, for the identification of *Campylobacter* spp. biochemical tests were performed which were also similar to the studies of a number of researchers (Shiramaru et al., 2012; Gblossi et al., 2012; Kabir et al., 2014a, 2014b).

PCR primers targeting 16S rRNA gene of *Campylobacter* spp. were amplified. 1530 bp fragments of DNA confirmed the identity of *Campylobacter* spp. Similar results were also observed by several researchers (Foster et al., 2004; Samosornsuk et al., 2007; Kabir, 2011). *Campylobacter* spp. were found in the collected samples of diarrhoeal patients in SK Hospital, Mymensingh Medical College, Mymensingh. Similar findings were reported in other studies by Awad et al. (2010); Malmuthuge et al. (2012) and Hossain et al. (2015).

Reliable and reproducible laboratory techniques are required for monitoring drug resistance among the *Campylobacter* isolates. *Campylobacter* present difficulties in antimicrobial susceptibility testing like other fastidious bacteria because of their unique growth requirements and test conditions. In this study, susceptibility profiles of *Campylobacter* isolates were determined through agar disk diffusion method. The methodology has proven to be a relatively accurate method to test antimicrobial susceptibilities of fastidious organisms including *Campylobacter* spp.

The current study was conducted to detect the antimicrobial resistance patterns by disk diffusion methods of *Campylobacter* spp. that is used by several researchers (Kabir, 2011; Gblossi et al., 2012; Wieczorek et al., 2013; Kabir et al., 2014a, 2014b). The high level of ciprofloxacin resistance (>80%) and tetracycline had been found by Senok et al*.* (2007) in human patients that suffered from diarrhoea. Hakanen et al. (2003) found 46% resistance to ciprofloxacin, tetracycline (46%) and ampicillin (17%) respectively from the diarrhoeal patients.

Higher frequency of erythromycin resistance in *C. coli* than in *C. jejuni* (0 to 11% in *C. jejuni* and 0 to 68.4% in *C. coli*) had been reported by Sjögren et al*.* (1997) for human that suffered from Campylobacterial diarrhoea. Resistance to erythromycin was low in Japan, Canada, and Finland but recent development of resistance in Thailand and Sweden has been reported by Hoge et al. (1998). The concomitant resistance rates among nalidixic acid resistant *C. jejuni* isolated from patients (exclusively children) were 2, 12, 12, 97 and 66% for gentamicin, erythromycin, clindamycin, tetracycline, ciprofloxacin respectively and 90% of the *C. coli* isolates were concomitantly resistant to clindamycin. The rates of resistance of 51 to 72 human strains of *C. jejuni* isolated annually from 1998 to 2001 in Montréal, Québec, Canada, varied from 1 to 12% for erythromycin, 43 to 68% for tetracycline and 10 to 47% for ciprofloxacin (Gaudreau, 2003).

For *Campylobacter* infections, Hoge et al*.* (1998) found 100% co-resistance between *Campylobacter* spp. isolates resistant to azithromycin and ciprofloxacin. Fluoroquinolone resistance in *C. jejuni, C. coli* or combination of *C. jejuni* and *C. coli* were 56.9%, 84%, and 75 to 88%, respectively which is supported by the findings of the present study.

This study recorded highest resistance rates to ampicillin, erythromycin, nalidixic acid and norfloxacin against *C. jejuni* and *C. coli*. Findings of this study suggest that multidrug resistant *Campylobacter* spp. isolated from human diarrhoeal samples might be an important concern for human health. This study has generated the first report on the antimicrobial resistance pathogens of *Campylobacter* spp*.* isolated from human diarrhoeal samples in Bangladesh.

Conclusion

The research project revealed that *Campylobacter* spp. infection is a common phenomenon in the study area. The findings of the study also revealed the presence of multidrug resistant *Campylobacter* species in human diarrhoeal samples in Mymensingh. Strictly hygienic measures with personal hygiene, food safety especially during consumption of food stuffs contaminated with *Campylobacter* spp. organism should strictly be prohibited. Antibiotics could be used after sensitivity test for the treatment of clinical cases of *Campylobacter* spp. infection in human. It is also necessary to avoid excessive use of antibiotics in food animals due to their antimicrobial resistance properties.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors like to sincerely thank the University Grants Commission (UGC) of Bangladesh (Project No. 2015/274/UGC) for giving financial and logistic support for this study.

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