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Article

Molecular identification and antimicrobial resistance profiles of *Campylobacter* strains of poultry origin in India with special emphasis on fluoroquinolone resistance

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Abstract: The current study aimed to identify the selected number of *Campylobacter* strains of poultry origin in India that were isolated in the Laboratory of International Prevention of Epidemics, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Japan to the species level with the aid of *cdtA/B/C* gene-based multiplex PCR assays as well as to investigate their antimicrobial resistance profiles. *C. jejuni* (4 strains) and *C. coli* (16 strains) were identified. The poultry isolates identified were subjected to susceptibility testing with the aid of disk diffusion method using 12 antimicrobial agents. Again, the resistant and intermediate isolates confirmed by the disk diffusion method were subjected to determination of minimum inhibitory concentration by agar dilution method. To elucidate the mechanism of quinolone resistance, a total number of 20 quinolone resistant strains were subjected to sequence determination and analysis of the *gyrA* gene in the quinolone-resistance determining region. The results of the disk diffusion method were consistent with the results of the agar dilution method with slight variation in case of ampicillin, levofloxacin, ofloxacin and fosfomycin. All *C. jejuni* and *C. coli* strains had the Thr 86 to Ile substitution in *gyrA.* Results of this study support the emergence of resistance of *C. jejuni and C. coli* strains of poultry origin in India to a variety of antimicrobials especially fluoroquinolones.

Keywords: identification; multiplex PCR; *Campylobacter* species; antimicrobial resistance

1. Introduction

Campylobacter, a gram negative, nonsporulating, motile bacterium, is commonly isolated as a pathogen associated with diarrhoea in many industrialized countries (Stanley and Jones, 2003). Chickens are frequently colonized by pathogenic *Campylobacter* species like *C. jejuni* and *C. coli* (Corry and Atabay, 2001). *Campylobacter* infections are primarily because of handling and consumption of raw or undercooked poultry and due to cross contamination (Stanley and Jones, 2003). Studies on isolation of *Campylobacter* from poultry meat have been carried out from the regions Tamilnadu and Calcutta using conventional method (Chowdhury *et al.*, 1984; Varma *et al.*, 2000). Although *Campylobacter* with resistance to antimicrobial agents has been reported worldwide (Looveren *et al.*, 2001; Isenbarger *et al.*, 2002), the situation seems to deteriorate more

rapidly in developing countries, where there is widespread and uncontrolled use of antibiotics (Hart and Kariuki, 1998). Moreover, *Campylobacter* infections pose a serious public health problem for which many countries are monitoring their infection and antimicrobial resistance patterns. The current study was aimed to identify the selected number of *Campylobacter* strains of poultry origin in Calcutta, India that were isolated in the Laboratory of International Prevention of Epidemics, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Japan to the species level with the aid of *cdtA/B/C* gene-based multiplex PCR assays as well as to investigate their antimicrobial resistance profiles.

2. Materials and Methods

2.1. Bacterial strains and media

A total of twenty *Campylobacter* strains isolated from poultry origin (cloacal swabs and raw poultry meat) in Calcutta, India were used in this study. *E. coli* ATCC 25922 was used as a quality control organism in antimicrobial susceptibility testing. All *Campylobacter* strains and *E. coli* ATCC 25922 were grown on blood base agar no. 2 (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood (Nippon Bio-Supp. Center, Tokyo, Japan) under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37 ^oC for 48 hr.

2.2. DNA preparation

Template DNA was prepared by the boiling method as described by Hoshino *et al*. (1998). Briefly, a loopful of bacteria collected from agar plate was suspended in 1 ml of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] followed by boiling of the suspension for 10 min, centrifugation at 12, 800 *g* for 5 min and 2 µl of supernatant was used as a PCR template.

2.3. *hipO* gene PCR

The hippuricase gene (*hipO*) was amplified by PCR using primers as described in Table 1 (Linton *et al*., 1997). The PCR reaction contained appropriate concentration of primer sets (Table 1), 0.2 mM each of dNTP mixture (dATP, dCTP, dGTP, and dTTP), 1X Ex *Taq* DNA polymerase buffer, and 1.0 U of Ex *Taq* DNA polymerase in 40 µl reaction volume. Amplification was performed on an Applied Biosystems GeneAmp PCR 9700 (Applied Biosystems). PCR products were analyzed by 1.5% agarose gel electrophoresis and bands were visualized with UV light after staining with ethidium bromide (1 µg/ml). Images were captured on a Bio-Rad Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Sequencing of the 16S rRNA gene

PCR primers for amplifying 16S rRNA gene are described in Table 1. Reaction mixture was as described above. PCR product was purified by a QIAquick PCR Purification Kit according to the manufacturer's instruction (QIAGEN GmbH, Hilden, Germany). The purified DNA was subjected to cycle sequencing reaction by using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the primers used for 16S rRNA gene amplification and four additional primers, 16S520F (5'-GAGTTTGATCCTGGCTC-3'), 16S1100F (5'-GCAACGAGCGCAACCC-3'), 16S741R (5'-GTATCTAATCCTGTTTGC-3') and 16S1240R (5'-CCATTGTAGCACGTGT-3'), which can specifically bind inner region of 16S rRNA gene were also used. Nucleotide sequences were determined by using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed using the DNA Lasergene software package (DNASTAR, Madison, WI, USA). Homology searches were performed against all sequences in the GenBank database by using the BLAST search engine, available through the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

2.5. Multiplex PCR

The multiplex PCR assay for detection of *cdtA*, *cdtB* and *cdtC* genes of *C. jejuni*, *C. coli* and *C. fetus* was performed by gene specific primers (Asakura *et al*., 2008) are summarized in Table 1. All reactions contained appropriate concentrations of three primer sets, 0.2 mM each of dNTP mixture, 1X Ex *Taq* DNA polymerase buffer, and 1.0 U of Ex *Taq* DNA polymerase in a 40-µL reaction volume. PCR products were analyzed by 2% agarose gel electrophoresis and visualization of bands and capturing of image were done as described above.

2.6. Antimicrobial agents and susceptibility testing

All *Campylobacter* strains were tested against ampicillin (10 µg), tetracycline (30 µg), azithromycin (15 µg), erythromycin (15 µg), chloramphenicol (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ofloxacin (5 µg), fosfomycin (50 µg) and sulphamethoxazole-trimethoprim (1.25 µg) by disk diffusion method as described by Luangtongkum *et al.* (2007) with some modifications. Again, all intermediately resistant and resistant *Campylobacter* strains confirmed by disk diffusion method except sulphamethoxazole-trimethoprim resistant strains were subjected to the determination of minimum inhibitory concentration (MIC) by agar dilution method as described by Luangtongkum *et al.* (2007) with some modifications. The antimicrobial resistance break points (Minimum inhibitory concentration: MIC) used were those established by national antimicrobial resistance monitoring system (NARMS) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines: ampicillin, ≥32 µg ml^{-1}; tetracycline, ≥16 µg ml^{-1}; azithromycin, ≥8 µg ml^{-1}; erythromycin, ≥8 µg ml^{-1}; chloramphenicol, ≥32 µg ml^{-1}; gentamicin, ≥16 µg ml^{-1}; nalidixic acid, ≥32 µg ml^{-1}; ciprofloxacin, ≥4 µg ml^{-1}; levofloxacin,≥8 µg ml^{-1}; ofloxacin, ≥8 µg ml^{-1}. Resistance to fosfomycin was considered when the MIC was ≥128 µg/ml **(**Andrews *et al.*, 1983). All susceptibility data were confirmed on at least two separate experiments.

2.7. PCR amplification of the *gyrA* gene

PCR amplification of the *gyrA* gene was performed by PCR primers and conditions as described by Zirnstein *et al.* (1999 & 2000).

2.8. Sequence determination

PCR products of the *gyrA* genes were purified by a QIAquick PCR Purification Kit according to the manufacturer's instruction (QIAGEN GmbH, Hilden, Germany). The purified DNA was subjected to cycle sequencing reaction by using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and primers used for the *gyrA* genes amplification. The reactions were conducted in a GeneAmp 9700 thermal cycler (Applied Biosystems) in accordance with the manufacturer's instruction. Nucleotide sequences were determined by using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Homology searches were performed against all sequences in the GenBank database by using the BLAST search engine, available through the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Nucleotide and amino acid sequence alignments were subsequently generated by MegAlign and ClustalW programs in the lasergene software package (DNASTAR, Madison, WI, USA). DNA Sequences of *C. jejuni* UA580 (GenBank accession number L04566) and *C. coli* RM 2228 (GenBank accession number NZ_AAFL01000007) were used for making comparison with the *gyrA* genes sequences obtained in this study.

3. Results and Discussion

The summary of the species identification of *Campylobacter* strains of poultry origin in India by molecular methods are presented in Tables 2 and 3. Among the 20 *Campylobacter* strains isolated from poultry origin in India, 4 were identified as *C. jejuni* and the rest 16 were identified as *C. coli* with the aid of hipO gene based PCR, 16S rRNA gene sequencing and *cdtA/B/C* gene based multiplex PCR assays. Detection of *C. jejuni* and *C. coli* by *cdtB* gene-based multiplex PCR are shown in Figures 1 and 2. *Cdt* gene-based multiplex PCR assays appeared to be best for the accurate identification of *Campylobacter* strains. Routine detection of *Campylobacter* species in most clinical laboratories is based on culture method using selective media and following phenotypic identification (Maher *et al*., 2003). However, phenotypic identification can be challenging because of the fastidious growth requirements, the asaccharolytic nature and possession of few distinguishing biochemical characteristics of campylobacters (Goossens & Butzler, 1992). The only biochemical test for discriminating between *C. jejuni* and *C. coli* is based on hippurate hydrolysis activity, which is time consuming, cumbersome and sometimes difficult to interpret the result when the enzymatic activity is impaired under the methodological conditions (Totten *et al*., 1987; Rautelin *et al*., 1999). Thus, genetic methods can be a possible alternative. Singh *et al*. (2009) isolated *C. jejuni* and *C. coli* in chicken meat and carcass swabs collected from local poultry farms and retail shops of Bareilly, Uttar Pradesh, India. The results of antimicrobial susceptibility of *Campylobacter* strains of poultry origin in India by disk diffusion method are summarized in Table 4. In case of C. jejuni, 100% isolates were sensitive to ampicillin, azithromycin, erythromycin, chloramphenicol and gentamicin. However, 100% isolates were resistant to tetracycline, nalidixic acid, ciprofloxacin, levofloxacin, ofloxacin and sulphamethoxazole-trimethoprim.

On the other hand, in case of *C. coli*, 100% isolates were sensitive to azithromycin, erythromycin, chloramphenicol, gentamicin and fosfomycin. However, 100% isolates were resistant to nalidixic acid, ciprofloxacin and sulphamethoxazole-trimethoprim. Susceptibility tests for *campylobacter* species are not standardized (there is no recommendation by the CLSI), so there are variabilities in the susceptibility reported (Vandenberg *et al*., 2006). However, we used disk diffusion method for the susceptibility testing of *C. jejuni* and *C. coli* strains used in this study followed by determination of MIC of the intermediately resistant and resistant strains by agar dilution method (Luangtongkum *et al*., 2007). Susceptibility testing of *Campylobacter* species is important to facilitate appropriate treatment where indicated and also for surveillance of emergence of drug resistance. The present findings were closely related with the findings of Jain *et al*., 2005.

The distribution of MICs for *C. jejuni* and *C. coli* isolates from poultry origin in India are presented in Table 5. In case of *C. jejuni* strains (n = 4), 100% isolates were resistant to tetracycline (MIC, 128 µg/ml), nalidixic acid (MIC, 128 µg/ml), ciprofloxacin (MIC, 64 µg/ml), sulfamethoxazole-trimethoprim, levofloxacin (MIC, 16 µg/ml) and ofloxacin (MIC, 32 µg/ml) whereas 25% isolates were resistant to fosfomycin (MIC, 32-64 µg/ml) and all *C. jejuni* strains had the Thr 86 to Ile and Asn 203 to Ser substitution in GyrA (Table 6). On the other hand, in case of *C. coli* strains (n =16), 100% isolates were resistant to nalidixic acid (MIC, 32-128 µg/ml), ciprofloxacin (MIC, 4-128 µg/ml) and sulfamethoxazole-trimethoprim whereas 93.75% isolates were resistant to levofloxacin (MIC, 2-16 µg/ml) and ofloxacin (MIC, 4-32 µg/ml) followed by 62.5% were resistant to ampicillin (MIC, 16-32 µg/ml) and 43.75% were resistant to tetracycline (MIC, 128 µg/ml) and one *C. coli* strains had the Thr 86 to Ile and Met 181 to Arg substitution in GyrA and the rest 15 strains had only Thr 86 to Ile substitution in GyrA (Table 6). For *Campylobacter* strains, high-level resistance to fluoroquinolones is mostly mediated by mutations within the QRDR of the *gyrA* gene, of which replacement of C256 with T leading to a Thr-86-Ile substitution in GyrA is predominant (Ge *et al*., 2003; Vacher *et al*., 2003; Payot *et al*., 2006; Bakeli *et al*., 2008).

Table 1. Primers used for the *cdt* genes based multiplex PCR, *hipO* gene PCR and 16S rRNA gene amplification.

Primer	Sequence (5'-3')	Target	Reference
Cj-CdtAU2	AGGACTTGAACCTACTTTTC	*Cj cdtA*	Asakura *et al*., 2008
Cj-CdtAR2	AGGTGGAGTAGTTAAAAACC		
Cc-CdtAU1	ATTGCCAAGGCTAAAATCTC	*Cc cdtA*	
Cc-CdtAR1	GATAAAGTCTCCAAAACTGC		
Cf-CdtAU1	AACGACAAATGTAAGCACTC	*Cf cdtA*	
Cf-CdtAR1	TATTTATGCAAGTCGTGCGA		
Cj-CdtBU5	ATCTTTTAACCTTGCTTTTGC	*Cj cdtB*	Asakura *et al*., 2008
Cj-CdtBR6	GCAAGCATTAAAATCGCAGC		
Cc-CdtBU5	TTTAATGTATTATTTGCCGC	*Cc cdtB*	
Cc-CdtBR5	TCATTGCCTATGCGTATG		
Cf-CdtBU6	GGCTTTGCAAAAACCAGAAG	*Cf cdtB*	
Cf-CdtBR3	CAAGAGTTCCTCTTAAACTC		
Cj-CdtCU1	TTTAGCCTTTGCAACTCCTA	*Cj cdtC*	Asakura *et al*., 2008
Cj-CdtCR2	AAGGGGTAGCAGCTGTTAA		
Cc-CdtCU1	TAGGGATATGCACGCAAAAG	*Cc cdtC*	
Cc-CdtCR1	GCTTAATACAGTTACGATAG		
Cf-CdtCU2	AAGCATAAGTTTTGCAAACG	*Cf cdtC*	
Cf-CdtCR2	GTTTGGATTTTCAAATGTTCC		
HIP400F	GAAGAGGGTTTGGGTGGTG	hippuricase	Linton *et al*., 1997
HIP1134R	AGCTAGCTTCGCATAATAACTTG	gene	
16S9F	GAGTTTGATCCTGGCTC	16S rRNA	Samosornsuk *et al*., 2007
16S1540R	AAGGAGGTGATCCAGCC	gene	

Cj, *C. jejuni* ; Cc, *C. coli* ; Cf, *C. fetus*

Table 2. The summary of the species identification of *Campylobacter* strains of poultry origin in India.

Screening methods	Total no. of strains tested	No. of PCR positive strains		No. of PCR negative strains
		C. jejuni	*C. coli*	
hipO gene PCR	20	4	-	16[A]
cdt4 gene based M-PCR	20	4	16	-
cdtB gene based M-PCR	20	4	16	-
cdtC gene based M-PCR	20	4	16	-

[A]These 16 strains were subjected to 16S rRNA gene sequencing and sequence data were trimmed and adjusted to 1,335 bp to perform homology searches and all were identified as C. jejuni / C. coli.

Table 3. 16S rRNA gene sequencing for the identification of *Campylobacter* strains of poultry origin in India.

Sample No.	Amplicon size (bp)	Query length (bp)	16S rRNA gene sequence
1-1a	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
1-2a	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
1-3b	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
1-4a	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
7-1a	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
7-2b	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
8-1a	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
8-2d	1,530	1,335	*C. jejuni* [3] / *C. coli* [4]
9-1b	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
9-2a	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
9-3c	1,530	1,335	*C. jejuni* [1] / *C. coli* [2]
9-4a	1,530	1,335	*C. jejuni* [3] / *C. coli* [4]
10-1d	1,530	1,335	*C. jejuni* [3] / *C. coli* [4]
10-2d	1,530	1,335	*C. jejuni* [5] / *C. coli* [6]
10-3a	1,530	1,335	*C. jejuni* [3] / *C. coli* [4]
10-4c	1,530	1,335	*C. jejuni* [3] / *C. coli* [4]

[1]Accession No. GQ167679, *C. jejuni* strain INN-73-83 094400, Identities = 1335/1335 (100%)
[2]Accession No. GQ167673, *C. coli* strain X10, Identities = 1335/1335 (100%)
[3]Accession No. GQ479817, *C. jejuni* strain SWUN0717, Identities = 1332/1335 (99%)
[4]Accession No. GQ167673, *C. coli* strain X10, Identities = 1332/1335 (99%)
[5]Accession No. GQ479819, *C. jejuni* strain SWUN1202, Identities = 1334/1335 (99%)
[6]Accession No. GQ167673, *C. coli* strain X10, Identities = 1334/1335 (99%)

Table 4. Antimicrobial susceptibility of *Campylobacter* strains of poultry origin in India.

Campylobacter	No. (%)											
spp.	ABPC	TC	AZM	EM	CP	GM	NA	CPFX	LVFX	OFLX	FOM	ST
C. jejuni (n=4)												
S	4 (100)	-	4 (100)	4 (100)	4 (100)	4 (100)	-	-	-	-	-	-
I	-	-	-	-	-	-	-	-	-	-	3 (75)	-
R	-	4 (100)	-	-	-	-	4 (100)	4 (100)	4 (100)	4 (100)	1 (25)	4 (100)
C. coli (n=16)												
S	6 (37.5)	9 (56.25)	16 (100)	16 (100)	16 (100)	16 (100)	-	-	1 (6.25)	1 (6.25)	16 (100)	-
I	-	-	-	-	-	-	-	-	-	-	-	-
R	10 (62.5)	7 (43.75)	-	-	-	-	16 (100)	16 (100)	15 (93.75)	15 (93.75)	-	16 (100)

ABPC: ampicillin; TC: tetracycline; AZM: azithromycin; EM: erythromycin; CP: chloramphenicol; GM: gentamicin; NA: nalidixic acid; CPFX: ciprofloxacin; LVFX: levofloxacin; OFLX: ofloxacin; FOM: fosfomycin; ST: sulphamethoxazole-trimethoprim; S: Susceptible; I: Intermediate; R: Resistant

Table 5. Distribution of MICs for *C. jejuni* and *C. coli* isolates from poultry origin in India.

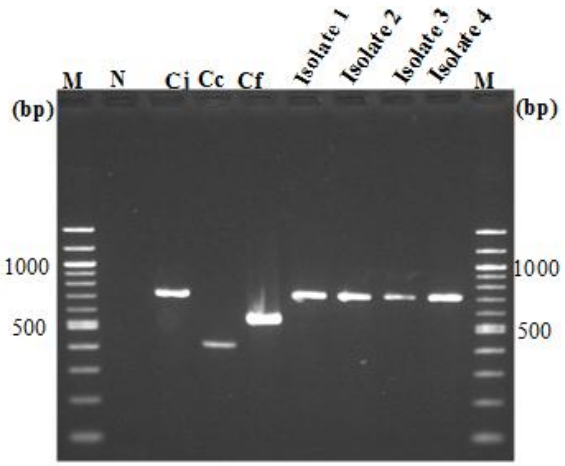
Antimicrobial agents	Species	Number of isolates with MIC (µg/ml) of												Number of resistant isolates
		0.25	0.5	1	2	4	8	16	32	64	128	256	>256	
Ampicillin	*C. jejuni*													-
	C. coli							4	6					6
Tetracycline	*C. jejuni*										4			4
	C. coli										7			7
Nalidixic acid	*C. jejuni*										4			4
	C. coli								1		15			16
Ciprofloxacin	*C. jejuni*									4				4
	C. coli					1	1		5	5	4			16
Levofloxacin	*C. jejuni*							4						4
	C. coli				1		7	7						14
Ofloxacin	*C. jejuni*								4					4
	C. coli					1		7	7					14
Fosfomycin	*C. jejuni*								3	1				00
	C. coli													-

Vertical lines (|) indicate breakpoints for resistance.

Highlighted regions are the dilution ranges used in the agar dilution.

Table 6. Point mutations observed in *gyrA* sequences of *C. Jejuni* and *C. coli* strains of poultry origin in India.

Species	*gyrA* mutation (s)			MIC of ciprofloxacin (µg/ml)	No. of strains
	Thr86Ile	Met181Arg	Asn203Ser		
C. jejuni	+	-	+	64	4
C. coli	+	+	-	128	1
	+	-	-	4-128	15

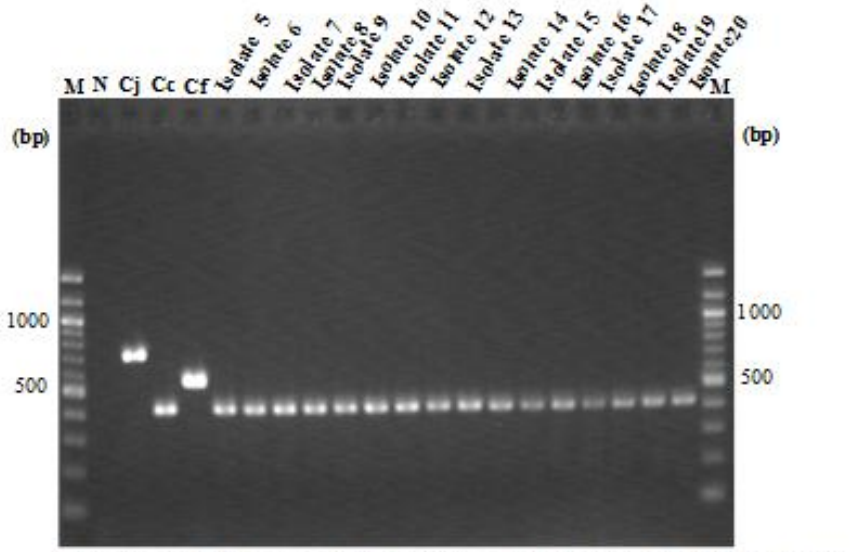


2 % SeaKem ME Agarose Gel (Cambrex Bio Science Rockland, Inc. Rockland, ME USA)

M: 100 bp ladder (New England Biolabs Inc., Beverly, MA, USA)

N: Negative control (*E. coli* C600)

Figure 1. Detection of *Campylobacter jejuni* by *cdtB* gene-based multiplex PCR.



2 % SeaKem ME Agarose Gel (Cambrex Bio Science Rockland, Inc. Rockland, ME USA)

M: 100 bp ladder (New England Biolabs Inc., Beverly, MA, USA)

N: Negative control (*E. coli* C600)

Figure 2. Detection of *Campylobacter coli* by *cdtB* gene-based multiplex PCR.

4. Conclusions

Results of this study support the emergence of resistance of *C. jejuni and C. coli* strains of poultry origin in India to a variety of antimicrobials especially fluoroquinolones. Erythromycin should still be the drug of choice in treatment since all C. *jejuni/coli* strains were sensitive to the drug in this study.

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Conflict of interest

None to declare.

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