Original Research

Antibiotic Resistance Profiling of *Campylobacter jejuni* Isolated from Poultry in India

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Abstract

The present study was conducted for detection of antibiotic resistance in Campylobacter jejuni isolates of poultry origin in India. Highest (100%) antibiotic sensitivity was observed for chloramphenicol and aminoglycosides (gentamicin, amikacin) whereas high antibiotic resistance was observed against β-lactam antibiotics and fluoroquinolone antibiotics (ciprofloxacin, ofloxacin, nalidixic acid and norfloxacin) in in vitro antibiotic susceptibility testing and MIC determination as well. The gyrB gene which codes for proteins responsible for fluoroquinolone resistance was detected in 100% of the isolates followed by gyrA gene (95.34%). The gyrA gene-based phylogeny represented close homology of our C. jejuni isolates with isolates from Europe and didn't represent homology with isolates from India in public domain.

Key words: Antibiotic Resistance, *Campylobacter jejuni*, Poultry

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Introduction

Campylobacter jejuni is considered as most emerging food borne zoonotic pathogen and had received serious attention due to high floroquinolones and macrolide resistance in many parts of the world (Epps *et al.*, 2013). Poultry are the major reservoir of this bacterium and responsible for transmission of infection through poultry products. Supplementation of antibiotics in poultry feed have evolved bacterial strains to be multidrug resistant (Mani *et al.*, 2018). *Campylobacter* species are intrinsically resistant to a number of antibiotics, including cefoperazone, cephalothin, bacitracin, vancomycin, rifampin and trimethoprim (Acheson and Allos, 2001), some of these are utilized in selective media for isolation. Resistance may be chromosomal or plasmid-borne, and represent a combination of endogenous and acquired genes *viz.*

modification of the antibiotic's target and/or its expression (DNA gyrase mutations), inability of the antibiotic to reach its target (expression of the major outer membrane protein or MOMP), efflux of the antibiotic (multidrug efflux pumps such as cmeABC), modification or inactivation of the antibiotic (β-lactamase production) (Iovine, 2013).

In last few decades, intensive rearing of poultry along with indiscriminate use of antibiotics such as fluoroquinolone as feed additives, growth enhancer and therapeutics has resulted in emergence of multiple antibiotic resistant *Campylobacter* strains in habitat (Wieczorekand Osek, 2013). The resistant bacteria can be transmitted through contaminated poultry meat and eggs into humans (El-baky *et al.,* 2014). World health organization has given advisory for limiting the use of floroquinolones in poultry sector as therapeutic and feed supplement in most of the western countries a decade ago. US Food and Drug Administration also have withdrawn use of fluoroquinolone from poultry since 2005 (Nelson *et al.,* 2007). Monitoring drug resistance pattern among the *Campylobacter* isolates not only gives vital clues to the clinician regarding the judicious therapeutic regime to be adopted against individual cases, but also an important tool to devise a comprehensive chemoprophylactic and chemotherapeutic drug schedule within a geographical area among human and animal origin isolates (Siddiqui *et al.,* 2015).

Antibiotic resistance patterns may vary on source of infection and geographical area. There is only few reports published from Indian origin isolates regarding antibiotic resistance of *C. jejuni* isolates in public domain (Chatur *et al.,* 2014). Therefore, present study was carried out for detection of antibiotic resistance profile of *C. jejuni* isolates from poultry origin in India.

Material and Methods

Determination of *In vitro* Antibiotic Susceptibility Test with MIC

A number of 43 *C. jejuni* were isolated from local poultry farms in and around Bikaner, Rajasthan, India (Yadav *et al.,* 2016). Antibiotic sensitivity testing was done against 24 antibiotics belonging to different class, generation and mechanism of action as per the method described by Bauer *et al.* (1966) following the guidelines of Clinical laboratory standard institute (Wayne, 2010; 2011) and European committee on antimicrobial susceptibility (Kahlmeter, 2006). Due to poor visibility of zone of inhibition on M-H plates; for the present study 0.5 McFarland concentrations of *Campylobacter* enrichment *broth culture* (*Preston* enrichment *broth* base, Himedia) supplemented with *Campylobacter* supplement IV (Himedia) and 7% lysed horse blood was then swabbed on modified charcoal cefoperazone deoxycholate agar (mCCDA) plate including *Campylobacter* supplement V (Himedia) with the help of sterile cotton swab. On this medium the visibility of zones of inhibition was clearer. Variations in standard disk diffusion method have been approved by Wayne (2010) for *Campylobacter* isolates (Beek *et al.,* 2010). MIC determination was done by Ezy MIC™ strips of six antibiotics (amikacin, erythromycin, chloramphenicol, gentamicin,

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ciprofloxacin and ofloxacin) with similar procedure as described above. Zone of inhibition for an antibiotic were interpreted as per the standards of *Enterobacteriaceae* (Shin *et al.*, 2015) defined by clinical laboratory standards institute (Wayne, 2011). All Multidrug resistant isolates were evaluated for their multiple antibiotics resistance (MAR) index (Krumperman, 1983).

Amplification of Virulence Associated Genes

Molecular detection of various antibiotic resistance genes *i.e.* tetracycline, aminoglycosides, multidrug resistance efflux and integrons were done using earlier reported primer sets and/or primer set designed for the present study (Table 1). The primers were designed by primer 3 tool of NCBI for *cme*RABC (multidrug efflux pump) and *aph*3 (aminoglycosides resistance) gene in this study. All PCR amplifications were performed in a mixture (25 µl) containing: 2.5µl of the 10X PCR buffer, 2.5µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs (10 mM), 1 µl of each primer (100 µM), 0.5 µl (1U) of the *Taq* DNA polymerase (Promega), 3 µl of the bacterial template DNA and 14 µl nuclease free water. The PCR products were analyzed by electrophoresis on 1.5% agarose gel for 1 h at 100V. The gel was then visualized under UVP gel documentation system (BioDoc-It Imaging System).

Sequence Analysis of *gyr*A Gene

PCR products of partial *gyr*A gene partial cds from nine representative of *C. jejuni* isolates were sequenced (DNA Sequencing Facility, Delhi University). The sequences obtained were subjected to NCBI nucleotide Basic Local Alignment Search tool (BLAST) to determine the similarity with the already prevalent gene sequences. The nucleotide sequences (accession number KY084915 to KY084923) were submitted to NCBI genebank. The sequences were also aligned using Bio edit and MEGA6 software to study the variations in the nucleotide sequences and their phylogenetic cluster analysis (Bikandi *et al.*, 2004).

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Table 1: PCR primers and conditions for detection of antibiotic resistance genes

S. no	Antibiotic resistance gene	Gene name	Primer sequence	A. temp (°C)	Size (bp)	Reference
Antibiotic Resistance genes						
1	**Tetracycline resistance genes**	*tet*O	F-AACTTAGGCATTCTGGCTCAC	56	515	Abdi-Hachesoo*et al.* (2014)
			R-TCCCACTGTTCCATATCGTCA			
2		*tetA*	F-GTAATTCTGAGCACTGTCGC	57	956	Wilkerson *et al.* (2004)
			R-CTGCCTGGACAACATTGCTT			
3		*tetB*	F-CTCAGTATTCCAAGCCTTTG	52	414	
			R-ACTCCCCTGAGCTTGAGGGG			
4		*tetC*	F-GGTTGAAGGCTCTCAAGGGC	62	505	
			R-CCTCTTGCGGGATATCGTCC			
5		*tetD*	F-CATCCATCCGGAAGTGATAGC	57	485	
			R-GGATATCTCACCGCATCTGC			
6		*tetE*	F-TGATGATGGCACTGGTCA	57	262	
			R-GCTGGCTGTTGCCATTA			
7		*tetG*	F-GCAGCGAAAGCGTATTTGCG	62	662	
			R-TCCGAAAGCTGTCCAAGCAT			
8	**Aminoglycoside resistance genes**	*aph*3	F-TTCTAGCCACGACCAAAAAG	56	363	Current study
			R-CGTGAGCCATAAAGTCTAGC			
9		*strA*	F-CCAATCGCAGATAGAAGGC	55	286	Scholz *et al.* (1989)
			R-CTTGGTGATAACGGCAATTC			
10		*aadA2*	F-ATTTGCTGGTTACGGTGACC	59	451	
			R-CTTCAAGTATGACGGGCTGA			
11	**Fluoroquinolone resistance genes**	*gyr*A	F -GAAGAATTTTATATGCTATG	50	235	Chatur *et al.* (2014)
			R-TCAGTATAACGCATCGCAGC			
12		*gyr*B	F-ATGGCAGCTAGAGGAAGAGA	53	382	
			R-GTGATCCATCAACATCCGCA			
13		*parC*	F-CTATGCGATGTCAGAGCTGG	59	285	
			R-TAACAGCAGCTCGGCGTATT			
Multidrug resistance determinants genes						
14	**Efflux pump (*cme*ABC operon) genes**	*cme*RABC, Strain	F-CAATCTTCAATCAGGGGCAA	56	625	Current study
			R-TCGCAAAAAGAGTGCACATA			
15	**Integron genes**	*int*1F	F-CCTCCCGCACGATGATC	55	280	Moura *et al.* (2007)
		*int*1R`	R-TCCACGCATCGTCAGGC			
16		*int*2F	F-TTATTGCTGGGATTAGGC	50	233	
		*int*2R	R-ACGGCTACCCTCTGTTATC			
17		*int*3F	F-AGTGGGTGGCGAATGAGTG	50	600	
		*int*3R	R-TGTTCTTGTATCGGCAGGTG			

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Results and Discussion

Determination of *In vitro* Antibiotic Susceptibility Test with MIC

All the 43 isolates were subjected to antibiotic sensitivity testing against 24 antibiotics of different classes and generation. Highest (100%) sensitivity was observed for polymxin-B followed by chloramphenicol (97.67%), gentamicin (95.35%), amikacin (88.37%), aztreonam (83.72%), meropenem and imepenem (76.74%), kanamycin (72.09%), ceftriaxone (65.12%), erythromycin and ampicillin (53.49%). Isolates were 100% resistant to Penicillin-G, methicillin and rifampcin. Relatively lower level of resistance was detected against cephalothin (95.35%), vancomycin (93.02%), ciprofloxacin (90.70%), ofloxacin (79.07%), nalidixic acid (74.42%) and norfloxacin (72.09%). High resistance was observed against β-lactam antibiotics. Similarly, a very high resistance (100%) against fluoroquinolone group of antibiotics (ciprofloxacin, ofloxacin, nalidixic acid and norfloxacin) was also seen.

In the present investigation, all 43 isolates were subjected to MIC determination for six antibiotics *i.e.* erythromycin (2 to 12 mcg/ml), gentamicin (0.38 to 6 mcg/ml), chloramphenicol (3 to 16 mcg/ml), amikacin (0.5 to 32 mcg/ml) by Ezy MIC™ Strip method. Erythromycin has highest average MIC value of 5.74 mcg/ml followed by chloramphenicol (4.80 mcg/ml), amikacin (0.86 mcg/ml) and gentamicin (0.23 mcg/ml). None of the isolate formed any zone of inhibition for ciprofloxacin and ofloxacin, considered them to be 100% resistant. On the basis of their average MIC value, isolates were detected as sensitive for amikacin and gentamicin, intermediate for erythromycin and chloramphenicol and resistant for ciprofloxacin and ofloxacin.

Resistance against multiple antibiotics was observed by multiple antibiotic resistances (MAR) index which is an epidemiological tool used to assess the risk analysis of environment for bacterial contamination and acquisition of drug resistance through use of multiple antibiotics. If MAR index is greater than 0.2; it implies that strains of such bacteria originated from an environment where several antibiotics have been used (Krumperman, 1983). The average MAR index of the 43 isolates under study was 0.45 demonstrates high prevalence of multiple antibiotic resistant *C. jejuni* isolates from India (Table 2).

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Table 2: Detection of multiple antibiotic resistance (MAR) index value among isolates

MAR Index Value Type (MAR)	Isolate I.D.	No. of Isolate	No. of antibiotic, which the isolate was resistant	Total no of antibiotics	MAR Index Value	Significance
MAR1	C1, C18, C29, C30	4	7	24	0.29	43 (100%) isolates had 0.2 or more than 0.2 MAR index value with high risk potential source of spread MDR
MAR 2	C25, C28	2	8	24	0.33	
MAR 3	C7, C12, C14, C16, C22, C23, C26, C31, C42	9	9	24	0.38	
MAR 4	C24, C27, C36, C39, C40, C41	6	10	24	0.42	
MAR 5	C4, C5, C21, C37, C43	5	11	24	0.46	
MAR 6	C2, C3, C8, C13, C17, C20, C32, C34	8	12	24	0.5	
MAR 7	C6, C9, C11, C19, C38	5	13	24	0.54	
MAR 8	C35	1	14	24	0.58	
MAR 9	C10, C15, C33	3	15	24	0.63	
TOTAL		**43**			**19.17**	
Average MAR Value					**0.45**	

In agreement to our observation, Ghimire *et al.* (2014) detected 77.8% of the isolates with MAR index value >0.2. Multiple mechanisms associated with antibiotic resistance have been identified in *Campylobacter*, but target mutations and drug efflux are most relevant to the resistance to fluoroquinolones and macrolides (Luangtongkum *et al.*, 2009).

Amplification of Virulence Associated Genes

PCR assay successfully amplify only seven genes (*tet*O, *aph*3, *gyr*A, *gyr*B, *cme*RABC, *int*1 and *int*2) out of 17 genes (Fig. 1). The *gyr*B gene which codes for proteins responsible for fluoroquinolone resistance was detected in 100% of the isolates followed by *gyr*A gene (95.34%), *tet*O and *aph*3 genes (74.41%) and *cme*RABC (72.09%) isolates. Out of three integron genes only *int*1 (30.23%) and *int*2 (6.97%) were amplified.

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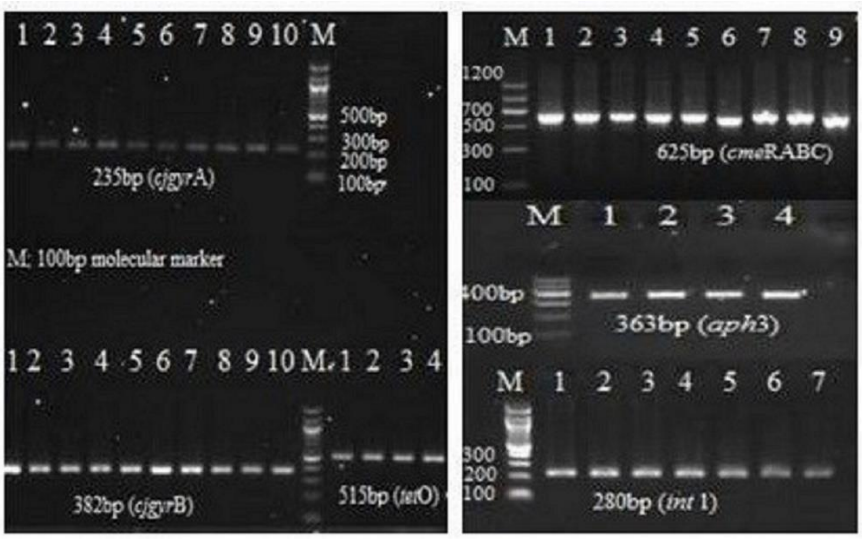

Fig. 1: Agarose gel electrophoresis image of various antibiotic resistance genes of *C. jejuni*

Campylobacter multidrug efflux pump (*cme*ABC) genes are major player in theefflux of bile acids and plays a critical role in facilitating *Campylobacter* colonization of the intestinal tract (Lin *et al.*, 2003; Elhadidy *et al.*, 2018). The isolates from present study harbored the *cmeABC* genes responsible for multidrug resistance. Integrons are not common in *Campylobacter* and do not considered to play a major role in the horizontal transfer of antibiotic resistance in *Campylobacter*. However, studies by Lee *et al.* (2002); O'Halloran *et al.* (2004) suggested the integrons-associated antibiotic resistance (aminoglycoside resistance genes (*aad*A2 and *aac*A4), in *C. jejuni* and *C. coli*. Usually resistance towards tetracycline antibiotic occurs due to expression of various *tet* genes types *i.e tet*A, *tet*B, *tet*C, *tet*E, *tet*g, *tet*O found in plasmid as well as chromosome of various Gram positive and Gram-negative organism. However, only *tet*O was reported as highly prevalent in *Campylobacter* species (Dasti *et al.*, 2007). We also didn't detect any of the tetracycline genes except *tet*O in present study.

Sequence Analysis of *gyr*A Gene

In addition, we also performed *gyr*A gene sequence based phylogenetic analysis. In addition to nine isolates from the current study, we selected 27 sequences (from across the world) of *gyr*A gene from the public domain. The phylogenetic analysis revealed three major clusters (Fig. 2). Five isolates from the present study grouped along with poultry and human isolates from Europe (Austria, Slovenia, Germany, Serbia, Bosnia and Herzegovina), New Zealand and Japan. Rest four isolates (C22, C25, C31, and C32) were grouped under separate cluster (cluster II) that has majority of isolates form USA. The previously reported *gyr*A gene sequences from India were grouped in separate cluster (Cluster III) and didn't represent close homology with the isolates from this study. Taken together *gyr*A gene-based phylogeny represented close homology of our *C. jejuni* isolates with isolates from Europe.

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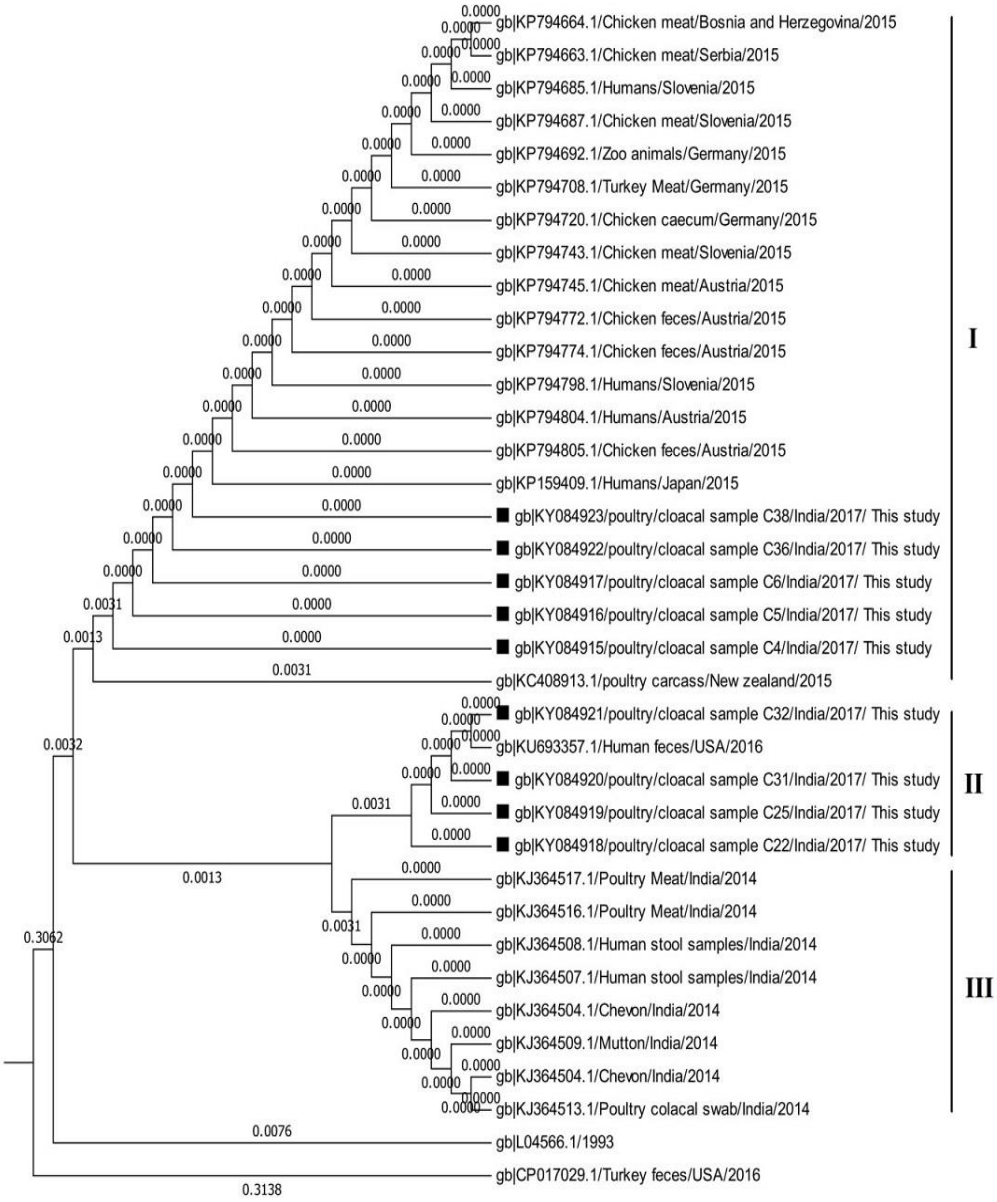

Fig. 2: Phylogenetic analysis of *gyr*A gene sequences

In this study, multidrug resistance was observed in more than 70% of the isolates and absolute resistance was determined for fluoroquinolones antibiotics by in vitro susceptibility testing, MIC determination, and detection of fluoroquinolones resistance genes i.e. *gyr*A and *gyr*B. While most microorganisms possess 2 fluoroquinolone-targets (DNA-Gyrase and Topoisomerase IV), *Campylobacter* spp. only possesses one of the DNA-Gyrases (Luangtongkum *et al.*, 2009). Resistance to fluoroquinolone is believed to develop more rapidly in *Campylobacter* spp, than in other Gram-negative bacteria, mainly attributed to single-step point mutation in Thr-86-Ile of *gyr*A gene (Luangtongkum *et al.*, 2009; Otigbu *et al.*, 2018). *gyr*A gene-based phylogenetic analysis of present study isolates showed close homology with isolates from Europe, rather

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than isolates from India. High fluoroquinoles resistance has been reported by Chatur *et al.* (2014) in India previously. The reason for the high prevalence of fluoroquinolone resistance in India is unknown, but it might be driven by the use of fluoroquinolone in animal and poultry production (Collado *et al.*, 2018). In this increasing trend of fluoroquinolone resistance in our and previous studies indicate the need of interventions to limit spread of resistant isolates.

Conclusion

Conclusively, *C. jejuni* isolates from present study are detected as multidrug resistant strains. High MAR values and MDR status of all the isolates is an indication of excessive use of antibiotics and is a point of concern. The antibiotic resistance patterns indicate that *C. jejuni* isolates have evolved themselves for resistance to fluoroquinolone group of antibiotics. The absolute fluoroquinolone resistance may acquire by cumulative effect of *gyr*A gene point mutation, efflux pump regulator *Cme*RABC gene and integrons. Such mutant strains are more stable as compared to fluoroquinolone sensitive strains and didn't carry fitness burden.

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Conflict of Interest

Authors don't have any conflict of interest.

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