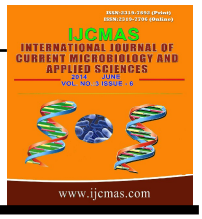
Int.J.Curr.Microbiol.App.Sci (2014) 3(6) 773-783

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 6 (2014) pp. 773-783**
http://www.ijcmas.com



Original Research Article

Fluoroquinolone resistance and detection of topoisomerase gene mutation in *Campylobacter jejuni* isolated from animal and human sources

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Keywords

Fluoro-quinolone, MIC, topoisomerase gene, direct DNA sequencing

A B S T R A C T

Antibiotic resistance has been the worldwide concern in Campylobacter, a leading bacterial diarrheal agent. Though it causes self limiting bacterial gastroenteritis, antimicrobial interventions are required in case of immune compromised, pregnant or elderly patients. Illegitimate use of antimicrobials in treatment and feed has been held responsible for increasing antimicrobial resistance. This study was undertaken to detect level of fluoroquinolone resistance in *Campylobacter jejuni* isolates in study area. A total of 28 *C. jejuni* isolates obtained from human and animal sources were subjected for determination of MICs of Ciprofloxacin and Nalidixic acid by microbroth dilution method. Direct sequencing of PCR products of topoisomerase genes (*gyr*A and *gyr*B) was performed to detect the mutation in quinolone resistance determining region (QRDR). All Campylobacter isolates were resistance to Ciprofloxacin and Nalidixic acid with MICs ranging from 4 to 16mg/L and 16 to ≥128mg/L, respectively. All the isolates were harbouring the missense mutation in QRDR of *gyr*A (Thr (86)→ Ile) while sequence analysis of *gyr*B gene revealed nonsense mutations. The evidence of 100% resistance in *C. jejuni* isolates indicates the wide spread of antibiotic resistant Campylobacters directing the need of extensive epidemiological surveillance and proper intervention for limiting their spread of antibiotic resistant Campylobacter isolates.

Introduction

Campylobacter is the leading human bacterial diarrheal pathogen in both developed and developing countries. Domestic animals, pets, wild birds, rodents and poultry act as a reservoir. In developing countries, most of the infection is food borne primarily due consumption of unpasteurized milk, contaminated water and meat especially poultry meat, rather than human to human transfer (Humphrey et al., 2007). Travel in developing countries has been identified as the source of Campylobacter infection transfer of antimicrobial resistant campylobacter has been reported by different workers (Allos, 2001; Hakanen et al., 2003; Kassenborg et al., 2004; Nelson et al., 2007; Feodoroff et al., 2011; Vlieghe et al., 2008). It causes an acute self-limited

disease characterized by diarrhea, fever and abdominal cramps (Allos, 2001). Although, Campylobacter is rarely identified in stools of healthy individuals, depending on the population studied, as many as 50% of persons infected during outbreaks are asymptomatic (Calva et al.,1988). Extraintestinal manifestation are rare and may include meningitis, endocarditis, septic arthritis, osteomyelitis, and neonatal sepsis (Skirrow et al., 1993; On, 2001; Humphrey et al., 2007). The most important postinfectious complication of *C. jejuni* is Guillian Barre Syndrome (GBS) and Miller Fischer Syndrome which is an acute demylenating disease of peripheral nervous system (Yuki, 2001).

Generally, the diarrhea caused is self limiting and no specific treatment is required (Allos, 2001; Engberg, 2006). But, the treatment is advised in old, young, pregnant and immunocompromised patients. Macrolides and fluoroquinolone has been the choice for antimicrobial interventions (Altekruse et al., 1999; Engberg et al., 2001). The increasing prevalence of antimicrobial resistant campylobacter is a concern worldwide and has been linked to the illegitimate use of antimicrobials in food animals, animal feeds and flock treatment of animals rather than individual approach (Endtz et al., 1991; Gaunf and Piddock, 1996; Kassenborg et al., 2004; Phillips et al., 2004; Nelson *et al.*, 2007; Vlieghe et al., 2008; Marshall and Levy, 2011). Flock treatment may lead to the transfer of resistant isolates to healthy animals or development of resistance in it.

High-level fluoroquinolone resistances in Campylobacter have been associated with a single point mutation in quinolone resistance-determining region (QRDR) of *gyr*A gene which is homologous to Ser-83-Leu in *E. coli*. Specific mutations at positions Thr-86, Asp-90 and Ala-70 in *Gyr*A have been associated with fluroquinolone resistance in *C. jejuni* but, Thr-86-Ile mutation in *gyr*A confers a high-level resistance to fluoroquinolone (Ge et al., 2005).

This study had been undertaken with objectives to detect minimum inhibitory concentrations (MIC) of Nalidixic acid and Ciprofloxacin for *Campylobacter jejuni* isolates and correlate with the mutation in quinolone resistance-determining region (QRDR) of topoisomerase (*gyr*A and *gyr*B) genes.

Materials and Methods

Source of Campylobacter isolates

A total of 28 *Campylobacter jejuni* isolates recovered from different sources in department of Veterinary Public Health and Epidemiology, Veterinary Science College, AAU, Anand, Gujarat, India were used in the study (Table 1). The approval for collection of diarrheal stool samples was taken from Human Resources Ethics Committee constituted in Shri Krishna Medical College, Karamsad, Anand, Gujarat, India. The isolates were confirmed by 16S rRNA sequencing in the prior study. The standard strain (ATCC 33560) was used for standardization of dilution and protocol for determination of minimum inhibitory concentration. Isolates were stored in Brucella broth supplemented with Campylobacter growth supplement and 15 percent glycerol at -70^{o}C.

Determination of minimum inhibitory concentration

The isolates were thawed at room temperature and subcultured in Mueller-Hinton Broth No. 2 Cation Controlled supplemented with Campylobacter growth supplement (HiMedia, Mumbai, India) with

incubation at 37^{o}C for 24 hours in microaerophilic environment. The inoculums were adjusted to 0.5 McFarland units before inoculating. MICs of Nalidixic acid and Ciprofloxacin were determined by microbroth dilution method as per Clinical and Laboratory Standards Institute (CLSI) guidelines (2005). The antibiotic powder (HiMedia, Mumbai, India) was dissolved and diluted as per antibiotic requirement; and working range of antibiotic was adjusted to 128-0.25mg/L and 64-0.125mg/L for Nalidixic acid and Ciprofloxacin, respectively as described by Andrews (2001). Nalidixic acid was dissolved in distilled water with drop wise addition of 1N NaOH solution till it get dissolve whereas ciprofloxacin was dissolved in distilled water.

Each 96 well cell culture plate was prepared with said dilutions of one antibiotic only in Mueller-Hinton broth No. 2. 100µl Mueller-Hinton broth No. 2 with 2.5% lysed sheep blood was added in each well of plates followed by addition of 256mg/L and 128mg/L working dilution of Nalidixic acid and Ciprofloxacin in first column of separate plates to make concentration of 128mg/L and 64mg/L, respectively. The solutions were mixed six times with multichannel pipette and transferred upto 10th column. 25µl sample isolates (set at 0.5 McFarland unit) were inoculated in each column till 11th column and plates were incubated at 37^{o}C for 48 hours in microaerophilic environment (85% N_2, 10% CO_2 and 5% O_2) build in CO_2 incubator (NUAIRE, Polymouth, MN, USA). 11th and 12th column was used as control to assure for suitability for culture and aseptic condition during working, respectively. Culture of ATCC 33560 was evaluated in each plate to check quality.

It was used ≥4mg/L as resistance to Ciprofloxacin as recommended by CLSI (2005). Though, cut off value of Nalidixic acid for *C. jejuni* was not given in the European Committee on Antimicrobial Susceptibility Testing EUCAST (2014) and CLSI (2005) document, the value >16mg/L as resistance was implemented as given by External Quality Assurance System (EQAS, 2013).

Detection of mutation in topoisomerase gene

Topoisomearse genes (*gyr*A and *gyr*B) was amplified and sequenced with primers described by (Piddock et al., 2003) with certain modifications. Primers were synthesized from Eurofins Genomics India Pvt. Ltd., Banglore, India. A 270 bp (codons 38–126) PCR product of *gyr*A gene was amplified using cjgyrA_F (5'-ACGCAAGAGAGATGGTT-3') and cjgyrA_R (5'-TCAGTATAACGC ATCGC AGC-3') whereas 235 bp (codons 50–126) PCR product was amplified using clgyrA_F (5'-GAAGAATTTTATATGCTATG-3') and cjgyrA_R (5'-TCAGTATAAC GCATCGC AGC-3'). A 382 bp fragment of the QRDR of *gyr*B (codons 387 – 499) was amplified and sequenced using primers forward (5'-ATGGCAGCTAGAGGAAGAGA-3') and reverse (5'-GTGATCCATCA ACATCC GCA-3'). The 50µl PCR reaction was performed in the ABI 2720 Thermal cycler with pre-heated lid (Lid temp. 103^{o}C). The reaction mixture was optimized to contain 25 µl 2 X PCR master mix (MBI Fermentas) (containing 0.05 unit/ µl Taq DNA Polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP), 10 pmole of each forward and reverse primer (10pmole/µl), 18.0 µl nuclease free distilled water and 5 µl of DNA template for both the genes. The PCR with cjgyr_F + cjgyr_R, clgyr_F + cjgyr_R (for *gyr*A gene) and *gyr*B primers was performed at annealing temperature of 45^{o}C, 53^{o}C and 53^{o}C for 50 sec,

respectively. Rest of reaction was set as, Initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 50 sec, extension at 30 sec and final extension at 72°C for 7 min.

Sequencing of amplification products

The direct sequencing of the PCR-generated product was performed. Both strands of the purified PCR products were submitted to the cycle sequencing reaction using respective amplification product by Sanger's dideoxy method using Applied Biosystem Big Dye® terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif.) as per manufacturer's recommendations and cycle sequencing products were resolved in ABI 3500 Genetic Analyzer platform.

Analysis of raw sequences

The forward and reverse raw sequences were aligned by using SeqScape Software v2.5 against the reference sequences. Complete coding sequences of DNA gyrase subunit A (*gyr*A) gene (Accession no. KC408913) and DNA gyrase subunit B (*gyr*B) gene (Accession no. KC408908) of *Campylobacter jejuni* subsp. *jejuni* strain P694a was used as the reference sequences for analysis and detection of mutation in our isolates. The *gyr*A gene sequence of this strain does not have mutation at 86 amino acid position. The aligned sequences were submitted in NCBI nucleotide database using Sequin v12.91 program.

Results and Discussion

Determination of minimum inhibitory concentration

All the 28 *Campylobacter jejuni* isolates (Table 1) isolated from meat, poultry cloacal swab, animal and human diarrheal samples were found resistant to Ciprofloxacin and Nalidixic acid. The MIC values for the Ciprofloxacin and Nalidixic acid of the isolates ranged from 4 to 16mg/L and 16 to ≥128 mg/L, respectively (Table 2).

Detection of mutation in topoisomerase genes

The 270 or 235bp PCR product of *gyr*A gene was amplified (Fig. 1A) and sequenced by direct sequencing. All *Campylobacter jejuni* isolates shown the missense mutation at nucleotide position 257 (c→t) which changed codon 86 from Threonine to Isoleucine. Along with missense mutation at nucleiotide position 257 (c→t), nonsense mutations were also recorded at nucleotide position 243 (t→c), 357 (c→t) and 361 (t→c). All *Campylobacter jejuni* isolates amplified 382bp PCR product of DNA *gyrase* subunit B gene (Fig. 1B) and sequence analysis of amplified products revealed the nonsense mutation at different locations except two isolates. Two *Campylobacter jejuni* isolate (HDJ3 and HDJ6) does not revealed any nucleotide change in *gyr*B gene sequence and on the contrary, MIC value was observed as 16 and 64 mg/L for Ciprofloxacin and Nalidixic acid, respectively (Table 2). The sites of nucleotide mutation in *gyr*B gene were as follows: 1143 (g→a), 1152 (t→a), 1167 (c→t), 1179 (t→c), 1182 (a→g), 1185 (c→t), 1209 (g→a), 1233 (a→g), 1239 (a→g), 1308 (c→t), 1428 (a→g) and 1449 (t→a). These mutations have been described by different authors suggesting normal polymorphism in the DNA *gyrase* gene which has not resulted in any amino acid sequence change.

All Campylobacter isolates were resistant to Nalidixic acid and Ciprofloxacin. Interestingly, all *C. jejuni* isolates in this study shown high MIC values ranging from 4 to 16mg/L and 16 to ≥128mg/L for Ciprofloxacin and Nalidixic acid, respectively. The previous work in the same department (Tayde, 2010) reported 41.86% (18/43) poultry *C. jejuni* isolates resistant to

Ciprofloxacin by disc diffusion method. The current status of 100% fluoroquinolone and quinolone resistance gives the alarming sign of spread of antimicrobial resistant Campylobacter isolates.

In India, fluoroquinolone resistant *Campylobacter* spp. have been isolated by different authors but their prevalence differed significantly like 2.7% in diarrheal patients and healthy chickens in northern India (Prasad et al., 1994), 71.4% in a north Indian rural community (Jain et al., 2005), 10.34% resistance and 62.1% intermediate resistance in animal and human isolates, respectively (Rajagunalan et al., 2012); and 97% in patients hospitalized with diarrhea at the Infectious Disease Hospital in Kolkata, India (Mukherjee et al., 2013). This increasing trend of fluoroquinolone resistance and 100% resistance *Campylobacter jejuni* isolates in our study indicate the need of interventions to limit spread of resistant isolates. Similar extremely high resistance to Ciprofloxacin in *Campylobacter jejuni* isolates was reported by Saenz et al (2000) in Spain from broilers (98.7%) and foods (74.4%) whereas 75% in human isolates along with cross resistance to Nalidixic acid. Taremi et al (2006) detected 75% and 69.4% resistance to Nalidixic acid and Ciprofloxacin in Campyloabcter isolates from retail raw chicken and beef meat in Iran, respectively. In the Czech Republic, 72% and 55% Ciprofloxacin resistant Campylobacter isolates from poultry and human were reported by Bardon et al (2009), respectively. In Germany, Luber et al (2003) detected significant increase of Ciprofloxacin resistance in chicken and human isolates from 27.3 and 4.9% to 45.6% and 45.1% between 1991 to 2001, respectively.

A lower incidence of fluoroquinolone resistance Campylobacter was observed in different countries. Iran, 45.5% (Rahimi, 2010); Polland, 38.3% resistance to each Nalidixic acid and Ciprofloxacin from cattle in slaughterhouses (Wieczorek et al., 2013) and in pig and cattle carcasses with 57.1% (Wieczorek and Osek, 2013); Ethiopia, 2.1% for Nalidixic acid (Chanyalew et al., 2013); Oklahoma, USA, 42 and 39% for Nalidixic acid and Ciprofloxacin, respectively (Noormohamed and Fakhr, 2013); Italy, 62.76% and 55.17% for Ciprofloxacin and Nalidixic acid, respectively (Di Giannatale et al., 2014). The difference of prevalence rate may be due to the geographical practices in animal husbandry, method of isolation or antimicrobial susceptibility technique implemented**.**

It was confirmed the fluoroquinolone resistance in all *C. jejuni* isolates by detecting the mutation in *gyrase* A gene by direct sequencing of PCR product as also seen by Mukherjee et al. (2013) whereas Sonnevend *et al*. (2006) and Said et al (2010) detected 85.4% and 89% Ciprofloxacin resistant *C. jejuni* strains with Thr-86 to Ile mutation in *gyr*A gene by mismatch amplification mutation assay (MAMA). The standard strain (ATCC 33560) used in this study was sensitive to Nalidixic acid and Ciprofloxacin with MIC value of 8 and 0.5 mg/L, respectively; and did not carry mutation at 86 codon site. Comparison of molecular signature of mutation in isolates and standard strain with phenotypic demonstration confirmed the role of mutation in *gyr*A gene for fluoroquinolone resistance. Observation confirms that the mutation at AA 86 (Thr→Ile) in *gyr*A gene of Campylobacter is responsible for increased quinolone resistance as two *Campylobacter jejuni* isolate (HDJ3 and HDJ6) does not revealed any nucleotide change in *gyr*B gene sequence and even showing the MIC value of 16 and 64 μg/ml for Ciprofloxacin and Nalidixic acid, respectively (Table 2).

Table.1 Source of *Campylobacter jejuni* isolates

Sr. No.	Samples source		Number of isolates
1	Diarrheal cases	Cattle (Fecal samples)	2
		Sheep (Rectal swabs)	2
		Dog (Rectal swabs)	2
		Poultry (Fecal samples)	5
2	Poultry	(Cloacal swabs)	4
3	Meat samples	Mutton	1
		Chevon	1
		Chicken	5
4	Human diarrheal samples		6
Total			28

Table.2 Minimum inhibitory concentration of *Campylobacter jejuni* isolates

Sr. No	Isolates		MICs		Sr. No.	Isolates		MICs	
	Source	**ID**	**CIP**	**NAL**		**Source**	**ID**	**CIP**	**NAL**
1	Cattle D_a	CDJ1	8	32	**16**	Mutton	MJ1	8	32
2		CDJ2	16	64	**17**	Chevon	CJ1	8	64
3	Sheep D_a	SDJ1	8	32	**18**	Chicken	PMJ1	8	32
4		SDJ2	16	128	**19**		PMJ2	4	32
5	Dog D_a	DDJ1	8	32	**20**		PMJ3	8	32
6		DDJ2	4	32	**21**		PMJ4	8	64
7	Poultry D_a	PDJ1	4	32	**22**		PMJ5	4	32
8		PDJ2	16	64	**23**	Human diarrheal samples	HDJ1	16	64
9		PDJ3	8	32	**24**		HDJ2	8	32
10		PDJ4	16	128	**25**		HDJ3	16	64
11		PDJ5	4	16	**26**		HDJ4	4	16
12	Poultry H_b	PHJ1	16	64	**27**		HDJ5	16	64
13		PHJ2	4	64	**28**		HDJ6	16	64
14		PHJ3	16	64	Note: $_a$-Diarrheal sample, $_b$-Cloacal swab of healthy birds				
15		PHJ4	4	64					

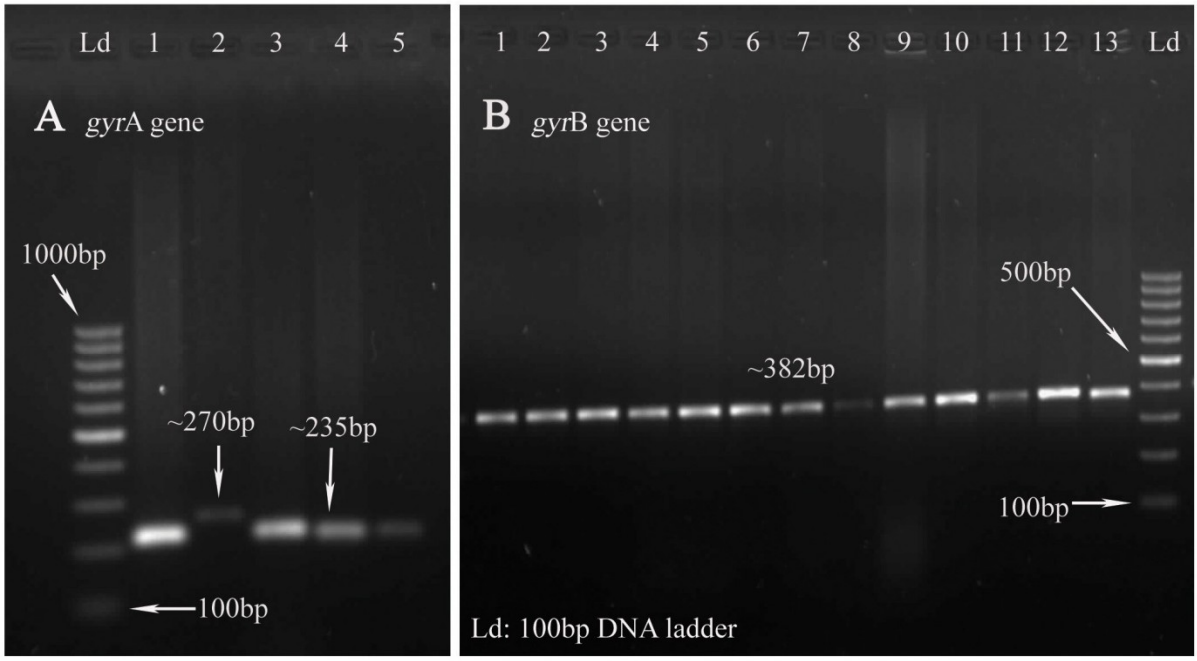

Fig. 1: Agarose gel showing the amplification products of gyrA (A) and gyrB (B) gene

Fluoroquinolone resistance in the Campylobacter isolates from different sources indicates the increases status of antimicrobial resistance in Campylobacter isolates. The isolates used in this were recovered from meat, diarrheic animals and human diarrheal cases in a yearlong cross sectional study which shows the homogenized widespread of these strains in study area. As per our search, earlier report of antibiotic resistance in Campylobacters was not recorded in Gujarat state except by Tayde (2010). The study indicates the probable reflection of illegitimate use of antimicrobials in poultry feeds, flock treatment and lack of biosecurity measures in animal husbandry practices in term of fluoroquinolone resistant *Campylobacter jejuni* isolates.

This study demands the need of extensive epidemiological surveillance of leading bacterial diarrheal pathogen in food of animal origin, assessment of impact of antibiotic resistant isolates on disease pattern and in feasible interventions to certain the spread as described by Chandy et al (2013).

Nucleotide Sequence Accession Numbers

The *Campylobacter jejuni* isolates used in our study had been characterized by 16S ribosomal RNA sequencing and the sequences were submitted with accession no. from KJ777712 to KJ777729. The aligned consensus sequences of DNA *gyrase* A gene of *C. jejuni* isolates has been deposited in NCBI nucleotide database under the accession numbers from KJ364503 to KJ364517 while sequences of *gyrase* B gene of *C. jejuni* isolates were submitted with accession numbers KJ461856 to KJ461859, KJ461863 and KJ461869.

Acknowledgements

We express sincere thank to Centre of Excellence for Plant Biotechnology, Anand Agricultural University, Anand,

Gujarat for providing facility to conduct DNA sequencing at 3500 Genetic analyzer platform and Govt. of Gujarat state for providing fund for project.

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