DOI: 10.7860/JCDR/2016/19034.7830

Original Article

Microbiology Section

Global DNA Methylation Level among Ciprofloxacin-Resistant Clinical Isolates of *Escherichia coli*

THIYAGARAJAN YUGENDRAN[1], BELGODE NARASIMHA HARISH[2]

ABSTRACT

Introduction: Fluoroquinolone resistant clinical isolates belonging to the family Enterobacteriaceae, is a major public health concern in India. Data analysis in JIPMER hospital revealed 10% rise in fluoroquinolone resistance within a span of three years suggestive of the possible involvement of mechanism/s other than QRDR capable of imparting fluoroquinolone resistance. DNA methylation regulates gene expression. Moreover, methylated cytosine is a mutational hotspot. Thus, DNA methylation can alter bacterial gene expression profile as well as facilitate the bacteria in accumulating mutations possibly leading to increased antimicrobial resistance. Therefore, the present study was carried out to identify the potential involvement of DNA methylation in ciprofloxacin resistance.

Aim: To elucidate and compare the methylation level of genomic and plasmid DNA among clinical isolates of *E. coli* sensitive and resistant to ciprofloxacin.

Materials and Methods: The study included 40 clinical *E. coli* isolates of which, 30 were ciprofloxacin-resistant and 10 were sensitive to ciprofloxacin. Genomic DNA (gDNA) and plasmid DNA were extracted and quantified. Methylation levels were elucidated using 5-mC DNA ELISA kit (Zymoresearch, California, USA) as per kit protocol and guidelines.

Statistical Analysis: Spearman correlation 2-tailed test was used. A p-value <0.05 was considered significant.

Results: The MIC values of sensitive and resistant strains against ciprofloxacin ranged from 0.125 µg/mL – 0.75 µg/mL and 8 µg/mL - >256 µg/mL respectively. No difference was found in plasmid DNA methylation level but, the gDNA methylation level of the resistant strains significantly differed from that of the sensitive strains. Based on Spearman correlation test gDNA methylation level of bacteria was found to be inversely proportional to its MIC against ciprofloxacin with p= -0.956 (p-value < 0.0001).

Conclusion: The influence of DNA methylation over plasmid-mediated quinolone resistance needs to be further confirmed by bisulphite DNA sequencing of the plasmid-borne genes. Extensive usage of ciprofloxacin has led to rise in ciprofloxacin resistance possibly induced by DNA methylation. Thus rational usage of ciprofloxacin in a clinical setting is essential to combat the further development of ciprofloxacin resistance. Hypomethylated genes and adenine methylation needs to be identified to fill up gaps in knowledge concerning the involvement of DNA methylation in fluoroquinolone resistance exhibited by *E. coli*.

Keywords: 5-mC methylation, Bacterial epigenetics, Enterobacteriaceae, Fluoroquinolone resistance

INTRODUCTION

Fluoroquinolone resistant clinical isolates belonging to the family Enterobacteriaceae form a major public health concern as it has increased the incidence of respiratory tract infections worldwide and that of UTI and intra-abdominal infections particularly in Asia [1]. Until 1998 accumulation of mutations within Quinolone-Resistance Determining Region (QRDR) alone, was considered responsible for fluoroquinolone resistance until a plasmid encoded gene *qnr* capable of imparting fluoroquinolone resistance was identified [2]. Similarly, little more plasmid-mediated mechanism such as enzymatic modification of fluoroquinolones, protection of DNA gyrase enzyme against the fluoroquinolone activity and efflux pumps have also been reported [3-5]. Of late the prevalence as well as the level of fluoroquinolone resistance among clinical isolates belonging to the family Enterobacteriaceae has increased [6,7]. The prevalence of fluoroquinolone resistance among clinical isolates of Enterobacteriaceae has gone high particularly in India, ranging from 65% to 70% [8,9]. We also observed similar findings in our laboratory, 75% of the Enterobacteriaceae species isolated in the Dept. of Microbiology, JIPMER were resistant to ciprofloxacin in the year 2012. But in 2015, it increased to 85%, with MIC values ranging from 2 µg/mL to >256 µg/mL (Unpublished Observations). Thus, some other resistance mechanism apart from QRDR could be associated with this steep increase in fluoroquinolone resistance.

DNA methylation was initially discovered in the context of the restriction-modification (R-M) system, but some of the bacterial DNA methylases are known to regulate cellular pathways (such as cell signalling, bacterial virulence and pathogenesis) other than the R-M system [10]. DNA methylation regulates gene expression; increase in DNA methylation lowers gene expression [11]. A significant increase in the genomic DNA (gDNA) methylation level of the *E. coli* isolates during the exponential to stationary phase transition has been reported [12]. The gDNA methylation level was found to be lowest in the exponential phase of the *E. coli* during which the cells are highly metabolically active [12]. As the metabolic activity of a bacterium is comparatively higher in the presence of an antibiotic, there could be a possible alteration in the DNA methylation profile of the resistant strains. This modified methylation level of the bacterial DNA could, in turn, alter the bacterial gene expression profile thereby, imparting antimicrobial resistance. Moreover, methylated cytosine is more prone to thymine mutation at a rate of 5.8×10 – 13 per second [13], these spontaneous mutations can lead to accumulation of mutations leading to increased antimicrobial resistance when compared with unmethylated cytosine within the bacteria. Thus, DNA methylation can also facilitate the bacterial cells to accumulate mutations. Apart from accumulating mutations and regulating the gene expression profile of the bacteria, DNA methylation is also involved in the regulation of protein-DNA interaction and DNA

replication [10]. This alternate regulation of DNA replication may be responsible for the higher survival rates of bacteria in the presence of fluoroquinolones. Thus, there can be a possible association between DNA methylation and fluoroquinolone resistance. However, this association needs to be identified experimentally.

Therefore, elucidating the DNA methylation level of the resistant isolates and comparing them with the methylation level of the sensitive strains would provide us an idea of the possible alteration in the bacterial resistance to ciprofloxacin.

AIM

Hence, the present study was carried to find out the potential involvement of methylation levels of plasmid and genomic DNA in ciprofloxacin resistance, by elucidating the association between the levels of DNA methylation and the increase in Minimum Inhibitory Concentration (MIC) values.

MATERIAL AND METHODS

Bacterial Strains

The cross-sectional study described herein, was conducted in Diagnostic Bacteriology Laboratory at JIPMER from June to November 2015. Due to the unavailability of previous literatures and CpG density index for other Enterobacteriaceae species we restricted our study to *E. coli* with a sample size of convenience. The bacterial isolates were prospectively isolated from general culture on sheep blood and MacConkey agar from clinical specimens like blood, sterile body fluids, pus, wound swabs, etc. The isolated bacterial cells were identified by standard biochemical test [14]. Only one confirmed *E. coli* culture per patient was included. The test isolates consisted of 30 ciprofloxacin-resistant isolates and 10 isolates sensitive to ciprofloxacin. The sensitive strains served as controls in the study.

Antibiotic Susceptibility Test

Ciprofloxacin resistance among the test isolates was identified by Kirby-Bauer disk diffusion method and further confirmed by E-test (Himedia Laboratories, Mumbai, India) following CLSI M100-S25 guidelines. *E. coli* ATCC 25922 was included as the quality control.

Template DNA Preparation

gDNA and plasmid DNA were extracted from bacterial cultures grown overnight using QIAamp® DNA Mini kit (Qiagen, Hilden, Germany) and Pure Yield TM Plasmid Miniprep system (Promega, Fitchburg, USA) respectively as per manufacturer's protocol. The extracted DNA was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) based on the 260/280 ratio.

Methylation ELISA

The difference in methylation profile of the resistant and sensitive bacteria was identified using 5-mC DNA ELISA kit (Zymoresearch, California, USA) based on the methodology of Chaturvedi et al., as a reference [15]. The methylation level of gDNA and plasmid samples were elucidated as per the protocol and guidelines mentioned in the kit. The negative and positive controls consisting of 100 ng/µl double-stranded DNA, was included in each run. The standard curve was prepared using multiple combinations of negative and positive controls provided with the kit in different proportions as prescribed by the manufacturer. The absorbance was measured at 450 nm using 680XR microplate reader (Biorad, California, USA). The results were validated using duplicate samples.

Ethics

The study was approved by Institute scientific advisory and human ethics committees (ECR/324/Inst/PY/2013).

STATISTICAL ANALYSIS

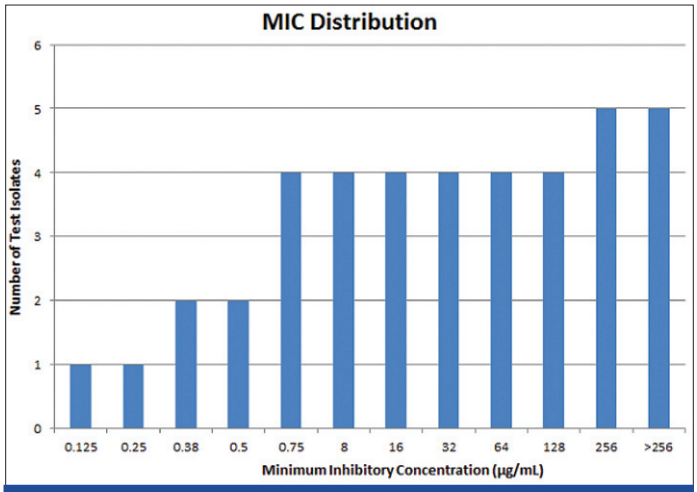
All statistical analyses were carried out using SPSS version 20.0 at 95 % confidence interval. All categorical data were expressed as numbers and percentages. Percentage of gDNA and plasmid methylation was expressed as mean±SD. Spearman correlation 2-tailed test was used to elucidate the association between DNA methylation level and the MIC values.

RESULTS

The MIC values of sensitive and resistant strains against ciprofloxacin ranged from 0.125 µg/mL – 0.75 µg/mL and 8 µg/mL - >256 µg/mL respectively [Table/Fig-1]. Out of 30 resistant isolates, five isolates were highly resistant to ciprofloxacin with MIC values >256 µg/mL.

The cut-off values of the positive and negative control used in 5-mC DNA methylation ELISA were within the permissible range. The percentage of gDNA and plasmid DNA methylated were elucidated based on the concentration of 5-mC, which, is directly proportional to the DNA methylation level.

No significant difference was found in plasmid DNA methylation level but, the gDNA methylation level of the resistant strains significantly differed from that of the sensitive strains [Table/Fig-2]. The methylation level of the sensitive strains ranged between 2.41% to 2.5%. On an average 2.2% of the gDNA, was methylated in the case of the resistant strains ranging from 2.02% to 2.37%. With an increase in the MIC value of the bacterial strains, there was a simultaneous decrease in their methylation level. gDNA of the isolates with MIC >256 were the least methylated with two of these isolates having methylation level as low as 2.02%. Based on Spearman correlation test, gDNA methylation level of a bacteria was found to be inversely proportional to its MIC with p = -0.956 (p-value <0.0001). Higher the MIC value of a bacterial strain, lower the methylation level of its gDNA [Table/Fig-3]. The fall in the methylation level of the gDNA among ciprofloxacin resistant isolates was ~0.25%.



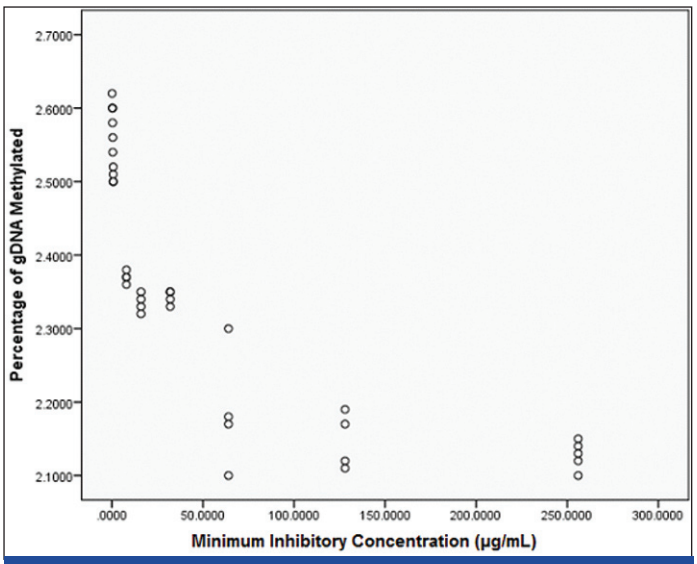
[Table/Fig-1]: Comparison of the genomic DNA methylation level and ciprofloxacin resistance among the test isolates.

Ciprofloxacin susceptibility of Bacterial Isolates	Percentage of DNA Methylated (Mean±SD)	
	Genomic DNA	Plasmid DNA
Resistant Isolates (n=30)	2.215±0.12283	1.154±0.06027
Sensitive Isolates (n=10)	2.457±0.03129	1.117±0.07747

[Table/Fig-2]: Distribution of Minimum Inhibitory Concentration (MIC) of the test isolates against ciprofloxacin.

DISCUSSION

DNA methylation induced drug resistance in cancer therapeutics is a well-established fact [16,17]. DNA methylation is also associated with platinum resistance in cancer therapy [18]. Moreover, a high-level plasmid mediated aminoglycoside resistance due to 16s rRNA



[Table/Fig-3]: Correlation between the MIC of an isolate and the methylation level of their genomic DNA.

methylation among Enterobacteriaceae isolates has also been reported [19]. Thus, the association of DNA methylation with drug resistance is a well-known phenomenon. But, there are no clear evidence-based studies available to elucidate the importance of DNA methylation in fluoroquinolone resistance. Hence, the present study was attempted to put forward the idea of epigenetics in fluoroquinolone resistance by elucidating the difference in the level of genomic DNA and plasmid DNA methylation among clinical isolates resistant and sensitive to ciprofloxacin. Ciprofloxacin an orally administered antibiotic being cost effective is of great use in developing country like India. At present, ciprofloxacin is being used extensively for the treatment of variety of bacterial infections and it has led to the increase in ciprofloxacin resistance [20]. But, the increase in MIC values and 10% rise in fluoroquinolone resistance within a span of three years which we have experienced in JIPMER tertiary-care setup (Unpublished Observations), were suggestive of the possible involvement of some mechanism other than QRDR capable of imparting fluoroquinolone resistance.

The resistant and sensitive strains were distinguishable by the percentage of total gDNA methylated, as the methylation level of the sensitive strains was comparatively higher. A difference of ~0.25% in the gDNA methylation of resistant strains identified in this study may appear minimal but in the context of DNA methylation it is very high. A previous study has reported a 0.2% increase in gDNA methylation level of *E. coli* during exponential phase to stationary phase transition [12] and the same was found to be statistically significant.

The findings of this study statistically correlate the increase in MIC value of a bacterial strain to the decrease in the methylation level of its genomic DNA opening up the possibility for the involvement of DNA methylation in ciprofloxacin resistance. This can be further explained by the fact that DNA methylation regulates gene expression; expression level of a gene is inversely proportional to its methylation level [9]. Thus, the decreased methylation of the DNA in presence of ciprofloxacin can alter the expression level of candidate genes (although not identified in this study) imparting fluoroquinolone resistance which, in turn can influence the level of resistance against ciprofloxacin exhibited by the bacteria.

Though adenine methylation does not regulate gene expression, it may turn out be crucial in fluoroquinolone resistance perspective. Dam methylase of enteric bacteria involves adenine methylation which controls DNA-protein interactions and DNA replication [10]. As fluoroquinolones inhibit DNA replication by targeting DNA gyrase enzyme that binds to DNA, this alternate DNA replication regulatory system may contribute to the increase in MIC against ciprofloxacin. However, we must admit that it is just a hypothesis and needs to be confirmed based on experimental findings. Therefore, identifying the adenine methylation profile of ciprofloxacin-resistant strains can throw more light on the mechanism underlying ciprofloxacin resistance.

There was no significant difference in the methylation level of the plasmid DNA, which may be due to the smaller size of the plasmids and comparatively lower GC content than that of the gDNA. Methylation ELISA is inconclusive about the potential involvement of plasmid DNA methylation as the assay is not gene specific. Thus, the bisulphite DNA sequencing of the plasmid-encoded genes is necessary for the confirmation of the possible involvement of plasmid DNA methylation in fluoroquinolone resistance.

Fluoroquinolone resistance among Enterobacteriaceae species has increased the incidence of respiratory tract infections, intra-abdominal and urinary tract infections [1]. Cross-resistance to different fluoroquinolone molecules among gram-negative bacteria has been identified [21]. Involvement of DNA methylation in ciprofloxacin resistance has further increased the seriousness associated with fluoroquinolone resistance. Fluoroquinolone resistance is a major public health concern in developing countries like India. Thus, ciprofloxacin needs to be used carefully in Indian clinical settings as ciprofloxacin resistance can also lead to resistance against other fluoroquinolones.

LIMITATION

The present study identified the cytosine methylation level of gDNA and plasmids in ciprofloxacin resistant *E. coli* isolates, but the level of adenine methylation in these isolates remains unknown. Identifying the adenine methylation profile of these clinical *E. coli* strains would have provided more information regarding DNA methylation with respect to ciprofloxacin resistance.

CONCLUSION

DNA methylation induced ciprofloxacin resistance due to extensive usage of this wonder drug will make it ineffective, and clinicians will have to resort to antibiotics which are costlier and require to be administered parenterally. Cross-resistance to fluoroquinolones being very common these findings reemphasize the fact that a prudent usage of ciprofloxacin is essential to combat the further development of fluoroquinolone resistance.

Future studies should focus on identifying the hypomethylated genes by genome-wide scan as well as adenine methylation to fill up the gaps of knowledge concerning the involvement of DNA methylation in ciprofloxacin resistance exhibited by *E. coli*.

Source of Funding

This study was supported by JIPMER Institute Research Council intramural grant.

REFERENCES

[1] Dalhoff A. Global fluoroquinolone resistance epidemiology and implictions for clinical use. *Interdiscip Perspect Infect Dis*. 2012;1:1-37.
[2] Martínez-Martínez L, Pascual A, Jacoby GA. Quinolone resistance from a transferable plasmid. *Lancet*. 1998;351:797-99.
[3] Robicsek A, Strhilevitz J, Jacoby GA, Macielag M, Abbanat D, Park CH, et al, Hooper DC. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat Med*. 2006;12(1):83-88.
[4] Strahilevitz J, Jacoby GA, Hooper DC. Plasmid-mediated quinolone resistance: a multifaceted threat. *Clin Microbiol Rev*. 2009;22:664-89.
[5] Robicsek A, Jacoby GA, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis*. 2006;6:629-40.
[6] Lautenbach E, Strom BL, Nachamkin I, Bilker WB, Marr AM, Larosa LA, Fishman NO. Longitudinal trends in fluoroquinolone resistance among Enterobacteriaceae isolates from inpatients and outpatients, 1989-2000: Differences in the emergence and epidemiology of resistance across organisms. *Clin Infect Dis*. 2004;38(5):655-62.
[7] Redgrave LS, Sutton SB, Webber MA, Piddock LJV. Fluoroquinolone resistance: mechanism, impact on bacteria, and role in evolutionary success. *Trends Microbiol.* 2014;22(8):438-45.

[8] Hariharan P, Bharani T, Franklyne JS, Biswas P, Solanki SS, Paul-Satyaseela M. Antibiotic susceptibility pattern of Enterobacteriaceae and non-fermenter Gram-negative clinical isolates of microbial resource orchid. *Journal of Natural Science, Biology and Medicine*. 2015;6:198–201.
[9] Varughese LR, Beniwal B. High quinolone resistance pattern among enteric pathogens isolated from patients with urinary tract infection. *Indian J Biotechnol.* 2015;14:167–71.
[10] Casadesus J, Low D. Epigenetic gene regulation in the bacterial world. *Microbiol Mol Biol Rev*. 2006;70(3):830-56.
[11] Philips T. The role of methylation in gene expression. *Nat Educ*. 2008;1:116–24.
[12] Kahramanoglou C, Prieto AI, Khedkar S, Haase B, Gupta A, Benes V, et al. Genomics of DNA cytosine methylation in *Escherichia coli* reveals its role in stationary phase transcription. *Nat Comm*. 2012;3:1-9.
[13] Shen, JC, Rideout WM, Jones PA. The rate of hydrolytic deamination of 5-methylcytosine in double-stranded DNA. *Nucleic Acids Res*. 1994;22:972-76.
[14] Forbes BA, Sahm DF, Weissfeld AS. Bailey & Scott's Diagnostic Microbiology. 12 ed. Missouri: Elsevier Mosby; 2007.
[15] Chaturvedi P, Kalani A, Givvimani S, Kamat PK, Familtseva A, Tyagi SC. Differential regulation of DNA methylation versus histone acetylation in cardiomyocytes during HHcy in vitro and in vivo: an epigenetic mechanism. *Physiol Genomics*. 2014;46:245-55.
[16] Jane AP, Gordon S, Julieann S, Stanley BK, Robert B. Reversal of drug resistance in human tumour xenografts by 2'-Deoxy-5-azacytidine-induced Demethylation of the hMLH1 Gene Promoter. *Cancer Res*. 2000;60:6039-44.
[17] Christian NA, Goel A, BOLAND RC. Role of hmlh1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. *Int J Cancer.* 2003;106:66–73.
[18] Martin LP, Hamilton TC, Schilder RJ. Platinum resistance: the role of DNA repair pathways. *Clin Cancer Res*. 2008;14:1291-95.
[19] Galimand M, Courvalin P, Lambert T. Plasmid-Mediated high-level resistance to aminoglycosides in enterobacteriaceae due to 16s rRNA methylation. 2003;47(8):2565-71.
[20] Geetha VK, Yugendran T, Srinivasan R, Harish BN. Plasmid-mediated quinolone resistance in typhoidal Salmonellae: A preliminary report from South India. *Indian J Med Microbiol.* 2014;32:31-34.
[21] Aibinu IE, Adenipekun EO, Nwaka D, Adelowotan AO, Ajekigbe AT, Adeyemi OF, et al. Emergence of Cross- Resistance to Fluoroquinolones in Gram-Negative Isolates from Cancer Infections in a Tertiary Hospital in Nigeria. *J Am Sci*. 2008;4:14-20.

PARTICULARS OF CONTRIBUTORS:
1. Research Scholar, Department of Microbiology, JIPMER, Puducherry, India.
2. Senior Professor and Head, Department of Microbiology, JIPMER, Puducherry, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Belgode Narasimha Harish,
Department of Microbiology, Second Floor, Institute Block, JIPMER, Gorimedu, Puducherry - 605006, India.
E-mail: drbnharish@gmail.com

FINANCIAL OR OTHER COMPETING INTERESTS: As declared above.

Date of Submission: **Jan 23, 2016**
Date of Peer Review: **Feb 08, 2016**
Date of Acceptance: **Apr 07, 2016**
Date of Publishing: **May 01, 2016**