Title:- Detection of mutations in *gyr*B using denaturing high performance liquid chromatography (DHPLC) among *Salmonella enterica* serovar Typhi and ParatyphiA.

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Abstract

Background:- Fluoroquinolone resistance is mediated by mutations in the quinolone-resistance determining region (QRDR) of the topoisomerase genes. Denaturing high performance liquid chromatography (DHPLC) was evaluated for detection of clinically important mutations in *gyr*B among *Salmonella.*

Method:- *S.* Typhi and *S*. ParatyphiA characterised for mutation in QRDR of *gyr*A, *par*C and *par*E were studied for mutation in *gyr*B by DHPLC and validated by sequencing.

Result:- The DHPLC analysis was able to resolve the test mutant from isolates with wild type *gyr*B and distinguished mutants from other mutant by peak profile and shift in retention time. Three sequence variants were detected at codon 464, and a novel mutation Ser→Thr was also detected. *gyr*B mutation was associated with non classical quinolone resistance (NAL^{S}-CIP^{DS}) in 34 isolates of *S.* Typhi only and was distinct from classical quinolone resistance associated with *gyr*A mutations (NAL^{R}-CIP^{DS}).

Conclusion: DHPLC is effective for the detection of mutation and can reduce the need forsequencing to detect clinically significant *gyr*B mutations..

Accession number:- (GenBank accession nos. KF993966, GenBank accession no. KF993965 and GenBank accession no. KF993964).

Key words: Decreased Ciprofloxacin Susceptibility, DHPLC, *gyr*B Mutation, *Salmonella* Typhi, *Salmonella* Paratyphi A ,

1. Introduction

Salmonella enterica serovar Typhi (*S.* Typhi) and *Salmonella enterica* serovar Paratyphi A (*S.* Paratyphi A) cause the major human infection, enteric fever. The current WHO guidelines state that fluoroquinolones (FQ) are the optimal antimicrobials for treatment of uncomplicated enteric fever.[1] Resistance to quinolones in *S.* Typhi and *S.* Paratyphi A can be caused by amino acid substitutions in the quinolone resistance-determining region (QRDR) of the DNA *gyrase* subunit *gyr*A, a key target of ciprofloxacin (CIP).[2] These isolates are typically resistant to nalidixic acid (NAL^R, MIC≥32µg/ml) and show decreased susceptibility to ciprofloxacin (CIP^{DS} MIC >0.064µg/ml). Mutations in the QRDR of the other subunit of DNA *gyrase (gyr*B) and both subunit of DNA topoisomerases IV (*par*C and *par*E) will also result in increased resistance to quinolones,[3] however the role of these mutations is not well studied in *S.* Typhi nor *S.* Paratyphi A. Another cause of decreased susceptibility to ciprofloxacin involves mutation in codons 464 (Ser to Phe) and 466 (Glu to Asp) of the DNA *gyrase* subunit *gyr*B. These isolates remain sensitive to the recommended screening agent, nalidixic acid,[4] but infected patients are predicted to show longer times to fever clearance with increased treatment failure following ciprofloxacin therapy.[2, 5]

In diagnostic microbiology laboratories, especially in typhoid endemic regions, the rapid detection of *Salmonella* with decreased susceptibility is important but the most common method, nalidixic acid resistance screening using disc diffusion, is no longer reliable.[6] To detect mutations in topoisomerase genes, the ultimate method is direct sequencing but this is labour intensive and expensive. Alternative methods to sequencing include: single-strand conformational polymorphism (SSCP), mismatch amplification mutation assay (MAMA-PCR), PCR-restriction fragment length polymorphism (RFLP), high resolution melt analysis[7] and Light Cycler based PCR-hybridization mutation assay. With the exception of SSCP these methods rely upon mutation-specific oligo-primers (MAMAPCR), mutation-specific oligonucleotide probes (Light Cycler), and mutation-specific enzymes (PCR-RFLP) and so only known mutations can be identified. SSCP relies on differential separation of DNA by gel electrophoresis and does not distinguish all mutations. A

method equivalent to direct sequencing in scope but simpler, cheaper and with high sample throughput is needed. Denaturing High Performance Liquid Chromatography (DHPLC) was developed in 1995 and has emerged as a rapid, high throughput screening method to detect mutations and polymorphisms.

Mutation detection using DHPLC involves subjecting PCR products, of wild type and test DNA sample in 1:1 mixture, to ion-pair reverse-phase liquid chromatography. Under conditions of partial heat denaturation, heteroduplexes form in PCR samples having internal sequence variation and display reduced column retention time relative to their homoduplex counterparts. Elution profiles for such samples are distinct from those having homozygous sequence, making the identification of samples harbouring polymorphisms or mutations a straight forward procedure. DHPLC is capable of detecting single nucleotide substitutions, small insertions and deletions by online UV or fluorescence monitoring within 10 minutes in unpurified amplicons as large as 1.5 kb. The objective of this study was to evaluate the application of DHPLC, in a developing country setting, as a high throughput tool for detection of clinically important mutations in bacteria using mutations associated with fluoroquinolone resistance in *Salmonella* as a proof of concept.

2. Material and methods:-

2.1 Selection of isolates:- Two hundred and six isolates of *S.* Typhi (*n*=162) and Paratyphi A (*n*=44), isolated between 2006-2011 were selected to represent diversity in terms of minimum inhibitory concentration (MIC) of nalidixic acid (NAL), ciprofloxacin (CIP), year of isolation and antibiotic resistance profile. MIC for NAL and CIP were determined by E-test (*AB Biodisk, Solna, Sweden).* Breakpoints for susceptibility and resistance for NAL and CIP were ≤16µg/ml and ≥32µg/ml and ≤0.064 and ≥1µg/ml respectively (CLSI guideline 2012). Decreased ciprofloxacin susceptibility (CIP^{DS}) was defined as MIC > 0.064 µg/ml. These isolates had been previously

100 characterised for mutation in QRDR of *gyr*A , *par*C and *par*E genes by PCR amplification and
101 confirmed by direct sequencing.

102 **2.2. PCR**

103 DNA was extracted from isolated bacterial colonies using the Wizard® Genomic DNA
104 Purification Kit (Promega) according to the manufacturer's protocol. PCR was used to amplify the
105 QRDR region of *gyr*B of test and control strains using primer FP: 5'-GCG CTG TCC GAA CTG
106 TAC C– 3' and RP: 5'-TGA TCA GCG TCG CCA CTT C-3' with amplicon size 169bp. The
107 primers used in this study were designed in-house using Generunner software (vesion 3.05) and
108 obtained from commercial source (Eurofins, Bengaluru, India). Thermocycler (Gradient
109 Eppendorf) with the following conditions: initial denaturation at 94ºC for 5 min, followed by 30
110 cycles of 45 sec at 94ºC (denaturation), 45 sec at 50ºC (annealing), 45 sec at 72ºC (extension), and
111 a final extension of 10 min at 72°C. PCR products were checked by 1.5% agarose gel
112 electrophoresis in 1X TAE buffer. For antibiotic susceptibility tests and PCR *S.* Typhi (Ty2) and *S.*
113 Paratyphi A (ATCC9150) strains were used as controls.

114

115 **2.3. DHPLC analysis**

116 The DHPLC analysis was performed using WAVE Nucleic acid fragment analysis system
117 (WAVE System 4500, Transgenomic Inc.). Briefly 5µl of hybridized amplified PCR product of test
118 and wild type strains (Ty2 and ATCC 9150) were mixed in 1:1 ratio. The above mixture of
119 amplimers was hybridized by heating at 95°C for 3min and then cooled gradually by ramping the
120 temperature down to 35°C in 1°C/min steps. The hybridized DNA was loaded on the
121 DNASepCartridge (Transgenomics) with 54% elutent A (0.1 M tri-ethyl-ammonium acetate
122 (TEAA) and 46% elutent B (0.1 M TEAA in 25% (vol/vol) acetonitrile). The predicted average

123 melting temperature over the whole 169bp *gyr*B fragment was 63.3°C. DHPLC analysis was
124 therefore performed at temperatures: 61.3°C, 62.3°C, 63.3°C and 64.3°C at flow rate of 0.9ml/min
125 to optimize formation of duplex DNA in *gyr*B gene. The DNA fragment elution profiles were
126 captured using Transgenomic WAVE MAKER software to determine the correct partial
127 denaturation temperature for mutation scanning based on the sequence of the wild-type DNA from
128 *S.* Typhi (Ty2) and *S.* Paratyphi A (ATCC9150) strains. Eluted DNA fragments were detected by
129 the system's ultraviolet detector. DNA sequence variant detection depends on heteroduplex
130 formation between wild-type and mutant DNA single strands. At elevated temperatures, the less
131 thermostable heteroduplexes start to melt at the mismatched region, and as a result the DNA elutes
132 earlier than corresponding homoduplexes. The optimal temperature to detect mutations in *gyr*B
133 gene was then confirmed empirically by comparing chromatograms (peaks) obtained with retention
134 time for PCR products from wild-type strains. Different peak profile to wild type or same peak
135 profile but with a shift in retention time at a specific temperature were considered to indicate the
136 presence of a mutation. Analyses of wild type DNA of *S.* Typhi (Ty2) and *S.* Paratyphi A
137 (ATCC9150) were performed ten consecutive times to test the reproducibility of retention time.
138
139 **2.4. Sequencing**
140 All isolates of *S.* Typhi and *S.* Paratyphi A had been previously sequenced for *gyr*A, *par*C
141 and *par*E (Table 1). Sequencing was also performed to validate predicted mutations in *gyr*B region
142 detected by DHPLC in 34 *S.* Typhi strains with heteroduplexes and 20 of *S.* Typhi and *S.* Paratyphi
143 A strains with homoduplexes.
144 For sequencing, the 50µl of amplified PCR product was run on 2% agarose gel. The desired
145 band of the DNA was excised. The DNA was extracted from the gel by Qiagen gel extraction kit
146 (QIAgen India Pvt. Ltd, New Delhi, India) and sequencing was done commercially by Macrogen
147 (Korea). For novel mutations sequencing was in duplicate. Sequences obtained were then

compared with available sequences on NCBI of *S.* Typhi strain Ty2 (Accession no. NC_004631) and *S*. Paratyphi A strain ATCC9150 (Accession no CP000026) using Fintch TV version 1.4.0 and MEGA version 5 software.

3. Results

3.1. Selection of isolates

206 *S*. Typhi and *S*. Paratyphi A isolates were assigned to different groups based on MICs of NAL and CIP, as shown in Table 1. Group 1 included NAL^{S}-CIP^{S} isolates, group 2 NAL^{R}-CIP^{DS} (classical quinolone resistance), Group 3 high level of CIP resistance and Group 4 NAL^{S}-CIP^{DS} (non classical quinolone resistance); 34/162 *S.* Typhi isolates and 0/44 *S.* Paratyphi A isolates.

3.2. Optimal temperature for mutation detection in *gyr*B by DHPLC

A sharp peak of DHPLC chromatograms were obtained at 62.3ºC which was identified as the optimal temperature for analysis of mutation in *gyr*B gene for both *S.* Typhi and Paratyphi A. Wild type isolates of both serovars gave rise to a single peak at this temperature (homoduplexes). The retention time for DNA for both *S.* Typhi (Ty2) and *S.* Paratyphi A (ATCC9150) isolates at 62.3°C was between 4.76 - 4.81 and 3.88 - 3.90 min respectively.

3.3. Detection of *gyr*B mutations by DHPLC

We analyzed the *gyr*B QRDR by DHPLC to assess its ability to detect point mutations. A total of 206 *isolates* of *Salmonella* were screened using this method. DHPLC detected 3 DNA sequence variants at Ser464 codon: Ser464-Phe, Ser464-Tyr and Ser 464-Thr which is a novel mutation,

Any changes from the single-peak profile characteristic of wild-type *S.*Typhi (Ty2) *S.* Paratyphi A (ATCC9150) resulted in formation of heteroduplexes and was indicative of at least one

mutation at *gyr*B within the test DNA fragment. Among *S.* Paratyphi A isolates irrespective of their MIC values for ciprofloxacin and nalidixic acid, only a single peak profile characteristic of wild type *gyr*B was observed (Figure 1).

Among *S.*Typhi isolates, DHPLC detected four different peak profiles including the wild type as shown in Figure 2. A single-peak profile characteristic of wild-type (Figure 2, peak A) *gyrB* was observed among groups 1, 2 and 3. Heteroduplexes were observed only among group 4 of isolates with non-classical quinolone resistance (NAL^{S}-CIP^{DS}) indicating the presence of mutation in *gyr*B. All samples containing the same single-base substitutions had identical peak profiles (shown by overlaying them using the WAVEMAKER software). Single mutations at the same point but which incorporated a different substitution were easily seen as different DHPLC peak profiles, for example Ser464-Phe (Figure 2 peak C, *n*=31, (GenBank accession no. KF993966), and Ser464-Tyr (Figure 2 peak D, *n*=2, (GenBank accession no. KF993965). A novel mutation was observed at Ser 464-Thr (Figure 2 peak B *n*=1, (GenBank accession no. KF993964). The retention time of these four peak profiles were also distinct (Table 2). Multiple mutations were ruled out by analysis at different temperature.

3.4. Correlation of NAL and CIP MIC with mutations in DNA *gyrase* and topoisomerase genes is shown in Table 1:-

S. Typhi and *S.* Paratyphi A group 1 isolates with wild type *gyr*A, *gyr*B and *par*C were susceptible to NAL and CIP (NAL^{S}-CIP^{S}). Group 2 isolates of both serovar were uniformly associated with mutation in *gyr*A (Table 1) which conferred classical quinolone resistant phenotype (NAL^{R}-CIP^{DS}). However among three *S.* Typhi isolates an additional mutation in *par*C gene was also observed. Group 3 isolates with high level of fluoroquinolone resistance were associated with double mutation in *gyr*A and a single mutation in *par*C gene. Mutation in *gyr*B conferred non

197 classical resistance phenotype (NAL^{S}-CIP^{DS}) among *S.*Typhi only. Although these isolates were
198 nalidixic acid susceptible, they were distinct from group 1 wild type isolates with NAL and CIP
199 MIC being 2-3 and 10 fold higher respectively. The decreased susceptibility to CIP (MIC 0.064-
200 0.5µg/ml) among *gyr*B mutants was twice fold less than decreased susceptibility (MIC CIP 0.064-1)
201 associated with *gyr*A and *par*C mutation among group 1 isolates. Ser 464 Phe was observed most
202 common mutation in *gyr*B. Mutations in *gyr*B did not co-exist with mutation in *gyr*A or *par*C gene
203 and were not observed in *S.* Paratyphi A. No mutations were observed in *par*E gene among both
204 serovars.

205 **4. Discussion:-**

206 DHPLC has been used as a medical research tool since the 1990s to detect polymorphisms
207 in human genes.[8] More recently DHPLC has also been used for the separation and identification of
208 PCR-amplified fragments from bacterial genes[9] including: 16S-23S intergenic spacer region (ISR)
209 and *gyrA* gene of *Bacillus anthracis*;[10] and single nucleotide polymorphisms (SNPs) in QRDR of
210 *gyrA* gene for ciprofloxacin resistance in *Salmonella*.[11] Methods have also been developed for
211 molecular screening and diagnosis of tumors,[12] and cystic fibrosis.[13] In this study we have used the
212 DHPLC method for the detect fluoroquinolone resistance in *S.* Typhi and *S.* Paratyphi A. Resistance
213 to quinolones is generally associated with mutation in *gyr*A and *par*C, [2, 5, 14] and the role of
214 mutation in DNA *gyrase* subunit *gyr*B is less well described. This is probably due to the cost
215 involved in screening all genes from several hundred clinical isolates. In the present study DHPLC
216 was evaluated for its ability to detect SNPs or sequence variation in QRDR of *gyr*B among *S.* Typhi
217 and Paratyphi A and results were validated by direct sequencing of amplicons. Major advantages of
218 DHPLC are that knowledge of the exact mutation is within the QRDR of the topoisomerases
219 involved is not necessary; automation is possible; and standard PCR can be used, so this is an ideal
220 method for screening large number of strains. Although sequencing remains a gold standard in

SNPs discovery, DHPLC analysis is cheaper and simpler; and novel mutations can be identified. The technique is robust enough to use only one assay for each sample. Mutations are identified by a shift in retention time or a characteristic separation of homo- or heteroduplex peaks. The auto run mode of DHPLC significantly decreases handling time without the loss of assay specificity making screening relatively quick and easy (8 minutes for total run per sample after PCR which includes sample injection, column equilibration, and cleaning). For large numbers of samples automation using 96 well plates can be programmed for repeated injection and analysis at more than one temperature; this makes it a rapid and cost effective. With sufficient evidence to satisfy clinical accreditation this method could be used to rapidly screen for resistance and so reduce the dependences on labour intensive culture based methods. The capital cost is high (approx 110000 USD) but running costs are relatively low USD 0.7. The wave maker utility software allows analysis by both overlay of profile (to confirm any subtle changes) and detailed peak data. This allows immediate recognition of identical peaks and those that are novel. Once a library of mutation is generated, sequencing will be necessary only when a novel mutation is observed as distinct peaks.

Using DHPLC we correctly identified mutations in *gyr*B in 34 NAL^S *S.* Typhi isolates (MIC ≤16µg/ml). The DHPLC analysis was sensitive and specific and was able to resolve the test mutant from isolates with wild type *gyr*B and distinguished all mutants (with specific changes) from other mutant on the basis of peak profile and shift in retention time.The sequencing data is shown in Figure 3. Sequencing confirmed the DHPLC predicted SNPs. Three sequence variants were detected at codon 464 (Table 2), being Ser464-Phe (*n*=31) the predominant and a novel mutation Ser-464-Thr was also detected. Negative DHPLC results were confirmed by sequencing (*n*=20). It confirms the presence of the wild type (susceptible) sequence rather than the absence of mutation as detected by qPCR methods. All NAL^S isolates with mutations at *gyrB* corresponded to non-classical quinolone resistance phenotype (NAL^S-CIP^{DS}). This is of clear significance in clinical practice as

different study groups have already reported that patients infected with isolates showing decreased ciprofloxacin susceptibility normally experience more frequent treatment failures and phenotypic screening with nalidixic acid would not detect *gyrB* mediated resistance. Further *gyrB* mutation was not associated with high level FQ resistance, occurred singly and did not co-exist with other mutations. Previous reports have shown that DHPLC has in addition the advantage of detecting mutations in several regions of the DNA in one test sample.[11] Mutations in *gyr*B are rare and multiple mutations were not found.

5. Conclusion:-

Our data show that DHPLC can be used to detect mutations in other genes conferring quinolone resistance including genes not normally associated with resistance (*gyr*B) and mutations outside the QRDR region which have not been thoroughly investigated. The high initial cost of the Transgenomic Wave DHPLC may be a factor preventing a wide application of this technique in diagnostic laboratories and further development is needed to perform multiplexed assay for simultaneous detection of mutations in different genes.

Authors' disclaimers (if required):- Nil

Authors' contributions:- JW , RG and BP conceived the study; Ruchi G and SFB designed the study protocol; Ruchi G performed laboratory experiment ; RG, Ruchi G and MD drafted the manuscript, LCS performed DHPLC analysis data; JW and SR edited the final draft of manuscript. RG and SFB are guarantors of the paper. RG, JW and BP prepared the reply for reviewers comments.

Acknowledgements (if required):- Nil

Funding:-

This work was supported by Indian Council of Medical Research, Govt. of India. [Grant No. 5/8-1(8)2010-11/ECD-II IRIS Cell (ID-2010-04020)] to Dr. Rajni Gaind.

Competing interests:- Nil

Ethical approval

This work was approved by ethics committee (No. 12-10-EC (4/17).

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Legends

Graph showing overlay of elution pattern of peaks with different retention time for *S.* Paratyphi A and *S.*Typhi with mutation in *gyr*B (Figure 1 and Figure 2)

Figure 1.

DHPLC analyses of *S.*Paratyphi A at 62.3˚C with mutations in *gyr*B – comparisons with ATCC9150 wild strain by the following peak pattern is showing WT ATCC9150 and test isolates, had no mutation.

Figure 2

DHPLC analyses of *S.*Typhi strains at 62.3°C with mutation in *gyr*B region – comparisons withTy2 Wild strain by the following elution of peak patterns: 1) Peak A:-wild type Ty2, had no mutation. 2) Peak B:- Ser 464 Thr, 3) Peak C:- Ser 464 Phe and 4) Peak D:- Ser 464 Tyr. Mutations were confirmed by sequencing.

Figure 3

Sequence data of showing mutations in *gyr*B at codon 464

1. Reference sequence of wild type *gyr*B *S*.TyphiTy2 Accession no. NC_004631.1,

2. Ty2 control strain, no mutation as Serine (S) (TCC, Peak A; green colour),

3. 780 july /08 sample with mutation Serine to Tyrosine (Y) (TCC→TAC: Peak D; red colour)

4. 40 may/10 sample with mutation Serine to Threonine (T) (TCC→ACC, PeakB; blue colour)

5. 22 aug/11 sample with mutation Serine to Phenylalanine (F) (TCC→TTC, PeakC; yellow colour)