Ciprofloxacin induced antibiotic resistance in *Salmonella* Typhimurium mutants and genome analysis

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Abstract
Antibiotic resistance of *Salmonella* species is well reported. Ciprofloxacin is the frontline antibiotic for salmonellosis. The repeated exposure to ciprofloxacin leads to resistant strains. After 20 cycles of antibiotic exposure, resistant bacterial clones were evaluated. The colony size of the mutants was small and had an extended lag phase compared to parent strain. The whole genome sequencing showed 40,513 mutations across the genome. Small percentage (5.2%) of mutations was non-synonymous. Four-fold more transitions were observed than transversions. Ratio of < 1 transition vs transversion showed a positive selection for antibiotic resistant trait. Mutation distribution across the genome was uniform. The native plasmid was an exception and 2 mutations were observed on 90 kb plasmid. The important genes like *dnaE*, *gyrA*, *iroC*, *meth* and *rpoB* involved in antibiotic resistance had point mutations. The genome analysis revealed most of the metabolic pathways were affected.

Keywords *Salmonella* · Ciprofloxacin · WGS · Mutation · Antibiotic resistance

Introduction

*Salmonella* Typhimurium causes approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths each year in the United States (CDC, 2019). A common first line of treatment is fluoroquinolones for elders and azithromycin in infants. However, due to increased resistance to fluoroquinolones, especially ciprofloxacin (3%) in the US, 56% in Mexico and > 91% in Thailand, ceftriaxone has become the drug of choice. However, now 5% bacteria are resistant to more than one antibiotic. Antibiotic resistance is a burden to the entire world, in the U. S. alone 99,000 deaths occur annually (Aslam et al. 2018). Antibiotics are also used as growth promoters in animal husbandry (poultry feeds) and agriculture. This has probably led to an increase in the number of infections caused by multidrug-resistant bacteria, which is a threat to human life according to a recent world economic global risk report (CDC, 2019). Antibiotic-resistant organisms resulting from human activity are controversial, because such organisms have been isolated from environments devoid of human intrusions supporting the hypothesis that antibiotic resistance is an ancient and genetically rich natural phenomenon (D’Costa et al. 2011).

The bacterial evolution studied by phenotypic tests like metabolic activity, drug resistance or genetic tests like variations in chromosomal structure or individual gene sequence were useful but had limitations as they gave information of very small variations and not complete picture or resolution. The recent advances in whole genome sequencing aided to understand evolution, antibiotic resistance, pathogenicity and virulence mechanisms of bacterial pathogens. Advances in sequencing technologies help to capture sequence variation for each sample under study, providing the potential to detect all changes at all positions in the genome from single nucleotide changes to large-scale insertions and deletions (Bryant et al. 2012; Pu et al. 2019). The WGS gives most precise structural details and definitive phylogenetic relationships thus helping researchers in understanding molecular mechanisms of antimicrobial resistance (Hendriksen

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et al. 2019). A vital step for this is detection of resistant determinants like single nucleotide polymorphism (SNP), insertions or deletions (INDELS) and frameshift mutations. SNP may produce synonymous mutation (sSNP) where an amino acid substitution may be silent, causing no change in the encoded protein or nonsynonymous mutation (nSNP) where the amino acid may change the function of a protein. INDELS and frameshifts may alter a protein function significantly or a stop codon may halt a function abruptly. A point mutation in a non-protein coding region may affect regulatory elements (Bryant et al. 2012).

In the present study, we addressed two questions. First, how many repetitions of ciprofloxacin exposure would cause resistant strain? Second, what kind of genotypic change would lead to resistance?

Materials and methods

Microbial culture

Salmonella Typhimurium MTCC 98 was purchased from Microbial Type Culture Collection Chandigarh, India.

Media and antibiotics

All the following media, antibiotics and antibiotic discs were purchased from Hi Media Laboratories, Mumbai, India: Muller Hinton Broth (MHB), Muller Hinton Agar (MHA), Luria Broth (LB), Luria agar (LA), Xylose-Lysine Deoxycholate Agar (XLDA), Hektoen Enteric Agar (HEA), Bismuth Sulphite Agar (BSA), ciprofloxacin, ampicillin, tetracycline, chloramphenicol, kanamycin powders. Discs of ciprofloxacin (10 µg/ml), ampicillin (10 µg/ml), tetracycline (30 µg/ml), chloramphenicol (30 µg/ml), kanamycin 30 µg/ml), enrofloxacin (10 µg/ml), gatifloxacin (5 µg/ml), lomefloxacin (5 µg/ml), ofloxacin (2 µg/ml), levofloxacin (5 µg/ml), sparfloxacin (5 µg/ml).

Minimum inhibitory concentration (MIC)

MIC of the cultures was determined according to the guidelines provided by Clinical and Laboratory Standards Institute (CLSI) by micro-dilution method (CLSI 2020). The overnight culture (approximately 1 × 10⁸ cfu/ml) was diluted in the fresh MHB (1:10). Hundred microliters of MHB was added to all wells of 96-well microtiter plate. Ten microliters of culture and 100 µl of 1:10 diluted stock concentration of ciprofloxacin were added to the first well of the 96-well microtiter plate (Corning, US). The solution from the first lane was diluted two-fold to proceeding wells to obtain concentration gradient of antibiotic. The well without antibiotic but culture served as positive control. The plates were incubated at 37 °C for 18 h. The lowest dilution of ciprofloxacin that showed no visible growth was noted as Minimal Inhibitory Concentration (MIC).

Antibiotic susceptibility test (AST)

AST was performed as per standard protocol CLSI (2020) method. Briefly, an overnight culture was diluted to obtain the McFarland standard of 0.5 at 600 nm. This was spread plated on previously prepared MHA plates. The plates were dried for 5 min. The required antibiotic discs ciprofloxacin (10 µg/ml), ampicillin (10 µg/ml), tetracycline (30 µg/ml), chloramphenicol (30 µg/ml), kanamycin 30 µg/ml), enrofloxacin (10 µg/ml), gatifloxacin (5 µg/ml), lomefloxacin (5 µg/ml), ofloxacin (2 µg/ml), levofloxacin (5 µg/ml), sparfloxacin (5 µg/ml) were placed on the bacterial lawn and incubated at 37 °C for 18 h. The zone of inhibition (mm) was measured and results interpreted as per the chart provided by CLSI (2020).

Laboratory development of ciprofloxacin resistance

The overnight culture was inoculated in LB for 3 h to obtain a logarithmic phase (approx. 4 × 10⁸ cfu/ml). The 5 µl of culture was then added to a fresh LB (5 ml) containing ciprofloxacin ten times the MIC and allowed to grow for 18 h at 140 rpm. The 5 µl inoculum was then suspended in fresh LB; allowed to grow for 3 h and again exposed to ciprofloxacin (10X MIC) as explained earlier. After each cycle, MIC was determined. After 20 cycles of growth and ciprofloxacin exposure, the culture was plated on LA plate with ciprofloxacin (10 µg/ml) incubated at 37 °C for 18 h. Three clones were selected and processed for phenotypic and genotypic analysis.

Stress experiments

Heat stress

An overnight culture supplemented with ciprofloxacin was washed with saline and inoculated in a fresh medium. These were heated at different temperatures (45 °C, 50 °C, 55 °C and 58 °C) for 10 min and immediately appropriate dilutions were spread plated on LA plates. The colony forming units (cfu/ml) were determined after incubation at 37 °C for 18 h.

Osmotic stress

An overnight culture supplemented with ciprofloxacin was washed with saline and inoculated in fresh LB containing different concentrations of NaCl (3, 5, 6, 12 & 15%). These were then incubated at 37 °C at 160 rpm for 18 h. After
overnight incubation, the appropriate dilutions were spread plated on LA plates. The colony forming units (cfu/ml) were determined after incubation at 37 °C for 18 h.

**Determination of lag phase**

A 96-well microtiter plate was used for the experiment. A logarithmic phase culture (0.5 at OD 600 nm) was added (5 µl) to 195 µl of LB. The growth analysis was carried out at an absorbance of 600 nm on Synergy H1 microplate reader, Biotek (Winooski, Vermont, USA). A medium shaking speed (120 rpm) was used and optical densities recorded after every 20 min for 20 h at 37 °C.

**Whole genome sequencing (WGS)**

The whole genome sequencing was carried out using Illumina HiSeq 4000 sequencer (Illumina, San Diego, USA), following manufacturer’s instructions at Genotypic Technologies Pvt. Ltd. Bangalore, India. The sequencing quality was assessed using FastQC v0.11.8 software. The adapter clipped, high-quality reads were aligned to the genome sequence of *S. Typhimurium* LT2 using Bowtie2 (Langmead and Salzberg 2012) and variant calling was done using Samtools 1.3 (Li et al. 2009). The significant variants were annotated and the consensus sequence obtained. The data obtained from the sequencing analysis was analysed in Jupyter notebook with python 3.7 backend in the Anaconda package. The Circos plot was made by compiling the chromosome coordinates and annotates mutations and plotting the data with circos-0.69–9 (Krzywinski et al. 2009). The genes with non-synonymous mutations were submitted to the Panther database public server (Mi et al. 2019) and DAVID database (Huang et al. 2009) for gene function classification. The protein–protein interaction of the DNA metabolic genes and resistance genes was obtained from STRING v11 public server (Szklarczyk et al. 2019). The 3D structure of gyrA was obtained by intensive modelling at PHYRE 2 server and string analysis performed using string database (https://string-db.org/). The genome of the variants was scanned for the presence of resistance genes at the Comprehensive Antibiotic Resistance Database (Alcock et al. 2020). Details of software used are provided in Online Resource 1.

**Results**

**Development of antibiotic resistance**

*S. Typhimurium* is sensitive to ciprofloxacin (MIC 0.05 µg/ml). When *S. Typhimurium* was exposed to ten times MIC concentrations of ciprofloxacin, 99% of the population was eliminated. The remaining 1% of the population was again treated with 10X MIC concentration of ciprofloxacin. This ciprofloxacin exposure was repeated up to 20 cycles. In each cycle, MIC of the *S. Typhimurium* was measured. The ciprofloxacin resistance was increased up to 100-fold (MIC 5 µg/ml) after the 10th cycle. There was no further increase in antibiotic resistance up to the 20th cycle. After 20th exposure, three resistant *S. Typhimurium* clones were selected for detailed evaluation. These clones were designated as A, B and C and were characterized in detail with respect to phenotype and genotype.

**Phenotypic characterization**

The clones exhibited similar characteristics as wild type, both were Gram-negative, lactose non-fermenters and showed typical dark centred colonies on selective media (XLD, HEA, BSA). However, the clones formed smaller colonies on LB (0.7 ± 0.5 mm) as compared to wild type (2 ± 0.5 mm). The clones had significantly longer lag phase (70 min) as compared to wild type (20 min) (Fig. 1). A, B and C were also resistant to diverse antibiotics belonging to different classes of antibiotics like beta-lactam, aminoglycosides, chloramphenicol and tetracycline, along with fluoroquinolones. This is shown by lower or no zone of inhibition of the clones as compared to the wild type (Table 1). However, the clones (A, B and C) were more sensitive to heat and osmotic stress than the wild type. This can be observed by 1.26-fold and 1.88-fold lower colony forming units of the clones compared to wild type for heat and osmotic stress, respectively (Table 1).

![Fig. 1 The difference in the lag phases of wild-type (Stm) and mutant (A) strains of *S. Typhimurium*. The lag phase was determined in the LB medium. The growth analysis was carried out in a 96-well microtiter plate at an absorbance of 600 nm on Synergy H1 microplate reader, Biotek (Winooski, Vermont, USA). A medium shaking speed (120 rpm) was used and optical densities were recorded after every 20 min for 20 h, at 37 °C. The Y-axis shows absorbance value at 600 nm and the X-axis represents the time interval in minutes (min.) The Clone A strain shows a lag of 70 min compared to the wild-type *S. Typhimurium* (Stm) strain. The inset represents a plot of 120 min where the actual lag phase is visible](https://example.com/fig1.png)
**Genotypic characterization**

The whole genome sequencing of three ciprofloxacin resistant clones of *S. Typhimurium* and parent strain was carried out. The sequences of all the clones were matched with the parent sequence and amongst themselves. All three clones had similar mutations, suggesting that they originated from one founder strain. Clones A and C were closely related and only 0.2% variation observed. However, A and B diverged at some point and a difference of 4.2% was observed while, the difference between B and C was 3.8% (Online Resource 2).

The detailed genomic analysis of clone A was carried out and the differences between clone A and B are discussed in a relevant section. The genome analysis revealed 40,858 single nucleotide polymorphisms (SNPs) and 113 INDELS distributed across the genome. There are total 4489 genes annotated in *S. Typhimurium* LT2. Total 3836 genes were mutated; that is 95% of *S. Typhimurium* genome was mutated. However, the majority of these mutations observed (15,344 mutations) were synonymous mutations. A total of 23.13% genes showed nonsynonymous mutations. Many genes showed multiple mutations comprising both synonymous and non-synonymous mutations (Online Resource 3). However, in these genes, the number of synonymous mutations were higher than non-synonymous mutations. This can be illustrated with one example. The gene *metH* had a total 61 mutations out of these, 57 were synonymous mutations and 4 mutations were non-synonymous. The mutations were also observed in intergenic regions. Total 4418 mutations were observed in intergenic regions. Current report is therefore the first report of high SNPs in a single isolate. The mutation caused by ciprofloxacin exposure was random. The Circos plot shows a uniform distribution of the SNPs and INDELS across the genome (Fig. 2A). One interesting observation was, the plasmid of 90 kb had only two mutations, which is significantly less than the genome. Transition mutations were four-fold compared to transversion mutations (Fig. 2B).

**Highly mutated genes and location of mutations**

The genes *metH, mukB, gcvP* and *gltB* showed over 45 mutations per gene. The interesting observation was that genes like STM4261 (*siiE*), the largest gene of 16,680 bp responsible for putative inner membrane protein showed the highest number (159) of SNP and nSNPs (58). However, gene STM2690 of length 1409 bp responsible for efflux transmembrane transport protein had ten SNPs but the number

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**Table 1** Phenotypic characteristics of wild type and Clones (A, B and C)

| Characteristics          | Wild type | Clones (A, B and C)a |
|--------------------------|-----------|----------------------|
| Colony size              | 2.0 mm    | 0.7 mm               |
| MIC                      | 0.05 µg/ml| 5.0 µg/ml            |
| Lag phase                | 20 min    | 70 min               |
| Antibiotic sensitivity   | Sensitive | Resistant to different classes of antibioticsb |
| Heat stress (55 °C—10 min) | 4.03 cfu/ml | 3.18 cfu/ml         |
| Osmotic stress (15% NaCl overnight) | 4.96 cfu/ml | cfu/ml               |

aThere was no significant difference between the three clones with respect to phenotypic characteristics and stress tolerance.

bAll the three clones were resistant to aminoglycoside, beta-lactam, chloramphenicol, tetracycline along with fluoroquinolone.
of nSNPs were only three. Another important gene muk B (4467 bp) involved in chromosomal condensation, segregation and cell cycle progression was mutated with 55 SNPs, it had two nSNPs (D294E and W945G). The location of SNPs in the present study is not specific but covers the entire chromosome as observed for the genes dnaE, gyrA, iroC, metH and rpoB (Fig. 3) point mutations which is against the observation that chromosomal location determines the type of mutation (Hudson et al. 2002).

The most probable genes that could contribute to antibiotic resistance

The mutants had 23 SNPs in gyrA with the common S83F nSNP. The 3D structure of gyrA shows Ser83 is replaced by bulky phenylalanine (Fig. 4) which creates a narrow, rigid pocket for DNA. Total 33sSNP were observed in gyrB. Besides gyrA we found nonsynonymous mutations in parC and polA. Mutations in parC are rare in Gram-negative organisms, yet we report 36SNPs in parC (with 3 nSNP T620A, A469S, A57S) which are different than those noted by the earlier researchers. Similarly, 14 SNPs with a single nSNP (nSNP P571S) in parE was observed; though parE mutation is seldom responsible for ciprofloxacin resistance. The genes acrA and acrB encoding efflux pump proteins were mutated with 14 and 26 SNPs, respectively, but only one nSNP at T270S was observed in acrA. The outer membrane channel protein encoding gene tolC had a total of 12 sSNPs and single nSNP at the position D457E. Moreover, two putative efflux pumps encoded by gene yegN and yegO also showed 41 and 45 SNPs with 7 and 2 nSNPs, respectively. Further, the efflux pump regulators marR, acrR and, soxR genes had 2, 2 and 3 sSNPs, respectively, but no non-synonymous mutations probably, showing efflux activity was important in the present study, which needs to be further explored. Genes encoding outer membrane protein OmpA and porins OmpC, OmpF show 5, 9 and 7 sSNPs with single nSNPs in ompA and ompF, respectively. The transcription and translation machinery also play a role in antibiotic resistance. The clone A showed 52 sSNPs in rpoC distributed across the gene except a narrow band of 400 bp devoid of mutation while a single nSNP and 32 sSNPs were observed in rpoB gene, probably indicating role of these genes in resistance as noted by other researchers. The ABC efflux pump gene metH (3771 bp) is responsible for iron transport. This gene had 4 nSNPs within 61 total mutations distributed over the entire gene but genes sulA, yggX important in a negative regulator of cell division and iron-sulphur binding and oxidative stress, respectively, showed only single sSNP each. The other genes with high mutations were sbcC, recD with 48 and 26 SNPs, respectively and 7 nSNPs; involved in SOS repair pathways. Two more genes invoC encoding ABC mediated involved in virulence and polA encoding an enzyme involved in DNA repair and proof-reading activity are highly mutated with 48 and 27 SNPs including 13 and 3 nSNPs, respectively. String analysis shows a relationship between sbcC, recD, gyrA and polA (Online Resource 4).

Functional analysis of the mutants

In most of the antibiotic resistant mutants, altered metabolism has been observed in DNA repair and replication pathways, oxidative stress management pathways and cell wall and cell membrane synthesis pathways. In the current study, we observed multiple proteins in these pathways were altered by non-synonymous mutations. Few of the important pathways are highlighted. Both Panther and David databases showed the following pathways were mutated (Fig. 5).
transcription regulation, 61–64% of genes analysed had mutation. The prominent genes found to be mutated were prpR, ssrB, arcB, rpoB and torB. Transmembrane transport mutations are common in antibiotic-resistant strains. We observed 40–53% of genes in this category were mutated (yajR, emrB, yeaN, ydhC, mdfA). Mutations were also observed in genes fimA, fimH, flID encoding proteins for cell adhesion; genes sopA, sopB, sseA, pipB encoding for pathogenesis and genes fimC, stiC, stiB responsible for pilus assembly pathways. Details of the genes described in the text with the position of nSNPs are given in Online Resource 3.

Comparison of mutations amongst three clones (A, B and C)

All the three clones developed maximum resistance after ten cycles but they gained this at different cyclic exposure (Online Resource 5). A gradual increase in MIC to 1.25 µg/ml in 4th cycle and 2.5 µg/ml in 6th cycle respectively was observed in clone A. Clone B showed a sudden increase within two cycles to 2.5 µg/ml and reached a maximum of 5 µg/ml after ten cycles while clone C showed a sudden increase to 5 µg/ml after four cycles. This shows clone B acquired resistance earlier but is less resistant to ciprofloxacin, as it shows a bigger zone of inhibition compared to clones A and C (Table 2). It is also less resistant to chloramphenicol, tetracycline and co-trimethazole (Online Resource 4). However, WGS shows it has higher SNPs and INDELs (Online Resource 2). It shows additional nSNP at T67S in parC and rpoC at P1026S amongst the genes important in antibiotic resistance, which need to be studied further.

Discussion

Many approaches are followed to understand the evolution of antibiotic resistance. The Adaptive Laboratory Evolution (ALE) is used in most of the studies (Oz et al. 2014; Munck et al. 2014; Kim et al. 2018). In ALE, two approaches were followed, first, incremental increase in the antibiotic concentration and second method stepwise exposure to antibiotics (Jahn et al. 2017). In the current study, stepwise antibiotic exposure was used. Ciprofloxacin was the antibiotic of choice. One of the most effective antibiotics used to treat P. aeruginosa infections is the second-generation fluoroquinolone, ciprofloxacin; introduced in 1987,
ciprofloxacin proved so effective at treating an infection that it rapidly joined the WHO list of medicines essential for basic healthcare (Wise et al. 1983). It has a broad spectrum of action, with good tissue penetration, oral absorption and favourable pharmacokinetics, making it ideal to treat a wide range of infections. Crucially, the cyclopropane moiety on the N atom of the heterocycle in ciprofloxacin increases its activity (compared with first generation fluoroquinolones such as norfloxacin) against *P. aeruginosa* by a factor of four (Jedrey et al. 2018). The second reason for the use of ciprofloxacin is its ability to induce more mutations by interfering with DNA replication and repair mechanisms (Maxwell et al. 2015; Bush et al. 2020). Suzuki et al. (2014) suggested that antibiotic resistance development in a gradual manner is unlikely. Evolution often happens in spurts (Neher 2013). In our experiments, we observed that the resistance increases by 25 and 50 folds after 4 and 6 cycles respectively in clone A. After 10th cycle, 100-fold increase in resistance was observed and this is the plateau of the resistance. *P. aeruginosa*, when exposed to constant sub-inhibitory concentrations or increasing concentration of quaternary ammonium compounds, showed a plateau in MIC after 10 cycles, leading to ciprofloxacin resistance (Kim et al. 2018; Voumard et al. 2020). This is probably because of regrowth of bacteria tolerant to antibiotic after initial kill of 90–95% population as we have noted the MIC after the growth up to approximately 10^7 cells. These tolerant bacteria remove ciprofloxacin by efflux, grow and also maintain the structure of the outer membrane showing involvement of different mechanisms simultaneously, i.e., initial stress...

Table 2 Antibiotic sensitivities of wild type and Clones A, B and C to different antibiotics

| Antibiotics       | Zone of inhibition (mm) |
|-------------------|-------------------------|
|                   | Wild | A   | B   | C   |
| Ampicillin (10 µg/ml) | 8.3  | Nil | Nil | Nil |
| Chloramphenicol (30 µg/ml) | 25   | Nil | Nil | Nil |
| Ciprofloxacin (10 µg/ml) | 36.3 | 10  | 15.6| 11.6|
| Kanamycin (30 µg/ml) | 17   | Nil | Nil | Nil |
| Tetracycline (30 µg/ml) | 23   | 8   | 8   | 8   |
| Enrofloxacin (10 µg/ml) | 27   | 13  | 13  | 16  |
| Gatifloxin (5 µg/ml) | 31   | 10  | 10  | 15  |
| Lomefloxacin (5 µg/ml) | 30   | 13  | 13  | 18  |
| Ofloxacin (2 µg/ml) | 32   | 10  | 10  | 20  |
| Levofloxacin (5 µg/ml) | 36   | 10  | 13  | 16  |
| Sparfloxacin (5 µg/ml) | 31   | 10  | 10  | 17  |

Overnight culture was diluted to obtain 5×10^5 cfu/ml of culture. It was spread plated on MHA plates. Antibiotic discs of different concentration of antibiotics were placed on the plates and incubated at 37 °C for 18 h. Zone of inhibition measured and compared with standard charts provides by CSLI (2020)
response of the surviving population. The tolerant bacteria probably pump out ciprofloxacin by efflux and grow (Moen et al. 2012). This shows higher concentrations of antibiotics present in the broth (10X MIC) as in our case or in industrial sewage or clinical environment which are more prone to development of antibiotic-resistant bacteria than the sub-inhibitory or lower concentrations used in food processing, animal husbandry or veterinary conditions as pointed out by Gullberg et al. (2011). Eagle (1948) noted that bacteria or fungi surviving above its bactericidal concentration had improved survival, leading to antibiotic resistances. Survival of *Staphylococci* 1000-fold above its MIC in penicillin was also observed (Kirby 1945; Prasetyoputri et al. 2019). However, MIC does not give any phenotypic or genotypic knowledge of the bacterium therefore; we have characterized the mutants (A, B, C). The observations from this study and previous study clearly suggest the randomness of the event. The founder mutation will lead to multiple mutations. However, in the current study, we have not sequenced the survivors of each cycle to screen for mutations. Our aim was to know what is the maximum resistance that could be achieved in ALE experiments.

The exposure of the wild-type strain to ciprofloxacin ten times its MIC showed lower growth rate as indicated with an increased lag phase (70 min) of the mutant strain (Fig. 1). This delay in growth probably is the strategy of bacteria to remain dormant, prevent itself from the harms of the antibiotic and prepare for the reproduction. The bacteria first become tolerant in the lag phase and this “tolerance by lag” leads to antibiotic resistance. (Fridman et al. 2014; Li et al. 2016). Fridman et al. (2014) observed that on exposing the *Escherichia coli* strain to ampicillin for 3, 5 and cycles increased the lag to 3.5, 6 and 10 h, respectively; therefore, authors presume lag phase is optimized to tolerate antibiotic stress. *S. Enteritidis* mutants (SE-M1, SE-M2), which presented reduced susceptibility to ciprofloxacin, exhibited the same growth as the parental strain; however, the mutants that gained resistance had longer lag phases than the parental strain and did not reach the same cell density in the stationary phase. On exposure to ciprofloxacin, a longer lag phase of approximately 12 h was observed in *S. Enteritidis* and *S. Typhimurium* mutants (Zhang et al. 2017).

All three mutants in the current study were sensitive to heat and osmotic stress (Table 1). Cellular responses, antimicrobial exposure, and other growth-compromising stresses, have all been linked to the development of antimicrobial resistance in Gram-negative bacteria resulting from the stimulation of protective changes to cell physiology, activation of resistance mechanisms, and induction of resistance mutations (Poole 2012). However, these mutants in present study were resistant to different antibiotics like ampicillin, kanamycin, tetracycline and chloramphenicol which belong to different classes of antibiotics viz: beta-lactam, aminoglycoside, tetracyclines and chloramphenicol along with fluoroquinolones having different modes of action. The resistance to different generations of ciprofloxacin was also observed (Table 2). Thus, ciprofloxacin induced antibiotic resistance causes multidrug resistance. The antibiotics probably have common targets or common mechanisms to develop resistance. Jahn et al. (2017) have shown that *E. coli* can adapt to resistant mutation in different antibiotics like amikacin, pipercillin and tetracycline irrespective of the method of selection or adaptation. Recently *E. coli* and *S. Typhimurium* mutants resistant to tetracycline (tigecycline), beta-lactam (mecillinam) and antimicrobial peptide (protease) showed increased sensitivity to antibiotic nitrofurantoin under laboratory condition (Roemhild et al. 2020).

Colony size of bacteria is phenotype variation to study genetic diversity and intermediate exposure to antibiotics may cause small size colonies (Lee et al. 2018). Small colony size and long lag phase have been associated with exposure to aminoglycoside stresses in *P. aeruginosa* (Wei et al. 2011). In clinical isolates of *S. aureus*, small colony variants have been linked to antibiotic resistant infections (Cao et al. 2017). The bacteria may show phenotypic switching of colony size shape or cell morphology to adapt to a hostile environment, and this needs to be studied further. In this study, small colony size has probably helped in ciprofloxacin resistance adaptation.

After 20 cycles of exposure to ciprofloxacin, 3 clones were selected for WGS. The aim was to understand what mutations led to resistance? Second aim was to find out whether all the clones have originated from the same parental strain or each evolved independently? There could be multiple evolutionary pathways which lead to each clone. However, sequencing each clone and its ancestor was beyond the scope of this work. The whole genome comparison of three clones suggests that they are derived from a single parental strain. This strain may have a founder mutation, probably in gyrase or in the DNA repair pathway. In an earlier study on evolution of resistance in *S. aureus* after 22 days of antibiotic exposure (trimethoprim, ciprofloxacin and neomycin), WGS was carried out. They sequenced 120 clones and found that treatments with alternating antibiotics changed the spectrum of resistance mutations. These genetic constraints affected the rate of evolution of mutations associated with the cross resistance amongst the drugs (Kim et al. 2014). *E. coli* on exposure to amikacin, tetracycline and pipercillin at the end of the study 96 clones were sequenced and found that cross resistance is similar in the 2 approaches used in the study leading to similar phenotypic and genotypic changes (Jahn et al. 2017).

This is the first report showing 40,000 SNPs in a single isolate after cyclic exposure to ciprofloxacin. Ten clinical *Pseudomonas aeruginosa* mutants resistant to amikacin showed 18,876 nSNPs and 81 nSNPs were identified.
Similarly, in 10 clinical isolates of Acinetobacter baumannii, 11,387 SNPs in the coding region, 42 INDELS and 33 antibiotic-related genes were observed. Recently from India, 74,713 SNPs have been reported in 60 isolates of MDR E. coli (Pu et al. 2019; Ragupathi et al. 2020) and in the current study the clone A showed 113 INDELS. Out of these, ten (nine insertions and one deletion) were in the coding region while others in the non-coding regions. These were present in genes not essential for antibiotic resistance. In Mycobacterium tuberculosis, short indels are shown to cause antibiotic resistance upon disruption (Godefroid et al. 2020). WGS of 25 antibiotic resistant clinical isolates of pneumococcal strains showed 131 to 171 INDELS and SNPs in the range 16,103–28,128 (Pan et al. 2018). However, the contribution of indels in antibiotic evolution is poorly understood.

All clones in the present study showed 80% transitions and 20% transversions. In earlier studies also, a similar ratio of 1:4 ratio of transversion: transition was observed in multidrug-resistant E. coli, M. tuberculosis and human genome (Guo et al. 2017; Payne et al. 2019; Ragupathi et al. 2020). In a previous study, it has been shown that fluoroquinolone (norfloxacin) antibiotics disrupt DNA repair pathways. This impaired mismatch repair pathway may also contribute to skewed ratios of transition to transversion (Jørgensen et al. 2013). The widespread biasness observed in transition: transversion ratios are unknown. Two main hypotheses to explain this phenomenon are; first the mutational hypothesis which shows there is transitional biasness and higher transitional rate in coding and noncoding regions therefore transition mutational rates of polymersases are higher than transversion rates (Zhang 2003; Jiang and Zhao 2006). The second selective hypothesis states that nonsynonymous transitions conserve important biochemical properties of original amino acids therefore natural selection does not favour transversions (Vogel and Kopun 1977; Miyata et al. 1979; Zhang 2000; Lyons and Lauring 2017). The synonymous mutations do not affect the encoded amino acid and therefore, have no role in adaptation or fitness of a bacterial cell. However, laboratory evolved populations of P. fluorescense and S. Typhimurium have shown increased fitness because of synonymous mutation (Lind et al. 2010; Bailey et al. 2014). This shows our knowledge about gene function and their role in bacterial genome evolution is still scarce, as pointed out by Bryant et al. (2012).

The ciprofloxacin induced mutation frequency across the genome was uniform in all three clones. The exception was the native plasmid (pSLT) (Fig. 2A). There are many genes in the current study that had multiple SNPs. However, the number of mutations per 1000 bp was uniform across the genome. Hence, bigger genes like STM_4261 (sitE) have more mutations compared to smaller genes like marR. However, there are many reports of mutation frequency biasness concentrated in a certain region of genome. In a recent study, Foster et al. (2013), reported that mutations are not randomly distributed along the chromosome. Instead, mutations fall in a wavelike pattern that is repeated in an almost exact mirror image in the two separately replicated halves (replicores) of the E. coli chromosome (Martincorena et al. 2012; Foster et al. 2013). Mutation is difficult to study because it is a highly random process. Mutations also affect variation in a manner that is highly entangled with the effects of natural selection. Future studies will undoubtedly take advantage of the increasing ability to examine variation at the whole-genome level to reveal much more about mutation and how it acts as an engine of evolution in bacteria (Hershberg 2015).

Mutations were uniformly distributed across the genome. Therefore, multiple metabolic pathways are affected. Since selection was based on the survival, all the three clones could grow on a rich medium, albeit slow. Most of the metabolic pathways are functioning or alternative pathways are functioning to keep the metabolism going. However, compared to wild type, the mutants grew slowly and formed small colonies. Survival and growth in high antibiotic concentration are the result of a contribution of multiple adjustments in many metabolic pathways. The mutant should have corrupted targets or targets that are not affected by antibiotics. All three mutants had mutations in DNA replication and repair pathways. The increase in MIC of ciprofloxacin is by alterations in primary and secondary drug targets gyrA, gyrB the DNA gyrases or parc parE the topoisomerases and regulator genes of efflux pumps like (marR, acrR and soxR); recently RNA polymerases rpoB, rpoC are also shown to improve import of ciprofloxacin in E. coli (Ricci et al. 2006; Pietsch et al. 2017). In previous studies, it has been shown that a single mutation in gyrA at S83 orA87 was observed in fluoroquinolone resistant Salmonella from human and animal origin. Similarly, resistance linking gyrB mutation at (S464T) and mutations at S80 or G78 of parc has been reported in ciprofloxacin resistant Salmonella (Chen et al. 2007). The second possibility is pumping out the antibiotic or restriction of the antibiotic entering into the cell. Active efflux and decrease in drug permeability also contribute to resistance to many antibiotics, including fluoroquinolones in clinical isolates of Salmonella, E. coli, Pseudomonas, etc.; (Toprak et al. 2011; Redgrave et al. 2014) acrAB-toIC efflux pump is the prominent one in S. Typhimurium. This pump consists of periplasmic accessory protein AcrA, transporter inner membrane protein AcrB and outer membrane channel protein (ToIC). The mutants had multiple mutations in transporter proteins. It has been shown that ciprofloxacin affects cell viability by causing oxidative stress. We observed at least 45 genes involved in oxidative stress were mutated. However, none of these mutations were biologically tested for their contribution to antibiotic resistance. The antibiotic resistance observed in the current study
may be the cumulative effect of mutations in many pathways or mutations in few genes. This needs further validation by detailed physiological studies. (Toprak et al. 2011; Redgrave et al. 2014).

There are few observations which we were unable to explain. The pSLT plasmid is a 90 kb native plasmid of S. Typhimurium. The entire plasmid had only two mutations. Since plasmid replication and maintenance are also carried out by genes on the genome and expected similar mutation rate as on the main genome. The transformation experiments in E. coli have suggested that probably the mechanism of replication and maintenance is different in chromosome and plasmid. The other reason could be that the dose of ciprofloxacin used in this study was high and the cost of maintaining resistant plasmid will be ameliorated in evolution experiments, as observed by Svara and Rankin (2011).

The important shortcoming of the current study was, we could not track the mutations from the first cycle to the 20th cycle of antibiotic exposure. This would have given an insight into the possible origin of hypermutation phenotype. However, clones A, B and C all share more than 98% of the mutations; suggests that all have originated from one hyper mutant.

**Conclusion**

A sensitive S. Typhimurium parent strain was treated with a high concentration of ciprofloxacin repeatedly. After ten cycles of ciprofloxacin treatment, ciprofloxacin resistant mutants were observed. The genome sequence of these mutants showed extensive mutations across the genome. Mutations were observed in gyrA and other DNA replication related genes. The initial mutations in replication and DNA repair machinery may have led to further mutations. It is necessary to study each metabolic pathway in depth. This kind of study may explain the resistance to ciprofloxacin and cross resistance to other antibiotics. This study will help to improve our understanding of antibiotic resistance mechanisms in bacteria.

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**Availability of data and material** The microbial culture is available with the corresponding author on request. The raw data of genome sequencing are available with GenBank with below mentioned accession numbers. Wild type S. Typhimurium and plasmid: local id: 1 and 2; Accession no. CP074092 and CP074093. Clone A and plasmid: local id SO_8752_A_cip & A_cip; Accession no. CP074094 and CP074095. Clone B and plasmid: local id SO_8752_Cip_2B and B_cip_Plasmid; Accession no. CP074096 and CP074097. Clone C and plasmid SO_8752_C_Cip and C_cip_Plasmid; Accession no CP074098 and CP074099.

**Code availability** Not applicable.

**Declarations**

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

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