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Fluoroquinolone Resistance in *Salmonella*: Mechanisms, Fitness, and Virulence

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Abstract

Fluoroquinolones are highly effective broad-spectrum antibiotics usually used for the treatment of human and animal infections, including salmonellosis. Fluoroquinolones act against *Salmonella* by inhibiting their DNA replication. However, several zoonotic serotypes of *Salmonella* have developed resistance or are less susceptible to fluoroquinolones. *Salmonella* presents its resistance by substituting amino acids within the topoisomerase subunits, overexpression of multidrug efflux pumps, or decreasing the expression of outer membrane porins. The resistance level is further increased with the plasmid-mediated quinolone resistance genes which could horizontally transfer the resistance from strain to strain. The development of resistance in *Salmonella* shows that it is a multifactorial process and the acquisition of fluoroquinolone resistance might have significant influences on the bacterial fitness and virulence. Due to the high level resistance against fluoroquinolones that has been observed in *Salmonella*, care needs to be taken to avoid misuse and overuse of this important class of antibiotics to minimize the occurrence and dissemination of resistance.

Keywords: fluoroquinolone, *Salmonella*, resistance, mechanism, fitness, virulence

1. Introduction

Zoonotic *Salmonella* infections are common causes of foodborne human infections worldwide [1]. Typhoid fever and gastroenteritis are the two main subtypes of salmonellosis. Typhoid fever, caused by *Salmonella* Typhi and Paratyphi, is a generalized infection and is fatal in about 10% of cases. The symptoms are usually very severe and show serious sequel. On the other hand, gastroenteritis is a localized infection of the gut leading to diarrhea, fever, nausea, and

headaches and usually caused by all other zoonotic *Salmonella* serotypes [1, 2]. Antimicrobial therapy is indicated in case of generalized infection with life-threatening situation. Presently, fluoroquinolones are the drug of choice for having the high level of clinical efficacy against most of the enteric pathogens including *Salmonella* [3, 4]. Probably, both human and veterinary uses have significantly contributed to the emergence of *Salmonella* strains with reduced susceptibility to fluoroquinolones [5–7]. In this chapter, the updates on the development and mechanisms of fluoroquinolone resistance in *Salmonella* and also the fitness and virulence changes after acquiring resistance are introduced.

2. Resistance

2.1. Mechanism of resistance

The genetic basis of fluoroquinolone resistance in *Salmonella* is the mutations in DNA gyrase (topoisomerase II) and topoisomerase IV, which are the intracellular targets of this class of antibiotics (**Figure 1**) [4, 8, 9]. Other mechanisms which contribute to the resistance of *Salmonella* to fluoroquinolone are overactivation of multidrug efflux pumps and decreased outer membrane permeability [10, 11]. In some clinical isolates of *Salmonella*, plasmid-mediated quinolone resistance (PMQR) genes also confer low-level quinolone resistance (**Figure 1**). Thus, the development of fluoroquinolone resistance in *Salmonella* is an endpoint result of the accumulation of several biochemical mechanisms [12].

2.1.1. Target mutations in DNA gyrase and topoisomerase IV

The quinolone resistance in *Salmonella* was firstly attributed to point mutations in the *gyrA* gene coding for the subunit A of DNA gyrase. In *Salmonella*, a single-point mutation in the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene, which have been clustered in a region of the protein between amino acids 67 and 106 [4], could mediate resistance to nalidixic acid and decrease susceptibility to ciprofloxacin [13]; however, for higher-level resistance to fluoroquinolones, the bacteria must attain additional mechanisms [14].

The most prevalent amino acid changes in nalidixic acid-resistant strains are Ser-83 (to Leu, Thr, Phe, Tyr, or Ala) and Asp-87 (to Gly, Lys, Asn, or Tyr) [6, 15–23]. In high-level resistant clinical *S. enterica* serovar Typhimurium isolates (e.g., MIC of ciprofloxacin, 32 µg/mL), double mutations at both residues 83 and 87 have been commonly observed [24]. Other than Ser-83 and Asp-87 amino acid substitution mutations at GyrA, *Salmonella* strains also have mutations at Ala-67 (to Pro), Gly-81 (to Ser, Asp, Cys, or His), and Leu-98 (to Val) (**Figure 2A**) [16, 18, 25]. Previously, Eaves et al. identified the mutations at Ala131 and Glu133 which are outside of the QRDR [26] which may have different types of mechanisms conferring resistance. Different serotypes may have different mutation positions in the *gyrA* gene. As reported by Giraud et al., the substituting amino acids at Ser83 and Asp87 were not equally distributed among different serotypes, and mutation in Asp87 prevailed in serovars Hadar and Kottbus and mutation at Ser83 were more prevalent in serovars Newport, Virchow, and Typhimurium

Figure 1. Mechanisms of quinolone resistance. Chromosomal mutations within the QRDRs of the genes encoding the subunits A and B of DNA gyrase and topoisomerase IV structurally change the target protein, reducing its drug-binding affinity. Chromosomal mutations lead to reduced outer membrane permeability and also increased expression of efflux pumps. Plasmid-encoded quinolone-resistant genes can produce Qnr target protection proteins and AAC(6')-Ib-cr acetyltransferase variants capable of modifying certain quinolones or QepA and OqxAB efflux pumps that actively extrude quinolones. The global regulatory proteins MarA, SoxS, and Rob are primarily responsible for activation of *acrAB* and *tolC* transcription.

[16]. These findings were further supported by the results documented by Allen and Poppe, who reported that all the *S. Bredeney* strains tested have a Ser83Tyr substitution, while all the *S. Senftenberg* strains tested have a mutation of Asp87Gly [27]. The acquisition of mutations in *gyrA* may play an important part in the dissemination of *Salmonella* of particular serotypes [28]. The source of the strains may also be the cause of differences in the distribution of mutations. Lindstedt et al. reported that the *S. Hadar* strains from Southeast Asia harbored mutation at Ser83, while *S. Hadar* strains from Southern Europe and North Africa have mutations at Asp87. They further explained that the differences might be due to the exposure of sublethal concentrations of quinolones in East Asia and Europe/North Africa [29]. In human strains of *S. Typhimurium* DT104 [22, 30] and farm animal isolates of *S. Hadar* and *S. Montevideo* [31], Asp87Asn was the most frequently detected mutation site, while Asp87Gly as the most common mutation in their panel of veterinary *Salmonella*, as reported by Piddock et al. [21]. In contradiction to these findings, the study of Griggs et al. documented that mutation at Ser83 is very common in veterinary isolates of *S. Newport* strains [18]. Strains having different substitutions at codons 83 and/or 87 and some other additional resistance mechanisms always show different susceptibility levels to quinolones. It might be due to the fact that sometimes

Figure 2. Homology modeling and the amino acid mutations of the subunit A (A, GyrA) and subunit B (B, GyrB) of DNA gyrase and subunit C (C, ParC) and subunit D (D, ParE) of the topoisomerase IV in *Salmonella*.

the same codon may have different substitutions which alter the binding capacity of quinolones to the DNA- gyrase complex. As reported by Levy et al., during selection process the nature of the FQ determines the *gyrA* mutation spectra [32]. For instance, selection with enrofloxacin appeared more likely to select for Ser83Phe substitutions, whereas selection with ciprofloxacin favored recovery of Asp87Gly mutants [16, 18, 22, 25, 26, 32, 33]. Levy et al. concluded that the emergence of quinolone resistance is usually because of the mutant strains being defective in methyl-directed mismatch repair [32].

As compared to *gyrA*, the mutations in *gyrB*, which encodes the B subunit of DNA gyrase, are less common (**Figure 2B**). Point mutation at codon 463 of *gyrB* with an amino acid substitution of Ser to Tyr has been reported in a quinolone-resistant post-therapy isolate of *S. enterica* serovar Typhimurium [34]. Complementation experiments provided evidence of the contribution of mutations in both *gyrA* and *gyrB* genes to the fluoroquinolone resistance [24]. For codon Ser464, it was considered as a mild spot since it was found altered (to Phe or Tyr) in a few independent FQ-resistant strains [33, 35–37].

The *parC* and *parE* genes of topoisomerase IV, which is the secondary target for quinolones, are homologous *gyrA* and *gyrB* in *Salmonella*. Generally, the quinolone-resistant mutations in *parC* occur at codon Ser80 and less frequently at codon Glu84 (**Figure 2C**). These codons are homologous to the Ser83 and Asp87 codons of DNA gyrase, respectively [33, 35, 38–40]. Studies showed that mutations in *parC* of *Salmonella* do not play an important role in quinolone

resistance as mutations in *gyrA* or they may only be required to achieve higher-level resistance [21, 23]. However, the experiment of transformation of *parC* mutants with wild-type *parC* shows an associated temporary reversal resistance to ciprofloxacin in *Salmonella* [37]. A study conducted by Piddock et al. reported that there are no *parC* mutants in 196 strains of veterinary isolates by using a Cip MIC of ≥ 0.5 mg/L as a cutoff value [21]. It was further supported by the study conducted by Giraud et al. who use in vitro and in vivo strains with Cip MICs of up to 16 mg/L [16]. Usually, mutant *parC* is detected in the *Salmonella* strains with two mutations in *gyrA*, while they have been observed in *E. coli* with only one *gyrA* mutation [24, 41–43]. In comparison to the strains without mutations, the Thr57Ser alone was able to increase the MIC of ciprofloxacin from 6 to 11 mg/L [38]. The Thr57Ser mutation which occurs outside the QRDR might have some different types of mechanism for quinolone resistance [29]. The substitution of amino acids (Ser458Pro) in *parE* of *Salmonella* was detected in human isolates from Hong Kong [38]. Mutations in ParE have been observed most rarely (**Figure 2D**) [44, 45].

2.1.2. Efflux pumps and porins

Different isolates may have same mutations in topoisomerases but present various quinolone-resistant phenotypes, other mechanisms such as overexpression of efflux pumps are also considered to contribute to the fluoroquinolone resistance [16]. Many studies have reported the contribution of overactivation of the efflux pumps to fluoroquinolone resistance in *Salmonella* (**Figure 1**) [11, 16, 40, 46].

In the past few years, many studies have been performed to investigate the role of efflux pumps to high- and low-level resistance in *Salmonella* [11, 40, 47]. The fluoroquinolone resistance level was decreased from 16- to 32-fold when the *acrB* gene (coding for the transporter) and *tolC* gene (coding for the outer membrane component of the efflux system) were inactivated or the AcrB efflux pump was inhibited by the inhibitor L-phenylalanine-L-arginine- β -naphthylamine (PA β N) [11]. AcrAB-TolC efflux system appears to be the main mechanism mediating quinolone resistance in *S. Typhimurium* DT104 strains with little contribution from *gyrA* mutations, while in *S. Typhimurium* DT204, both active efflux and accumulation of target gene mutations are required for the higher level of resistance to fluoroquinolone [47]. In a comparative study among the *S. Typhimurium* with *acrAB* operon mutation with its parent and AcrAB-overproducing strains, the results showed that the AcrAB efflux pump conferred significant resistance to a number of antimicrobials [48]. Giraud et al. reported that the resistance level of *S. Typhimurium* strains was strongly correlated with the expression of the AcrAB efflux pump [49]. In addition, the overexpression of efflux pumps (AcrEF and MdlAB) in a fluoroquinolone-resistant *Salmonella* Typhimurium strain S21 was also reported by Chen et al., but they are not contributed to the elevation of MIC to fluoroquinolones [50]. However, another study reported that when the AcrAB is out of function, the AcrEF can be recruited to efflux fluoroquinolones [51]. It is generally observed that the level of the increase of the susceptibility of bacteria is dependent on the specific FQ antibiotic used [40].

The *soxRS* and *marRAB* operons are also present in *Salmonella* (**Figure 1**) [46, 52–56]. Recently, it came to know that the mutations in the *acrAB* and *acrEF* operons also play an important role in

FQ resistance. In an in vitro study conducted on FQ-resistant strain of *S. Typhimurium*, substitutions at amino acids Ile75 and Glu76 were described in *acrR*, which is the local repressor of *acrAB* [57]. A study of whole genome sequencing identified a mutation of Gln78Stp on *acrR* in a resistant clinical *S. Choleraesuis* strain with *acrAB* consistently overexpressed [58]. However, the author further found that this internal stop codon in *acrR* was also present in susceptible isolates, and it may be a genetic diversity in the *Choleraesuis* serotype rather as FQ resistance. Some studies have shown that strains with wild-type topoisomerase genes and *mar*, *sox*, or *acrR* regulatory loci, yet exhibit the low level of FQ susceptibility and overexpression of *acrAB*, suggest that some other regulators may be involved. The *ramA*, from *S. enterica* serovar Typhimurium and other enterobacteria (but is absent in *E. coli*), may be the regulator locus, whose product is homologous to the *acrAB* transcriptional activators SoxS and MarA [59]. Experimentally, overexpression of *ramA* in *S. Typhimurium* can lead to multidrug-resistant (MDR) phenotype, and the *ramA* might act by direct activation or MarA-controlled genes [60]. However, it was further reported by the authors that their MICs in 15 clinical strains were never affected by the inactivation of *ramA* and finalized that *ramA* was not a common MDR mechanism in *Salmonella* [60]. In a study by Koutsolioutsou et al. [53], during the clinical usage of fluoroquinolones, resistant *S. Typhimurium* emerged with a mutation in *soxRS* gene, whose overexpression leads to the increase of the resistance level [53]. Neither was *marA* induced by a number of antimicrobials, salicylate did also induce *marA* [61]. It has been found that the treatment of aspirin might lead to high plasma concentrations and induces MarA overexpression [62]. Coban et al. documented that the medication of aspirin and ibuprofen during clinical treatment of salmonellosis could lead to development of resistance [63].

It is thought that quinolones particularly hydrophilic ones penetrate the cells through porin [8]. But it is not clear yet whether the absence of OmpF has any role in decreasing the levels of quinolone accumulation in cells. In a study by Piddock et al., the decrease or absence of OmpF or any other OMP was not associated with the reduced accumulation of quinolones in several strains [63]. As described by Lewin et al. and Ruiz et al., in comparison of the nalidixic acid-resistant and acid-susceptible strains of *Salmonella*, no difference was found between the OMP [23, 64], and Giraud et al. also reported that the expression level of porins in their *S. Typhimurium* MAR mutants was not reduced [49]. In contradiction to the previous studies, Howard et al. reported substantially the reduced level of OmpF expression in a *S. Typhimurium* strain which was resistant to ciprofloxacin, and Toro et al. reported an isolate of *S. Typhimurium* that lacked OmpF and presented MAR phenotype [65, 66].

Some previous studies reported that in quinolone-resistant *Salmonella*, there is an alteration in the expression of outer membrane protein or lipopolysaccharide [17, 21, 49]. However, the role of these alterations in decreasing the outer membrane permeability and association with quinolone resistance is not clear. Although the role of lipopolysaccharide composition on the accumulation of quinolones has been studied in several bacterial species, it remains unclear, and sometimes contradictory results have been reported [67–70]. It has been assumed that in quinolone-resistant *Pseudomonas aeruginosa* isolates, the amount of lipopolysaccharide increases and forms a permeability barrier which acts preferentially against hydrophilic quinolones [68]. The lengthening of the O-chains in the quinolone-resistant *Salmonella* mutants also contributes to the reduction of permeability of the outer membrane [49].

2.1.3. PMQRs

Transferable nalidixic acid resistance had been sought unsuccessfully in the 1970s [71], and plasmid-mediated resistance was thought unlikely to exist since quinolones are synthetic compounds and adequate resistance can arise by chromosomal mutations [72]. However, a plasmid-mediated quinolone resistance (PMQR) mechanism was firstly reported by Martinez-Martinez et al. in 1998 [73], 31 years after nalidixic acid began to be used clinically and 12 years after modern fluoroquinolones were approved for use [74]. Presently, there are five Qnr families which differ in sequence (QnrA, QnrB, QnrC, QnrD, and QnrS) about 40% or more from each other [75]. In addition, the substitutions of amino acids within each family lead to numerous variants, e.g., with more than 20 alleles, and *qnrB* is the most varied [75]. The first PMQR that could transfer low-level ciprofloxacin resistance to a variety of Gram-negative bacteria was discovered in a multiresistant urinary isolate of *K. pneumoniae* from Alabama. After the responsible gene (*qnr* and later *qnrA*) was cloned and sequenced [76], *qnr* was soon found at low frequency on plasmids in Gram-negative isolates around the world [77]. The mechanism of Qnr protein is on the basis of protecting the quinolone target [4]. The *qnr* can encode for a 219 amino acid protein which belongs to pentapeptide repeat family and has the ability to bind to and protect both DNA gyrase and topoisomerase IV from fluoroquinolones [76, 78, 79]. Structural study of a pentapeptide repeat protein from mycobacteria (MfpA) that contributes to quinolone resistance revealed that it formed a rodlike dimer with surface charge and dimensions similar to double-stranded DNA and could thus act as a DNA mimic [80]. The Qnr protein might have similar structure with MfpA [80, 81], but it can only protect targets when the concentration of quinolones is very low [76, 81, 82], and it has a glycine residue which separates the Qnr protein into two parts. Generally, Qnr genes located on plasmids carrying multiresistant determinants, especially those having genes encoding extended-spectrum β -lactamases [83], e.g., *qnrA* and *qnrB*, are commonly found as a part of complex *sul1*-type integrons [84].

The production of a modified aminoglycoside acetyltransferase (AAC(6')-Ib-cr) is another mechanism of resistance to ciprofloxacin. It can modify the drug and reduce the antimicrobial activity [85]. Based on an epidemiology study of human clinical strains, the detection frequency of the *aac(6')-Ib-cr* gene varied from 0.4 to 34% [86] and mostly from *E. coli* and *K. pneumoniae* strains. Recently, it has been identified in *Salmonella* spp. isolated from chickens in Japan and in *E. coli* of poultry origin in Spain or of pig origin in China [87–89]. The *aac(6')-Ib-cr* gene is distributed worldwide, stable in the environment over time, and prevalent in both FQ-susceptible and FQ-resistant isolates [90].

A conjugative plasmid with a multidrug efflux pump OqxAB was detected in clinical *E. coli* strains isolated from swine, and it contributes to the resistance of olaquinox [91, 92]. Recently, Wong and Chen [93] reported that *oqxAB* was found in *Salmonella* spp. isolated from retail meats in Hong Kong and it confers resistance to multiple antibiotics (olaquinox quinolones and chloramphenicol). Other isolates characterized in this study carried the *qnrS* and *aac(6')-Ib-cr* genes. Another important plasmid-mediated efflux pump (QepA) was found in a clinical strain of *E. coli* in Japan and presents MAR phenotype including aminoglycosides, fluoroquinolones, and broad-spectrum β -lactams [94].

PMQR genes facilitate the development of higher-level quinolone resistance and have been detected in various bacterial species in many countries around the world [77]. A previous study conducted on *Salmonella* (n = 1215) and *E. coli* (n = 333) isolates shows that six *qnrB* variants were found in 138 *qnrB*-positive isolates and majority of these isolated from turkeys [95]. Another study from Spain and Italy reported that the *qnrD* gene was identified in 22 *Salmonella* isolates of eight different serotypes [96]. A multiplex study about 107 strains of non-Typhi *Salmonella* isolated in the USA from 1996 to 2003 showed that *Salmonella* Bovismorbificans carried *qnrS1*, *qnrS2* was identified in *S. Anatum*, *qnrB2* was reported in *Salmonella* Mbandaka, and a new variant, *qnrB5*, was reported in seven *Salmonella* Berta isolates [84]. An international collaborative study conducted in 13 European countries showed that among isolates of *Salmonella enterica* of various origins (environment, food, humans, pigs, fowl, reptiles, sheep, turkeys), 59% (288/485) carried PMQR genes. The *qnrS1* gene was found in six isolates with one strain bearing the *aac(6')-1b-cr* gene. *qnrB19* and *qnrD* genes were found in two and one isolates, respectively [85]. A survey conducted on 13 nalidixic acid-resistant *Salmonella* spp. strains isolated from food animals in Colombia from 2004 to 2007 shows that 30.8% of the strains were positive for *qnrB*, while *qnrB19* was found in all cases [97]. A study performed in the Henan Province of China reported that four *Salmonella enterica* isolates were slightly resistant to ciprofloxacin. These isolates were obtained from humans, and the resistance was transferable by a 4.3 kb plasmid bearing the *qnrD* gene. It increased the MIC of ciprofloxacin about 32-fold in *E. coli* [98]. The *qnrD* gene has been identified in 22 out of 1215 *Salmonella* isolates obtained from different European countries, being either of human or animal isolates [95].

2.2. Development of resistance

The order of the implementation of different mechanisms in the process of resistance development has attracted broad attention. The background of highly resistant isolates is not clear, and the parent-susceptible strain cannot be obtained; thus, multiple studies have attempted to use the in vitro multistep selections to trace the development of resistance [12]. In in vitro selection of FQ-resistant *E. coli*, the first-step mutants may have a mutation in *gyrA* [99], the second-step mutants show overexpression of efflux pumps and multiresistant phenotype, and the third-step mutants present further enhanced efflux expression and more mutations in the DNA gyrase or topoisomerase IV. In clinical isolates of *E. coli*, the development process seems to be the same, and several mutations are needed for the high resistance [41, 100]. The in vitro selection of high-level FQ-resistant *Salmonella* is also a multistep process [49], but the sequence of mechanisms may be different from *E. coli*, where active efflux caused by the overactivation of AcrAB efflux pump appears before mutation in the *gyrA* gene [49] and no mutations were detected in *parC* in the third-step mutants; only the further overexpression of AcrAB efflux pump was found.

The emergence order of each individual mechanism may somewhat depend on the particular bacteria strains to which the antibiotic is imposed [12]. Luria-Delbruck dogma reported that mutations may occur prior to the exposure of antimicrobials. Under the drug concentrations within the mutant selection window (MSW), which was defined by Drlica, the bacteria with

specific mutation can be selected [101]. In a parent-susceptible bacterial population, there may be two types of resistant bacteria, topoisomerase mutants and efflux mutants. The number of topoisomerase mutants is far less than the diverse efflux mutants, since only specific substitutions in target topoisomerase can increase resistance and may induce fitness cost in bacteria [102]. The efflux mutants usually mediate low-level FQ resistance; thus, for the drug concentrations near the bottom of the MSW, most of the selected mutants would be efflux mutants [101]. When the drug concentration increased, the topoisomerase mutants would be selected and become prevalent. In a treated animal, the drug concentration may be changed temporally and spatially, so that the highly resistant strains may be easily obtained. The initial efflux mutants facilitated the further step of selection of topoisomerase mutants. Mutations in *gyrA* are frequently detected in clinical-resistant *Salmonella* isolates, but the sequence of the mutation is not clear till now [16, 33]. There are also studies reported that the efflux mutations can be induced in *gyrA* mutants [49]. Olliver et al. revealed that the AcrEF efflux would be activated when the IS1 or IS10 elements were inserted in promoter regions. However, this phenomenon was only observed in *S. Typhimurium* DT204, but not in *S. Typhimurium* phage-type DT104 [51]. The efflux mechanisms would present in specific strain according to the characteristics of the IS elements [12].

In clinical settings, underdosing seems to be inevitable and tends to easily select for resistance [103]. It was supported by Giraud et al., who conducted an in vivo experiment on chicken, and the results showed that a single low dose of enrofloxacin was enough to select resistant isolates [16]. Fluoroquinolones are usually used for population medication of sick animals by feed or water. The variations of drug intake among each animal lead to the underdosing and selection for resistance. In addition, the salmonellosis in swine and poultry is usually self-limited without symptoms, when the fluoroquinolones are medicated for treating other diseases; *Salmonella* is also under the antibiotic pressure and resistance selection may occur [1].

3. Fitness

Understanding the fitness effects of antimicrobial resistance evolution is crucial for controlling the spread of resistance, as the fitness cost induced by antimicrobial resistance is one of the few biological features of resistant organisms that can be leveraged against them [104]. The FQ resistance in *Salmonella* is not as frequent as it is in other members of *Enterobacteriaceae*. It might be due to the different FQ resistance mechanisms in *Salmonella*, which may have a prohibitive fitness cost which restrains the spread of resistance [16, 105]. Nevertheless, the emergence and spread of highly resistant strains were observed in the early 1990s in Europe with *Salmonella enterica* serovar Typhimurium phage-type DT204 and presently reoccurred in various serovars, such as Typhimurium, Choleraesuis, or Schwarzengrund [38, 106, 107]. This strongly stresses the necessity of further surveillance of FQ resistance and the prudent use of FQs.

In contrast to the wealth of information available on the mechanisms leading to high-level fluoroquinolone resistance in *Salmonella*, few studies to date have investigated the fitness costs associated with this phenotype [105]. Data from these studies suggest that mutations in

antibiotic target genes and overexpression of multidrug resistance (MDR) efflux pumps have been associated with fitness costs, including reduced growth rates and virulence, which may limit the survival of resistant strains in the absence of antibiotic selective pressure [108–110]. However, stabilization of resistance can occur through the development of compensatory mutations that restore fitness without loss of the original level of resistance [111].

In vitro selected FQ-resistant *Salmonella* by Giraud et al. showed smaller colony size on solid media than the susceptible counterparts [16]. Further experiments indicated that FQ-resistant mutants selected in vitro or in vivo (chicken) varied dramatically in the level of resistance to FQs and the growth characteristics in culture medium and in chickens in the absence of FQ antimicrobials. The in vitro selected mutants were highly resistant to FQs, showed significantly reduced growth rate in culture medium, and could not colonize chickens. In contrast, the in vivo selected resistant isolates exhibited intermediate susceptibility to FQs, had normal growth in liquid medium (slow growth on solid medium), and were able to colonize chickens at the extent comparable to or lower than that of the wild-type strains [105]. The fitness was restored partly after several passages *in vitro* or *in vivo* without antibiotics [105]. Another study described the fitness costs associated with high-level fluoroquinolone resistance for phenotypically and genotypically characterized ciprofloxacin-resistant *Salmonella enterica* serotype Enteritidis mutants (104-cip and 5408-cip, MIC >32 g/ml) [112]. Mutants 104-cip and 5408-cip displayed altered morphology on agar and by electron microscopy, reduced growth rates, motility and invasiveness in Caco-2 cells, and increased sensitivity to environmental stresses. Microarray data revealed decreased expression of virulence and motility genes in both mutants. Reverted clones for mutant 104-cip were obtained from separate lineages after several passages on antibiotic-free agar. All fitness costs, except motility, were reversed in the reverted strains. The altered porin and lipopolysaccharide (LPS) profiles observed in 104-cip were reversed, and additional mutations in SoxR and ParC were observed in the reverted strain. Randall et al. reported that the disinfectant-exposed *S. Typhimurium* strains, although MAR, were less fit, were less able to disseminate than the parent strain, and were not preferentially selected by therapeutic antibiotic treatment [113].

However, using in vitro competition experiments, Baker et al. assayed the fitness of 11 isogenic *S. Typhimurium* strains with resistance mutations in the FQ target genes, *gyrA* and *parC* [104]. The results showed that in the absence of antimicrobial pressure, 6 out of 11 mutants carried a selective advantage over the antimicrobial-sensitive parent strain, indicating that FQ resistance in *S. Typhimurium* is not typically associated with fitness costs. Double mutants exhibited higher expected fitness cost as a result of synergistic epistasis, signifying that epistasis may be a critical factor in the evolution and molecular epidemiology of *S. Typhimurium*.

The measurement of fitness can also be influenced by a number of factors. In classical competition assays [114, 115], antimicrobial-susceptible and antimicrobial-resistant organisms are competed over many generations, and their sensitivity and resistance are noted at various stages; hence, the fitness of the resistant strain to the sensitive strain can be calculated from the population trajectories [116–118]. For competitive growth assay, the selection of relative strain is critically important [119, 120]. It would be difficult to measure the effect of a specific mutation when using imperfectly isogenic strains [112, 117, 121, 122]. The enumeration and

culturing of bacteria may also be inaccurate due to the spontaneous mutations after exposed to low concentrations of antibiotics. Usually, *S. Typhimurium* disseminate through the macrophages after invading the intestinal epithelial cells (M cells). Intracellular assay using epithelial cell or macrophage as models can provide a suitable method for measuring fitness in *S. Typhi* [123]. Nevertheless, the antibiotic exposure, uptake, and cellular replication and division would affect the experimental accuracy and reproducibility. The *in vivo* competition experiment using animals as models is a well-described method. But it is hard to control the brief duration of infection, which may result in small variations in bacterial numbers and generations [104].

4. Virulence

There is an increase in the knowledge about the virulence mechanisms of *Salmonella* which led to a broad study of the *Salmonella* pathogenicity islands (SPIs) [124, 125] and other virulence determinants, such as virulence plasmid, adhesins, flagella, and biofilm-related proteins [126–130]. These virulence factors are controlled by an extensively complicated regulatory system, which correlates and synchronizes all the elements [131].

Several studies have investigated the impact of acquisition of fluoroquinolone resistance on the virulence of *Salmonella*. In a classical study by Bjorkman et al. investigating the virulence of nalidixic acid-resistant strain of *Salmonella* Typhimurium, they found that the virulence was reduced after acquiring resistance, but compensatory mutations occurred rapidly to restore the virulence without losing the resistance [132]. Other studies showed that the *acrB* gene [133] and *tolC* gene [8] may associate with virulence in *Salmonella*. The *acrB* mutant showed a reduced ability to colonize the intestine of mice. The *tolC* mutant was a virulent factor for mice when administered by the oral route. Fabrega et al. [134] documented that the activation of efflux, production of biofilm, and bacterial fitness are interrelated. The FQ resistance was linked to the reduction of biofilm production and decreased expression of *csgB* gene. Giraud et al. [135] reported that the *ramRA* mutations may reduce the invasiveness ability of clinical FQ-resistant *S. Typhimurium* strains, but this is strain-dependent. In a registry-based cohort study performed by Helms et al. [136], in comparison with infections by pansusceptible strains, the infections with FQ-resistant *S. Typhimurium* was associated with a 3.15-fold higher risk of invasive illness or death within 90 days of infection.

5. Conclusions

Fluoroquinolones are one of the most valuable antibiotics used for the treatment of a variety of infections in both humans and animals, especially salmonellosis. However, the usage has led to the prevalence of FQ resistance among different serotypes of *Salmonella*, and ultimately the clinical efficacy has been compromised. To preserve the efficiency of fluoroquinolones, the drugs should be used prudently, the residues in foods need to be monitored, and comprehensive

surveillance should be implemented to the resistance of bacteria from both animals and humans. Efflux pump inhibitors can be applied as new therapeutics and combined with fluoroquinolones to minimize the emergence of high-level resistance in different pathogens, including *Salmonella*.

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Competing financial interests

The authors declare no competing financial interests.

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