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Quinolone Resistance Mechanisms among Extended-Spectrum Beta-Lactamase (ESBL) Producing *Escherichia coli* Isolated from Rivers and Lakes in Switzerland

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Abstract

Sixty extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolated from rivers and lakes in Switzerland were screened for individual strains additionally exhibiting a reduced quinolone susceptibility phenotype. Totally, 42 such isolates were found and further characterized for their molecular (fluoro)quinolone resistance mechanisms. PCR and sequencing analysis were performed to identify chromosomal mutations in the quinolone resistance-determining regions (QRDR) of *gyrA*, *gyrB*, *parC* and *parE* and to describe the occurrence of the following plasmid-mediated quinolone resistance genes: *qepA*, *aac*(6')-*ib*-cr, *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*. The contribution of efflux pumps to the resistance phenotype of selected strains was further determined by the broth microdilution method in the presence and absence of the efflux pump inhibitor phe-arg-b-naphthylamide (PAβN). Almost all strains, except two isolates, showed at least one mutation in the QRDR of *gyrA*. Ten strains showed only one mutation in *gyrA*, whereas thirty isolates exhibited up to four mutations in the QRDR of *gyrA*, *parC* and/or *parE*. No mutations were detected in *gyrB*. Most frequently the amino-acid substitution Ser83→Leu was detected in GyrA followed by Asp87→Asn in GyrA, Ser80→Ile in ParC, Glu84→Val in ParC and Ser458→Ala in ParE. Plasmid-mediated quinolone resistance mechanisms were found in twenty isolates bearing QnrS1 (4/20), AAC-6'-ib-cr (15/20) and QepA (1/20) determinants, respectively. No *qnrA*, *qnrB*, *qnrC* and *qnrD* were found. In the presence of PAβN, the MICs of nalidixic acid were decreased 4- to 32-fold. (Fluoro) quinolone resistance is due to various mechanisms frequently associated with ESBL-production in *E. coli* from surface waters in Switzerland.

Introduction

Quinolones and fluoroquinolones (FQ) are broad-spectrum antimicrobial compounds and of critical importance in clinical use. They are the antimicrobials of choice in the treatment of acute gastrointestinal infections, urinary tract infections and respiratory tract infections. Resistance to FQ drugs increased in past years following the extensive and widespread use.

The mechanisms of action of FQs is due to inhibition of DNA replication and resistance to FQs is either based on (i) mutational alterations in the FQ target enzymes: DNA topoisomerase II (DNA gyrase) and topoisomerase IV, or (ii) plasmid-mediated quinolone resistance (PMQR) mechanisms or simply (iii) the decreased uptake of the drug due to the loss of a membrane-bound porin and/or drug extrusion via efflux pumps [1,2]. PMQR mechanisms include target protection proteins of the *qnr* gene family, enzymatic modification gene *aac*(6')-*ib*-cr and the efflux pump gene *qepA*. So far, five different *qnr* genes with a number of variations have been described: *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* [3–7]. The Qnr proteins belong to the pentapeptide family of proteins and share common structural properties. AAC(6')-*ib*-cr differs from the aminoglycoside acetyltransferase, AAC(6')-Ib, by two specific codon exchanges Trp102→Arg and Asp179→Tyr, which have been found to be necessary and sufficient for the ciprofloxacin (CIP) resistance phenotype [8]. The PMQR mechanisms confer only low level resistance to quinolones and thus, they were thought to enable the occurrence of chromosomal mutation which then lead to clinically relevant resistance levels [9].

In a previous study [10], 42 of 60 extended-spectrum β-lactamase-producing *Escherichia coli* strains isolated from rivers and lakes additionally displayed increased levels of nalidixic acid (NA) and/or CIP resistance.

The spread of *E. coli* co-expressing (fluoro)quinolone resistance along with extended-spectrum β-lactamases into the environment, especially into water bodies such as rivers, streams, wastewater effluents and lakes is worrisome. Rivers are considered to be of special importance as a reservoir of resistance genes since they are recipients of bacteria from different sources, e.g., wastewater plants, water of urban or industrial effluents, or agricultural activities [11].

The aim of this study was to identify and characterize quinolone and fluoroquinolone resistance mechanisms in higher generation cephalosporin resistant *E. coli* isolated from rivers and lakes in Switzerland.

Materials and Methods

Bacterial Isolates

Forty-two of 60 extended-spectrum β-lactamase-producing *Escherichia coli* strains isolated from rivers and lakes during a
previous study and additionally displaying increased levels of nalidixic acid (NA) and/or CIP resistance were selected for this study. The protocol for collecting the water samples and isolating the strains as well as the species identification, antibiotic sensitivity testing and the characterization of the ESBL-types were described in the previous study [10].

**MIC Determination of Nalidixic Acid and Ciprofloxacin**

MICS for NA and CIP of all 42 strains were determined using Etest strips (bioMe´rieux, Marcy l’Etoile, France). Of selected strains, MICs for NA (Sigma-Aldrich, Switzerland) were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) [12], in the absence and presence of 40 \( \mu \)g/ml phe-arg-b-naphthylamide (PA\( \beta \)N) (Sigma-Aldrich, Switzerland). As recommended by the CLSI, the \( E. coli \) ATCC25922 was included as quality control strain.

**Identification of Quinolone Resistance Determinants and DNA Sequencing**

DNA was extracted by a standard heat lysis protocol. Amplification of the quinolone resistance-determining regions (QRDRs) of the \( \text{gyrA} \), \( \text{gyrB} \), \( \text{parC} \) and \( \text{parE} \) genes and PCR based detection of the plasmid-mediated quinolone resistance (PMQR) markers (\( qnrA, qnrB, qnrC, qnrD, qnrS, \text{aac}(6\beta)-\text{Ib-cr} \) and \( \text{qepA} \)) was performed using primers listed in Table 1. Custom-sequencing was performed by Microsynth (Balgach, Switzerland) and the nucleotide and translated protein sequences were analyzed with CLC Main Workbench 6.6.1. For database searches the BLASTN and BLASTP programs of NCBI (http://www.ncbi.nlm.nih.gov/blast/) were used.

**Transferability of the PMQR Determinants**

Conjugation experiments were performed with the plasmid-free recipient strain \( E. coli \) HK225 strain (Strp\( ^{\text{R}} \), Rif\( ^{\text{R}} \)) [13] in order to assess the transferability of the plasmid-mediated quinolone resistance markers \( \text{qnrS1}, \text{aac}(6\beta)-\text{Ib-cr} \) and \( \text{qepA} \). Four \( \text{qnrS1} \) (OW1E2, OW54E2, OW60E1 and OW95E1), five \( \text{aac}(6\beta)-\text{Ib-cr} \) (OW8C2, OW29E1; OW55C1, OW77E1 and OW89E1) and one \( \text{qepA} \) (OW54E1) positive isolates were selected at random as putative conjugation donors. Briefly, single colonies of the donor and recipient were inoculated in LB broth (Difco Laboratories) and grown overnight at 37\( ^{\circ} \)C. Subsequently, equal volumes of the donor and recipient cultures were mixed and incubated overnight at 37\( ^{\circ} \)C without shaking. Serial dilutions were then plated on LB agar selection plates supplemented with a combination of 600 \( \mu \)g/ml streptomycin (Sigma-Aldrich, Switzerland), 100 \( \mu \)g/ml rifampicin (Sigma-Aldrich, Switzerland) and either 10 \( \mu \)g/ml cefotaxime (Sigma-Aldrich, Switzerland) or 20 \( \mu \)g/ml tetracycline (Sigma-Aldrich, Switzerland). The colonies from the selection plates were examined by pulsed field gel

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**Table 1. Target genes, primers and PCR reaction conditions used for strain characterization.**

| Target        | Primer       | Sequence (S’-3’)                  | Annealing temperature (°C) | Product size (bp) | Reference |
|---------------|--------------|-----------------------------------|----------------------------|------------------|-----------|
| Detection primers |              |                                   |                            |                  |           |
| \( qnrA \)    | QnrAm-F      | AGAGGATTTCCTACGGCCAGGG            | 54                         | 516              | [39]      |
|               | QnrA_R       | GCCATACCTACGGCGATACC              |                            |                  | [40]      |
| \( qnrB \)    | qnrB_F       | GATCGTGAAGGGCCGAGAGG              | 54                         | 476              | [41]      |
|               | qnrB_R       | ATGAGCAAGATGCGCTTTGA             |                            |                  | [41]      |
| \( qnrC \)    | qnrC-F       | GGGTTGTACATTTATGAAATC            | 47                         | 447              | [6]       |
|               | qnrC-R       | TCCACTTTACAGATTCTC               |                            |                  | [6]       |
| \( qnrD \)    | qnrD fw      | CGAGATCAATTTACGGCGGAA           | 54                         | 582              | [7]       |
|               | qnrD rev     | AACAGCGTACGGCGCTG                |                            |                  | [7]       |
| \( qnrS \)    | QnrSm-F      | GCAAGTTCTATTGAACAGGT             | 54                         | 428              | [39]      |
|               | QnrSm-R      | TCTAACCCTCGGTCGGCGGG             |                            |                  | [39]      |
| \( \text{aac}(6\beta)-\text{Ib-cr} \) |              |                                   |                            |                  |           |
|               | aac(6\beta)-Ib_For | TTGCGATGCTCCTATGAGCGTTA | 55                         | 482              | [42]      |
|               | aac(6\beta)-Ib_Rev | CTCAAGTGGCTGCGCGTGT             |                            |                  | [42]      |
| \( \text{qepA} \) | QEPfor       | TGGTCTACCCGATGCGACCTCA         | 56                         | 1137             | [43]      |
|               | QEPrev       | TGGATCCTGAGCCACTGGCCTGG          |                            |                  | [43]      |

**Sequencing primers**

| Target | Primer       | Sequence (S’-3’)                  | Annealing temperature (°C) | Product size (bp) | Reference |
|--------|--------------|-----------------------------------|----------------------------|------------------|-----------|
| \( \text{gyrA} \) | gyrA WF     | AAATCTGCCCCTGTTGCTTGGT          | 55                         | 344              | [41]      |
|        | gyrA WR      | GCCATACCTACGGCGTACC              |                            |                  | [41]      |
| \( \text{gyrB} \) | gyrB-F     | GAAATGACC CGCCGTAAA           | 55                         | 272              | [44]      |
|        | gyrB-R       | AGCGGCCATACCCACACCC             |                            |                  | [44]      |
| \( \text{parC} \) | parC WF     | CTGAATGCGCAGCGCGAATT            | 55                         | 168              | [41]      |
|        | parC WR      | GCGAAGTATTCGGCTGCTGTC          |                            |                  | [41]      |
| \( \text{parE} \) | parE-F     | CTGAATGCGCAGCGCGAATT            | 60                         | 483              | [44]      |
|        | parE-R       | GCGGTCCTCGGCGCGCTGGA           |                            |                  | [44]      |
| \( \text{qnrS} \) | qnrS1_orf_f | GTGTTAGTGTGTTGATGTAACGG        | 52                         | 825              | This study |
|        | qnrS1_orf_r  | CCTATGTCTATTATTGCAAGG           |                            |                  | This study |

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Table 2. Quinolone resistance, amino acid substitutions in the QRDR of GyrA, ParC and ParE proteins in terms of amino acid positions and PMQR determinants in extended-spectrum β-lactamase (ESBL) producing *Escherichia coli* isolated from rivers and lakes in Switzerland.

| Isolate a) | Origin | blaESBL | NA MIC (mg/ml) | CIP MIC (mg/ml) | Substitutions in the QRDR | PMQR determinants | Additional resistance profile |
|------------|--------|---------|----------------|-----------------|--------------------------|-------------------|-------------------------------|
| OW60E1     | Reuss  | CTX-M-27| 12             | 0.5             | –                        | –                 | AM, CF, CTX, TE, S             |
| OW54E2     | Aare   | CTX-M-55| 24             | 0.5             | –                        | –                 | AM, CF, CTX, TE, SMZ           |
| OW2E1      | Eibach | CTX-M-15| 256            | 0.19            | S83L                     | –                 | AM, AMC, CF, CTX, GM, TE, TMP  |
| OW64E2     | Rhein  | CTX-M-15| 256            | 0.19            | Ser83L                   | –                 | AM, CF, CTX, S, SMZ, TMP       |
| OW65E1     | Rhein  | CTX-M-15| 256            | 0.19            | Ser83L                   | –                 | AM, AMC, CF, CTX, SMZ          |
| OW67E1     | Rhein  | CTX-M-15| 256            | 0.19            | S83L                     | –                 | AM, CF, CTX, TE, S, SMZ, TMP   |
| OW7E1      | Marbach| CTX-M-3  | 256            | 0.25            | S83V                     | –                 | AM, CF, CTX, SMZ, TMP          |
| OW65E2     | Limmat | CTX-M-15| 256            | 0.25            | –                        | –                 | AM, AMC, CF, CTX, TE, S, C, SMZ, TMP |
| OW95E1     | Looze  | CTX-M-15| 256            | 1.5             | S83L                     | –                 | AM, CF, CTX, TE, S, SMZ, TMP   |
| OW1E2      | Birs   | CTX-M-5  | 256            | 2               | S83L                     | –                 | AM, CF, CTX, TE, S, C, SMZ, TMP |
| OW18E1     | Reuss  | CTX-M-15| >256           | >256            | S83L                     | –                 | AM, CF, CTX, TE, S, SMZ, TMP   |
| OW5E1      | Aare   | SHV-12  | >256           | >256            | S83L                     | S80R              | AM, AMC, CF, CTX, TE, S, C, K, SMZ, TMP |
| OW3E1      | Emme   | CTX-M-3  | >256           | >256            | S83L, D87N               | S80I              | AM, CF, CTX, TE, S, K, SMZ, TMP |
| OW4E1     | Aabach | CTX-M-1  | >256           | >256            | S83L, D87N               | S80I              | AM, CF, CTX, SMZ, TMP          |
| OW3E1      | Birs   | SHV-12  | >256           | >256            | S83L, D87N               | S80I              | AM, CF, CTX, TE, S, C, SMZ, TMP |
| OW6E2      | Rhein  | CTX-M-1  | >256           | >256            | S83L, D87N               | S80I              | AM, CF, CTX, TE, S, SMZ, TMP   |
| OW7E2      | Thur   | CTX-M-14 | >256           | >256            | S83L, D87N               | S80I              | AM, CF, CTX, GM, TE, S, SMZ, TMP |
| OW9E2      | Rotbach| CTX-M-27| >32            | >32             | S83L, D87N               | S80I, E84V        | AM, CF, CTX, TE, S, SMZ, TMP   |
| OW4E2      | Ergolz | CTX-M-27| >32            | >32             | S83L, D87N               | S80I, E84V        | AM, CF, CTX, TE, S, SMZ, TMP   |
| OW1E1      | Rhein  | CTX-M-14| >32            | >32             | S83L, D87N               | S80I, E84V        | AM, CF, CTX, SMZ               |
| OW1S1E2    | Rhein  | CTX-M-27| >32            | >32             | S83L, D87N               | S80I, E84V        | AM, CF, CTX, SMZ, TMP          |
| OW1E2      | Reuss  | CTX-M-15| >32            | >32             | S83L, D87N               | S80I, E84V        | AM, CF, CTX, TE, S, SMZ, TMP   |
| OW5E1      | Aare   | CTX-M-55| >32            | >32             | S83L, D87N               | S80I, E84G        | AM, CF, CTX, TE, S, C, SMZ, TMP |
| OW7E1      | Goldach| CTX-M-15| >32            | >32             | S83L, D87N               | S80I, E84G        | AM, CF, CTX, TE, K, SMZ, TMP   |
| OW8E1      | Reuss  | CTX-M-15| >32            | >32             | S83L, D87N               | S80I, E84V        | AM, CF, CTX, TE, K, SMZ, TMP   |
| OW11E2     | Birs   | CTX-M-15| >32            | >32             | S83L, L112V, L127M, A128S, K154R | S80I, S458A | AM, AMC, CF, CTX, GM, K, SMZ, TMP |
| OW3C1      | Birs   | CTX-M-15| >32            | >32             | S83L, D87N               | S80I, S458A       | AM, AMC, CF, CTX, GM, K, SMZ   |
| OW5E1      | Ergolz | CTX-M-14| >32            | >32             | S83L, D87N               | S80I, S458A       | AM, CF, CTX, GM, S, SMZ, TMP   |
| OW8E1C1    | Marbach| CTX-M-15| >32            | >32             | S83L, D87N               | S80I, S458A       | AM, AMC, CF, CTX, TE, K, SMZ, TMP |
| OW4E2      | Rhein  | CTX-M-15| >32            | >32             | S83L, D87N               | S80I, S458A       | AM, AMC, CF, CTX, TE, S, SMZ, TMP |
| OW1E2      | Rhein  | CTX-M-15| >32            | >32             | S83L, D87N               | S80I, S458A       | AM, AMC, CF, CTX, GM, TE, K, SMZ, TMP |
Results

Of the total of 60 ESBL-producing *E. coli* isolates, 42 co-expressing (F)Q resistance were further characterized. Based on the Etest assay, two strains showed intermediate resistance to NA, nine were classified as resistant to NA, two were resistant to NA and showed intermediate resistance to CIP and 29 were resistant to NA and CIP.

In 40/42 (95.2%) of the investigated isolates, at least one point mutation leading to amino acid substitution in any of the quinolone resistance-determining regions (QRDR) of *gyrA*, *parC* and *parE* was detected (Table 2). No mutations were identified in *gyrB*. High-level resistance to NA was always attributed to a single mutation in *gyrA*, whereas strains resistant to both tested (F)Qs exhibited at least one point mutation in two different topoisomerase genes; most frequently in *gyrA* and *parC*. A total of ten strains showed only one point mutation, nine had the predominate substitution in GyrA Ser83→Leu and one a Ser83→Val exchange. One strain (OW55E1) showed one substitution in GyrA (Ser83→Leu) and one in ParC (Ser80→Arg). Six strains had the following set of substitutions: GyrA (Ser83→Leu and Asp87→Asn), and ParC (Ser80→Ile). OW86E2 showed the same substitutions in GyrA, but in ParC (Glu84→Val). Twenty-one strains showed four substitutions in at least two different proteins: fourteen had substitutions in GyrA (Ser83→Leu, Asp87→Asn) and in ParC (Ser80→Arg) and in ParE (Ser139→Ala), five strains in GyrA (Ser83→Leu, Asp87→Asn) in ParC (Ser80→Ile, Ghu84→Val), OW54E1 in GyrA (Ser83→Leu, Asp87→Asn) in ParC (Ser80→Ile, Ghu84→Val) and OW60C2 in GyrA (Ser83→Leu, Asp87→Asn) in ParC (Ser80→Ile) and ParE (Glu160→Asp), respectively. Strain OW1C2 exhibited unique substitutions in GyrA: besides the frequently substituted position Ser83, here (Ser83→Thr), also positions Ile112→Val, Leu127→Met, Ala128→Ser and Lys154→Arg were affected. Additionally, substitutions in ParC (Ser80→Ile) and in ParE (Ser148→Ala) were detected.

Twenty isolates (48%) harbored plasmid-mediated quinoline resistance (PMQR) genes. Most frequently the *aac-6'-Ib-cr* gene (15/42 (36%)) was found, followed by *qnrS1* (14/42 (10%) and *qepA* (4/42 (10%)). The *qnrA*, *qnrB*, *qnrC* and *qnrD* genes were not detected in this study.

Intermediate resistance to NA was associated either with the presence of the plasmid-mediated resistance protein QnrS1 since in two strains (OW54E2 and OW60C1) chromosomal mutations and other PMQR determinants were absent, or with the presence of general efflux pumps.

Conjugation experiments revealed that two of the four *qnrS1*, three of five selected *aac-6'-Ib-cr* but not the *qepA* bearing plasmid were transferable to the recipient strain *E. coli* HK225.

Plasmid incompatibility typing of the transconjugants revealed that both the *qnrS1* and *aac-6'-Ib-cr* genes were present on IncF plasmids. Furthermore, all transferred PMQR determinants were detected on the same plasmids as the *blaESBL* genes. The contribution to FQ resistance of the plasmids bearing the PMQR determinants was measured by the difference in the MICs (NA and CIP) of the recipient strain (*E. coli* HK225) compared to the
transconjugants. The effect on the resistance to NA was minimal since the MICs increased only 1-3-fold. However, the resistance to CIP increased by factors of 4 to 16 in the presence of PMQR determinant harboring plasmids (Table 3).

In the presence of the efflux pump inhibitor PAßN, the MICs of NA decreased 4 to 32-fold. This finding confirmed that also efflux pump activity is involved in quinoline resistance (Table 4).

### Discussion

So far, there are only few studies that have investigated the occurrence of (fluoro)quinoline resistant Enterobacteriaceae in surface waters [15–18]. Rivers and lakes are known to be a relevant putative reservoir of multiresistant bacteria since they collect surface waters containing material from different origins, e.g., wastewater plants, water from urban or industrial effluents, agricultural activities, and they harbor an immense antibiotic resitome, distributed to pathogenic and nonpathogenic bacteria [19].

Quinolone resistance in E. coli is associated predominantly with DNA topoisomerase II (DNA gyrase) and topoisomerase IV involving specific mutations in the QRDRs of gyrA and parC genes [2]. A single mutation in the QRDR of gyrA seems to be sufficient to generate a high-level quinoline, and a double mutation even an high-level fluoroquinolone resistance phenotype [20]. It is supposed that these mutations occur in a stepwise manner [2]. The effect of these mutations may alter the formation of hydrogen bonds, and the negative charge of the amino acids seem to be important for quinolone interactions with the DNA-gyrase-DNA complex [21].

As in previous studies, we mainly detected the substitutions in terms of amino acid positions GyrA Ser83→Leu and GyrA Asp87→Asn, which are located within the QRDR. Interestingly, OW1C2, besides harboring a substitution at position GyrA Ser83→Val, contained additional substitutions at position 112, 127, 128 and 154. So far, these four substitutions have been found in a *Raoiella planticola* and a *Klebsiella oxytoca* isolate (except for position 154), as shown by sequence alignments using the NCBI BLASTP program (accession-numbers: BAN58234.1 and CAQ76691.1). Hüthero, very few substitutions have focused upon the role of amino acid substitutions outside the QRDR [22], hence little is known about their effect on resistance levels. Our finding suggests that in addition to the standard QRDR, additional sites in GyrA seem to play a role in FQ resistance. Further studies thus need to be conducted in order to further characterize the role of rare substitutions in GyrA.

In clinical *E. coli* isolates, quinolone resistance is mainly associated with mutations in gyrA whereas mutations in the topoisomerase IV genes, parC and parE, play a secondary role [2]. To date, mutations within parC and parE are always linked to mutations in gyrA, and it is supposed that mutations within these genes only occur if the strain already possesses a reduced sensitivity to fluoroquinolones by mutations in DNA gyrase. Therefore, additional mutations in parC and parE are supposed to play an important role in the formation of highly resistant strains [2,23]. As many as 71% of the isolates described in this study, in addition to showing one to two substitutions in GyrA, harbored substitutions in ParC. Interestingly, the substitutions in ParC were found predominantly at position Ser80, which, apart from substitutions in GyrA, is one of the most common alterations in FQ-resistant clinical isolates [24]. This finding is suggestive of the anthropogenic origin of these strains that were isolated from the aquatic environment.

PMQR is a phenomenon of increasing importance. The most relevant PMQR genes known to date are the acetyltansferase gene *aac(6’)-Ib-cr*, the *qnr*-like genes and genes encoding efflux pumps, such as *qepA* [25,26].

In the isolates described in this study, PMQR determinant *aac(6’)-Ib-cr* was the most prevalent (36%), followed by *qnrS1* (10%) and *qepA* (2%). The relatively high prevalence of *aac(6’)-Ib-cr* is not surprising since several reports showed that this gene is often present on the same plasmid as *bla* _ESBL_. [27,28]. *QnrS*-producing strains were frequently described in aquatic environments, and - as shown by a study by Cattoir and Co-workers [29] - *Vibrio splendidus*, an aquatic Gram-negative bacteria, was identified as a possible source for *qnrS*-like genes. Interestingly, in a previous study by Picão et al. [30], a *qnrS2* bearing *Aeromonas allosaccharophila* strain was isolated from Lake Lugano in the southern part of Switzerland. *QnrS* is found very rarely and only sporadically in clinical isolates in Europe [31]. Further, Liassine et al. [32] investigated human _ESBL-*producing Enterobacteriaceae_ isolates for *qnrA, qnrB* and *qnrS* collected by a Swiss clinical microbiology laboratory. However, from 155 analyzed isolates, they found only *qnrB* (5 *Citrobacter freundii*, 1 *Klebsiella pneumonia* and 1 *E. coli*) and *qnrB2* (1 *E. coli*). In contrast to our data, no *qnrS* variants were found in that study, indicating that *qnrS* is not characteristic for FQ-resistant human isolates in Switzerland.

Our conjugation experiments revealed that *qnrS1*, and *aac(6’)-Ib-cr* harboring plasmids were transferable to FQ-susceptible strains. The resistance phenotypes of the resulting transconjugants confirmed previous observations that the contribution of PMQR

### Table 3. Plasmid-mediated quinoline resistance mechanisms, replicon types of transferred plasmids and quinolone resistance levels of the transconjugants of selected *E. coli* isolates.

| Strain | Transferred gene | Replicon types | Etest MIC (µg/mL) [Fold-MIC increase vs. donor] |
|--------|-----------------|----------------|-----------------------------------------------|
| E. coli HK225 Strep*Rif* | – | – | NA (0.25) |
| TC_OW54E2 | qnrS1 | F | 12 [3] 0.38 [16] |
| TC_OW95E1 | qnrS1 | F | 8 [2] 0.25 [10] |
| TC_OW88C2 | aac(6’)-Ib-cr | F | 4 [1] 0.094 [4] |
| TC_OW29E1 | aac(6’)-Ib-cr | F | 6 [1.5] 0.094 [4] |
| TC_OW77E1 | aac(6’)-Ib-cr | F | 4 [1] 0.094 [4] |

*E. coli* HK225 Strep*Rif*, recipient strain resistant to streptomycin and rifamycin. TC_OW54E2, transconjugant receiving the plasmid from the donor strain OW54E2. doi:10.1371/journal.pone.0095864.003
### Table 4. MIC values of nalidixic acid in the presence and absence of Phe-Arg-β-naphthylamide in selected *E. coli* isolates.

| Isolate   | NA     | NA +PAβN | Fold-decrease | PMQR genes | gyrA | parC | parE |
|-----------|--------|----------|---------------|------------|------|------|------|
| OW1E2     | 128    | 16       | 8             | qnrS1      | 1    | –    | –    |
| OW1C2     | >1024  | 64       | >16           | aac-6'-lb-cr | 5    | 1    | 1    |
| OW3E1     | >1024  | 128      | >8            | –          | 2    | 1    | –    |
| OW8E1     | 512    | 16       | 32            | –          | 1    | –    | –    |
| OW14E2    | >1024  | 256      | >4            | aac-6'-lb-cr | 2    | 2    | –    |
| OW5E1     | >1024  | 256      | >4            | qepA       | 2    | 2    | –    |
| OW5E2     | 32     | 2        | 16            | qnrS1      | –    | –    | –    |
| OW5SC1    | >1024  | 64       | >16           | aac-6'-lb-cr | 2    | 1    | 1    |
| OW5SE1    | >1024  | 128      | >8            | –          | 1    | 1    | –    |
| OW6E1     | 16     | 2        | 8             | qnrS1      | –    | –    | –    |
| OW6E2     | 256    | 16       | 16            | –          | 1    | –    | –    |
| OW6SE2    | >1024  | 128      | >8            | –          | 2    | 1    | –    |
| OW7E1     | >1024  | 128      | >8            | aac-6'-lb-cr | 2    | 2    | –    |
| OW8E2     | >1024  | 128      | >8            | –          | 2    | 1    | –    |
| OW9SE1    | 256    | 16       | 16            | qnrS1      | 2    | 2    | –    |
| ATCC25922 | <1     | <1       | 1             | –          | –    | –    | –    |

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determinants to fluoroquinolone resistance levels of the plasmid-bearing strains is minor compared with the chromosomal mutations in DNA gyrase. The proposed role of these genes is that they enhance resistance to fluoroquinolones, supplementing chromosomal resistance genes [33]. Poor transfer of qnrS-encoding plasmids is confirmative of a previous study describing a qnrS-harboring clinical _E. coli_ isolate [31]. Nevertheless, our results demonstrate that _qnrS_ determinants are indeed transferable and capable of conferring very low level resistance, also in the absence of chromosomally-encoded (F)Q-resistance determinants.

Replicon typing of the transconjugants revealed that both _qnrS_ and _aac(6\')-Ib-cr_ are present on IncF plasmids. Additionally, the genes coding for ESBL were detected on the same plasmids. CTXM-M-15, the most prevalent CTX-M-type isolated from water bodies in Switzerland, is often associated with _aac(6\')-Ib-cr_ resistance genes and has been located frequently on IncF group plasmids [34]. IncF type plasmids carrying CTX-M-15 are an inhomogeneous group of plasmids with sizes that vary between 50–200 kb and often carry more than one replicon (e.g., _repFII_, _repFIA_ and/or _repFIB_) [34]. This multi-replicon plasmid-genotype was also observed in our study.

Besides chromosomal mutations and PMQR determinants, efflux pumps can also contribute in a more unspecific way to the (F)Q resistance phenotype. The effect of members of the resistance-nodulation-division (RND) family of membrane transporters can be investigated by inhibiting them with PA [35,36]. In this study, the MICs of NA decreased 4 to 32-fold in the presence of the inhibitor, which clearly confirmed that these efflux pumps play an important role in quinolone resistance. Sænz et al. [36] could already show that a single amino acid exchange in GyrA was not enough to confer a high level NA resistance phenotype in _E. coli_.

Recently, Sato et al. [37] investigated an _E. coli_ isolate whose MICs of CIP, levofloxacin and norfloxacin exceed the respective CLSI breakpoints, although it had no mutations in the QRDRs of either DNA gyrase or topoisomerase. The conclusion of their study was that FQ resistance can also arise due to concomitant acquisition of several PMQR determinates (in their study: _QnrS_ and _OqxAB_, in combination with overexpression of _acrAB-TolC_ and other chromosome-encoded efflux pumps). Our study yielded no isolate of this kind.

In summary, the acquisition of a high-level (F)Q resistance phenotype often includes combination of multiple mechanisms such as acquisition of mutations in DNA gyrase and topoisomerase genes as well as enhanced efflux and these processes can be accelerated by the presence of PMQR-determinants [2]. Our findings suggest that this holds true also for our FQ-resistant strains isolated from the aquatic environment.

It is possible that as a consequence of excretion of unmetabolized quinolones into wastewater by humans and animals, the environmental quinolone accumulation has contributed to the high prevalence of quinolone resistant _E. coli_ in the water environment [9,38].

This study provides further evidence for the importance of the water-related environment as a reservoir for this emerging threat of (F)Q resistance. Moreover, the resistance characteristics of the isolates described in this study indicate that multilateral exchange of genetic material between bacteria of both anthropogenic and environmental origins is currently occurring and presents a phenomenon of growing importance. It is, finally, worrisome that more than two thirds of ESBL producers carry resistance determinants against (F)Qs, thus defying the two currently most popular antibiotic options for human therapy, β-lactams and FQs.

**Author Contributions**

Conceived and designed the experiments: RS HH KZ. Performed the experiments: KZ HA. Analyzed the data: RS KZ MI. Contributed reagents/materials/analysis tools: RS KZ. Wrote the paper: KZ MI RS.

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