High Carriage of Plasmid-Mediated Quinolone Resistance (PMQR) Genes by Cefotaxime-Resistant *Escherichia Coli* Recovered from Surface-Leaking Sanitary Sewers

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Research Article

Keywords: Cefotaxime-resistant bacteria, *Escherichia coli*, Sewage, Sanitary sewers, Antibiotic resistance, Plasmid-mediated quinolone resistance

Posted Date: July 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-699642/v1>

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Version of Record: A version of this preprint was published at Archives of Microbiology on January 9th, 2022. See the published version at <https://doi.org/10.1007/s00203-021-02627-6>.

Abstract

There is a rapid rise in the incident of quinolone resistant bacteria in Nigeria. Most studies in Nigeria have focused on isolates from the clinical settings, with few focusing on isolates of environmental origin. This study aimed to investigate the antibiogram and carriage of plasmid-mediated quinolone resistance (PMQR) genes by quinolone-resistant isolates obtained from a pool of cefotaxime-resistant *Escherichia coli* (*E. coli*) recovered from sewage leaking out of some broken sanitary sewers in a University community in Nigeria. Isolation of *E. coli* from the sewage samples was done on CHROMagar *E. coli* after enrichment of the samples was done in Brain Heart Infusion amended with 6µg/mL of cefotaxime. Identification of presumptive *E. coli* was done using molecular methods (detection of *uidA* gene), while susceptibility to antibiotics was carried out using disc-diffusion method. Detection of PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, *qepA* and *oqxAB*) was done using primer-specific PCR. A total of 32 non-repetitive cefotaxime-resistant *E. coli* were obtained from the sewage, with 21 being quinolone-resistant. The quinolone-resistant isolates showed varying level of resistance to the tested antibiotics, with imipenem being the only exception with 0% resistance. The PMQR genes: *aac(6')-Ib-cr*, *qnrA*, *qnrB*, *qnrS* and *qepA* and *oqxAB* were detected in 90.5%, 61.9%, 47.6%, 38.1%, 4.8% and 0% respectively of the isolates. The findings of this study showed a high level of resistance to antibiotics and carriage of PMQR genes by quinolone-resistant *E. coli* obtained from the leaking sanitary sewers, suggesting a potential environmental and public health concern.

Introduction

The wastewater environment, specifically composed of high concentrations of bacteria, nutrients, and suspended solids is a known potential hub for the inception of antibiotic resistance and the stimulation of horizontal gene transfer [1]. The high levels of antibiotics typically discharged in human wastes into the environment, is known to sufficiently inhibit susceptible bacteria and provide a selective advantage to the resistant ones [2], making sewage and other wastewaters a favorable environment for bacterial growth and the likely spread of antibiotic resistance [3]. The typical end products (effluent or bio-solids), or non-typical routes like seepage through cracks in tanks, flooding or animal contact, and also aerosols, are channels by which bacteria in wastewater may enter the into the environment [4].

The use of quinolone antibiotics in human and veterinary medicine is on the rise due to their efficacy as important weapons against pathogenic bacteria via their ability to selectively inhibit the synthesis of DNA. These antibiotics constitute about 17% of the antibiotic global consumption [5], and are frequently encountered in domestic and hospital sewage because they are largely excreted unchanged in urine and faecal materials. [6]. Quinolones are not easily degraded, and are adsorbed to sewage sludge during biological treatment [7]. In a study carried out by Lindberg et al. [8] and Fink et al. [9], they found out that fluoroquinolones are hardly removed in effluent treatment plants, with approximately 70% remaining attached the sludge. In addition to this, the low effectiveness of treatment plants cannot be overlooked in the continuous spread of multidrug-resistant (MDR) bacteria into water aquifers [10].

The occurrence of quinolones in the environment has caused some substantial effects on the microbial structure and community, due to the exposure of these flora to the antibiotic at sub-lethal concentration, making them develop resistance to these agents. This eventually makes the environment a repository of antibiotic resistant bacteria carrying several resistance genes and other mobile genetic elements (MGE) [11, 12].

The detection of PMQR determinants in the late 1990s has opened the doors of research into the mechanisms of quinolone resistance in bacteria and their transferability. Different resistance mechanisms have so far been described and they range from the qnr gene families which encode proteins that protect DNA gyrase and topoisomerase IV from the destructive action of quinolones; *aac(6')-Ib-cr* gene, coding for aminoglycoside acetyltransferase, which are involved in the acetylation of fluoroquinolones such as ciprofloxacin and norfloxacin; and two active efflux pumps, *qepA* and *oqxAB*, which confer decreased susceptibility to fluoroquinolones [13, 14].

Most data on quinolone resistance in Nigeria have focused majorly on the occurrence of these genes in clinical isolates, therefore making data related to the environment very few. This study therefore aimed at investigating the carriage of PMQR by *Escherichia coli* isolated from broken sanitary sewers within a University community.

Materials And Methods

Study site and sample collection

The study was carried out within the environment of an institution of higher learning in South-west Nigeria. Sanitary wastes from the students' hall of residence, staff quarters, other residential areas and the business pools are being channeled to a treatment plant via sanitary sewers located underground all across the University premises. A common challenge with this system of waste transportation is the breakage of conveying pipes as a result of several factors. This often times leads to the outflow of the content of the pipes into the environment, creating a very horrible sight and foul-smelling odour. Although this situation is remedied by the concerned unit of the University, it takes time before a positive action is taken on such breakage. The horrible sight of such breakage was a major motivating factor that stimulated the execution of this study. Sewage (SW) from the burst pipes were collected aseptically at different dripping points and transported to the laboratories on ice for microbiological analysis. Samples were analyzed about one hour after collection.

Isolation of cefotaxime-resistant *Escherichia coli* from the samples

Aliquot (2mL) of each sewage sample was inoculated into 3mL Brain Heart Infusion, to which a disc of 30µg cefotaxime (Oxoid, UK) has been added, giving a final concentration of 6 µg of the antibiotic in the tube. The tubes were incubated overnight at 35±2°C. Using the streak plate technique, a loopful of each of the cefotaxime-amended samples was inoculated onto the surface of already prepared CHROMagar *E.*

coli (CHROMagar, France) and incubated for overnight at 35±2°C. Blue colonies presumptive of *Escherichia coli* were picked, purified and stored in glycerol stock (15% glycerol).

Molecular characterization of cefotaxime-resistant *Escherichia coli*

The genomic DNA of presumptive *Escherichia coli* was extracted using the boiling lysis method according to the modified method of Gugliandolo et al. [15]. The identity of the isolates was confirmed by detecting the presence of *uidA*, a housekeeping gene in *Escherichia coli*. The method of Janezic et al. [16] was used for the PCR amplification of the gene. *Escherichia coli* OG1a possessing the *uidA* gene as reported by Adekanmbi et al. [17] was used as the positive control. Isolates possessing the *uidA* gene were selected for further studies. The details of the *uidA* primers are shown in Table 1.

Susceptibility to antibiotics

The confirmed *Escherichia coli* were subjected to a panel of eight antibiotics using the Kirby-Bauer disc diffusion method [20]. The procedure used for the testing, including the choice of medium, inoculum standardization, reading and interpretation of the zone diameter were as described by CLSI [21]. Isolates that were resistant to ciprofloxacin (a fluoroquinolone), were selected for the detection of the PMQR genes which encode quinolone resistance.

Detection of PMQR genes

The detection of six genes encoding PMQR in confirmed ciprofloxacin-resistant *Escherichia coli* was done using the methods of Wu et al. [18] and Park et al. [19]. The detection of *qnrA*, *qnrB* and *qnrS* and *qepA* was carried out using a duplex PCR, while a monoplex PCR was used for the detection of *aac(6')-Ib-cr* and *oqxAB*. The PCR reaction volume was 25µL consisting of 5× PCR Master Mix (Jena Bioscience, Germany), forward and reverse primers (0.25 µL each, DNA template (1 µL), with PCR quality water making up the volume. The oligonucleotide primers used in this study are highlighted in Table 1.

Results

Thirty-two non-repetitive cefotaxime-resistant *Escherichia coli* isolates were recovered from sewage obtained from the burst sanitary sewers. Twenty-one of the isolates representing 65.6% of the total isolates were resistant to ciprofloxacin (CIP), while 34.4% (11/32) were sensitive to the antibiotic. The frequency of CIP-sensitive and CIP-resistant *E. coli* obtained in this study is shown in Figure 1.

The susceptibility of the isolates to eight antibiotics is highlighted in Table 2. There was varying level of resistance to the antimicrobials, with the only exception being imipenem, to which no resistance was observed. Six of the isolates (28.6%) showed resistance to amoxicillin-clavulanate, seven (33.3%) were resistant to ceftazidime, 10 (47.6%) showed resistance to gentamicin and 19 (90.5%) were resistant to trimethoprim/sulfamethoxazole. There was 100% resistance to each of cefotaxime, cefpodoxime and cefuroxime.

Table 3 is showing the distribution of PMQR genes in the CIP-resistant *Escherichia coli* obtained from the sanitary sewer while Table 4 is showing the frequency of occurrence of the PMQR genes detected. All the targeted PMQR were detected in varying number of isolates, with the notable exception being *oqxAB* gene, which was not detected at all in any isolate. Of the six PMQR genes targeted for detection in this study, *qnrA* was detected in 13 (61.9%) of the isolates, making it the second most prevalent PMQR gene, after *aac(6')-Ib-cr* (which was the most prevalent) with 19 (90.5%) of the isolates harbouring it. The frequency of occurrence of *qnrB*, *qnrS* and *qepA* was 47.6% (10/21), 38.1% (8/21) and 4.8% (1/21) respectively. *oqxAB* was not detected in any of the twenty-one CIP-resistant *Escherichia coli*. One of the isolates (*E. coli* SWS2a), did not carry any of the six PMQR genes targeted, while six isolates carried one PMQR gene only. Four isolates carried two and three PMQR genes respectively, while four of the six PMQR genes were carried by 6/21 of the isolates. There was co-occurrence of PMQR genes in 14 (66.7%) of the isolates.

Discussion

The fluoroquinolone antibiotics have been used extensively in the treatment of human and animal infections, mainly because of their selective inhibition of the synthesis of DNA in bacterial cells. These antibiotics also account for about 17% of the global use of antibiotics [5]. Ciprofloxacin, a second-generation fluoroquinolone has been widely used in the treatment of bacterial infections of human origin and is the one of the most abundant antibiotics detected in municipal biosolids, owing to their widespread use and the fact that it is excreted mostly unchanged in urine and faecal materials, making them available in high concentration in wastewater from hospitals and sewage [5, 6, 22]. The major difference between the quinolones of the first and second generation is the possession of a fluorine atom at sixth carbon of the quinolone moiety by the latter, and they exhibit a high level of potency against gram-negative bacteria and several gram-positive genera. In addition to this however, ciprofloxacin shows a high level of activity against *Pseudomonas aeruginosa*, making it a drug of choice in the treatment of a variety of infections [23, 24].

Resistance to fluoroquinolones has increased worldwide over the past few years and this has limited available treatment options, resulting in several cases of resistance and treatment failure [25–27]. The rising tide of antimicrobial resistance has been majorly linked to the widespread and continuous use of antibiotics in agriculture, human medicine and veterinary practices. This has placed a lot of selective pressure on bacteria, making it difficult to curtail the geometric rise in antibiotic resistance over the years [28–30]. Sewage and wastewater on the other hand have been taunted as ideal environment for the acquisition of antibiotic resistance genes (ARG) and this has been largely linked to influence from anthropogenic activities [31, 32].

In our study, a total of 21 CIP-resistant *E. coli* was obtained from the pool of 32 cefotaxime-resistant isolates recovered from the sanitary sewers. Bacteria showing resistance to quinolones, especially *E. coli* have been well reported in several studies. Osinska et al. [32] reported the isolation of FQRB from sewage and surface water samples in Poland, while studies carried out by Guillard et al. [33], Yang et al. [34] and

Ranjbar et al. [35], pointed out the fact that *Escherichia coli* ranked as the most frequently occurring FQ-resistant bacteria from water, wastewater, animals, food and the clinical environment. A study carried out in Nigeria by Ehwarieme and his colleagues [36], reported the occurrence of FQ-resistant enteric bacteria from samples of human and animal origin, with *Escherichia coli* being the predominant organism obtained.

In this study, the target PMQR genes with the exception of *oqxAB*, were detected in twenty of the twenty-one CIP-resistant isolates obtained, with one isolate devoid of any of the six PMQR genes. The *qnr* determinants, which have been reported to occur on chromosomal DNA in many environmental strains [37] are considered exclusively plasmid-borne in the *Enterobacteriaceae* [38]. These genes are proteins of the pentapeptide repeat family and are concerned with the protection of DNA gyrase and topoisomerase (IV) from the deleterious effect of the quinolone antibiotics. They are very diverse group of PMQR determinants and have been found resident in bacteria of animal, human and environmental origin. In this current study, three variants of the *qnr* gene were targeted with varying degree of detection observed in the CIP-resistant isolates. Of the *qnr* determinants, *qnrA* was the most prevalent in this study, with thirteen of the isolates (61.9%) harbouring it, followed by *qnrB* with 10 isolates (47.6%) and *qnrS* 8 (38.1%). The relative occurrence of the *qnr* genes in this study is higher than the frequency of occurrence in a study carried out by Ranjbar et al. (2019), where they reported the detection of *qnrA*, *qnrB* and *qnrS* in 1%, 9% and 28% of the total isolates obtained from water sources in Iran, while a study on hospital wastewater in the same country by Ranjbar et al. [39], reported the detection of *qnrB* and *qnrS* in eleven (45.8%) and seven (29.2%) respectively of the *E. coli* isolates obtained. However, none of the isolates in their study harboured *qnrA* gene, which is contrary to this study.

The quinolone efflux pump (*qepA*) which was first described in a study by Yamane et al. [40], confers on the bacterial cell, the ability to reduce quinolone accumulation in the cell. It has been detected widely in many gram-negative genera in most countries of the world. In our study, one isolate (4.8%) of the entire twenty-one CIP-resistant bacteria, harboured this particular gene. The low frequency of occurrence of the gene has been reported in several studies, and could be linked to its limited host spectrum. Chen et al. [41] reported a percentage occurrence of 2.6% for *qepA* in 1022 *E. coli* isolates obtained in their study, while Cattoir et al. [42] and Yamane et al. [43] detected *qepA* in 0.8% and 0.3% of the total isolates obtained in their respective studies.

There is limited data on *oqxAB*, an efflux pump-mediating gene, which was discovered in the late 2000s. Its role as a PMQR was not identified until then, thereby making data available on the gene quite limited. It confers resistance to the veterinary growth-promoting quinoxaline-di-*N*-oxide olaquinox, and was first discovered in *Escherichia coli* from swine [44–46]. The frequency of occurrence of the gene in most studies is very low in comparison with other PMQR and this could explain the absence of the gene in all the quinolone-resistant isolates obtained in this study.

Aminoglycoside acetyltransferase (*aac(6')-Ib-cr*) acts by causing the acetylation of quinolones, causing the modification of the antibiotics, which eventually leads to its inactivation. On its own, the resistance

conferred on bacteria by aminoglycoside acetyltransferase is typically low, but it becomes elevated with the presence of *qnr* gene in the cell. In most reports on quinolone resistance in bacteria, *aac(6')-Ib-cr* appears to be the commonest PMQR determinants [46–48], and this corroborates the observation in this study, where 90% (19/21) of the quinolone resistant bacteria carried the gene. This observation is in agreement with several other reports where the occurrence of the gene and its high prevalence in clinical samples and wastewater of hospital origin have been widely reported. In a study on hospital wastewater carried out by Chandran et al. [49], they detected *aac(6')-Ib-cr* in 89% of their isolates, while Guillard et al. [33] and Piekarska et al. [27] detected the gene in 96.7% (118/122) and 85.7% of isolates obtained from samples and wastewater of clinical origin in their respective studies. In a different twist to the aforementioned, Su et al. [50] reported the detection of *aac(6')-Ib-cr* in 5.1% of the total 452 quinolone-resistant isolates from a municipal treatment facility in China, making it the least occurring PMQR determinant in their study.

Limitations Of The Study

Despite the abundance of PMQR genes in the isolates obtained in this study, the limitations stem from the fact that the sampling process stopped as soon as the repair works on the burst sewers commenced, making it difficult to get more samples for the isolation of more resistant isolates. Despite this limitation, other studies could venture into the detection of PMQR in other bacterial genera and the genomic characterization of isolates obtained from such environment using advanced molecular biology tools. This will enrich the few available reports on PMQR in isolates of environmental origin.

Conclusion

This present study has provided some information on the occurrence of *Escherichia coli* carrying PMQR genes in sewage samples, with *aac(6')-Ib-cr* being the most predominant PMQR, while *oqxAB* was not detected in any of the isolates. There was different level of resistance shown to the tested antibiotics, with imipenem (a carbapenem) being the only antibiotic, to which resistance was not observed to. This suggests that the receiving environment of these untreated leaky wastewater is a potential repository of these resistant bacteria and PMQR genes, which could expose the University community to a potential public health challenge. This calls for a holistic approach to the concept of antibiotic resistance, which is majorly being looked at from the clinical perspective, as the environment is not spared of this menace. There is also a need for more proactive and urgent mechanisms to be put in place to address the issues of broken sewers within the community.

Declarations

Funding

The study was funded by the authors

Acknowledgments

The authors would like to acknowledge the postgraduate students, who assisted with sample collection.

Conflicts of interest/Competing interests

No conflict of interest as regards this study.

Availability of data and materials

All the data generated or analysed during the execution of this study are included in this published article.

Code availability

Not applicable

Authors' contributions

Abimbola Olumide Adekanmbi developed the original idea and the protocol. All authors performed the experiments, and were involved in the collection of data collection. Abimbola Olumide Adekanmbi wrote the preliminary draft and analyzed the data. All authors read, revised and approved the manuscript for publication.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

All authors agreed to have this article published

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Tables

Table 1: Oligonucleotide primers and amplicon sizes of PMQR genes targeted in this study

Target gene	Primer sequence (5' to 3')	Amplicon size (bp)	Reference
<i>qnrA</i>	TTCAGCAAGAGGATTTCTCA GGCAGCACCATTACTCCCAA	608	[18]
<i>qnrB</i>	CCTGAGCGGCACTGAATTCAT GTTTGCTGCTCGCCAGTCGA	389	[18]
<i>qnrS</i>	CAATCATACATATCGGCACC TCAGGATAAACAACAATACCC	621	[18]
<i>qepA</i>	GCAGGTCCAGCAGCGGGTAG CTTCCTGCCCGAAGTATCGTG	218	[18]
<i>oqxAB</i>	CTCGGCGCGGATGATGCT CCACTCTTCACGGGAGACGA	392	[18]
<i>aac(6')-Ib-cr</i>	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCTTGGCGCGTTT	482	[19]

Table 2: Resistance of the ciprofloxacin-resistant *Escherichia coli* to antibiotics (n=21)

Antibiotics (Concentration)	Sensitivity (%)	Intermediate (%)	Resistance (%)
SXT (25 µg)	2 (9.5)	0 (0)	19 (90.5)
CN (15µg)	5 (23.8)	6 (28.6)	10 (47.6)
IPM (5µg)	21 (100)	0 (0)	0 (0)
CTX (30µg)	0 (0)	0 (0)	21 (100)
AMC (30µg)	10 (47.6)	5 (23.8)	6 (28.6)
CPD (30µg)	0(0)	0 (0)	21 (100)
CAZ (30µg)	8 (30.1)	6 (28.6)	7 (33.3)
CXM (30µg)	0 (0)	0 (0)	21 (100)

KEY: n: Total number of ciprofloxacin-resistant bacteria obtained; SXT: trimethoprim/sulfamethoxazole (25 µg) CN: gentamycin (15µg); IMP: imipenem (5µg); CTX: cefotaxime (30µg); CPD: cefpodoxime (30µg); AMC: amoxicillin-clavulanate (30µg); CAZ: ceftazidime (30µg); CXM: cefuroxime (30µg)

Table 3: Distribution of PMQR genes in the ciprofloxacin-resistant *Escherichia coli*

Lab Code	<i>qnrA</i>	<i>qnrB</i>	<i>qnrS</i>	<i>qepA</i>	<i>oqxAB</i>	<i>aac(6')-Ib-cr</i>
SW1b	-	-	-	-	-	+
SW1d	-	-	-	+	-	+
SW2a	+	-	-	-	-	-
SW2e	-	-	-	-	-	+
SW3a	-	-	-	-	-	+
SW3b	+	-	+	-	-	+
SW3c	-	-	-	-	-	+
SWS1b	-	-	-	-	-	+
SWS1c	+	+	+	-	-	+
SWS1d	-	+	+	-	-	+
SWS1e	+	+	+	-	-	+
SWS2a	-	-	-	-	-	-
SWS2b	+	-	-	-	-	+
SWS2c	+	+	+	-	-	+
SWS2d	+	+	-	-	-	+
SWS3a	+	+	+	-	-	+
SWS3c	+	+	+	-	-	+
SWS3d	+	+	+	-	-	+
SWS4c	+	+	-	-	-	+
SWS4d	+	+	-	-	-	+
SWS4e	+	-	-	-	-	+

KEY: +: Target gene present; -: Target gene absent.

Table 4: Frequency of occurrence of PMQR genes in the ciprofloxacin-resistant *Escherichia coli*

PMQR gene	Frequency	Percentage Occurrence (%)
<i>qnrA</i>	13/21	61.9
<i>qnrB</i>	10/21	47.6
<i>qnrS</i>	8/21	38.1
<i>qepA</i>	1/21	4.8
<i>oqxAB</i>	0/21	0
<i>aac(6')-Ib-cr</i>	19/21	90.5

Figures

Figure 1

Frequency of CIP-sensitive and CIP-resistant E. coli isolated in this study