Dissemination of the Transmissible Quinolone-Resistance Gene qnrS1 by IncX Plasmids in Nigeria

Eric T. Sumrall¹, Elizabeth B. Gallo¹, Aaron Oladipo Aboderin², Adebayo Lamikanra³, Iruka N. Okeke¹*

¹ Department of Biology, Haverford College, Haverford, Pennsylvania, United States of America, ² Department of Medical Microbiology and Parasitology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria, ³ Department of Sciences, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria

Abstract
The plasmid-encoded quinolone resistance gene qnrS1 was recently found to be commonly associated with ciprofloxacin resistance in Nigeria. We mapped the qnrS1 gene from an Escherichia coli isolate obtained in Nigeria to a 43.5 Kb IncX2 plasmid. The plasmid, pEBG1, was sufficient to confer ciprofloxacin non-susceptibility, as well as tetracycline and trimethoprim resistance, on E. coli K-12. Deletion analysis confirmed that qnrS1 accounted for all the ciprofloxacin non-susceptibility conferred by pEBG1 and tetracycline and trimethoprim resistance could be attributed to tetAR and dfrA14 genes respectively. While it contained a complete IncX conjugation system, pEBG1 was not self-transmissible likely due to an IS2 element inserted between the pilX5 and pilX6 genes. The plasmid was however efficiently mobilizable. pEBG1 was most similar to another qnrS1-bearing IncX2 plasmid from Nigeria, but both plasmids acquired qnrS1 independently and differ in their content of other resistance genes. Screening qnrS1-positive isolates from other individuals in Nigeria revealed that they carried neither pEBG1 nor pNGX2-QnrS1 but that IncX plasmids were prevalent. This study demonstrates that the IncX backbone is a flexible platform that has contributed to qnrS1 dissemination in Nigeria.

Introduction
When antibacterial quinolones were first introduced into clinical practice, it was thought that resistance would be slow to appear and that transmissible resistance was improbable [1]. Initial reports of quinolone resistance were due to point mutations in the genes encoding their gyrase and topoisomerase targets that made them less sensitive to the drug. In 1998, Martinez-Martinez et al [2] described a plasmid-borne gene, now termed qnrA, which conferred four-to-sixteen-fold resistance to quinolones on Enterobacteriaceae. qnrA is a pentapeptide repeat protein that protects DNA gyrase from quinolone binding and inhibition [3]. Other qnr genes have since been reported and many can be transmitted horizontally. Transmissible quinolone resistance is also attributable to genes encoding plasmid-encoded efflux pumps, such as aac(6′)-Ib-cr. While plasmid-encoded quinolone-resistance genes generally confer low-level resistance, their overall impact is great because they shield otherwise susceptible bacteria from the lethal effects of the quinolones, allowing them greater time and opportunity to evolve higher-level resistance.

Until recently, reports of quinolone resistance were almost nonexistent from West Africa [4]. However, in the past decade Nigeria has seen a very rapid increase in fluoroquinolone use, due to the recent expiration of patents protecting ciprofloxacin and perfleroxacin. Introduction of ciprofloxacin into Nigerian clinics was temporally associated with a significant rise in resistance among gut commensals. Five years after fluoroquinolones were introduced in a community in Western Nigeria, Escherichia coli strains showing quinolone-specific resistance mechanisms were isolated [5]. Although the majority of these isolates carried point-mutations in the quinolone-resistance determining regions of gyrA and parC, six strains bore the plasmid-encoded resistance gene qnrS1. In this study, we characterized a mobile element from one of these isolates in order to understand the mode of qnrS1 dissemination in Western Nigeria.

Methods
Strains
Strain 09/22a and other qnrS1-bearing E. coli strains used in this study were isolated in 2009 during an earlier survey of quinolone resistance [5]. Other strains used in this study are listed in Table 1. Strains were maintained at −70°C in Luria broth: glycerol 1:1.

General molecular biology procedures
Genomic DNA was extracted using the Promega Wizard kit. Small-scale extractions of large, naturally occurring plasmids were
Table 1. Strains used in this study.

| Genotype and description | Reference or Source |
|-------------------------|---------------------|
| 09/22a qtnS1-positive E. coli isolate from Nigeria | [5] |
| DH5α | F− e808delOAcZM15 ΔlacZΔ8-argFU169 deoR recA1 endA1 hsdR17(k2 mK α) phoA supE44 Δ(λ thi-1 gyrA96 relA) Invitrogen |
| TOP10 | F− mcrA (mr−hsrRM5-mcrBC) O80lacZM15 lacX74 deoR recA1 araD139Δ1 (ara−leu)7697 galU galK rpsL (StrR) endA1 umpG Invitrogen |
| C600 Nalr | Nalidixic acid-resistant derivative of C600. [47] |
| SM10 λpir | λpir E. coli strain harboring an IncP conjugation system in the chromosome [35] |
| UMNO26 | Uropathogenic E. coli strain bearing IncP and IncX1 plasmids [36] |
| NCTC 10418 | Susceptibility testing control |
| ATCC 35218 | Susceptibility testing control |
| LMG194 | F− lacX74 galE thi−rpsL phoA (Pvu II) ara714 leuc::Tn10 Invitrogen [48] |
| EC1502 | Rifampicin resistant E. coli strain University of Bradford |
| J53 | F− met pro A2 Invitrogen [49] |

doi:10.1371/journal.pone.0110279.t001

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing by disc diffusion was performed using the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) [14]. Antimicrobial discs and control strain E. coli ATCC 35218 were obtained from Remel. The antimicrobial discs used contained ampicillin (10 μg), streptomycin (10 μg), trimethoprim (5 μg), tetracycline (30 μg), nalidixic acid (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg) and sulfonamide (300 μg). Inhibition zone diameters were interpreted in accordance with CLSI guidelines with WHONET software version 5.3 [15]. Minimum inhibitory concentrations (MICs) to nalidixic acid were measured by the E-test (Biomerieux), in accordance with manufacturer’s instructions and by the broth dilution technique in Mueller-Hinton broth as recommended by the CLSI and using E. coli ATCC 35218 as control [16].

In vitro conjugation

In vitro conjugation experiments were performed by solid and liquid mating. Donors and recipients were cultured in LB with appropriate selective antimicrobials. For liquid mating, 50 μl and 200 μl of mid-logarithmic phase LB cultures from donor and recipient respectively were mixed in 1 mL of LB, pre-warmed to 37°C. After one hour mating at 37°C, the cells were placed on ice to terminate the reaction. For solid mating, 0.5 mL of donor and recipient culture, grown overnight with selection, was spun down gently and resuspended in 20 μl of LB without antibiotics. The suspension was spotted onto dried LB plates, allowed to dry at room temperature for 15 minutes and then incubated at 37°C for three hours or overnight. The mating reaction was suspended in 1 mL of LB with vortexing and placed on ice to terminate conjugation. After mating, serial ten-fold dilutions of each terminated reaction was made in cold phosphate buffered saline and plated onto plates containing tetracycline (or other appropriate antimicrobials for controls) to select for the plasmid and nalidixic acid, resistance to which is conferred chromosomally in the recipient. Transconjugant colonies were counted after overnight incubation at 37°C and verified by plasmid profiling, phenotype on MacConkey and Eosin methylene blue agars, PCR-RFLP for the blc allele [17], and PCR for donor- and recipient-specific markers. Viable counts of donors and recipient were also performed. The number of transconjugant colonies per donor
colony-forming units was computed as the plasmid transfer efficiency [18].

**Plasmid stability**

We serially passaged pEBG1 in its source strain, 09/22a and in DH5αE essentially as described by Sandegren et al [19]. Triplicate starting cultures were grown overnight at 37°C in 1 mL of LB supplemented with tetracycline (25 mg/L). Bacterial cells were washed by spinning down overnight cultures and resuspending the pellet in 1 mL of LB without antibiotics. An aliquot of 100 μL of washed cells was transferred into 1 mL of LB and incubated overnight at 37°C. Serial passaging of 100 μL of overnight culture to 1 mL of LB was performed daily, approximating 10 generations of growth per passage. Every 20 generations (48 hours), samples were diluted and plated on MacConkey and tetracycline plates. Once counts began to drop off, colonies were screened through replica plating on plates containing tetracycline, trimethoprim and ciprofloxacin to confirm that cells lost the plasmid.

**Results**

Quinolone-non-susceptible strain 09/22a from Nigeria carries its qnrS1 gene on an IncX plasmid

Strain 09/22a is a quinolone-non-susceptible *E. coli* isolate from Nigeria that tested positive for *qnrS1* by PCR in a recent study [5]. The strain was unable to conjugate quinolone resistance to *E. coli* C600, TOP10 or EC1502. We therefore extracted plasmids from 09/22a by boiling and electroporated the extract into electrocompetent *E. coli* DH5αE (Invitrogen). Selection on nalidixic acid plates (100 mg/mL) yielded no transformants. Selection on plates containing 1 mg/mL of ciprofloxacin produced transformants resistant to ciprofloxacin, trimethoprim and tetracycline, all of which carried a single large plasmid. The plasmid, termed pEBG1,

| Target gene | Sequences | Application | Reference |
|-------------|-----------|-------------|-----------|
| topB        | F 5’ AGACTCTTTATGCTGAAAA ACCATC 3’; R 5’ TTTTTAGGCAATTC AAAACCTTATTTT 3’ | Amplification of topB from pEBG1 for cloning into pBAD/Thio-TOPO | This study |
| flic        | F 5’ ATGGCACAAGCTTATACACAAGGAC 3’; R 5’ CCTAACCTGAGCAGAGACA 3’ | flic PCR-RFLP | [17] |
| qnrS5       | F 5’ CAATCTATATATCGCACC 3’; R 5’ TCAGGATAACAAACAAATACC 3’ | Amplification of qnrS1 | [28] |
| IncX1 (taxC) | F 5’ GCTAGACTTTGTTTACGATG 3’ | Amplification of taxC from IncX1 plasmids | [20] |
| IncX2 (taxC) | F 5’ GCGAAGAAATCAAGAAGCT 3’ | Amplification of taxC from IncX1 plasmids | [20] |
| qnrS1comp   | F 5’ TGAGGGTTGTAATGTTTGAT 3’; R 5’ TGCAAGTGAATATATTATTTCTTTT 3’ | Cloning qnrS1 | This study |
| BlpIFrag    | F 5’ ATGGTGTCTCTGATGATCAGA 3’; R 5’ CTAGGTTTTCGTTTTCCTTT 3’ | Verifying qnrS1 deletion | This study |
| BlpI-2      | F 5’ ATCCGCGGACTTTGACGCT 3’; R 5’ TTCCGATAGGCGCCGCTTT 3’ | Verifying qnrS1 deletion | This study |
| pilX2       | F 5’ ACCGTGTCTCTGATGATCAGA 3’; R 5’ ACCAGTGGAGCCAATAGCCC 3’ | Amplification of pilX2 from IncX plasmids | This study |
| pilX8       | F 5’ GCACCTTTGAGCCGCTTTCC 3’; F 5’ CCTGTACTCTTTGTATCA 3’ | Amplification of pilX8 from IncX plasmids | This study |

**Table 2. Oligonucleotide primers used for PCR.**

**Table 3. Plasmids used in this study.**

| Plasmid | Description | Size | Reference or source |
|---------|-------------|------|---------------------|
| pEBG1   | WT qnrS1-containing plasmid derived from *E. coli* strain 09/22a, confers resistance to ciprofloxacin, tetracycline and trimethoprim | 43,530bp | This study |
| pETS4   | pEBG1 plasmid with excised region containing qnrS1 | 41,387 | This study |
| pNKC2002| qnrS1 cloned into the Tet gene of pACYC184 | 5,038 bp | This study |
| pETStop3| Expression vector (pBAD/Thio-TOPO vector) containing a topB insert | 6,773 bp | This study |
| pBAD/Thio-TOPO | Arabinose inducible expression vector | 4,454 bp | Invitrogen |
| p1ECUMN | IncP plasmid derived from *E. coli* strain UMN026 | 122,302 bp | [36] |
| p2ECUMN | IncX1 plasmid derived from *E. coli* strain UMN026 | 33,809 bp | [36] |
| pMG306  | Naturally occurring qnrS1-bearing plasmid from a *Salmonella* isolate | | [32] |
| pMB2    | Naturally occurring aac(6’)-Ib-cr-bearing plasmid | 125 Kb | This study |
| pMB80-2 | Naturally occurring conjugative plasmid from enteropathogenic *E. coli* strain | >100 Kb | [34] |
| pGEM-T  | Amp<sup>+</sup>; TA-cloning vector | 3,000 bp | Promega |
| pACYC184| Tet and Cm resistant cloning vector | 4,244 bp | NEB |

doi:10.1371/journal.pone.0110279.t002
doi:10.1371/journal.pone.0110279.t003
was purified and shotgun sequenced. pEBG1 was found to be to be 43,534 bp, with an overall G+C content of 46.72%. Its sequence has been deposited in Genbank (Accession number KF738053). pEBG1 is most similar to IncX2 plasmid pNGX2-QnrS1 (Genbank accession no. JQ269335) [20] and the two plasmids have 100% identical taxC genes. IncX plasmids are subclassified based on TaxC, which agrees with subclassifications based on polymorphic sites across whole plasmid sequences [20]. All other taxC genes are less than 90% identical to those of pEBG1 and pNGX2-QnrS1 and therefore pEBG1 is an IncX2 plasmid (Figure 1). The IncX2 plasmids demonstrate significant homology to other IncX plasmids, including the prototype R6K, with the most similarity occurring in the conjugative pilus operon [21].

As was pEBG1, pNGX2-QnrS1 was isolated in western Nigeria (Genbank accession no. JQ269335) [20] and the two plasmids currently represent the only completely sequenced IncX2 plasmids in the database. 71% of pEBG1 is 99% identical to 90% of pNGX2-QnrS1 and both plasmids carry the qnrS1 gene (Figure 2). The backbones, including the region encoding the conjugation system, are nearly identical between the two plasmids, strongly suggesting that they share a recent ancestor. Their resistance modules are integrated at the same site but as shown in Figure 2, the plasmids diverge in the predicted mobile elements and resistance genes they carry and their resistance modules, based on the orientation of the qnrS1 gene, are inserted in opposite orientations.

Like other IncX plasmids, pEBG1 contains alpha and beta origins of replication, and carries the necessary genes to facilitate plasmid replication and partition: pl, a replication initiation protein, ddp3, a DNA distortion polypeptide which alters the topology of the DNA to allow for transcription initiation similar to taxA and taxC, and parA and parG which are plasmid partition proteins involved in segregation and stability [21]. As shown in Figure 3, the pEBG1 plasmid backbone contains all other core genes common to IncX plasmids, in the following order: taxA, encoding a DNA-distortion polypeptide, which is necessary for altering the DNA helix of plasmids that contain two replication origins [22]; taxC, encoding a relaxase required for replication and conjugative transfer of the plasmid [21]; taxB, containing a NusG transcription termination motif and probably the last gene in that operon; the IncX-type pilus synthesis operon (pilX1-pilX11); putative relaxase, taxB; and putative nuclease parB. pEBG1 contains an IS3 element in its pilus synthesis operon between pilX5 and pilX6. This insertion sequence is not present in pNGX2-QnrS1 (Figures 2a and 3).

pEBG1 also carries open reading frames (orfs) not found in all IncX plasmids, including orfEBG036, predicted to encode a H-NS histone family protein, EBG071c, predicted to encode a PecM-like membrane protein associated with the tetAR genes, and a putative DnaJ-domain protein that is 39% similar to the N-terminal J-Domain of the E. coli heat-shock chaperone DnaJ/Hsp40. DnaJ-domain proteins are often encoded on plasmids, including pNGX2-QnrS1, but their function in this context is unknown. The function of the putative ABC transporter permease (PecM)-like protein is also unknown. Proteins belonging to this family are involved in small molecule efflux. Plasmid-borne H-NS family proteins are known to compete with chromosomal H-NS, reducing repression of A-T rich sequences present on the plasmid [23]. (The region containing the 657 bp qnrS1 orf and 570 bp of intergenic sequence upstream, conserved among qnrS1-bearing plasmids, has a GC% of 44.42%, compared to 46.72% for the whole plasmid and 50.8% for the E. coli chromosome [24].) Another gene found on pEBG1, which is also found in many but not all, other IncX-type plasmids, is the 2.3 Kb topB, coding for bacterial type III topoisomerase. topB is truncated at the 5’ end in pNGX2-QnrS1 by an antimicrobial resistance gene module but pEBG1 contains a full-length gene. Type III topoisomerases decatenate DNA during replication [23] but the function of plasmid-encoded topB genes, which are only partially conserved, has not been elucidated.

pEBG1 confers resistance to trimethoprim, tetracycline and the quinolones. The plasmid’s trimethoprim resistance gene, dfrA14, probably originated in a class 1 integron. The 5’ end of this integron, including the intI1 gene is intact. The integron also includes a truncated aadA gene. The 3’ end of the integron is truncated by an IS26 element that is 99% identical to the element downstream of qnrS1. Although both carry qnrS1, the region containing the dfrA14 gene is not present on the similar plasmid.
PNGX2-QnrS1, suggesting that it has been recently mobilized, perhaps via the IS
26 element. The resistance module encompassing qnrS1 and its flanking resistance and transposase genes is nearly identical to that of other plasmids, including E. coli plasmid pT078 and pKOX105, from a Klebsiella oxytoca strain, with the important exception that beta-lactamase genes present on these plasmids are absent in pEBG1 [26,27]. Although the qnrS1 modules of pEBG1 and PNGX2-QnrS1 are identical, the plasmid backbones are also highly similar, and the two plasmids were recovered from geographically proximal areas, it is clear that qnrS1 was acquired independently by each plasmid. The location of qnrS1 and its flanking IS26 element varies between the plasmids, being at the 5’ end of topB in PNGX2-QnrS1 but with the resistance region downstream of topB in pEBG1. qnrS1 is often adjacent to a tnpA transposase in other plasmids, and it has been hypothesized that the qnrS1 gene is usually part of an IS26 element [28]. IS26 contains two 14 bp perfect terminal repeats. We identified four IS26-associated inverted repeats [29] adjacent to the qnrS1 gene. These 14 bp sequences begin at coordinates 23,698, 24,504, 27,142 and 29,074, marking out a putative transposable element of 5,376 bp encompassing dfrA14 and qnrS1.

In addition to qnrS1 and dfrA14, pEBG1 also contains the genes tetR and tetA, which lie adjacent to the pecM-like gene EBG71 and another tnpA gene in a high G+C content region, whose collective sequence was also not found on PNGX2-QnrS1 (Figure 2). These genes are bordered by a putative relaxase and Tn3 transposase, which outline the boundaries of the elevated GC

---

**Figure 2.** a. Dot plot of pairwise alignment between incX2 plasmids pEBG1 on the x-axis and pNGX2-QnrS1 on the y-axis. The plasmids are highly conserved overall but differ in the location and content of their antimicrobial resistance modules. The pEBG1 conjugation system is interrupted by an IS3 element not present in pNGX2-QnrS1. b. schematic of the resistance region of plasmid pEBG1 and pNGX2-QnrS1. qnrS1 is colored brown and other antimicrobial resistance genes are shaded blue. The flanking core plasmid regions are colored yellow. Note that the insertions are in different orientations.

doi:10.1371/journal.pone.0110279.g002
content area, suggesting that the plasmid’s tetracycline resistance also recently came into the plasmid via a transposable element. The existence of this module on other resistance plasmids [30], and its absence from homologous plasmid pNGX2-QnrS1 support this idea. No other areas of the plasmid show a significant difference in G+C content, save for the fact that the backbone of the plasmid has an overall lower G+C content than the rest of the plasmid (Figure 3).

qnrS1 is the sole quinolone resistance-conferring gene on pEBG1

pEBG1 encodes a number of hypothetical ORFs as well as ORFs that could contribute to topoisomerase activity (TopB) or antimicrobial efflux (PecM-like EBG071c). To verify that qnrS1 is the only quinolone resistance gene on pEBG1, we deleted the gene by restricting the plasmid with BlpI (which cuts at positions 28,514 and 30,657 in our annotation effectively removing 2,143 bp, which includes the 3’ half of qnrS1 as well as two other hypothetical orfs, Figure 3). The resulting plasmid, pETS4 was confirmed by PCR analysis with the qnrS1F and R primer pair and restriction analysis with NruI, which has 10 sites in pEBG1 including a site at position 30,360, within the deleted region. pETS4 conferred a six-fold lower MIC to ciprofloxacin than pEBG1 on DH5α and a greater than 32-fold lower MIC on TOP10. Resistance was restored upon trans-complementation with qnrS1 clone pINK2002. Deleting qnrS1 from pEBG1 however produced little effect (about 2-3 fold) on MICs to nalidixic acid. We then determined the level of resistance conferred by aac(6’)-Ib-cr, a gene that confers ciprofloxacin resistance but not to other quinolones [31], using plasmid pMB2, which carries this gene and no other known plasmid-mediated quinolone resistance gene. We found that pMB2 provided no significant alteration to the nalidixic acid MIC of DH5α, but increased resistance to ciprofloxacin three-fold. As qnrS1 has previously been reported to confer significant resistance to multiple quinolones, including nalidixic acid MIC of DH5α, but increased resistance to ciprofloxacin three-fold. Similar results were seen with this plasmid in the DH5α and J53 backgrounds. Thus qnrS1 principally confers ciprofloxacin resistance. In addition to demonstrating the pivotal role that qnrS1 plays in ciprofloxacin resistance, these data
demonstrate that the host background is an important determinant of the effect of qnrS1 (Table 4).

topB is a large, partially conserved gene that is present in most IncX plasmids, but the function of plasmid-encoded topoisomerases is unknown. Chromosomally encoded DNA gyrase and topoisomerase are the normal targets of the quinolones. While topB genes, encoding type III topoisomerases, should not normally be inhibited by antibacterial quinolones, which are better inhibitors of type II topoisomerases, bacterial topB genes have not previously been evaluated in this regard. Since over-expression of chromosomal topoisomerases can compensate for absence of gyrase [25], it is possible that plasmid-encoded TopB could confer resistance to the quinolones by supplementing the activity of inhibited chromosomal topoisomerases. As it is possible that topB is not expressed under conditions we used to test for quinolone resistance in vitro, we cloned the gene and placed it under the control of the arabinose promoter, using the vector pBAD/Thio-TOPO (Invitrogen). The resulting clone, pETS-Topo3 produced copious amounts of the TopB protein when induced with 0.2% arabinose. Expression was abrogated in the presence of 2% glucose (data not shown). MICs of the clone to nalidixic acid and ciprofloxacin were identical in arabinose and glucose, confirming the topB is not sufficient to confer quinolone resistance. Because it is known some topB alleles can promote homologous recombination by stabilizing and resolving Holliday junctions [33], we hypothesized that topB might enhance mutation rates and that this might serve as a mechanism of evolution to antimicrobial resistance by strains carrying IncX plasmids like pEBG1. However, tests with the pETS-Top3 clone in a TOP10 and LMG194 background did not reveal enhanced mutation rates leading to resistance to rifampicin determined by plating cultures on LB plates containing 50 μg/mL rifampicin (data not shown).

pEBG1 is mobilizable but not self-transmissible

We performed conjugation experiments on solid and in liquid media, using pMB80-2 as a control plasmid [34]. We were unable to conjugate pEBG1 into any of three recipient strains, C600, TOP10 or EC1502 even when mating was extended to 24 h. To determine whether pEBG1 could be mobilized in vitro, we electroporated it into strains carrying self-transmissible conjugation systems. Strain SM10, which contains conjugation genes from the broad host range IncP-type plasmid RP4 integrated into its chromosome [35] was unable to mobilize pEBG1. We then transferred two plasmids, IncP plasmid pECUMN and IncX1 plasmid p2ECUMN, from UM026 [36], into C600 (NalR) by conjugation. The presence of both plasmids in strain C600 was confirmed by plasmid profiling and PCR. C600 (p2ECUMN, p1ECUMN, pEBG1) could mobilize pEBG1 into TOP10 (StrepR). The conjugation rate for pEBG1 after a 3-hour mating was 1.7×10⁻³. At 24 hours it was 3.38×10⁻⁷.

Other qnrS1-bearing isolates from Western Nigeria carry non-identical plasmids

We screened genomic DNA from other qnrS1-bearing isolates recovered in the same study for pEBG1 plasmids. All these strains show lowered susceptibility to ciprofloxacin but only one meets the CLSI breakpoint criteria for resistance. The strains were screened using taxC-based primers designed by Johnson et al (2012) [20], as well as primers specific for qnrS1, topB, pilX2 and pilX8. As shown in Table 5, all five pEBG1 markers were detected in another strain isolated from the same individual but not in the other isolates. As we were able to obtain two or more of the pEBG1 marker amplicons from three other strains, we screened them for the IncX1 taxC gene and found that only two strains, including 09/22a carried IncX1 plasmids.

### Table 4. MICs to quinolones conferred by pEBG1, its derivatives and other Qnr-encoding plasmids.

| Strain  | Resistance to non-quinolone antimicrobials | Nalidixic acid MIC | Ciprofloxacin MIC |
|---------|-------------------------------------------|--------------------|------------------|
| 09/22a  | Amp Chl Str Sul Tet Tmp                    | 12                 | 0.38             |
| C600    | -                                         | >256               | 0.5              |
| DH5α    | -                                         | 24                 | 0.032            |
| DH5α (pEBG1) | Tet Tmp                        | 12                 | 0.125            |
| DH5α (pETS4) | Tet Tmp                        | 6                  | 0.032            |
| DH5α (pETS4, pINK2002) | Chl Tet Tmp                    | 8                  | 0.016            |
| DH5α (pETS4, pACYC184) | Chl Tet Tmp                    | 6                  | 0.008            |
| DH5α (pMG306) | Chl                                 | 24                 | 0.25             |
| DH5α (pMB2) | Tet Tmp                          | 24                 | 0.094            |
| TOP10   | Str                                       | 0.75               | <0.002           |
| TOP10 (pEBG1) | Str Tet Tmp                    | 2                  | 0.064            |
| TOP10 (pETS4) | Str Tet Tmp                        | 0.75               | <0.002           |
| TOP10 (pETS4, pINK2002) | Chl Str Tet Tmp                      | 1                  | 0.016            |
| TOP10 (pETS4, pACYC184) | Chl Str Tet Tmp                      | 0.75               | <0.002           |
| TOP10 (pMG306) | Chl                                 | 3                  | 0.19             |
| J53     | Azi                                       | 4                  | 0.008            |
| J53 (pMG306) | Azi Chl                         | 24                 | 0.75             |
| J53 (pMG282) | Azi Chl                          | 16                 | 0.38             |

doi:10.1371/journal.pone.0110279.t004
pEBG1 is propagated in the medium term without selection

We studied the stability of pEBG1 following serial passage. pEBG1 was stably inherited by its original host strain 09/22a and by DH5α for over 200 generations without selection (Figure 4). However, extended subculture lead to a significant proportion of those lines loosing the plasmid.

Discussion

Plasmids harboring qnrS genes tend to be on average smaller than plasmids carrying other qnr subtypes, and are less likely to be self-transmissible [1,37]. They vary greatly, belonging to N, L, M, H, R and X incompatibility groups and residing in diverse enterobacterial genera [22,37]. Multiple studies have also noted that qnrS1 tends not to be associated with integrons, and are instead associated with intA transposase genes, which likely contribute to intermolecular mobility [22,28,38].

In this study, we sequenced and functionally characterized a qnrS1-bearing plasmid obtained from an E. coli isolate in southwest Nigeria. At the time of isolation, qnrS1 was the sole plasmid-encoded quinolone resistance gene identified in that locality and since the isolates were not clonal, we hypothesized that one or a few plasmids might account for the recently emerged quinolone resistance seen in the community. We identified and characterized pEBG1, a 43.5 Kb IncX2 plasmid. We find that other strains from Nigeria carry similar plasmids but that pEBG1 does not appear to have clonally expanded.

The IncX2 plasmid we characterized carries, in addition to qnrS1, genes conferring resistance to tetracycline and trimethoprim. The resistance gene repertoire is the most common variable among qnrS1-bearing IncX plasmids in and beyond Nigeria. This finding is worrisome, since it suggests that IncX backbones may be good scaffolds for incorporating new resistance genes. For example, in addition to plasmid-encoded quinolone resistance genes, extended-spectrum beta-lactamase genes have also been recently found on IncX plasmids [39,40]. Although the plasmid we identified in this study was from a human isolate, IncX plasmids, including one IncX2 plasmid, bearing qnrS1 have also been found in isolates from migratory birds in Europe [41] and pNGX2-QnrS1 is a poultry isolate, pointing to possible animal reservoirs and global reach of these elements.

Qnr proteins confer low-level resistance by altering the interaction between the quinolones and their targets. What is known about Qnr function and spectrum has been gleaned from mechanistic studies with QnrA and QnrB. In this study, we found that qnrS1 from pEBG1 conferred significant fluoroquinolone but only modest quinolone resistance on laboratory E. coli isolates. The finding that qnrS1 confers ciprofloxacin non-susceptibility but very little nalidixic acid resistance on these strains is not wholly surprising since a previous study has indicated that qnrS1 gene products sometimes confer no nalidixic acid resistance [42]. A separate study found that qnrS confers moderate nalidixic acid and ciprofloxacin resistance in E. coli strain HB101, but confers no resistance at all in Shigella strains [43]. The qnrS1 gene was originally reported from a 47 Kb conjugative plasmid, pAH0376, from Japan in 2005 [43]. The plasmid was reported to confer on E. coli HB101 resistance to nalidixic acid (MIC, 16 μg/mL) and ciprofloxacin (MIC, 0.25 μg/mL). The following year, plasmid-borne qnrS1 was reported from Germany and although the responsible plasmid pINF1 was not identical to pAH0376, the qnrS1 genes on both plasmids were flanked by a Tn3 transposon carrying a blaTEM-1 gene. Other contexts for qnrS1 have since been described [37,41,42]. Most of these reports demonstrate that
qnrS1 confers low- to intermediate resistance to ciprofloxacin (0.25–0.5 μg/mL). Altogether, the findings of this and earlier studies indicate that the nature and degree of quinolone resistance conferred by qnrS1 is dependent on host strain background [42]. In Nigeria, most of the quinolone resistance selective pressure comes from ciprofloxacin, as in Vietnam [37], where qnrS1 is also highly prevalent, and it therefore makes sense that genes preferentially conferring resistance to this agent will be selected. This research, along with work from other laboratories [20,44], points to multiple instances of qnrS1-bearing IncX plasmids in Nigeria. In this study, we determined that plasmid pEBG1 is highly mobilizable, though not self transmissible. The model IncX plasmid R6K is reported to conjugate at a rate of $1.7 \times 10^{-3}$ into E. coli strain MC1061 [45], the same as the mobilization rate for pEBG1. The most likely explanation for the inability of pEBG1 to mediate its own conjugation is the insertion of an IS3 element between pilX5 and pilX6. Although this element does not disrupt a gene, it does interrupt the pilX operon and is likely polar on downstream genes. As the IS3 element is absent from pNGX2-QnrS1, we anticipate that self transmissible IncX plasmids are also in circulation in Nigeria. Resistant strains in Nigeria frequently carry multiple plasmids [46]. Therefore the lack of a fully functional conjugative system is unlikely to greatly compromise the success of this qnrS1-bearing plasmid as long as it remains mobilizable. Overall, the high transmission rate and modular content observed by us and others [20,45] illustrates that IncX plasmids are good candidates for mediating the dissemination of resistance genes. pEBG1 was stably inherited without selection in the medium term but was lost after 16–18 passages. This suggests that IncX2 plasmids bearing qnrS1 may be maintained by antibacterial drug selection and therefore their frequent occurrence in Nigeria may be linked to overuse of antibacterial drugs there, among which ciprofloxacin, trimethoprim and tetracycline are prominent.

Acknowledgments

We are grateful to Babatunde Odetoyin, Jose Otero-Vera and Erin Remaly for technical assistance. We thank the David C Hooper laboratory at Massachusetts General Hospital Boston and the University of Bradford for Strains.

Author Contributions

Conceived and designed the experiments: ETS EBG AL INO. Performed the experiments: ETS EBG AOA INO. Analyzed the data: ETS EBG AL INO. Contributed reagents/materials/analysis tools: AL INO. Wrote the paper: ETS EBG AOA INO.

References

1. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A (2009) Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev 22: 664–689.
2. Martinez-Martinez L, Pascual A, Jacoby GA (1998) Quinolone resistance from a transferable plasmid. Lancet 351: 797–799.
3. Tran JH, Jacoby GA (2002) Mechanism of plasmid-mediated quinolone resistance. Proc Natl Acad Sci U S A 99: 5638–5642.
4. Robicsek A, Jacoby GA, Hooper DC (2006) The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect Dis 6: 629–640.
5. Lamikanra A, Crowe JL, Lijek RS, Odetoyin BW, Wain J, et al. (2011) Rapid evolution of fluoroquinolone-resistant Escherichia coli in Nigeria is temporally associated with fluoroquinolone use. BMC Infect Dis 11: 312.
6. Kado CI, Liu ST (1981) Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 145: 1365–1373.
7. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning; a laboratory manual. New York: Cold Spring Harbor Laboratory Press.
8. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.
9. Rutherford K, Parkhill J, Crook J, Horne D, Rice P, et al. (2000) Artemis: sequence visualization and annotation. Bioinformatics 16: 944–945.
10. Carver T, Thomson N, Bleasby A, Berriman M, Parkhill J (2009) DNAPlotter: circular and linear interactive genome visualization. Bioinformatics 25: 119–120.
11. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
12. Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, et al. (2012) The Pfam protein families database. Nucleic Acids Res 40: D290–301.
13. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739.
14. NCCLS (2003) Performance standards for antimicrobial disk susceptibility tests, 8th Edition; Approved standard. Villanova, PA: National Committee for Clinical Laboratory Standards. NCCLS Document M2-A7. NCCLS Document M2-A8. 130 p.
15. O’Brien TF, Stelling JM (1993) WHONET: an information system for monitoring antimicrobial resistance. Emerg Infect Dis 1: 66.
16. CLSI (2006) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 7th Edition; Approved standard. Wayne, PA: Clinical and Laboratory Standards Institute. CLSI Document M7-A7. CLSI Document M7-A8.

Figure 4. Stability of pEBG1 in the absence of selection in its natural host, 09/22a, and in laboratory strain DH5α.

doi:10.1371/journal.pone.0110279.g004
17. Fields P, Bloom K, Hughes H, Helsel L, Feng P, et al. (1997) Molecular characterization of the gene encoding H antigen in Enterobacter cloacae and development of a PCR-restriction fragment length polymorphism test for identification of E. cloacae O157:H7 and O157:NM. Journal of Clinical Microbiology 35: 2340–2347.

18. Lu J, Manchak J, Klimke W, Davidson C, Firth N, et al. (2002) Analysis and characterization of the IncFV plasmid pE268 transfer region. Plasmid 48: 24–37.

19. Sandegren L, Linkevicius M, Lytsy B, Melhus A, Andersson DI (2012) Transfer of a novel ISEcp1-bearing plasmid pET168-1 from Escherichia coli to Salmonella enterica. J Antimicrob Chemother 63: 273–281.

20. Johnson TJ, Bielak EM, Fortini D, Hansen LH, Hasman H, et al. (2012) Interplay between Type 1A Topoisomerase A and Gyrase in Chromosome Segregation in Escherichia coli. J Bacteriol 195: 1738–1768.

21. Nunez B, Avila P, de la Cruz F (1997) Genes involved in conjugative DNA processing of plasmid R6K. Mol Microbiol 24: 1157–1160.

22. Garcia-Fernandez A, Forini D, Veldman K, Mevis D, Carattoli A (2009) Characterization of plasmids harbouring qnrS1, qnrB2 and qnrB19 genes in Salmonella. J Antimicrob Chemother 63: 273–281.

23. Doyle M, Fookes M, Ivins A, Mangan MW, Wain J, et al. (2007) An H-N-like stealth protein aids horizontal DNA transmission in bacteria. Nature 452: 231–234.

24. Blattner FR, Plunkett G 3rd, Burland V, Perna NT, Kocher T, et al. (1997) The complete genome sequence of Escherichia coli K-12. Science 277: 1453–1474.

25. Usongo V, Tanguay C, Nolent F, Bessong JE, Drolet M (2013) Interplay of plasmid-encoded enzymes in resistance to ciprofloxacin and nalidixic acid. Antimicrob Agents Chemother 57: 2493–2500.

26. Carattoli A, Aschbacher R, March A, Larcher C, Livermore DM, et al. (2010) Characterization of plasmids associated with AmpC and expanded-spectrum beta-lactamase production in Enterobacteriaceae isolated from pigs and poultry in Italy. Antimicrob Agents Chemother 54: 4098–4103.

27. Huang SY, Zhu XQ, Wang Y, Liu HB, Dai L, et al. (2012) Co-carriage of qnrS1, iroX, and blaCTX-M-14 on a multidrug-resistant plasmid in Enterobacter cloacae isolated from pigs. Foodborne Pathog Dis 9: 896–901.

28. Wu JJ, Ko W-C, Tsai S-H, Yan JJ (2007) Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of Enterobacter cloaceae in a Taiwanese hospital. Antimicrob Agents Chemother 51: 1223–1227.

29. Mollet B, Iida S, Shepherd J, Arber W (1983) Nucleotide sequence of IS26, a new prokaryotic mobile genetic element. Nucleic Acids Res 11: 6319–6330.

30. Schützer A, Heuer H, Szczepanowski R, Forney LJ, Thomas CM, et al. (2003) Identification of the type I trimethoprim-resistant dihydrofolate reductase specified by the Escherichia coli R-plasmid R483: comparison with procarboxyl and eucaryotic dihydrofolate reductases. Journal of Bacteriology 185: 1001–1008.

31. Lechat M, Callebo A, Hoede C, Barbe V, Touchon M, et al. (2009) A module located at a chromosomal integration hot spot is responsible for the multdrug resistance of a reference strain from Escherichia coli clonal group A. Antimicrob Agents Chemother 53: 2285–2290.

32. Vien LT, Alex-Oun M, Morrison V, Thomson N, Campbell JI, et al. (2011) Differential phenotypic and genotypic characteristics of qnrS1-harboring plasmids carried by hospital and community commensal Enterobacteriaceae. Antimicrob Agents and Chemotherapy 55: 1788–1802.

33. Kelebergen C, Hopkins KL, Thrall EF, Schwarz S (2007) Complete nucleotide sequence of a small qnrS1-carrying plasmid from Salmonella enterica subsp. enterica Typhimurium DT193. Journal of Antimicrobial Chemotherapy 60: 903–905.

34. Hu-Tu, Wang J-F, Fu Y, Zhao F, Chen Y, et al. (2013) Genetic characteristics of blaNDM-1-positive plasmid in Citrobacter freundii isolated from a clinical infectious patient. J Med Microbiol 62: 1332–1337.

35. Partridge SR, Ellen JA, Tetu SG, Zong Z, Paulden ET, et al. (2011) Complete sequence of pHE143, a pI-type plasmid carrying IS576-CTX-M-15 from an Escherichia coli ST131 isolate. Antimicrobial Ag Chemother 55: 5933–5935.

36. Leterik I, Dekiej M, Janoszowska D, Hrusakova J, Meissner W, et al. (2010) Antibiotic-Resistant Escherichia coli Bacteria, Including Strains with Genes Encoding the Extended-Spectrum Beta-Lactamase and QnrS, in Waterbirds on the Baltic Sea Coast of Poland. Appl Environ Microbiol 76: 8126–8134.

37. Gunell M, Wedderb MA, Kottlaien P, Li P, Dacke JM, et al. (2009) Mechanisms of resistance in nonypothiodial Salmonella enterica strains exhibiting a nonclassical quinolone resistance phenotype. Antimicrob Agents Chemother 53: 3832–3836.

38. Hata M, Suzuki M, Matsumoto M, Takahashi M, Sato K, et al. (2005) Cloning of a novel gene for quinolone resistance from a transferable plasmid in Nigella floweri 2b. Antimicrob Agents Chemother 49: 801–803.

39. Chah KF, Agbo IC, Eze DC, Somalo S, Estepa V, et al. (2010) Antimicrobial resistance, integrons and plasmid replication typing in multiresistant clinical Escherichia coli strains from Enugu State, Nigeria. J Basic Microbiol 50: 810–824.

40. Grzmiński AM, Karczewska-Dowjat A, Wolska KJ, Wild J (2007) Conjugal transfer of plasmid R6K gamma ori mimeriplenon derivatives from Escherichia coli to various genera of pathogenic bacteria. Curr Microbiol 55: 549–553.

41. Labar AS, Millman JS, Ruebush E, Opintan JA, Bishar RA, et al. (2012) Multimodal transmission and high-level expression by vectors containing the arabinose promoter. PLoS ONE 7: e38142.

42. Gunell M, Wedderb MA, Kottlaien P, Li A, Dacke JM, et al. (2009) Mechanisms of resistance in nonypothiodial Salmonella enterica strains exhibiting a nonclassical quinolone resistance phenotype. Antimicrob Agents Chemother 53: 3832–3836.

43. Sata M, Suzuki M, Takahashi M, Sato K, et al. (2005) Cloning of a novel gene for quinolone resistance from a transferable plasmid in Nigella floweri 2b. Antimicrob Agents Chemother 49: 801–803.

44. Chah KF, Agbo IC, Eze DC, Somalo S, Estepa V, et al. (2010) Antimicrobial resistance, integrons and plasmid replication typing in multiresistant clinical Escherichia coli strains from Enugu State, Nigeria. J Basic Microbiol 50: 810–824.

45. Grzmiński AM, Karczewska-Dowjat A, Wolska KJ, Wild J (2007) Conjugal transfer of plasmid R6K gamma ori mimeriplenon derivatives from Escherichia coli to various genera of pathogenic bacteria. Curr Microbiol 55: 549–553.

46. Labar AS, Millman JS, Ruebush E, Opintan JA, Bishar RA, et al. (2012) Multimodal transmission and high-level expression by vectors containing the arabinose promoter. PLoS ONE 7: e38142.

47. Appleby RK (1954) Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from Escherichia coli K12. Genetics 39: 440–452.

48. Guzmán LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promoter. J Bacteriol 177: 4121–4130.

49. Yi H, Cho Y-J, Yong D, Chun J (2012) Genome sequence of Escherichia coli J53, a reference strain for genetic studies. Journal of Bacteriology 194: 3742–3743.