Isolation of novel IncA/C and IncN fluoroquinolone resistance plasmids from an antibiotic-polluted lake

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Objectives: Antibiotic-polluted environments may function as reservoirs for novel resistance plasmids not yet encountered in pathogens. The aims of this study were to assess the potential of resistance transfer between bacteria from such environments and Escherichia coli, and to characterize the conjugative elements involved.

Methods: Sediment samples from Kazipally lake and Asanikunta tank, two Indian lakes with a history of severe pollution with fluoroquinolones, were investigated. Proportions of resistant bacteria were determined by selective cultivation, while horizontal gene transfer was studied using a GFP-tagged E. coli as recipient. Retrieved transconjugants were tested for susceptibility by Etest and captured conjugative resistance elements were characterized by WGS.

Results: The polluted lakes harboured considerably higher proportions of ciprofloxacin-resistant and sulfamethoxazole-resistant bacteria than did other Indian and Swedish lakes included for comparison (52% versus 2% and 60% versus 7%, respectively). Resistance plasmids were captured from Kazipally lake, but not from any of the other lakes; in the case of Asanikunta tank because of high sediment toxicity. Eight unique IncA/C and IncN resistance plasmids were identified among 11 sequenced transconjugants. Five plasmids were fully assembled, and four of these carried the quinolone resistance gene qnrVC1, which has previously only been found on chromosomes. Acquired resistance genes, in the majority of cases associated with class 1 integrons, could be linked to decreased susceptibility to several different classes of antibiotics.

Conclusions: Our study shows that environments heavily polluted with antibiotics contain novel multiresistance plasmids transferrable to E. coli.

Introduction

Many microorganisms produce antibiotic substances to compete for space and nutrients. Consequently, bacteria have evolved various defence mechanisms to withstand this chemical warfare long before mankind started to use antibiotics to treat bacterial infections. Recent studies have shown that genes conferring resistance to antibiotics can be found in 30000-year-old permafrost sediments, and tolerance to antibiotics is common also in bacterial isolates from deep caves protected from any human influence. In several cases identical, or nearly identical, antibiotic resistance genes (ARGs) have been found in environmental bacteria and pathogens. It is, therefore, increasingly recognized that the external environment constitutes a vast reservoir of ARGs and that many ARGs found in pathogens today have been acquired via horizontal gene transfer (HGT) from harmless bacteria in and around us. Bacteria carrying ARGs seem to be ubiquitous, although their relative frequencies differ widely between habitats.

Such bacteria are also expected to increase in relative numbers whenever a sufficiently high antibiotic selection pressure is applied. An increased abundance of bacteria carrying ARGs is likely to lead to increased opportunities for HGT of such genes, either directly to human pathogens or indirectly via other bacteria that are able to colonize the human body. Release of anthropogenically produced antibiotics to the external environment has, therefore, been identified as a risk factor for increased antibiotic resistance development in human and animal pathogens. Man-made antibiotics can reach the external environment in different ways, out of which releases from manufacturing sites have been associated with the highest concentrations. The role of antibiotic pollution in selection/enrichment of antibiotic-resistant bacteria and HGT-mediated spread of ARGs to other bacteria including human pathogens is, however, scarce, especially regarding the latter process. For some time, we have investigated polluted environments in the vicinity of one of the world’s largest production hubs for antibiotics, located northwest of
Hyderabad, India. Extensive and severe pollution with fluoroquinolone antibiotics has been documented, both in a river receiving the effluent from a treatment plant processing the waste water from a large number of antibiotic manufacturers as well as in two nearby lakes, Asanikunta tank and Kazipally lake.11,14,15 Subsequent studies on environmental samples from polluted sites have shown high levels of ARGs, particularly those conferring resistance to quinolones and sulphonamides.7,15 – 18 However, studies exploring opportunities for transfer of resistance to human pathogens in environments polluted by industrial discharges have to date not been performed. Based on the prevailing antibiotic selection pressure and the presence of a wide diversity of genetic factors promoting mobilization of genes, these extreme environments could conceivably harbour as yet undiscovered ARGs and resistance vectors.9

In this study, we aimed to characterize the level of antibiotic resistance in sediments from Asanikunta tank and Kazipally lake, both with a history of heavy fluoroquinolone pollution. We also aimed to investigate if bacterial communities from these environments are able to transfer resistance to a human pathogen, using Escherichia coli tagged with GFP as recipient. Finally, we wanted to characterize any captured mobile genetic elements (MGEs)—both in terms of resistance conferred as well as their genetics—with the aims to explain the resistance, provide clues to their potential host range, and possibly also identify novel MGEs harbouring ARGs.

Materials and methods

Sediment samples
Lake sediment samples were collected during 2012 from the Indian lakes Kazipally lake (17°34′25.32”N 78°21′21.54”E) and Asanikunta tank (17°33′9.9”N 78°19′59.4”E) on 31 January (one sample per lake) and 11 October (five samples per lake), and from Himayat sagar (17°19′52.86”N 78°21′50.10”E) and Osman sagar (17°22′23.76”N 78°19′7.92”E) on 19 November (one sample per lake). We have earlier reported severe pollution with fluoroquinolone antibiotics in the two former lakes, both located in the vicinity of Patancheru, where several major industries manufacture bulk drugs for the global market.14 The latter two lakes, which were included for comparative purposes, are located in the outskirts southwest of Hyderabad and have previously served as drinking water reservoirs. For additional comparison, sediment samples were collected from the Swedish lakes Harlandatjärn (57°42′27.36”N 12°2′50.28”E) on 6 March (one sample) and 29 October (five samples) 2012, and from Axlemosse (57°40′06.58”N 11°56′55.52”E) on 10 April (one sample) and 27 October (five samples) 2012, in Gothenburg, Sweden, both of which do not receive any municipal or industrial discharges. All samples were taken from the top 5 cm of sediment from different parts of the respective lake.

Analysis of phenotypic antibiotic resistance in sediment samples
One gram of each sediment sample was mixed with 9 mL of 0.85% NaCl by vortexing. The tubes were left for sedimentation for 1.5 min before serial dilutions were prepared and plated on R2 agar (R2A) (Oxoid, Basingstoke, UK) supplemented with cycloheximide (100 mg/L, Sigma-Aldrich, St Louis, MO, USA) and either ciprofloxacin (2 mg/L, AppliChem GmbH, Darmstadt, Germany) or sulfamethoxazole (100 mg/L, Sigma-Aldrich) as well as on control plates supplemented with cycloheximide only. The plates were incubated at 28°C before cfu were counted 4 days later and expressed as percentage growth of control. Differences in bacterial growth between different groups of samples were evaluated by one-way ANOVA with Tukey’s post hoc test.

Capture of MGEs carrying ARGs
Exogenous isolation of conjugative resistance plasmids from bacterial communities was, with minor modifications, conducted as described previously.19,20 A GFP-tagged and kanamycin- and rifampicin-resistant E. coli CV601 strain was mixed with bacterial fractions from the lake sediment samples. In order to activate and extract the bacterial fraction from each sample, 2 g of lake sediment was resuspended in 8 mL of 0.1× tryptic soy broth (TSB) (Lab M, Lancashire, UK) and incubated under gentle shaking (50 rpm) at 28°C overnight. After incubation, particles were allowed to settle for 5 min before the upper part of the supernatant (5 mL) was centrifuged at 4000 g for 5 min. The pellet was washed three times in 0.1× TSB before being resuspended in 500 µL of TSB and served as donor bacterial community.

The recipient strain was cultured in LB broth supplemented with kanamycin (50 mg/mL) at 28°C. The overnight culture was washed three times and resuspended in LB broth (OD400 of 1.0).

Equal volumes of the recipient and donor bacteria from the sediment samples were mixed and applied onto a 0.22 µm filter (Millipore, Bedford, MA, USA) placed on tryptone glucose yeast (TGY) agar (Scharlau, Barcelona, Spain) supplemented with cycloheximide (100 mg/L). Controls with either recipient or donor bacterial community alone were also prepared. After incubation at 28°C for 20 h the bacteria were detached from the filters in 0.85% NaCl by vortexing. Serial dilutions were plated on TGY agar supplemented with cycloheximide (100 mg/L), kanamycin (50 mg/L) and rifampicin (50 mg/L) to determine the total number of recipients, as well as on plates further supplemented with either ciprofloxacin (0.2 mg/L), carbenicillin (50 mg/L) or sulfamethoxazole (100 mg/L) to select for transconjugants. Carbenicillin was included as a selective agent since plasmid-mediated quinolone resistance is often co-transferred with β-lactam resistance.21 After 2 days of incubation at 28°C transconjugants were picked and streaked on identical selection plates and verified to originate from the recipient strain by detection of a green fluorescent signal. Transconjugants obtained by using ciprofloxacin, sulfamethoxazole or carbenicillin as selective antibiotic were termed CIP-TC, SMX-TC and CAR-TC, respectively, followed by a number. For validation purposes, the mobile elements from the transconjugants selected for WGS were transferred to another E. coli recipient, the tetracycline-resistant MH598 strain (a kind gift from Professor Malte Hermansson), by filter mating. The secondary transconjugants were selected with tetracycline (20 mg/L) and either sulfamethoxazole (100 mg/L) or trimethoprim (20 mg/L).

Isolation of plasmid DNA and restriction analysis
Plasmid DNA was isolated with Qiagen’s Plasmid Mini Kit (Qiagen, Hilden, Germany) with the modifications that all buffer volumes were doubled and a 15 min incubation on ice was added after the neutralization of the lysate. The eluted DNA was precipitated with isopropanol, washed with 70% ethanol and dissolved in 10 mM Tris/HCl, pH 8.

Approximately 0.5 µg of DNA from the plasmid preparations was digested with PstI (5 U) and Bst1107 I (5 U) (Fermentas GmbH, St Leon-Rot, Germany). After incubation (3 h, 37°C) the samples were analysed, together with undigested samples, on agarose gel (1%).

Antibiotic susceptibility determinations
The MICs of indicated antibiotics were determined for recipients and transconjugants using Etest® strips (bioMérieux SA, Marcy-l’Étoile, France) according to instructions from the provider. E. coli ATCC 25922 and Staphylococcus aureus ATCC 29213 were used as quality control strains.
Detection of ARGs by PCR
Total DNA was isolated from recipient and transconjugants using the DNeasy® Blood and Tissue Kit (Qiagen). DNA targets were PCR amplified (an initial activation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 40 s, before a final elongation step at 72°C for 7 min) using AmpliTaq® polymerase (Applied Biosystems, Foster City, CA, USA) and primers specific for sulphonamide (sul1 or sul2) or plasmid-mediated quinolone resistance genes (qnrA, qnrB, qnrC, qnrD, qnrS, qnrVC1, qnrVCA, aac6′-Ib-cr or qepA) (Table S1, available as Supplementary data at JAC Online).

WGS and bioinformatic analysis
Total DNA was prepared as described above and sent for sequencing at the facilities of Science for Life Laboratories (Stockholm, Sweden). TruSeq DNA libraries were generated for each sample, multiplexed, and sequenced on one Illumina MiSeq flowcell producing 2×250 bp paired-end reads. Quality filtering and trimming of adaptor sequences was done using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with default values for paired data, except for the stringency, which was set to 10 (parameter –s 10). Reads losing their paired read in the filtering step were retained (parameter –retain_unpaired). De novo assembly for each genome was performed with Spades 2.5.1 using mismatch correction (parameter --careful). Quality statistics of the resulting draft assemblies were obtained using Quast (Table S2).23 Generated contiguous sequences (contigs) were manually assembled into complete plasmid sequences. The manual assembly was confirmed by Sanger sequencing of PCR products that covered the junctions between the assembled contigs.

Genes involved in conjugation machineries were annotated by HMM profiles,24,25 using HMMer,26 and mobile resistance genes were identified by mapping against the ResFinder database (version 1.1, http://www.1928diagnostics.com/resdb) using BLAST.27 The contigs were also mapped against the ResFinder and PlasmidFinder databases to validate the ARG identification and for molecular plasmid replicon typing, respectively. To ensure that there were no spontaneous non-synonymous mutations in chromosomal genes commonly associated with increased quinolone or macrolide resistance, reference protein sequences of GyrA, GyrB, ParC, ParE, AcrR, AcrA, AcrB, TolC, MarR and MarA as well as ribosomal proteins L4 and L22, belonging to E. coli K-12 substr. MG1655, were downloaded from the Pathosystems Resource Integration Center (Patric)28 NCBI GenBank locus tags b2231, b3699, b3019, b3030, b0464, b0463, b0462, b3035, b1530, b1531, b3319 and b3315), and mapped to the draft assemblies using BLAST in blastmode. To identify resistance mutations in the genes encoding ribosomal RNA subunits 16S and 23S, all trimmed reads were mapped against reference sequences from E. coli K-12 MG1655 for both genes (GenBank locus tags b3968 and b4009) using Bowtie (v. 1.0.0).29 The Bowtie output was printed in SAM format (parameter –s) and converted to the VCF format for SNP detection, using the SAMtools (v. 0.1.19) commands view (parameter –bS), sort and mpileup.30 The results of the transconjugants were compared with the recipient strain to determine acquired resistance genes and mutations.

The raw sequencing data were submitted to the sequence read archive (NCBI SRP037860) and the sequences of plasmids pKAZ1, pKAZ2, pKAZ3, pKAZ4 and pKAZ5 were submitted to GenBank (accession numbers KM506769, KR827391, KR827392, KR827393 and KR827394, respectively).

Results
Elevated and transferable antibiotic resistance in sediments from lakes with a history of heavy antibiotic pollution
Analysis of cultivable bacteria from lake sediment samples showed considerably higher proportions of sulfamethoxazole- and ciprofloxacin-resistant bacteria in Kazipally lake and Asanikunta tank compared with the Indian and Swedish lakes included for comparison (Figure 1a). The proportion of resistant bacteria ranged between 23% and 97% and between 12% and 95% for sulfamethoxazole and ciprofloxacin, respectively, in the polluted lakes, whereas the corresponding intervals were 1%–18% and <1%–6% in lakes included for comparison.

When a GFP-tagged E. coli recipient was mixed with bacterial fractions from Kazipally lake, all samples gave rise to

Figure 1. High levels of antibiotic-resistant bacteria and transferable resistance in lakes with a history of severe antibiotic pollution. Lake sediment samples were collected from two Swedish lakes, Axlemosse (A) and Härlandatjärn (H), two Indian water reservoir lakes, Osman sagar (OS) and Himayat sagar (HS), and two Indian lakes where heavy contamination with antibiotics has been documented, Kazipally lake (KL) and Asanikunta tank (AT). (a) The sediment bacteria were cultured on R2A supplemented with 2 mg/L ciprofloxacin (CIP) or 100 mg/L sulfamethoxazole (SMX). The number of cfu was related to cfu on plates without antibiotics. Data are shown as mean values; error bars represent SEM. Significant differences in the percentage of resistant bacteria were assessed by one-way ANOVA: *P<0.05, **P<0.01 or ***P<0.001. (b) A GFP-tagged E. coli recipient strain was mated with bacterial fractions from the different lake sediments. The transconjugant frequencies (transconjugants per recipient) obtained after selection with 0.2 mg/L ciprofloxacin (CIP), 100 mg/L sulfamethoxazole (SMX) or 50 mg/L carbenicillin (CAR) were determined. Data are shown as mean values; error bars represent SEM.
transconjugants. Ciprofloxacin-, sulfamethoxazole- and carbenicillin-resistant transconjugants were retrieved at frequencies of \(4 \times 10^{-5}\), \(1 \times 10^{-4}\) and \(5 \times 10^{-6}\), respectively (Figure 1b). However, although samples collected at two different time-points from Asanikunta tank were used, no transconjugants could be obtained since the recipient bacteria were killed during filter mating despite extensive washing of the donor bacteria, most likely due to toxicants present in the sediments. No transconjugants were obtained from any of the other lakes included as comparison. Neither did control experiments with recipient alone generate any colonies on the selection plates.

**Plasmids captured from Kazipally lake confer multiresistance**

Thirty ciprofloxacin-resistant, 16 sulfamethoxazole-resistant and 8 carbenicillin-resistant transconjugants, termed CIP-TC, SMX-TC and CAR-TC, respectively, were randomly selected for further analyses. Apart from the expected increase in sulfamethoxazole MIC, none of the SMX-TC showed elevated MICs of any of the six additional antibiotics investigated compared with the recipient strain, even though isolated plasmids gave rise to at least five different restriction patterns (data not shown). Among the CAR-TC, two restriction patterns were observed. CAR-TC 2 displayed a unique pattern whereas the remainder appeared identical. This was also reflected in the antibiotic-susceptibility profiles, showing that the MIC of ceftazidime was markedly lower for CAR-TC 2 compared with the other CAR-TC (Table 1). However, the MICs of ampicillin, ceftazidime, sulfamethoxazole and ciprofloxacin were increased for all of the CAR-TC compared with the recipient. The CIP-TC could be divided into seven different groups based on restriction patterns. The indicated relationships between different CIP-TC were further supported by the antibiotic susceptibility tests, although no differences in the resistance profiles could be determined between the groups with the two most-common restriction patterns (Table 1). In addition to being less susceptible to ciprofloxacin, different CIP-TC showed an increased resistance to all of the tested antibiotics. CIP-TC 30, 32 and 33 and CAR-TC 2 showed the most pronounced multiresistance phenotypes.

**Captured plasmids belong to the IncA/C and IncN groups and carry a diversity of ARGs**

Based on the antibiotic susceptibility tests and the restriction fragment analyses, 11 representatives among the transconjugants showing decreased susceptibility to ciprofloxacin were, together with the recipient strain, subjected to WGS: CIP-TC 2, 9, 13, 23, 24, 29, 30, 31 and 33 as well as CAR-TC 2 and 6. The generated contigs were screened for the presence of ARGs as well as relaxase and type 4 secretion system (T4SS) genes involved in conjugation machineries (Table 2). The detected ARGs explained the decreased susceptibility to ciprofloxacin (qnrS1, qnrS2 and qnrVC1), sulfamethoxazole (sul1 and sul2), ampicillin and ceftazidime (blaOXA-10, blaSHV-12 and blOVEB-9), streptomycin (ant(3’)-Ia) and tetracycline (tet(C)) determined for the transconjugants. A tet(A) variant, whose deduced gene product aligned to amino acids 55–393 (97% identity) of the 399 amino acid-long protein encoded by the tet(A) gene described by Waters et al.\(^{31}\) (GenBank: X00006.1), was also observed in four of the transconjugants. Based on the antibiotic susceptibility tests, the identified tet(A) gene did not seem to confer increased tetracycline resistance in these specific genetic contexts. In addition, seven of the investigated transconjugants carried trimethoprim resistance genes (dfrA) and two carried the rifampicin resistance gene arr-3. The samples were also screened for the presence of plasmid-mediated quinolone resistance genes and sul genes with PCR, analyses that validated the shotgun sequencing (data not shown). A majority of the acquired ARGs were associated with class 1 integrons (Table 2). When compared with the recipient strain, none of the transconjugants carried any non-synonymous mutations in gyrA, gyrB, parC, parE, acrR, acrA, acrB, tolC, marR or marA, chromosomal genes known to be associated with increased quinolone resistance.\(^{32}\) Although decreased susceptibility to azithromycin was observed for CIP-TC 2 and 24, no specific macrolide resistance genes were found in the genomes from these isolates, neither were any mutations in the 23S rRNA genes detected. However, CIP-TC 2 and 24 were the only transconjugants carrying an acquired putative resistance–nodulation–division (RND) efflux system; the individual components of the system showed 77%, 67% and 72% identity with MexD, MexC and OprJ (GenBank: U57969), respectively.\(^{33}\) To further evaluate possible contributions of spontaneous mutations to the observed decrease in ciprofloxacin and azithromycin susceptibility, the captured plasmids were transferred to another E. coli recipient (the tetracycline-resistant MH598 strain). The secondary transconjugants showed similar fold changes in ciprofloxacin and azithromycin MICs as the primary ones (Table S3). Antibiotic susceptibility testing of the secondary transconjugants also showed that plasmids carrying the identified dfrA and arr-3 genes conferred trimethoprim and rifampicin resistance, respectively, and confirmed the previously observed resistance patterns for sulfamethoxazole, ampicillin, ceftazidime and streptomycin (Table S3).

Using the classification system proposed by Garcillan-Barcia et al.\(^{34}\) and Smillie et al.,\(^{25}\) the captured plasmids could be divided into two categories: those that carried a mobility (MOB)\(\alpha\) relaxase together with a mating-pair formation (MPF); T4SS and those with a MOB\(\beta\) relaxase in combination with a MPF; T4SS. However, the plasmid seen in both CIP-TC 2 and 24 also carried, in addition to its MPF; T4SS components, of an MPF; T4SS. Molecular replicon typing classified the captured elements as IncN and IncA/C plasmids, which was manifested by the presence of their corresponding repA genes.

**Genetic context for the captured qnr genes is diverse**

The DNA sequences for the IncA/C plasmids were fully assembled and named pKA21–5. Four of these plasmids carried qnrVC1, which has not been described on any plasmid before. On the closely related pKA21 (found in CIP-TC 31 and CAR-TC 6) and pKA22 (found in CIP-TC 29), qnrVC1 and hpa2 gene cassettes with complete att sites were detected downstream of a class 1 integron structure with a duplicated qacE.A1/sul1-containing 3’ conserved segment (3’CS). One ISCR1 transposase and one truncated ISCR2 transposase fused to a 5’ segment of an ISCR1 transposase gene flanked the two gene cassettes (Figure 2). On pKA23 harboured by CIP-TC 33, the same gene cassettes were found between two identical ISCR1 transposases and in close proximity to, but not embedded in, a tentative InT1 transposon (Figure 2). On pKA4, found in both CIP-TC 30 and CAR-TC 2, an almost identical qnrVC1 cassette (a single base deleted in a
non-coding region) was identified in an In2 integron embedded in a Tn21 transposon (Figure 2). This latter gene cassette was identical, or only differing by a single synonymous substitution, to qnrVC1 cassettes earlier described in chromosomal locations. On the pKAZ5 plasmid, found in both CIP-TC 2 and 24, qnrS2 was found in a mobile insertion cassette element. The largest assembled plasmid-associated contigs from CIP-TC 9, 13 and 23 (43.1–43.7 kb) were very similar to each other (>99% identity) and contained all ARGs as well as repA and conjugation machinery genes identified in the respective transconjugant. These contigs were closely related (99% identity) to the IncN plasmid pOW16C2 (GenBank: KF977034), although devoid of one of the class 1 integron structures. In all cases qnrS1 was located in close proximity to a DNA segment that showed 100% identity with the right end of the IS2-like element ISEcI2 (GenBank: AM234722).

### Table 1. Characterization of transconjugants by antibiotic susceptibility tests and restriction fragment analyses of plasmid DNA

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<th>Strain</th>
<th>MIC (mg/L)a</th>
<th>Restriction fragment pattern</th>
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CIP, ciprofloxacin; SMX, sulfamethoxazole; AMP, ampicillin; CAZ, ceftazidime; STR, streptomycin; TET, tetracycline; AZM, azithromycin.
aMIC values that were at least 4-fold increased compared with those for the recipient control are in bold.
Discussion

The present study is the first to capture and characterize conjugative resistance elements from environments polluted by discharges from antibiotic manufacturing. We have assessed phenotypic antibiotic resistance and resistance transferability in sediment bacteria from Kazipally lake and Asanikunta tank, two Indian lakes with a history of severe antibiotic pollution. We show that these lakes constitute environments where maintenance and enrichment of antibiotic-resistant bacteria as well as dissemination of resistance plasmids are facilitated. From one of the lakes we could isolate a variety of resistance plasmids, including several not previously described.

Our findings of high abundances of resistant bacteria in polluted lakes are in accordance with previous studies demonstrating elevated levels of antibiotic-resistant bacteria in aquatic environments impacted by industrial waste from antibiotic manufacturers. The application of GFP-labelled recipient...
bacteria gave us the additional opportunity to study transferable antibiotic resistance in the environmental samples. The approach has previously been used with success for exogenous isolation of a variety of plasmids from various environmental samples. In this study, at least eight different conjugative plasmid variants carrying qnr genes were captured from Kazipally lake, which reflect the history of extreme fluoroquinolone contamination. In two cases, identical plasmids were captured when either ciprofloxacin or carbencillin was used as selective agent. This is in agreement with the frequently reported co-localization of qnr and ESBL genes on various plasmids. In this study, all analysed plasmids carrying qnrVC1 also encoded ESBLs, either bla<sub>ESBL</sub> alone or a combination of bla<sub>SHV-12</sub> and bla<sub>OXA-10</sub>, whereas no ß-lactamase genes were detected on the qnrS-containing plasmids. The qnr genes confer moderate levels of quinolone resistance, but can facilitate the selection of mutants showing high-level resistance. Accordingly, significant proportions of the cultivable bacteria from the impacted lakes could tolerate a ciprofloxacin concentration of 2 mg/L. Many of the qnr-carrying transconjugants also showed a ciprofloxacin MIC very close to the resistance clinical breakpoints (CLSI >2 mg/L; EUCAST >1 mg/L). This is most likely a consequence of the relatively high MIC observed for the recipient strain used for plasmid capture and not due to chromosomal mutations. The observed fold changes in ciprofloxacin MICs are in the range of what have been observed for qnr genes before.

All captured plasmids that conferred decreased susceptibility to ciprofloxacin harboured either qnrS or qnrVC1. This is the first study, to our knowledge, to describe qnrVC1 as plasmidborne, although other members of the qnrVC family have been found on plasmids previously. Earlier studies have described qnrVC1 in chromosomal integrons and SXT elements in Vibrio cholerae. Fonseca et al. hypothesized that the qnrVC1 gene cassette might originate from Vibrio parahaemolyticus although the gene itself most likely has a different origin. In this study, the same qnrVC1 gene cassette was found on pKAZ4<sub>up</sub> embedded in a class 1 integron (In2). In the remaining qnrVC1-containing transconjugants, an almost identical gene cassette was detected between two IS2 transposases in close association with integron (pKAZ1 and pKAZ2) or In2 (pKAZ3) structures. This indicates that integrons and ISCR elements have been involved in the mobilization of qnrVC1 from chromosomes to conjugative plasmids. In contrast, qnrS genes have never been described in attC-containing gene cassettes. In accordance, the detected qnrS genes in this study were either localized to mobile insertion cassettes or associated with a truncated ISace2 element. ISace2 has earlier been shown to be associated with qnrS1 on plasmids from Enterobacteriaceae isolates, whereas qnrS2, as well as qnrD, have been identified as parts of mobile insertion cassettes located on plasmids from Aeromonas spp. and Proteae isolates, respectively. Altogether, this emphasizes the role of various genetic elements in the dissemination of qnr genes. Based on the genetic context of all detected ARGs, class 1 integrons seem to be particularly important for the acquisition of resistance among bacteria residing in Kazipally lake. This is supported by a recent study showing high abundance of intI1 in this lake. In addition, 74% of isolates collected inside the waste water treatment plant receiving process water from the nearby antibiotic manufacturers carried class 1 integrons.

Apart from the decreased azithromycin susceptibility, the changed resistance profiles of all transconjugants could be linked to acquired ARGs detected after WGS. However, a tentative efflux system related to MexCD-OprJ was detected in the two transconjugants showing increased resistance to azithromycin. MexCD-OprJ has previously been shown to decrease susceptibility to macrolides as well as other antibiotics and biocides, but such a system has previously not been shown to be plasmid-encoded.

The captured plasmids belong to the Inca/C and Incn incompatibility groups. The types of plasmids captured are inevitably affected by the choice of recipient bacteria, thus only plasmids that can be transferred to and maintained in E. coli could be detected with the protocol used here. However, Inca/C and Incn plasmids are currently regarded as epidemic resistance plasmids being involved in the worldwide dissemination of multidrug resistance among Enterobacteriaceae. In addition, the host range of Inca/C plasmids also comprise Vibrioaceae bacteria. Consequently, the captured novel plasmids have the potential to spread among a diversity of pathogens and thus be a concern for human and animal health. It has been demonstrated that long-term persistence of Inca/C plasmids in E. coli, where these plasmids are often found, requires a strong selection pressure. Johnson and Lang hypothesized that other non-Enterobacteriaceae bacteria may serve as reservoirs for these plasmids when resistance traits are not needed and proposed that certain soil and water bacteria have been the most recent long-term host of these plasmids. From this study we cannot conclude from which bacterial species the conjugative plasmids were captured, nor their evolutionary history. However, previously described Inca/C plasmids isolated from the aquatic bacteria Photobacterium damselae subsp. piscicida (pP91278) and Aeromonas hydrophila (pR148), both belonging to Vibrioaceae, have backbones closely related to those of pKAZ1-5. Thus, it is tempting to speculate that the captured plasmids have evolved in an aquatic environment under a selection pressure from quinolones, such as Kazipally lake. In accordance, a recent shotgun metagenomics analysis of sediments from Kazipally lake showed the presence of bacteria from Photobacterium and Aeromonas as well as Enterobacteriaceae genera.

To conclude, our study supports the hypothesis that sites contaminated with industrial waste from antibiotic manufacturers constitute risk environments for the development of antibiotic-resistant bacteria. We also highlight that such risk environments can be exploited to identify novel mediators of antibiotic resistance. Functional metagenomics as well as combining metagenomic sequencing and computational pipelines have proven effective in identifying novel ARGs, but without acquiring knowledge of their wider genetic context. As shown here, the combination of capturing MGEs conferring antibiotic resistance in tagged recipient bacteria and WGS of the generated transconjugants can thus complement the above-mentioned methods by identifying and characterizing novel resistance vectors.

Acknowledgements

We would like to thank Gamana, Patrik Sköna and Tomas Nilsson for valuable help with sampling, and Dr Carolin Rutgersson, Klara Karling and Helen Nygren for excellent technical assistance.

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SUPPLEMENTARY MATERIALS


**Funding**

This work was supported by the Swedish Research Council (grant numbers 523-2008-5711 and 2011-4744), FORMAS (grant number 521-2010-3142), MISTRA (grant number 2004-147), the Wallenberg Foundation and the Adlerbertska Research Foundation.

**Transparency declaration**

None to declare.

**Supplementary data**

Tables S1–S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**


Isolation of novel fluoroquinolone resistance plasmids


