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Quinolone resistance genes (*qnrA* and *qnrS*) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes

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Objectives: This study quantifies quinolone antibiotic resistance genes (*qnrA* and *qnrS*) in DNA of phage particles isolated from faecally polluted waters and evaluates the influence of phage inducers on the abundance of antibiotic resistance genes in packaged DNA.

Methods: *qnrA* and *qnrS* were quantified by qPCR in DNA of phage particles isolated from 18 raw urban wastewater samples, 18 river samples and 28 archived samples of animal wastewater. The bacterial fraction of the samples was treated with mitomycin C, ciprofloxacin, EDTA or sodium citrate under different conditions, and the number of resistance genes in DNA of phage particles was compared with the non-induced samples.

Results: *qnrA* was more prevalent than *qnrS*, with 100% of positive samples in urban wastewater and river and 71.4% of positive samples in animal wastewater. Densities of *qnrA* ranged from 2.3×10^2 gene copies (GC)/mL in urban wastewater to 7.4×10^1 GC/mL in animal wastewater. *qnrS* was detected in 38.9% of urban wastewater samples, in 22.2% of river samples and only in one animal wastewater sample (3.6%). Despite the lower prevalence, *qnrS* densities reached values of 10^3 GC/mL. Both *qnr* genes and other resistance genes assayed (bla_{TEM} and $bla_{CTX\text{-}M}$) showed a significant increase in DNA of phage particles when treated with EDTA or sodium citrate, while mitomycin C and ciprofloxacin showed no effect under the different conditions assayed.

Conclusions: This study confirms the contribution of phages to the mobilization of resistance genes and the role of the environment and certain inducers in the spread of antibiotic resistance genes by means of phages.

Keywords: phages, EDTA, sodium citrate

Introduction

Quinolones and fluoroquinolones are antimicrobials commonly used in clinical and veterinary medicine.[1] The first quinolone, nalidixic acid, was introduced into clinical use in 1962 and in the mid-1980s ciprofloxacin, a fluoroquinolone with a wide spectrum of *in vitro* antibacterial activity, first became available clinically.[2] Resistance to quinolones in enterobacteria has become common and is dramatically increasing worldwide.[3,4]

The main mechanism of quinolone resistance involves the accumulation of mutations in the genes coding for the target bacterial enzymes of the fluoroquinolones, DNA gyrase and DNA topoisomerase IV,[5] which protect against the inhibitory activity of quinolones. However, the traditional understanding of quinolone resistance as a mutational phenomenon has not provided a completely satisfactory explanation for the frequency with which it has arisen. Such a phenomenon might be better accounted for by horizontally transferable elements. These supply a degree of reduced quinolone susceptibility, enough for microorganisms to survive in the presence of quinolones, while resistance mutations occur sequentially rather than simultaneously. Since 1998, when *qnrA1* was discovered,[6] several plasmid-mediated quinolone resistance (PMQR) genes have been described.[7] More recent findings suggest that the *qnr* genes in circulation could have originated in the chromosomes of water-dwelling or other environmental organisms.[8] In the face of intense quinolone pressure, such genes have entered circulation on mobile genetic elements.[9]

Acquired Qnr proteins belong to a pentapeptide repeat family. To date, six families of Qnr proteins have been described: QnrA, QnrB, QnrC, QnrD, QnrS and QnrVC. *qnr* genes are highly diverse, with 7 *qnrA*, 73 *qnrB*, 1 *qnrC*, 2 *qnrD*, 9 *qnrS* and 5 *qnrVC* genes

identified (http://www.lahey.org/qnrStudies/). Qnr genes have been reported worldwide from unrelated enterobacterial species and are usually associated with mobile elements.[10,11]

A series of studies have identified the environment as a reservoir of PMQR genes, with farm animals and aquatic habitats being significantly involved. Recent observations suggest that the aquatic environment might constitute the original source of PMQR genes, which then spread among animal or human isolates by means of several mobile genetic elements.[12,13]

Besides plasmids, other studies highlight the abundance of phage particles as mobile genetic elements carrying antibiotic resistance genes (ARGs) in the environment.[14–16] Metagenomic analysis confirms the abundance of ARGs in viral DNA in diverse biomes.[17] However, there is still little information on phages carrying ARGs in environmental settings.

Several factors could promote gene transfer among bacteria in an aquatic environment.[18] The lytic cycle of temperate bacteriophages could be activated by various environmental factors and chemicals. Activation of the phage lytic cycle that leads to prophage excision from the bacterial chromosome is called phage induction, and continues with the generation of new phage particles until the bacterial host lyses or bursts, releasing the phages outside the cell. Among others, some antibiotics, and specifically fluoroquinolones, cause phage induction and subsequent gene transduction.[19,20]

In this study we focused first on the qPCR quantification of two quinolone resistance genes (*qnrA* and *qnrS*) in DNA in phage particles isolated from environmental samples. These genes were selected because they are widely distributed in our region and clinically relevant.[21] We also wanted to evaluate the influence of phage-inducing factors on the number of quinolone-resistance genes in DNA in phage particles after induction of the bacterial populations in the water samples. For this purpose, we quantified by qPCR four ARGs (bla_{TEM}, $bla_{CTX\text{-}M}$, *qnrA* and *qnrS*) in the phage DNA fraction of wastewater samples treated with phage inducers: mitomycin C as an inducer of the SOS response, ciprofloxacin as a quinolone antibiotic and EDTA and sodium citrate as chelating agents. Mitomycin C is a compound commonly used for induction of temperate phages.[22,23] In addition, some quinolones, such as ciprofloxacin, have also been used for induction of phages.[24–26] Both compounds activate the SOS response through RecA activation. EDTA and sodium citrate were also applied because they have been reported to increase the number of copies of Shiga toxin gene (*stx*) in temperate Stx phages when a culture of a strain lysogenic for a Stx phage is treated with 20 mM EDTA, even in the absence of RecA.[27]

Materials and methods

Samples

Eighteen raw urban wastewater samples were collected between autumn 2009 and spring 2013 from the influent of a wastewater treatment plant in the Barcelona area, including a number of cities and towns, with ~500 000 inhabitants. Eighteen river samples were collected monthly between winter 2010 and summer 2012 from the Llobregat river near the Barcelona area, which is subject to anthropogenic pressure.[15] Twenty-eight archived wastewater samples from animals (27 from cattle and 1 from poultry) that were collected from several slaughterhouses and farms in Spain were included in this study.[16]

All samples were collected in sterile containers, transported to the laboratory at 5 ± 2°C within 2 h of collection and processed immediately for bacterial counts and further experiments. Archived samples had been stored at −70°C for >1 year.

Bacterial strains and media

The clinical *Escherichia coli* strain 266 was used as a control for *qnrA* and the environmental *Enterobacter cloacae* strain 565 was used as a control for *qnrS*. *E. coli* WG5 (ATCC 700078) was used as a host for evaluation of somatic coliphages.[28] Luria–Bertani (LB) agar or broth was used for routine bacterial propagation. Tryptic soy agar (TSA) and Chromocult® Coliform Agar (Merck, Darmstadt, Germany) were used for the detection of heterotrophic bacteria and *E. coli* respectively. For the detection of resistant bacteria, media were supplemented with 32 mg/L ampicillin (Sigma-Aldrich, Steinheim, Germany), 25 mg/L nalidixic acid (Sigma-Aldrich) or ciprofloxacin at 4, 1, 0.4 and 0.1 mg/L (Sigma-Aldrich). A self-inducible Cdt bacteriophage was used as negative control for phage induction.[29]

Microbiological parameters

The extent of faecal contamination in the samples was established by counting the total heterotrophic bacteria grown at 37°C and *E. coli* as bacterial indicators. Heterotrophic bacteria and *E. coli* were analysed by the membrane filtration method, in line with previously standardized methods.[30] Briefly, serial decimal dilutions of urban wastewater and river water were filtered through 0.45 mm pore membrane filters (0.45 μm and 47 mm white-gridded EZ-Pak® Membrane Filters, Millipore). Membranes were placed upside up on the respective agar media and incubated at 37°C for 18 h. To evaluate the presence of bacteria resistant to antibiotics, samples were processed as described above and incubated in TSA or Chromocult® coliform agar for 2 h at 37°C. Then, membranes were transferred to TSA or Chromocult® coliform agar containing the respective antibiotics and further incubated at 37°C for 18 h.

Somatic coliphages, proposed as faecal viral indicators, were included to get an indication of the levels of virulent bacteriophages in the samples,[28] and to evaluate the effect of the inducers on the presence of virulent phages that could have been induced from the bacterial population. This method allows detection of many virulent infectious phages from a faecal sample with a single host strain (*E. coli* WG5) and through the double agar layer plaque assay.[28]

Standard PCR procedures

Conventional PCRs for ARGs bla_{TEM} and $bla_{CTX\text{-}M}$ were performed as described in previous studies.[15] For *qnr* amplification, *qnr*-positive strains were used as positive controls and PCR was performed using the oligonucleotides described in Table S1 (available as Supplementary data at *JAC* Online) with a GeneAmp PCR system 2700 (Applied Biosystems, Barcelona, Spain). An aliquot of 5 μL of each PCR product was analysed by agarose (0.8%) gel electrophoresis and bands were viewed by ethidium bromide staining. When necessary, PCR products were purified with a PCR Purification Kit (Qiagen Inc., Valencia, USA).

qPCR procedures

For quantification of each ARG in DNA in phage particles (phage DNA), TaqMan qPCR assays using standards prepared as previously described for bla_{TEM} and $bla_{CTX\text{-}M}$ genes were used.[15]

A real-time qPCR assay for *qnrA* and *qnrS* was developed in this study to detect both the quinolone resistance genes in phage DNA isolated from urban wastewater samples. The qPCR oligonucleotides for *qnrA* detected seven variants (*qnrA*1–7) of the gene and showed a detection limit of 3.1 gene copies (GC)/μL (threshold cycle of 33.5). The new qPCR developed for *qnrS* detected six variants (*qnrS*1–6) and showed a detection limit of 8.3 GC/μL (threshold cycle of 30) (Figure 1).

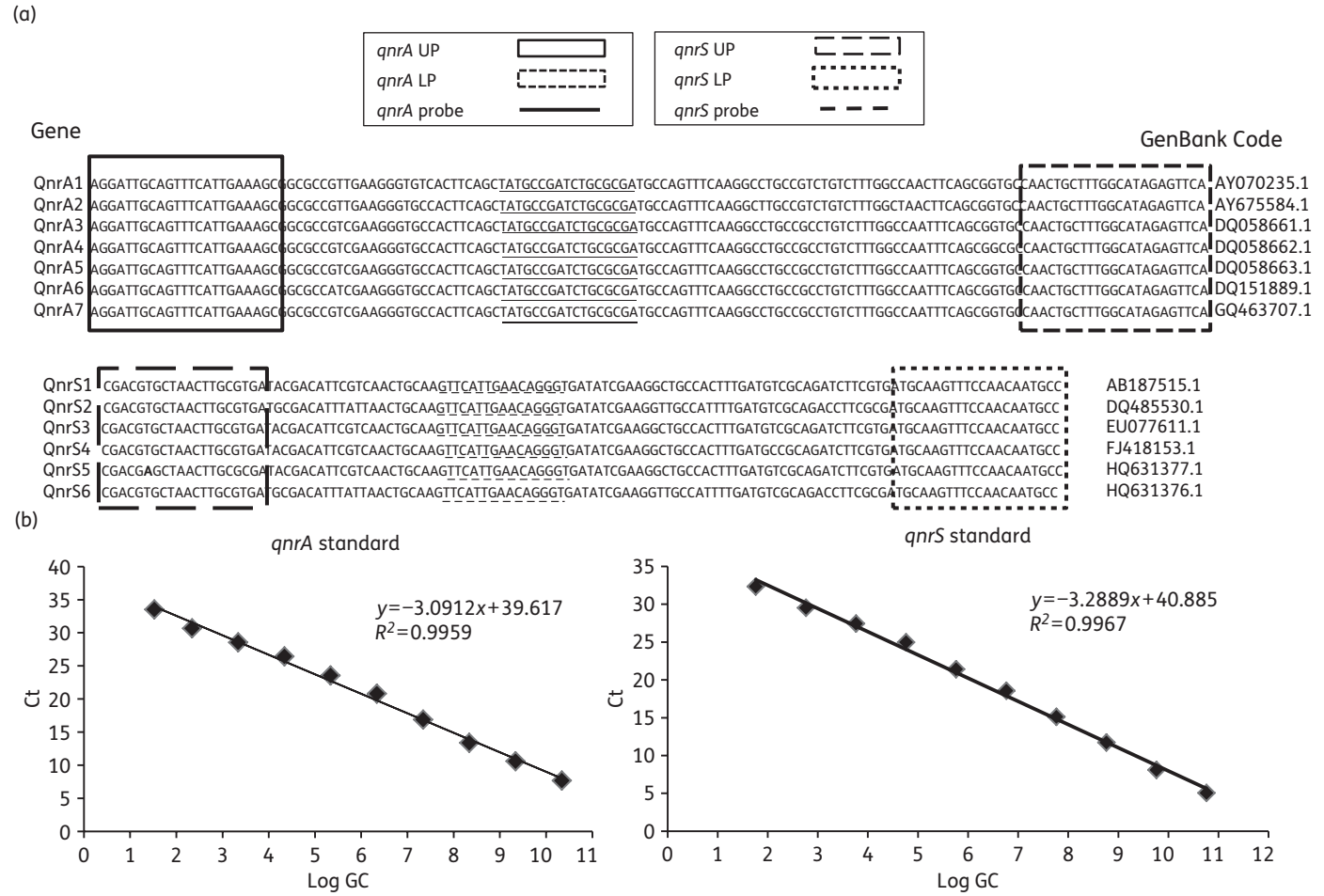

Figure 1. (a) Primers and TaqMan probe resulting from the alignment of *qnrA* genes and *qnrS* genes. Sequences of the probe, upper primer (UP) and lower primer (LP) are shown for each gene. The column on the right indicates the GenBank accession numbers of some of the genes used in the alignment. (b) Standard curves for *qnrA* and *qnrS* qPCR.

For the generation of standards for the qPCR assays, a plasmid construct was used. The 565 bp fragment of *qnrA* and the 425 bp fragment of *qnrS*, both obtained by conventional PCR as described above and purified using a gel extraction kit (PureLink Quick Gel Extraction and PCR Purification Combo Kit, Invitrogen Carlsbad, USA), were cloned with a pGEM-T Easy Vector (Promega, Barcelona, Spain) for insertion of PCR products, following the manufacturer's instructions. The construct was transformed by electroporation into *E. coli* DH5α electrocompetent cells. Cells were electroporated at 2.5 kV, 25 F capacitance and 200 Ω resistance in a BTX ECM 600 Electroporation System (Harvard Apparatus, Inc., MA, USA). Colonies containing the vector were screened by conventional PCR to evaluate the presence of the vector containing each insert. The presence of the insert in the vector and its orientation were assessed by PCR and sequencing. The vector containing the insert was purified from the positive colonies using the Qiagen Plasmid Midi purification kit (Qiagen Inc., Valencia, USA) and the concentration of the vector was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermoscientifics, Wilmington, USA). The reaction product was linearized by digestion with XmnI restriction endonuclease (Promega Corp., Madison, USA). The restricted product was purified and quantified for use in the qPCR assays.

The GC/μL value of the stock prepared was calculated for each gene and the standard curve for qPCR was prepared as previously described.[15] Three replicates of each dilution were added to each qPCR.

Using the software tool Primer Express 3.0 (Applied Biosystems), primers and probes were selected for use in a standardized TaqMan amplification protocol. Primers and 6-carboxyfluorescein (FAM)-labelled fluorogenic probes were commercially synthesized by Applied Biosystems (Spain). *qnrS* and *qnrA* probes were minor-groove binding (MGB) probes with an FAM reporter and a non-fluorescent quencher (NFQ). Primers and probes were used under standard conditions in a StepOne Real-Time PCR System (Applied Biosystems, Spain). Primer and probe specificities were determined with sequence alignments using BLAST and NCBI data entries. They were tested for cross-reactions with the respective susceptible strains. They were amplified in a 20 μL reaction mixture with TaqMan Environmental Real-Time PCR Master Mix 2.0 (Applied Biosystems, Spain). The reaction contained 7 μL of the DNA sample or quantified plasmid DNA. Thermal cycling was performed under standard conditions as indicated by the manufacturer of the StepOne Real-Time PCR system. Briefly, an initial setup of 10 min at 95°C was followed by 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing/extension at 60°C. All samples were run in duplicate, as well as the standards and positive

and negative controls. The GC value was defined as the average of the duplicate data obtained.

To screen for PCR inhibition, dilutions of the standard were spiked with environmental DNA and the experimental difference was compared with the known number of copies of the target genes in the standards. Inhibition of PCR by environmental DNA was not detected.

Sequencing

The amplimers cloned to prepare the qPCR standards were sequenced with an ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Spain), following the manufacturer's instructions and with the primers shown in Table S1. All sequencing was performed at least in duplicate.

Purification of DNA in phage particles

To purify DNA in phage particles from the samples (phage DNA), 50 mL of each sample was centrifuged at 3000 **g** for 10 min, passed through low protein-binding 0.22 μm pore membrane filters (Millex-GP, Millipore, Bedford, MA) and then 100-fold concentrated at 3000 **g** by means of protein concentrators (100 kDa Amicon Ultra centrifugal filter units, Millipore, Bedford, MA), following the manufacturer's instructions. The concentrate was recovered from the tube and treated with chloroform to rule out the presence of possible vesicles containing DNA, and then treated with DNase (100 U/mL of the phage lysate) to eliminate any free DNA that might be present in the samples outside the phage particles. After heat inactivation of the DNase, and to confirm that bacterial or free DNA containing the target genes had been removed from the sample, an aliquot of the phage lysate at this stage was amplified for eubacterial 16S rDNA by conventional PCR and the different ARGs by qPCR (Table S1).

DNA from the phage fraction was isolated from phage lysates as previously described.[15,16] The concentration and purity of the DNA extracted were determined with a NanoDrop ND-1000 spectrophotometer.

Assays with inducing agents

To evaluate the effects of different compounds on phage induction from the bacterial populations in the samples, 50 mL of raw urban wastewater was incubated with the addition of mitomycin C (0.5 mg/L), ciprofloxacin (4, 1, 0.4 and 0.1 mg/L), EDTA (20 mM, pH = 7.2) or 0.2 M sodium citrate, in aerobic conditions under agitation (180 rpm). When indicated, the samples were supplemented with glucose (0.1 mg/mL) and analysed at 37°C for 18 h or at 22°C for 48 h. Phage DNA was purified from the samples with or without inducing agent and used as a template for the quantification of the ARGs by qPCR. For anaerobic conditions, samples were incubated in anaerobic jars.

Statistical analyses

Data and statistical tests were performed using the Statistical Package for Social Science (SPSS) software. One-way analysis of variance (ANOVA) was used to evaluate the differences between the resistance genes detected in phage DNA and the difference in the densities of virulent phages in the induction experiments. Evaluations were based on a 5% significance level in both cases, where a P value of <0.05 was considered to denote a significant difference.

Results

Prevalence of heterotrophic bacteria and E. coli in urban wastewater and river water samples

The samples showed levels of bacterial and viral indicators that were relatively homogeneous in all the urban and river water samples tested (Table 1) and similar to those previously reported from the same source.[15,31] River water samples showed significantly lower numbers ($P<0.05$) than urban wastewater ones; the differences were attributed to the lower faecal input received by river water. The numbers of resistant bacteria were lower than the numbers of bacteria cultured without antibiotics, as expected, though the numbers of resistant bacteria were still quite high, suggesting that bacteria resistant to some antibiotics that were common in the water samples of our study.

Total heterotrophic bacteria showed average values of 7 $\log_{10}$ cfu/mL and 5 $\log_{10}$ cfu/mL of *E. coli* in urban wastewater (Table 1). Most heterotrophic bacterial isolates were resistant to ampicillin and nalidixic acid and showed densities of <1 logarithm below those of the colonies obtained without antibiotic selection. River water samples showed values at least 3 $\log_{10}$ units lower than urban wastewater for all microbiological parameters.

Table 1. Samples analysed and microbiological parameters

	Urban wastewater (n=18)		River water (n=18)	
	average $\log_{10}$ cfu/mL (SD)	differences between absence and presence of antibiotics	average $\log_{10}$ cfu/mL (SD)	differences between absence and presence of antibiotics
Heterotrophic bacteria	7.33 (6.81)	—	4.16 (3.72)	—
E. coli	5.58 (5.21)	—	1.76 (1.63)	—
Heterotrophic bacteria AMP^R	7.11 (6.96)	0.22	4.04 (4.08)	0.12
Heterotrophic bacteria CIP^R	5.30 (5.09)	2.03	1.83 (2.00)	2.33
Heterotrophic bacteria NAL^R	6.99 (6.83)	0.34	3.59 (3.08)	0.57
E. coli AMP^R	5.10 (4.77)	0.48	1.63 (1.41)	0.13
E. coli CIP^R	4.61 (4.19)	0.97	0.81 (0.62)	0.95
E. coli NAL^R	4.98 (4.45)	0.60	1.04 (1.06)	0.72
Somatic coliphages	4.41 (0.51)	—	2.39 (0.44)	—

AMP^R, ampicillin resistant; CIP^R, ciprofloxacin resistant; NAL^R, nalidixic acid resistant.

Resistant *E. coli* also showed values close to those of the bacteria grown without antibiotic selection, particularly when using ampicillin. Ciprofloxacin was the antibiotic showing the largest difference (>2 $\log_{10}$ cfu/mL), in both heterotrophic bacteria and *E. coli*, compared with the wastewater and river samples without antibiotics.

It should be noted that the antibiotic concentrations used in these studies were in the low range, in accordance with other reports analysing resistance in the environment,[32] in order to avoid inhibition of the growth of stressed or damaged bacterial cells.

Prevalence of qnrA and qnrS in the DNA of the phage fraction of the samples

The *qnrA* gene was more prevalent than *qnrS* in the phage DNA of the samples analysed. All urban wastewater and river samples were positive for *qnrA* in the phage DNA as were 71.4% of the archived animal wastewater samples. On average, river water and animal wastewater showed *qnrA* densities less than one order of magnitude below those of urban wastewater (Figure 2).

*qnr*S was less prevalent, detected in 38.9% of urban wastewater, 22.2% of river water and 3.6% of animal wastewater samples (Figure 2). The densities of the genes detected in the few positive samples were, however, higher than the values observed for *qnr*A and were higher in urban and animal wastewater than in river water.

For *qnrA*, reference values obtained from bacterial DNA in urban wastewater samples were on average 1.8×10^3 versus 1.3×10^2 GC/mL in river water samples. On average, *qnrS* showed densities of 2.0×10^5 GC/mL in urban wastewater and 6.9×10^3 GC/mL in river water samples.

Controls described in the Materials and methods section, designed to rule out the presence of non-encapsidated DNA and DNA in vesicles, showed negative values, which indicated that the results obtained were due to amplification of the DNA contained within viral particles.

Comparing the differences between urban wastewater and river water for the prevalence of *qnr* genes in phage DNA with the differences observed for quinolone-resistant bacteria (Table 1), wider differences between resistant bacteria were found. This could be attributable to several facts. First, resistance in bacteria is caused by genes other than *qnr*; second, we were detecting a fraction of quinolone-susceptible autochthonous bacteria in river water, while a larger fraction of faecal bacteria from humans is present in urban wastewater; third, there is stronger persistence of *qnr* in phages, protected within the phage capsid in the river environment, while bacterial DNA could have been degraded or bacteria could have been grazed.

Phage inducers: mitomycin C, ciprofloxacin and EDTA

Mitomycin C, ciprofloxacin and EDTA were assayed in wastewater samples to study whether these compounds could induce phages carrying ARGs from the bacterial population in the sample and hence increase the number of ARGs in the phage DNA fraction of the induced samples. Since these experiments were intended to induce phages from the bacterial population occurring in the samples, glucose was added to the samples to stimulate bacterial metabolism. Assuming that part of the bacterial population in the samples consisted of allochthonous (faecal) bacteria but also autochthonous (environmental) bacteria, the experiments were performed at 37°C for 18 h, but also at 22°C for 48 h for optimal growth of both types of population.

The GC/mL values of each gene in the phage DNA of the samples induced were compared with those of the non-induced samples assayed under the same conditions. The most striking result was that samples incubated with EDTA (and, as shown later, with sodium citrate), with or without glucose showed a significant (ANOVA, $P<0.05$) increase in *qnr* GC/mL in phage DNA when compared with the non-induced samples (Table 2). *qnrS* showed the highest number of GC/mL in phage DNA, reaching differences of up to 4.46 $\log_{10}$ when EDTA was present and the samples were incubated at 22°C without glucose. Under the other conditions, the increase in the density of *qnr*S with EDTA varied from 2.6 to 4 $\log_{10}$ units (Table 2).

Regarding the presence or absence of glucose and the influence of temperature, there were no significant differences (ANOVA $P>0.05$) between the number of GC/mL in phage DNA when samples were incubated at 37 or 22°C or when glucose was added.

In this study, we decided to seek ARGs other than *qnr* to verify whether the inducing agents would cause a similar effect. bla_{TEM}, $bla_{CTX\text{-}M}$ and *mecA* were analysed (Table 2). bla_{TEM} and, to a lesser extent, $bla_{CTX\text{-}M}$ showed a significant ($P<0.05$) increase in the GC/mL value in phage DNA after addition of EDTA, with differences of 2.34–3.31 $\log_{10}$ for bla_{TEM} and 0.82–1.60 $\log_{10}$ for $bla_{CTX\text{-}M}$. However, there was no significant ($P>0.05$) increase in GC/mL for the *mecA* gene when the inducers were present. Whether this was due to the gene itself or because this gene was located in Gram-positive bacteria could not be decided from our data.

Remarkably, the differences (increase or reduction in GC numbers) were not significant ($P>0.05$) (Table 2) when samples were incubated with mitomycin C or ciprofloxacin, known to be inducers of temperate phages by means of activation of RecA and therefore the SOS pathway. In some cases densities after induction were lower than those of the non-induced samples, probably due to the inherent uncertainty of the method. Given the results obtained with ciprofloxacin, we assayed different concentrations of the antibiotic (0.4, 1 or 10 mg/L) but, as observed at 4 mg/L, there were no significant ($P>0.05$) differences in the increase in GC/mL of any of the ARGs in phage DNA. Results given are for 4 mg/L only.

To rule out a possible effect of EDTA on the qPCR assay or on the extraction method that might cause the increase in GC number, we also evaluated as a control another temperate phage available within our research group. This phage harbours the cytolethal distending toxin (Cdt-V).[29] The phage has been reported as self-inducible, since it does not increase the number of phages induced after treatment with any of the inducing agents assayed here. No significant ($P>0.05$) increase in the GC number of Cdt phages in phage DNA after EDTA treatment was observed in this study (data not shown), and was reported previously.[29] These results confirm that the effect of the chelating agents was not due to any interference in the extraction or amplification process.

Sodium citrate

Since the effect observed by EDTA was attributable to its chelating properties,[27] we attempted to assay whether the chelating effect

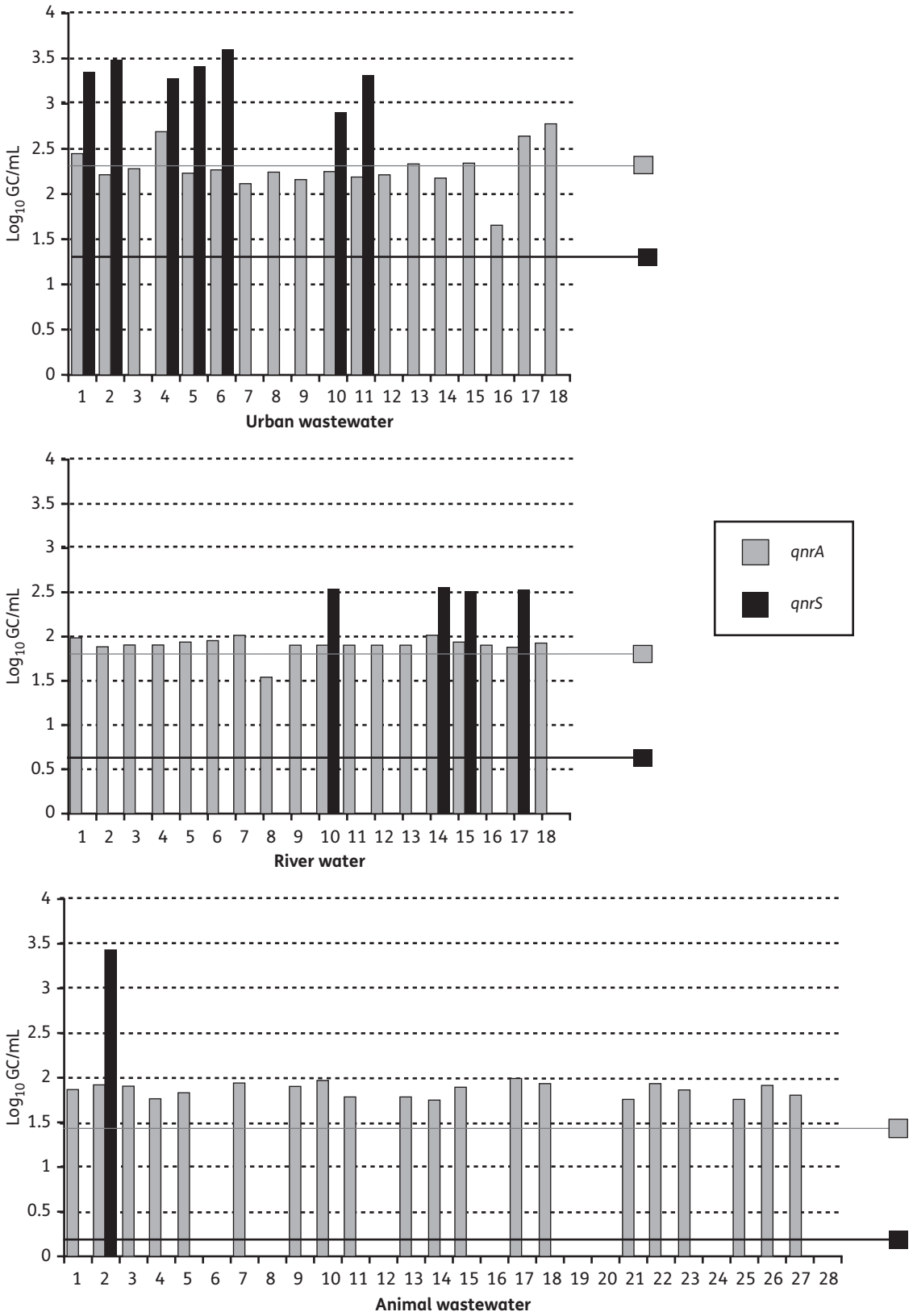

Figure 2. Densities of *qnrA* and *qnrS* genes (GC/mL) in phage DNA of raw urban wastewater, river and animal wastewater samples. Horizontal lines show the averaged values for *qnrA* and *qnrS*.

Table 2. Differences expressed as $\log_{10}$ GC/mL of bla_{TEM}, $bla_{CTX\text{-}M}$, *qnrA*, *qnrS* and *mecA* in phage DNA obtained from induced versus non-induced samples with and without glucose and incubated at 37°C and 22°C under aerobic conditions

		Differences between induced and non-induced samples ($\Delta\log_{10}$ GC/mL)											
		mitomycin C−control				ciprofloxacin (4 mg/L)−control				EDTA−control			
		−glucose		+glucose		−glucose		+glucose		−glucose		+glucose	
Gene		37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C
bla_{TEM}	inducer−control	−0.04	0.06	0.01	−0.53	−0.12	0.21	0.23	−0.46	**2.34**	**3.31**	**2.58**	**2.60**
	standard error	0.22	0.03	0.21	0.11	0.18	0.21	0.04	0.02	0.30	0.28	0.37	0.55
$bla_{CTX\text{-}M}$	inducer−control	0.12	−0.38	−0.12	0.10	0.16	0.44	0.49	−0.62	**0.82**	**1.01**	**1.60**	**1.27**
	standard error	0.06	0.55	0.01	0.12	0.28	0.30	0.34	0.17	0.20	0.35	0.15	0.69
qnrA	inducer−control	−0.24	−0.54	0.21	0.18	−0.52	−0.88	−0.76	−0.48	**0.88**	**1.01**	**1.52**	**1.50**
	standard error	0.52	0.10	0.56	0.05	0.53	0.24	0.07	0.07	0.41	0.52	0.43	0.53
qnrS	inducer−control	−0.14	0.31	−0.28	0.40	−0.47	0.57	−0.22	0.09	**2.85**	**4.46**	**2.60**	**4.00**
	standard error	0.25	0.10	0.06	0.11	0.17	0.23	0.18	0.07	0.27	0.13	0.38	0.24
mecA	inducer−control	0.13	0.08	0.45	0.08	0.05	0.49	−0.27	0.02	0.17	0.19	0.06	0.22
	standard error	0.07	0.04	0.25	0.04	0.11	0.30	0.22	0.07	0.12	0.01	0.09	0.03

		sodium citrate−control			
		−glucose		+glucose	
Gene		37°C	22°C	37°C	22°C
bla_{TEM}	inducer−control	**2.74**	**2.73**	**2.14**	**0.95**
	standard error	0.21	0.27	0.45	0.51
$bla_{CTX\text{-}M}$	inducer−control	**1.06**	**0.93**	**0.86**	**0.67**
	standard error	0.10	0.08	0.04	0.01
qnrA	inducer−control	**0.85**	**1.77**	**0.65**	**1.95**
	standard error	0.29	0.20	0.03	0.01
qnrS	inducer−control	**1.90**	**2.00**	**0.61**	**0.88**
	standard error	0.44	0.45	0.22	0.30
mecA	inducer−control	ND	ND	ND	ND
	standard error	—	—	—	—

ND, not detected.
Results are expressed as the mean of five independent experiments and the standard errors are indicated. Bold font indicates a significant increase (ANOVA $P<0.05$).

was the cause of the increase in ARGs in phage DNA. For this purpose samples were treated with another chelator, sodium citrate, which, like EDTA, is a chelator of Ca^{2+} and Mg^{2+}. Results observed with sodium citrate in *qnrA*, *qnrS*, bla_{TEM} and $bla_{CTX\text{-}M}$ showed the same effect as EDTA. Sodium citrate significantly ($P>0.05$) increased the number of ARGs in phage DNA of induced samples compared with non-induced samples (Table 2). The highest increase in GC number was observed for bla_{TEM}. As observed previously when using EDTA, no increase in the GC number of *mecA* in phage DNA was observed, and the use of Cdt phage as control did not result in any increase in the number of induced phages.

Anaerobic conditions

Samples incubated under anaerobic conditions did not show a significant ($P>0.05$) increase when using mitomycin C or ciprofloxacin (Table 3). In contrast, when using chelating agents there was a clear effect, shown by a significant ($P<0.05$) increase in the GC number of bla_{TEM} and *qnr* with EDTA. $bla_{CTX\text{-}M}$ genes showed an increase with EDTA, but it was not significant. Only *qnrS* with sodium citrate showed a significant increase. Results showed the same trends as assays under aerobic conditions.

Effect of inducing agents on virulent phages

We assayed the influence of the inducing agents on infectious somatic coliphages. The infectivity of the phages was confirmed by their ability to generate plaques of lysis on a monolayer of *E. coli* WG5 used as a host strain. Five urban wastewater samples were treated with mitomycin C, ciprofloxacin, EDTA and sodium citrate, and somatic coliphages present in the samples were evaluated before and after treatment with the four inducing agents (Figure 3). The differences between induced samples and controls were mostly not significant ($P>0.05$). On the few occasions when the difference was significant (e.g. with EDTA or sodium citrate) the induced sample showed lower density than the control. Only samples treated with mitomycin C and glucose showed a non-significant ($P<0.05$) increase, of <0.5 $\log_{10}$ unit.

Discussion

The mobilization of ARGs described in phages could be caused by generalized transduction.[33] The phage particles causing generalized transduction do not contain the phage genome, but fragments of bacterial DNA. As these particles are unable to cause lysis in a host strain, they might not be detectable by plaque assay or leave traces of phage DNA in the recipient cell. Nevertheless, they can infect a susceptible host and transduce the genes that they are mobilizing. A plausible interpretation of our results would be that the phages carrying *qnr* (as well as other ARGs) could be mainly generalized transducing particles. One argument to support this hypothesis is that the inducing agents have no effect on the densities of virulent phages and that these densities are not influenced by the amount of temperate phages that could be induced from the bacteria present in the sample, if any. This is either because the phages induced by chelating agents were not producing visible lytic plaques or because the increase in the number of phages did not overcome the number of virulent phages already present in the samples.

Table 3. Differences expressed as $\log_{10}$ GC/mL of bla_{TEM}, $bla_{CTX\text{-}M}$, *qnrA*, *qnrS* and *mecA* in phage DNA obtained from induced versus non-induced samples at 37°C and 22°C under anaerobic conditions

		Differences between induced and non-induced sample ($\Delta\log_{10}$ GC/mL)							
		mitomycin C−control		ciprofloxacin (4 mg/L)−control		EDTA−control		sodium citrate−control	
Gene		37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C
bla_{TEM}	inducer−control	−0.05	−0.18	0.01	−0.23	**1.36**	**1.31**	0.34	0.22
	standard error	0.14	0.29	0.26	0.01	0.39	0.01	0.25	0.19
$bla_{CTX\text{-}M}$	inducer−control	0.02	−0.18	−0.29	−0.21	0.34	0.19	0.23	0.22
	standard error	0.10	0.17	0.58	0.59	0.09	0.10	0.16	0.29
qnrA	inducer−control	−1.31	−0.59	−1.62	−1.10	**1.50**	**2.67**	0.22	0.34
	standard error	0.14	0.02	0.16	0.04	0.17	0.02	0.04	0.06
qnrS	inducer−control	0.07	−0.24	−0.26	−0.28	**3.31**	**3.27**	**1.55**	**1.58**
	standard error	0.01	0.12	0.15	0.06	0.08	0.26	0.54	0.32
mecA	inducer−control	0.03	0.25	−0.30	0.01	−0.08	−0.33	0.11	0.17
	standard error	0.12	0.00	0.01	0.10	0.10	0.15	0.07	0.07

Results are expressed as the mean of five independent experiments and the standard errors are indicated. Bold font indicates a significant increase (ANOVA $P<0.05$).

A second argument to support the hypothesis of generalized transducing particles is that the numbers of phages carrying *qnr* did not increase after addition of agents inducing the lytic cycle of temperate phages, which work well on many specialized transducing phages, such as mitomycin C or ciprofloxacin, though they did increase after addition of EDTA or sodium citrate. It is not clear whether the mechanisms of EDTA-mediated phage induction are similar to those of other inducing agents and whether the differences in induction observed with EDTA and mitomycin C may be attributed to activation of different pathways that lead to activation of the phage lytic cycle.[27] Considering its chemical structure, it is not to be expected that EDTA enters the bacterial cell,[34] and the uptake of EDTA is limited to a few strains able to use it as the sole source of carbon, nitrogen and energy.[34] Thus, its effect must be due to external influence, very likely on the envelope of bacteria, since EDTA is known to induce envelope stress responses.[35,36] Chelation of membrane cations from outside the cell should cause stress in the bacterial envelope.[36] This was suspected since a similar effect was observed when using another chelating agent, sodium citrate. In contrast, mitomycin C is known to activate the SOS response through RecA activation, leading to suppression of lysogeny control and induction of lytic cycles in temperate phages.[22,23,27] The inducing properties of EDTA are attributed to its chelating effects and have been reported to be independent of RecA.[27] However, there is no evidence that the particles induced by EDTA or citrate are completely functional temperate phages in all cases. It is known that phages performing generalized transduction, such as P22,[37] can give altered packaging specificity, leading to the formation of transducing particles at increased frequencies. This could be a possible cause of the increase in ARGs in DNA from phage particles observed when chelating agents were used.

Recently, Modi *et al.*[38] demonstrated that treatment with antibiotics increases the number of ARGs in the intestinal phageome of mice. As in our study, these authors found that phage encapsulation was stimulated by stress, plausibly as a mechanism to increase the robustness of the populations in the gut under these circumstances. It is reasonable to assume that other stressing factors, such as chelators, could lead to a similar effect. The higher number of gene copies in the bacterial cell after treatment with chelators would result in better chances for these genes to be later mobilized by transduction. If our assumption is correct and chelating agents stimulate generalized transducing particles, then this is consistent with no increase in the densities of virulent infectious phages observed, since phage-derived generalized transducing particles will not be able to cause plaques of lysis, although they will be able to transduce the ARGs to susceptible bacterial recipients.

We ran the induction experiments in this study because we believed that the use of quinolones, used as inducers of temperate phages, could stimulate the induction of Qnr-encoding phages and thus contribute to the transference of their own resistance. However, this was not confirmed by our experiments. A non-significant increase in the densities of *qnr* copies in phage DNA was observed only under a few conditions. In theory, Qnr prophage induction may occur in the presence of quinolones, and this has been demonstrated with intestinal populations.[38] However, it was not observed using the natural bacterial populations in our samples, even when using different ciprofloxacin concentrations, which may be because the density or physiological state of the bacteria was different.

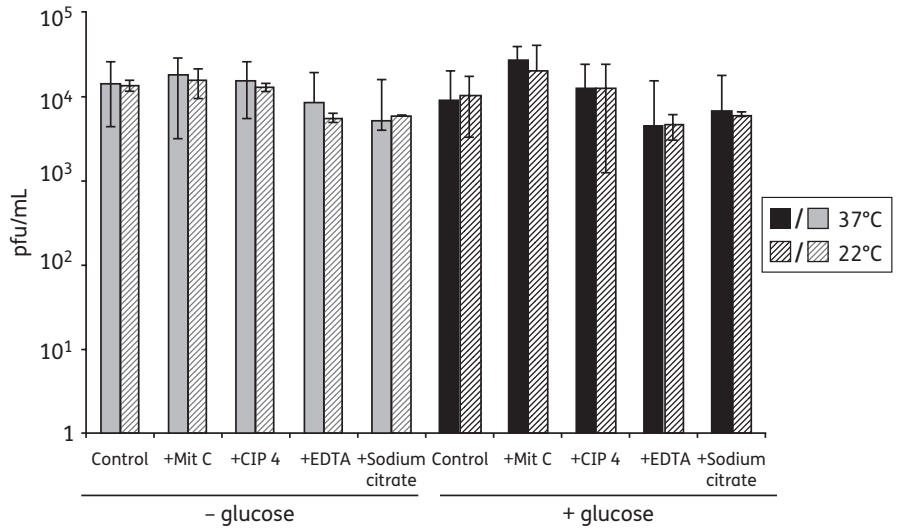

Figure 3. Effect of inducers on the densities of virulent somatic coliphages in raw urban wastewater, evaluated with and without inducing agents (mitomycin C, ciprofloxacin, EDTA and sodium citrate) at 22°C and 37°C. The pfu/mL values were determined following the ISO 10705-2 method for determination of somatic coliphages. Data presented are the means of five independent experiments. Error bars indicate SD. Mit C, mitomycin C; CIP 4, ciprofloxacin (4 mg/L).

EDTA is a common chelating agent (Mg^{2+} and Ca^{2+}) and antioxidant that is used as a food additive and has applications in medicine, cosmetics and pharmaceutical products.[39] It is also used to treat acute hypercalcaemia and lead poisoning.[39,40] As an antimicrobial agent, EDTA acts by disrupting the structure of the outer membrane of bacteria, so making this more permeable and therefore accessible to other antimicrobial agents.[35] Similarly, sodium citrate has several applications in food as a preservative (in Europe as food additive E331; https://webgate.ec.europa.eu/sanco_foods/main/index.cfm) and in medical usage as an anticoagulant. The effect of EDTA, also observable with sodium citrate, on the densities of ARGs in general and of quinolones in particular in the bacteriophage DNA fraction must be considered when applying them, since these agents could increase the number of particles harbouring these genes, increasing the possibilities of gene transfer and the generation of new resistant clones.

As indicated previously for other ARGs,[15,16,41] mobilization of quinolone resistance genes from their chromosomal or plasmid location and transduction to new clones could contribute to the spread of resistance and to a quick emergence of new resistant clones, already observed in practice,[9] which will represent the greatest challenge and the most important concern for the treatment of microbial infections in the future.

Conclusions

In summary, we showed that quinolone-resistance genes *qnrA* and *qnrS* are found in the bacteriophage DNA fraction isolated from urban wastewater, river water and animal faecal samples. Due to the unknown nature of these phage particles, we evaluated the potential induction of these phages by known inducing agents. There was no increase in GC number in DNA of phage particles when treated with typical inducers of temperate phages (mitomycin C or ciprofloxacin), but a significant increase when treated with EDTA or sodium citrate, probably due to their chelation properties. No increase in infectious lytic coliphages was found with any treatment. This, together with the fact that inducers of temperate phages did not cause an increase in Qnr phages, suggests that the *qnr*-encoding phages could be in fact generalized transducing particles. The presence of *qnr*-encoding phage particles and the effect caused by some chelating agents strongly increase the spread of some ARGs and also create the possibility of new transduction events that might cause the emergence of new resistant strains.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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