

Characterization of plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes in *Salmonella*

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Objectives: The aim of this study was to identify and characterize plasmids carrying *qnrS1*, *qnrB2* and *qnrB19* genes identified in *Salmonella* strains from The Netherlands. The identification of plasmids may help to follow the dissemination of these resistance genes in different countries and environments.

Methods: Plasmids from 33 *qnr*-positive *Salmonella* strains were transferred to *Escherichia coli* and analysed by restriction, Southern blot hybridization, PCR and sequencing of resistance determinants. They were also assigned to incompatibility groups by PCR-based replicon typing, including three additional PCR assays for the IncU, IncR and ColE groups. The collection included isolates from humans and one from chicken meat.

Results: Five IncN plasmids carrying *qnrS1*, *qnrB2* and *qnrB19* genes were identified in *Salmonella enterica* Bredeney, Typhimurium PT507, Kentucky and Saintpaul. *qnrS1* genes were also located on three further plasmid types, belonging to the ColE (in *Salmonella* Corvallis and Anatum), IncR (in *Salmonella* Montevideo) and IncHI2 (in *Salmonella* Stanley) groups.

Conclusions: Multiple events of mobilization, transposition and replicon fusion generate the complexity observed in *qnr*-positive isolates that are emerging worldwide. Despite the fact that the occurrence of *qnr* genes in bacteria from animals is scarcely reported, these genes are associated with genetic elements and located on plasmids that are recurrent in animal isolates.

Keywords: QNR, LAP-2, quinolone resistance, replicon-typing, animal reservoir

Introduction

Plasmid-mediated quinolone resistance is emerging worldwide in Enterobacteriaceae, including *Salmonella enterica*.^{1,2} Salmonellosis is treated with fluoroquinolones only in elderly or immunocompromised patients, but these drugs are also used for treating patients with enteric fever, invasive disease or long-term salmonellae carriage. Recent studies on *Salmonella* showed that plasmid-located *qnr* genes confer decreased susceptibility to fluoroquinolones (MIC >0.06 mg/L) and nalidixic acid (MIC 8–16 mg/L), without association with mutations in the topoisomerase genes.^{3–9} Recently, we reported the first *qnrB* and *qnrS* genes in *Salmonella* isolates from patients and a broiler

chicken in The Netherlands.⁵ These genes were previously described in *Salmonella* from the USA, Asia, Africa and Europe, but scarce information is available on the structure and circulation of plasmids carrying the different *qnr* gene variants.^{3–9} Currently, there are two completely sequenced plasmids carrying the *qnrS1* gene: one is named pTPqnrS-1a, a 10 kb plasmid obtained from a multiresistant *S. enterica* Typhimurium DT193 in the UK;³ the other is pK245, a 98 kb multireplicon plasmid identified in a clinical *Klebsiella pneumoniae* from Taiwan.¹⁰ DNA sequences of these plasmids highlighted some peculiar features that can be helpful to trace them and also provide information on the mechanisms responsible for the horizontal transfer of the *qnr* genes among different isolates.

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Plasmids harbouring *qnr* genes in *Salmonella*

Plasmid pTPqnrS-1a exhibited 89% nucleotide sequence identity to the ColE-plasmid pEC278 isolated from a pathogenic *Escherichia coli* strain (GenBank accession number AY589571) and the region adjacent to the origin of replication (*oriV*) showed 99% identity to plasmid pINF5 from *Salmonella* Infantis isolated from chicken carcasses in Germany.⁶ The pK245 plasmid structure was also complex, being composed of four main scaffolds: (i) a region deriving from an IncF plasmid; (ii) a region deriving from the IncQ plasmid RSF1010; (iii) a region encoding the RepA replication initiator protein found in *Pantoea stewartii* plasmid pSW800 (70% similarity); and (iv) a region encoding the *bla*_{LAP-2} and *qnrS* genes, and the *repB* gene of the *K. pneumoniae* pGSH500 plasmid (96% similarity).¹⁰

Plasmids of the IncU (p37) and IncQ (pGNB2) groups were associated with the *qnrS2* gene: in *Aeromonas punctata* from France and in plasmid DNA obtained from a wastewater treatment plant in Germany, respectively.^{11,12} Finally, the *qnrA1* gene associated with the *bla*_{VEB-1} gene emerged worldwide located on IncA/C2 plasmids,¹³ while little information is available on plasmids carrying the *qnrB* variants.

The aim of this study was to identify and characterize plasmids harbouring *qnrS1*, *qnrB2* and *qnrB19* genes identified in quinolone-resistant *Salmonella* strains from The Netherlands, with the final objective to provide a set of specific PCR assays, useful for monitoring the dissemination of these resistance traits in different countries and environments.

Materials and methods

Strains

A total of 33 *qnr*-positive *Salmonella* strains were analysed in this study. Most of the isolates were from patients from The Netherlands, and one strain (137.25) was from chicken meat (Table 1). The presence of *qnr* genes in these strains was previously described.⁵ *qnrS1*-positive salmonellae belong to serotypes Corvallis (25 isolates), Kentucky ($n = 2$), Anatum ($n = 1$), Montevideo ($n = 1$), Stanley ($n = 1$) and Saintpaul ($n = 1$) (Table 1). Comparison of pulsed-field gel electrophoresis (PFGE) patterns of Corvallis strains suggested that they were clonally related (>90% of similarity), with the exception of two recent isolates of 2006 (strains 162.58 and 163.43), showing unrelated PFGE profiles (<80% similarity). One *Salmonella* Bredeney and one Typhimurium PT507 were positive for *qnrB2* and *qnrB19* (formerly referred to as *qnrB5'*)⁵ genes, respectively. The *qnrB5'* gene name was updated by DNA sequencing of the amplicon generated by the primers QnrB10/19Fw and QnrB10/19Rv listed in Table 2 identified as *qnrB19*¹⁴ by comparison with the GenBank database and Lahey Clinic web site. The DNA sequence of the quinolone resistance determining region (QRDR) of the *gyrA* and *parC* genes was analysed for all the strains.¹⁵

Antimicrobial susceptibility

The resistance patterns were determined by broth microdilution according to EUCAST guidelines (www.eucast.org) using microtiter trays (TREK Diagnostic Systems, UK).

MIC breakpoints used for susceptibility and resistance to ciprofloxacin were ≤ 0.5 and > 1 mg/L, respectively, and for resistance to nalidixic acid it was > 16 mg/L as recommended by EUCAST.

Plasmid-mediated quinolone resistance transferability

Plasmid DNA was purified by the Qiagen Plasmid Midikit (Qiagen Inc., Milan, Italy). Purified plasmids were used to transform MAX Efficiency DH5 α *E. coli* chemically competent cells (Invitrogen, Milan, Italy). DH5 α was chosen as the recipient because it is capable of being transformed efficiently with large plasmids.¹⁶ However, this strain is resistant to nalidixic acid (MIC > 64 mg/L) due to mutations in the *gyrA* gene, but it is fully susceptible to ciprofloxacin with an MIC of 0.03 mg/L (Table 1). Consequently, transformants (T) were selected on LB agar plates containing 0.06 mg/L ciprofloxacin.

Conjugation experiments were performed at 25°C by liquid mating assay using a rifampicin-resistant *E. coli* CSH26 as recipient and selecting transconjugants (Tc) on LB agar supplemented with 100 mg/L rifampicin and 0.06 mg/L ciprofloxacin.¹⁷

Undigested [Figure S1; see Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>)] and *PvuII* restricted plasmids in the original and recipient strains were analysed by Southern blot hybridization using the digoxigenin-labelled *qnrS*, *qnrB* and *repN* amplicons as probes (PCR DIG probe synthesis kit, Roche Diagnostics GmbH, Mannheim, Germany).^{18,19} Hybridization and detection were performed according to the manufacturer's instructions.

Plasmid typing

Plasmids from parental and transformant/transconjugant strains were assigned to incompatibility groups by PCR-based replicon typing (PBRT) performed on total DNA using previously described primers and conditions.¹⁹ Total DNA was obtained by the Wizard Genomic DNA Purification System (Promega, Madison, WI, USA). Plasmids that were negative for the 18 replicons of the PBRT scheme were tested for three additional targets: the *oriV* of ColE-like plasmids (colE PCR), the *repA* gene of the pRA3 plasmid from *Aeromonas hydrophila* (IncU PCR) and the *repB* gene of the *K. pneumoniae* *qnrS1*-plasmid pK245. Since plasmid pK245 was not assigned to any known Inc group, this assay was named IncR PCR (Table 2). Amplicons were sequenced for confirmation. The colE PCR was devised to amplify all the colE-like plasmids. A colE_{TP} amplification was devised to specifically detect the subset of ColE-positive plasmids showing a different *oriV* sequence (74% of homology with the other ColE-like variants), but 100% identical to that of the pTPqnrS-1a plasmid from *Salmonella* Typhimurium DT193 (GenBank accession number AM746977). Primers are listed in Table 2.

ColE_{TP}-positive strains were further analysed by the *qnrS*-colE_{TP} PCR (Table 2) to confirm the co-linearity of the *qnrS1* gene with the colE_{TP} *oriV*, as described in the pTPqnrS-1a plasmid. Furthermore, the *qnrS1* and colE_{TP} *oriV* containing region of plasmid 138.31(T) was cloned and fully sequenced by ligating the *PstI* digested plasmid into the pZero-2.1 kanamycin-resistant vector (Invitrogen). Ligation mixture was introduced by transformation into the MAX Efficiency *E. coli* DH5 α chemically competent cells (Invitrogen). Transformants were selected on LB agar plates, containing 100 mg/L kanamycin and 0.06 mg/L ciprofloxacin. Recombinant plasmids were extracted by the Qiagen Plasmid Midikit (Qiagen Inc.) and inserts were sequenced on both strands by standard and walking primers.

Plasmids assigned by PBRT to the IncHI2 group were further typed applying the previously described HI2-plasmid typing scheme, consisting of 10 PCRs, devised on the IncHI2 R478 and pAPEC-O1-R plasmids (Table 2).²⁰

All plasmids were also screened for the presence of the *bla*_{LAP}, *bla*_{OXA}, *bla*_{TEM}, *aac(6')*-*Ib*-cr and *qepA* genes and for the presence of IS2 flanking the *qnrS1* gene (Table 2).^{21–24}

Table 1. Characteristics of *qnr*-positive *S. enterica* isolated in The Netherlands and their relative transformants/transconjugants (T/Tc)

Strain	Resistance pattern	Sample	Serotype	MIC (mg/L)		<i>qnr</i>	β-lactamase	PBRT+IncR+ColE	<i>qnr</i> -colE _{TP} PCR
				CIP	NAL				
137.25	SMX, TET, TMP, CIP _L	chicken	Bredeney	0.25	16	<i>qnrB2</i>	neg	N, colE	neg
137.25(T)	SMX, TET, TMP, CIP _L			0.25	64	<i>qnrB2</i>	neg	N	neg
152.40	CIP _L	human	Typhimurium PT507	0.5	16	<i>qnrB19</i>	neg	N, colE	neg
152.40(T)	CIP _L			0.25	64	<i>qnrB19</i>	neg	N	neg
128.12	AMP, CIP _L	human	Kentucky	1.0	16	<i>qnrS1</i>	TEM	N, I1, P	neg
128.12(T)	AMP, CIP _L			0.5	64	<i>qnrS1</i>	TEM	N	neg
168.27	AMP, STR, TET, CIP _L	human	Saintpaul	0.5	16	<i>qnrS1</i>	TEM	N, colE	neg
168.27(T)	AMP, STR, TET, CIP _L			0.5	64	<i>qnrS1</i>	TEM	N	neg
174.70	AMP, STR, CIP _L	human	Kentucky	0.5	16	<i>qnrS1</i>	TEM	N	neg
174.70(T)	AMP, STR, CIP _L			0.5	64	<i>qnrS1</i>	TEM	N	neg
146.71	AMP, CHL, GEN, KAN, SMX, STR TET, CIP _L	human	Stanley	1.0	16	<i>qnrS1</i>	LAP-2	HI2 _{pAPEC-O1-R} , P, colE	neg
146.71(Tc)	CHL, GEN, KAN, SMX, STR TET, CIP _L			0.5	16	<i>qnrS1</i>	LAP-2	HI2 _{pAPEC-O1-R}	neg
172.23	SMX, TMP, CIP _L	human	Montevideo	0.5	8	<i>qnrS1</i>	neg	R, colE _{TP}	neg
172.23(T)	SMX, TMP, CIP _L			0.5	64	<i>qnrS1</i>	neg	R	neg
138.31	SMX, CHL, TET, CIP _L	human	Anatum	1.0	16	<i>qnrS1</i>	neg	A/C, colE _{TP}	pos
138.31(T)	CIP _L			0.5	64	<i>qnrS1</i>	neg	colE _{TP}	pos
131.17	CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	B/O, colE _{TP}	pos
131.17(T)	CIP _L			0.5	64	<i>qnrS1</i>	neg	colE _{TP}	pos
73.75	TET, CIP _L	human	Corvallis	0.25	8	<i>qnrS1</i>	neg	colE _{TP}	pos
93.29	TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}	pos
102.46	SMX, TET, CIP _L	human	Corvallis	0.25	8	<i>qnrS1</i>	neg	colE _{TP}	pos
108.04	SMX, TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}	pos
117.43	SMX, TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}	pos
117.76	SMX, TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP} , I1	pos
120.52	SMX, TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}	pos
126.41	SMX, TET, CIP _L	human	Corvallis	0.25	8	<i>qnrS1</i>	neg	colE _{TP}	pos
130.08	CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP} , I1	pos
141.44	SMX, TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}	pos
143.23	SMX, TET, CIP _L	human	Corvallis	0.5	8	<i>qnrS1</i>	neg	colE _{TP}	pos
143.42	TET, CIP _L	human	Corvallis	1.0	16	<i>qnrS1</i>	neg	colE _{TP}	pos
144.12	CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}	pos
145.50	AMP, GEN, SMX, TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	TEM	colE _{TP} , I1	pos
145.62	AMP, GEN, SMX, TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	TEM	colE _{TP} , I1	pos
147.28	CIP _L	human	Corvallis	1.0	16	<i>qnrS1</i>	neg	colE _{TP}	pos
155.08	AMP, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	TEM	colE _{TP} , I1	pos
162.58	SMX, TET, CIP _L	human	Corvallis	0.25	8	<i>qnrS1</i>	neg	colE _{TP}	pos
163.43	SMX, TET, CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}	pos
168.20	CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}	pos

Plasmids harbouring *qnr* genes in *Salmonella*

Table 1. Continued

Strain	Resistance pattern	Sample	Serotype	MIC (mg/L)				<i>qnr</i>	β -lactamase	PBRT + IncR + ColE	<i>qnr</i> -colE _{TP} PCR
				CIP	NAL	<i>qnrS1</i>	<i>qnrS1</i>				
I71.04	CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}		pos	
I71.22	CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP}		pos	
I77.29	CIP _L	human	Corvallis	1.0	16	<i>qnrS1</i>	neg	colE _{TP}		pos	
I75.40	CIP _L	human	Corvallis	0.5	16	<i>qnrS1</i>	neg	colE _{TP} , I1		pos	
DH5 α	NAL	recipient		0.03	64	neg	neg	neg		neg	
CSH26	—	recipient		0.03	<4	neg	neg	neg		neg	

AMP, ampicillin; CHL, chloramphenicol; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; SMX, sulfamethoxazole; STR, streptomycin; TET, tetracycline; TMP, trimethoprim; CIP_L, reduced susceptibility to ciprofloxacin.

Results

Localization of *qnr* and β -lactamase genes on *Salmonella* plasmids

Qnr-positive plasmids were successfully transferred by transformation from all the parental strains to the recipient *E. coli* DH5 α strain (the 131.17 strain was chosen as representative for the *Salmonella* Corvallis clonal strain), with the exception of the *Salmonella* Stanley strain 146.71 that did not produce transformants; however, it positively transferred the plasmid by conjugation (Table 1). PCR and DNA sequencing experiments confirmed the *qnr*-gene presence in all transformants/transconjugants obtained (Table 1). MICs of ciprofloxacin and nalidixic acid were determined for the parental and recipient strains and for the empty CSH26 and DH5 α *E. coli* recipient strains (Table 1). Parental and recipient strains were PCR negative for the presence of the *aac(6')-Ib-cr* and *qepA* genes, conferring reduced susceptibility to fluoroquinolones. No mutations previously described to be associated with quinolone resistance were identified in the QRDR of the parental strains (data not shown).

Ampicillin resistance was associated with the presence of the *bla*_{TEM} gene in all strains, except strain 146.71(Tc), which was positive for the *bla*_{LAP} gene, identified by DNA sequencing of the amplicon as the *bla*_{LAP-2} gene variant.²⁵

Typing of both *qnrB2* and *qnrB19* plasmids

Plasmids from both parental and recipient strains were tested for 21 replicons (listed in Table 2 and reference 19).

Both *qnrB2* and *qnrB19* genes were located on IncN plasmids (100% identity to the *repA* gene of the R46 IncN reference plasmid, GenBank accession number AY046276). ColE plasmids were also identified in the parental strains but they were *qnr*-negative and were not transferred to the recipient strain (Table 1).

IncN plasmids in the transformant strains were further analysed by *PvuII* RFLP and the localization of the *qnr* gene was confirmed by Southern blot hybridization experiments (Figure 1).

Typing of *qnrS1* plasmids

The *qnrS1* genes were located on four different plasmid types.

(i) ColE-like plasmids of ~10 kb were identified in all *Salmonella* Corvallis and *Salmonella* Anatum strains. Several strains also showed co-resident I1, B/O or A/C type plasmids, which were not transferred by transformation. DNA sequencing of the ColE-amplicons obtained from strains 138.31, 131.17 and 120.52 revealed that these plasmids had the origin of replication identical to that of the pTP_{qnrS}-1a plasmid of *Salmonella* Typhimurium DT193.³ The presence of this type of *oriV* was then confirmed in all the *Salmonella* Anatum and *Salmonella* Corvallis strains by PCR assay specific for the ColE_{TP} *oriV* (Table 1). Furthermore, all these strains were also positive for the *qnrS*-colE_{TP} PCR indicating the localization of the *qnrS1* gene on pTP_{qnrS}-1a-like plasmids. This observation was confirmed by cloning and fully sequencing the 4375 bp *PstI* fragment from plasmid 138.31(T). This region contained the *mobC*

Table 2. Primers used in this study

Primer name	Sequence	Nucleotide positions	Accession no.	PCR target	Amplicon size (bp)
oricoLE FW ^a	5'-GTT CGT GCA TAC AGT CCA-3'	3797–3983	AM746977	ColE plasmids [Generic]	187
oricoLE RV	5'-GGC GAA ACC CGA CAG GAC T-3'				
oricoLE FW ^a	5'-GTT CGT GCA TAC AGT CCA-3'	3878–3983	AM746977	ColE _{TP} -like pTPqnrS-1a	106
oricoLE _{TP} RV ^b	5'-GGT TTA CCG GTG TCA TTC C-3'				
qnrS ^c	5'-TAA ATT GGC ACC CTG TAG GC-3'	6628–3878	AM746977	<i>qnrS1</i> -ColE _{TP} localization	2751
oricoLE _{TP} RV ^b	5'-GGT TTA CCG GTG TCA TTC C -3'				
IncR FW	5'-TCG CTT CAT TCC TGC TTC AGC -3'	19 367–19 617	DQ449578	IncR plasmids	251
IncR RV	5'-GTG TGC TGT GGT TAT GCC TCA -3'				
IncU FW	5'-TCA CGA CAC AAG CGC AAG GG-3'	51–893	DQ401103	IncU plasmids	843
IncU RV	5'-TCA TGG TAC ATC TGG GCG C-3'				
QnrB10/19FW	5'-CGG GTT TGA CGC ATA AC-3'	2779–2038	EU523120	<i>qnrB5</i> -like genes	742
QnrB10/19RV	5'-CAA ACG CAT CTC CCG GT-3'				
deltaresIS2FW	5'-TCA TAA TGC GAT ACA CCC GC-3'	5082–6628	AM746977	truncated IS2 and <i>qnrS1</i>	1547
qnrS ^c	5'-TAA ATT GGC ACC CTG TAG GC-3'				
R478 vs pAPEC-O1-R IncHI2 subtyping ^d					
10 FW	5'-AAT CGC CTG AAT CAG CTG G-3'	6655–7713	BX664015	HtdV protein	1059
11 RV	5'-TTC TTT ACT ACA CCA GAG CC-3'				
17 FW	5'-AAC TCT TGA AAA TCG TGG-3'	18 211–19 101	BX664015	SMR0017 lipoprotein	891
18 RV	5'-CTT CAG GCT ATC GTT TCG-3'				
Ter FW	5'-ATG CAG GCT CAA GGA ATC GC-3'	80 270–81 163	BX664015	tellurium resistance	894
Ter RV	5'-TTC ATC GAT CCA CGG TCT G-3'				
92 FW	5'-CTA TGT AAG CAA TGA TCC TC-3'	88 861–89 862	BX664015	SMR0092-93 protein	1002
93 RV	5'-TAT AGA GAG CAC CGA AGG-3'				
TnsDA FW	5'-AAT CCT TGT TCA GCC GG-3'	119 360–120 825	BX664015	Tn7 transposon	1466
TnsDA RV	5'-CAA AAG CCA GCC ATG CCC-3'				
136A FW	5'-TAC GAA AAT GAA TTG TGG C-3'	120 906–121 768	BX664015	SMR0136 protein	863
136A RV	5'-AAT TTA CAA TCT GCA GCC C-3'				
ArsB FW	5'-AGT GAA AGA CAG ACG AAG CG-3'	159 735–160 870	BX664015	arsenical pump membrane protein	1136
ArsB RV	5'-GGC AGA TAG TGT GGA ATG CG-3'				
201 FW	5'-TGT CAG GCT AAG TCA CTG G-3'	180 398–181 466	BX664015	SMR0201 protein	1069
201 RV	5'-ATT ATA CGG TAG ATC C-3'				
207 FW	5'-TTT CCC AAA TAG GCG ACG C-3'	190 238–191 131	BX664015	SMR0207–208 proteins	894
208 RV	5'-ATG TGA AAT TAC TAT ACC GG-3'				
239 FW	5'-TGG AAC GCG TGG TAT GTG G-3'	219 372–220 364	BX664015	SMR0239–240 proteins	993
240 RV	5'-ATA CCT GCC GTT TAC CC-3'				

^aThe oricoLE FW primer is used in combination with oricoLE RV for the generic detection of ColE-like plasmids and in combination with oricoLE_{TP} RV for the detection of the ColE_{TP} plasmids showing *oriV* identical to the pTPqnrS-1a plasmid.

^bThe oricoLE_{TP} RV is used in combination with qnrS for the localization of the *qnrS1* gene on ColE_{TP} plasmids.

^cThe qnrS primer is described by Robicsek *et al.*¹⁸

^dPrimers and conditions used for the IncHI2 subtyping are described in García *et al.*²⁰

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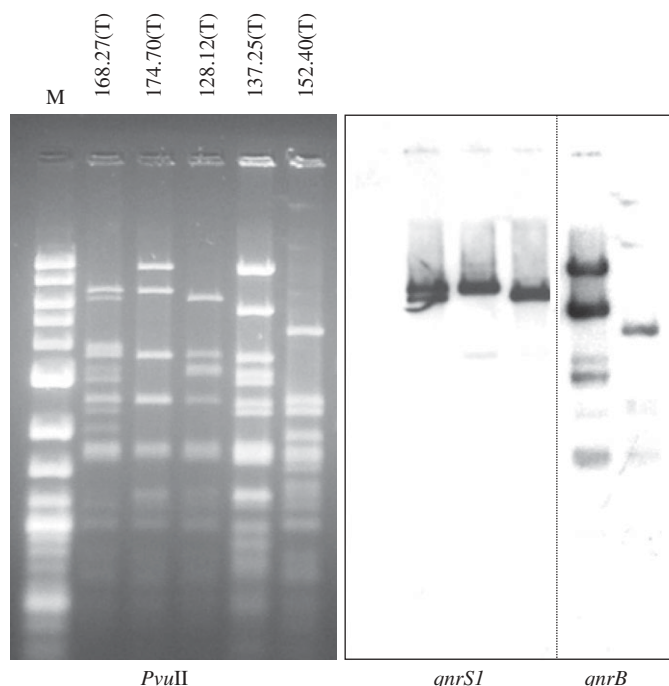


Figure 1. Restriction analysis of plasmids obtained from IncN transformants restricted by *PvuII*. Southern blot hybridization with digoxigenin-labelled *qnrS1* and *qnrB* probes. M: 1 kb DNA Extension Ladder (Invitrogen).

gene, the *oriV* and the *qnrS1* gene in an array identical to that previously described for the pTPqnrS-1a plasmid.

(ii) The *qnrS1* gene was identified on IncN plasmids of ~50 kb in the *Salmonella* Kentucky and Saintpaul strains. Replicons of the I1- and P-type were also detected in the *Salmonella* Kentucky parental strain but they were not transferred to the recipient strain. A co-resident ColE plasmid was identified in the Saintpaul strain but it was *qnr*-negative and not transferred by transformation. IncN plasmids from the transformants were further analysed by *PvuII* RFLP and the localization of the *qnr* gene was confirmed by Southern blot hybridization (Figure 1).

(iii) The *qnrS1* gene was also identified on an IncHI2 plasmid in *Salmonella* Stanley strain 146.71 and its relative transconjugants (Table 1). Plasmid DNA from this strain could not be purified (the estimated minimal size for IncHI2 plasmids is >250 kb), but the transferred plasmid was further characterized applying the previously described HI2-typing scheme, discerning the two reference IncHI2 R478 and pAPEC-O1-R plasmids (Table 2).²⁰ This analysis identified the IncHI2 plasmid from *Salmonella* Stanley as a pAPEC-O1-R-like plasmid. In fact, it lacked three genes that are present in the R478 but are missing in pAPEC-O1-R (*smr92*, *smr93* and *smr201*) and it was positive for the O1R_160 locus, which is disrupted by the Tn10 insertion in R478.²⁶ The *qnrS1*-HI2_{pAPEC-O1-R} plasmid conferred resistance to ampicillin, aminoglycosides, sulphonamides, streptomycin, tetracycline and chloramphenicol and it was positive for the *bla*_{LAP-2} gene.

(iv) The *qnrS1* gene was located on an IncR plasmid of ~50 kb in the *Salmonella* Montevideo strain. This plasmid conferred a multidrug-resistant phenotype, including aminoglycosides, chloramphenicol and tetracycline resistance. The parental strain was also positive for a *qnr*-negative ColE plasmid.

All the *qnrS1* genes, regardless of their locations on the different plasmid scaffolds, were flanked by truncated IS2 elements as previously described.^{3,27}

Discussion

This study describes plasmids harbouring *qnr* genes identified in eight different *Salmonella* serovars from The Netherlands. The sample included isolates from humans and one from chicken meat. IncN plasmids were the most recurrent plasmid type since the three *qnr* gene variants, *qnrS1*, *qnrB2* and *qnrB19*, were located on this kind of plasmid in four different *Salmonella* serotypes, from both human and the animal sources. Four of the five IncN plasmids showed a common scaffold and variable *PvuII* fragments corresponding to the variable region containing the *qnr* gene. It is interesting to note that *qnrS1*-positive IncN plasmids were also identified in *Salmonella* Virchow isolated in the UK in 2004–2005, causing an outbreak associated with imported cooked meat from Thailand⁷ and the *qnrS* plasmid pINF5 from *Salmonella* Infantis was hypothesized to have an IncN plasmid-like ancestor (pMUR050).⁶ The association *qnr*-IncN is likely a fortuitous event of integration of the Qnr determinant into a very common plasmid species. IncN was the most frequently identified (26%) plasmid type in a collection of 58 multidrug-resistant *S. enterica* strains from animals and food of animal origin isolated in Italy in 2000–2001.^{19,28} Furthermore, in a study performed on a large collection of *E. coli* isolates from the USA, the prevalence of IncN plasmids was 10.9% and 16.1% in avian faecal and pathogenic *E. coli*, respectively, but interestingly, *E. coli* strains from the faeces of healthy humans and from human urinary tract infections were all negative for IncN plasmids.²⁹ Therefore, it is plausible that IncN plasmids are common in zoonotic enterobacterial pathogens, but rarer in bacteria from humans, suggesting that IncN *Salmonella* plasmids could have acquired the *qnr* gene by transposition events occurring in animals.

The *qnrS1* gene was also located on small ColE-like plasmids in *Salmonella* Corvallis and Anatum strains. The Corvallis serotype carrying the *qnrS1* gene recently emerged in Denmark, associated with the consumption of imported food products from Thailand. Twenty-three isolates showing related PFGE patterns were obtained from humans from Denmark and Thailand and from chicken, pork and beef imported from Thailand.³⁰ The *Salmonella* Corvallis PFGE patterns were also very similar to those described in that study (data not shown). It could be speculated that the ColE_{TP}-*qnrS1* plasmid is present also in *Salmonella* Corvallis from Denmark and Thailand and contributed to the worldwide spread of *qnrS1* gene in this clone. However, the association *Salmonella* Corvallis-ColE_{TP}-*qnrS1* is not exclusive, since the same plasmid was identified in *Salmonella* Typhimurium and Virginia in the UK, and in Anatum in this study. Recently, a small *qnrS1*-positive plasmid showing 99% homology with the pTPqnrS-1a plasmid was also identified in a *Salmonella* Typhimurium isolated in Taiwan (GenBank accession number EU715253), suggesting that this plasmid is very common worldwide, but it seems to have a preference for the *Salmonella* species. More in general, ColE-like plasmids are largely present in Enterobacteriaceae and they are not self-conjugative, but they can be mobilized by co-resident conjugative plasmids, through the presence of the *mobABC* genes.³¹ The simultaneous presence of the ColE_{TP}-*qnrS* plasmid with additional plasmids belonging

to the II, B/O or A/C groups within the same parental strain strongly suggests that the latter plasmids can participate in the mobilization of the small ColE plasmids, promoting their circulation in different *Salmonella* serotypes.

In this study, the *qnrS1* gene was, for the first time, located on an IncHI2 plasmid identified in a *Salmonella* Stanley strain. The plasmid scaffold resembled the pAPEC-O1-R plasmid previously described in avian pathogenic *E. coli* in the USA.²⁶ It is important to note that HI2_{pAPEC-O1-R} plasmid variants were recently recognized in *Salmonella* Virchow producing the extended-spectrum β -lactamase (ESBL) CTX-M-2 from poultry flocks, poultry meat and humans in Belgium and French Guyana.³² The chronology of isolation of those strains suggested that these bacteria were transmitted to humans via the food chain, specifically by poultry meat. The *bla*_{CTX-M-2} gene was not present on the original pAPEC-O1-R plasmid²⁶ and it was not identified on the *qnrS1*-HI2_{pAPEC-O1-R} plasmid either. In conclusion, the HI2 plasmid variant associated with avian pathogenic *E. coli* in the USA has evolved by acquisition of ESBL or *qnr* genes in *Salmonella* in Europe.

The fourth plasmid type carrying the *qnrS1* gene was the IncR plasmid identified in the *Salmonella* Montevideo strain. The replicase gene of this plasmid was the same as that of the previously described pK245 plasmid from *K. pneumoniae*, showing the association *qnrS1*–*bla*_{LAP-2} genes. The IncR plasmid from *Salmonella* Montevideo was not positive for the *bla*_{LAP-2} gene, which was identified on the IncHI2 plasmid from the *Salmonella* Stanley.

From our findings, a complex figure of variably assorted plasmid replicons and resistance determinants clearly appears. Common genetic traits are organized on different scaffolds in the various strains. Multiple events of mobilization, transposition, illegitimate recombination, replicon fusion and resolution can generate the apparent complexity observed in the different *qnr*-positive isolates that are emerging worldwide, but these are in fact, mosaics of recurrent genetic traits. Despite the low prevalence of *qnr* genes in bacteria from animals in comparison with isolates from humans, these genes were located on plasmids frequently associated with *E. coli* and *Salmonella* isolates from animals and rarely occurring in humans. This aspect would merit a further investigation of the prevalence of *qnr* genes in the faecal flora from animals as a potential unexpected source for these resistance genes.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

References

1. Robicsek A, Hooper DC. The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis* 2006; **6**: 629–40.
2. Robicsek A, Strahilevitz J, Jacoby GA *et al.* Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat Med* 2006; **12**: 83–8.
3. Kehrenberg A, Hopkins KL, Threlfall EJ *et al.* Complete nucleotide sequence of a small *qnrS1*-carrying plasmid from *Salmonella enterica* subsp. *enterica* Typhimurium DT193. *J Antimicrob Chemother* 2007; **60**: 903–5.
4. Gay K, Robicsek A, Strahilevitz J *et al.* Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. *Clin Infect Dis* 2006; **43**: 297–304.
5. Veldman K, van Pelt W, Mevius D. First report of *qnr* in *Salmonella* in The Netherlands. *J Antimicrob Chemother* 2008; **61**: 452–3.
6. Kehrenberg C, Friederichs S, de Jong A *et al.* Identification of the plasmid-borne quinolone resistance gene *qnrS* in *Salmonella enterica* serovar Infantis. *J Antimicrob Chemother* 2006; **58**: 18–22.
7. Hopkins KL, Wootton L, Day MR *et al.* Plasmid-mediated quinolone resistance determinant *qnrS1* found in *Salmonella enterica* strains isolated in the UK. *J Antimicrob Chemother* 2007; **59**: 1071–8.
8. Cattoir V, Weill FX, Poirel L *et al.* Prevalence of *qnr* genes in *Salmonella* in France. *J Antimicrob Chemother* 2007; **59**: 751–4.
9. Ibadene H, Messai Y, Ammari H *et al.* Dissemination of ESBL and Qnr determinants in *Enterobacter cloacae* in Algeria. *J Antimicrob Chemother* 2008; **62**: 133–6.
10. Chen YT, Shu HY, Li LH *et al.* Complete nucleotide sequence of pK245, a 98-kilobase plasmid conferring quinolone resistance and extended-spectrum-beta-lactamase activity in a clinical *Klebsiella pneumoniae* isolate. *Antimicrob Agents Chemother* 2006; **50**: 3861–6.
11. Cattoir V, Poirel L, Aubert C *et al.* Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas* spp. *Emerg Infect Dis* 2008; **14**: 231–7.
12. Bönemann G, Stiens M, Pühler A *et al.* Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, *qnrS2*, isolated from the bacterial community of a wastewater treatment plant. *Antimicrob Agents Chemother* 2006; **50**: 3075–80.
13. Poirel L, Villa L, Bertini A *et al.* Expanded-spectrum beta-lactamase and plasmid-mediated quinolone resistance. *Emerg Infect Dis* 2007; **13**: 803–5.
14. Cattoir V, Nordmann P, Silva-Sanchez J *et al.* ISEcp1-mediated transposition of *qnrB*-like gene in *Escherichia coli*. *Antimicrob Agents Chemother* 2008; **52**: 2929–32.
15. Eaves DJ, Randall L, Gray DT *et al.* Prevalence of mutations within the quinolone resistant-determining region of *gyrA*, *gyrB*, *parC* and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. *Antimicrob Agents Chemother* 2004; **48**: 4012–5.
16. Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 1983; **166**: 557–80.
17. Miller JH. Experiments in molecular genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1972.
18. Robicsek A, Strahilevitz J, Sahn DF *et al.* *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. *Antimicrob Agents Chemother* 2006; **50**: 2872–4.

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19. Carattoli A, Bertini A, Villa L *et al.* Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005; **63**: 219–28.
20. García A, Navarro F, Miró E *et al.* Acquisition and diffusion of *bla*_{CTX-M-9} gene by R478-IncHI2 derivative plasmids. *FEMS Microbiol Lett* 2007; **271**: 71–7.
21. Mabilat C, Goussard S, Sougakoff W *et al.* Direct sequencing of the amplified structural gene and promoter for the extended-broad-spectrum beta-lactamase TEM-9 (RHH-1) of *Klebsiella pneumoniae*. *Plasmid* 1990; **23**: 27–34.
22. Poirel L, Cattoir V, Soares A *et al.* Novel ambler class A β -lactamase LAP-1 and its association with the plasmid-mediated quinolone resistance determinant QnrS1. *Antimicrob Agents Chemother* 2007; **51**: 631–7.
23. Park CH, Robicsek A, Jacoby GA *et al.* Prevalence in the United States of *aac(6)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. *Antimicrob Agents Chemother* 2006; **50**: 3953–5.
24. Yamane K, Wachino J, Suzuki S *et al.* Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. *Antimicrob Agents Chemother* 2008; **52**: 1564–6.
25. Huang Z, Mi Z, Wang C. A novel beta-lactamase gene, LAP-2, produced by an *Enterobacter cloacae* clinical isolate in China. *J Hosp Infect* 2008; **70**: 95–6.
26. Johnson TJ, Wannemuehler YM, Scaccianoce JA *et al.* Complete DNA sequence, comparative genomics, and prevalence of an IncHI2 plasmid occurring among extraintestinal pathogenic *Escherichia coli* isolates. *Antimicrob Agents Chemother* 2006; **50**: 3929–33.
27. Hu FP, Xu XG, Zhu DM *et al.* Coexistence of *qnrB4* and *qnrS1* in a clinical strain of *Klebsiella pneumoniae*. *Acta Pharmacol Sin* 2008; **29**: 320–4.
28. Pezzella C, Ricci A, Di Giannatale E *et al.* Tetracycline and streptomycin resistance genes, transposons, and plasmids in *Salmonella enterica* isolates from animals in Italy. *Antimicrob Agents Chemother* 2004; **48**: 903–8.
29. Johnson TJ, Wannemuehler YM, Johnson SJ *et al.* Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. *Appl Environ Microbiol* 2007; **73**: 1976–83.
30. Cavaco LM, Hendriksen RS. Plasmid-mediated quinolone resistance determinant *qnrS1* detected in *Salmonella enterica* serovar Corvallis strains isolated in Denmark and Thailand. *J Antimicrob Chemother* 2007; **60**: 704–6.
31. Ilyina TV, Koonin EV. Conserved sequence motifs in the initiator proteins for rolling circle DNA replication encoded by diverse replicons from eubacteria, eucaryotes and archaebacteria. *Nucleic Acids Res* 1992; **20**: 3279–85.
32. García-Fernández A, Cloeckert A, Bertini A *et al.* Comparative analysis of IncHI2 plasmids carrying *bla*_{CTX-M-2} or *bla*_{CTX-M-9} from *Escherichia coli* and *Salmonella enterica* strains isolated from poultry and humans. *Antimicrob Agents Chemother* 2007; **51**: 4177–80.