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Clinical *Salmonella* Typhimurium ST34 with metal tolerance genes and an IncHI2 plasmid carrying *oqxAB-aac(6′)-Ib-cr* from Europe

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Sir,

Fluoroquinolones are critical antibiotics for treating severe *Salmonella* infections, and the widespread of resistant isolates included in diverse epidemiological scenarios and carrying plasmid-mediated quinolone resistance (PMQR) is a global threat.[1,2] Among PMQR mechanisms, those encoded by *oqxAB* and *aac(6′)-Ib-cr* genes are of special concern as they also confer reduced susceptibility to other antibiotics (*oqxAB*: chloramphenicol, trimethoprim, olaquindox; *aac(6′)-Ib-cr*: aminoglycosides) and biocides [*oqxAB*: quaternary ammonium compounds (QACs)].[2,3] Although *oqxAB*± *aac(6′)-Ib-cr* are prevalent and widespread in Asia, where olaquindox is still widely used in animal production, they remain scarce in Europe.[1,2,4–6] Here we describe the molecular characterization of clinical ciprofloxacin-resistant *Salmonella enterica* Typhimurium with concomitant presence of *oqxAB* and *aac(6′)-Ib-cr* recovered for the first time in Europe.

Two ciprofloxacin-resistant (MIC 2 mg/L) *S. enterica* Typhimurium isolates carrying *oqxAB* and *aac(6′)-Ib-cr* genes were isolated from faeces of two young children (1 and 2 years old) admitted to a Portuguese hospital in September 2012. Further characterization included the study of susceptibility to 11 other antimicrobial agents and β-lactamase and carbapenemase production by disc diffusion, Etest and/or microdilution methods (EUCAST/CLSI guidelines) and the Blue-Carba test. Screening of genes encoding resistance to antibiotics (including quinolone resistance-determining region mutations and PMQR) or tolerance to biocides/metals found in the animal setting (e.g. feed/disinfectants) was performed by PCR and/or sequencing.[7–9] Determination of transferability of PMQR genes (conjugation at 25/30/37°C and transformation) and characterization of integrons and plasmid backbones were performed by PCR, plasmid-based replicon typing, plasmid MLST (pMLST; http://pubmlst.org/plasmid/), hybridization (I-CeuI/S1-PFGE nuclease) and/or sequencing.[1,7,8] The *oqxAB-aac(6′)-Ib-cr* genetic environment was characterized by PCR mapping based on known sequences.[1] Clonal analysis was assessed by XbaI-PFGE[8] and MLST (http://mlst.warwick.ac.uk/mlst/dbs/Senterica).

The two *Salmonella* Typhimurium isolates were assigned to the widespread ST34 (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) and presented indistinguishable PFGE patterns (Figure S1, available as Supplementary data at *JAC* Online). Clonal expansion of *Salmonella* Typhimurium, including the ST34 clone, with PFGE profiles closely related to those of our isolates, has been associated with the spread of *oqxAB-aac(6′)-Ib-cr* genes in Asian human clinical and food-producing animal isolates.[1,6] However, the isolates reported here could not be related to any outbreak of foodborne infection or to particular food products/animal contact and foreign travel. Additionally, a single *gyrA* mutation (D87N) found in our *Salmonella* Typhimurium strain was identical to that detected in most *oqxAB*-positive *Salmonella* Typhimurium isolates from Asia.[1,6] The strain was co-resistant to amoxicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline and trimethoprim (Table 1), and carried diverse metal tolerance genes (*merA*, mercury; *silA*, silver/copper; *pcoD*, copper; *terF*, tellurite). Transferability of *oqxAB-aac(6′)-Ib-cr* was achieved by transformation, with an 8-fold increase in ciprofloxacin MIC and acquisition of resistance to other antibiotics (amoxicillin, chloramphenicol, gentamicin, kanamycin, sulfamethoxazole and trimethoprim) (Table 1). A 2-fold increase in benzalkonium chloride MIC was observed, highlighting the role of the OqxAB efflux pump in the decreasing susceptibility to diverse compounds with antimicrobial activity, including QACs.[3] The concomitant presence of genes encoding resistance to antibiotics (e.g. quinolones, quinoxalines, florfenicol) and biocides/metals (e.g. QACs, copper) widely used in farm animals could contribute to the successful spread of *Salmonella* carrying *oqxAB-aac(6′)-Ib-cr* genes by different events of co-selection.

The *oqxAB* genes were linked to an incomplete class 1 integron containing the *aac(6′)-Ib-cr-bla*$_{OXA-1}$*-catB3-arr3-qacEΔ1-sul1* gene cassette array and flanked by two IS*26* (Figure 1), widely spread mobilizing elements responsible for the dissemination of *oqxAB*[9] and other clinically relevant antibiotic resistance genes.[2] The *oqxAB* genetic environment was identical to that found in Asian *Salmonella* Typhimurium isolates from food-producing animals.[1] In other bacteria (including different *Salmonella* serotypes) similar genetic platforms were observed, although differing in the linkage of *oqxAB* with *bla*$_{CTX-M}$ alleles instead of the gene cassette array.[2,4,5] This IS*26-oqxAB*-IS*26-aac(6′)-Ib-cr-bla*$_{OXA-1}$*-catB3-arr3-qacEΔ1-sul1*-IS*26* was located on a 180 kb IncHI2 plasmid

Table 1. Antimicrobial susceptibility phenotypes and genotypes of *Salmonella* Typhimurium isolates (234 and 248), the selected transformant (248_T) and the recipient (*Escherichia coli* DH5α)

	MIC (mg/L)				
Isolate	CIP[a]	NAL[b]	BZK	Resistance phenotype[c]/genotype to other antimicrobial agents	Metal tolerance genes
234	2	>256	32	AMX, CHL, GEN, KAN, STR, SUL, TET, TMP/bla_{OXA-1}-bla_{TEM}, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA-strA-strB*, *sul1-sul2-sul3*, *tet*(B), *dfrA12*	*merA*, *silA*, *pcoD*, *terF*
248	2	>256	32	AMX, CHL, GEN, KAN, STR, SUL, TET, TMP/bla_{OXA-1}-bla_{TEM}, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA-strA-strB*, *sul1-sul2-sul3*, *tet*(B), *dfrA12*	*merA*, *silA*, *pcoD*, *terF*
248_T	0.25	128	16	AMX, CHL, GEN, KAN, SUL, TMP/bla_{OXA-1}, *catB3-cmlA-floR*, *aac(3)-IV*, *aphA1*, *aadA*, *sul1-sul2-sul3*, *dfrA12*	*terF*
E. coli DH5α	0.03	64	8		

[a]PMQR genes screened: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *aac(6′)-Ib-cr* and *oqxAB*.
[b]*Salmonella* isolates presented a single *gyrA* mutation D87N.
[c]Susceptibility to amoxicillin (AMX), benzalkonium chloride (BZK), ciprofloxacin (CIP), chloramphenicol (CHL), gentamicin (GEN), imipenem, kanamycin (KAN), nalidixic acid (NAL), streptomycin (STR), sulfamethoxazole (SUL), tetracycline (TET) and trimethoprim (TMP) was determined.

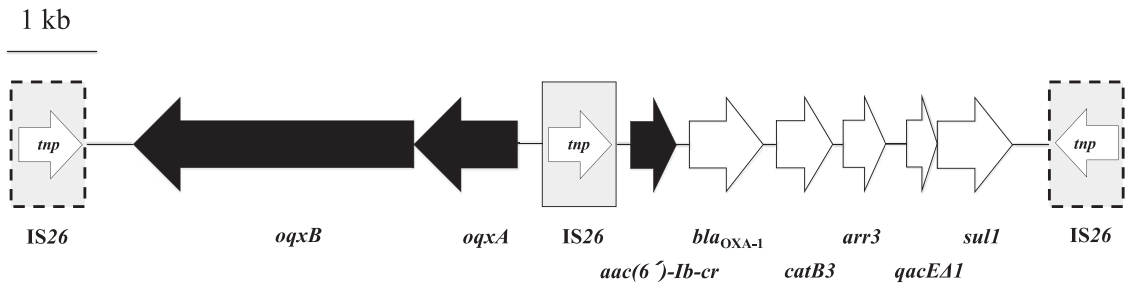

Figure 1. Genetic environment (12 110 bp) of *oqxAB-aac(6′)-Ib-cr* genes in an IncHI2 plasmid from an ST34 *Salmonella* Typhimurium strain. Horizontal arrows represent the positions and transcriptional directions of the ORFs. The IS*26* elements are shown as light grey boxes and dotted outlines indicate incomplete sequences.

carrying a *rep* gene 100% identical to Asian *Salmonella* Typhimurium pHXY0908 bearing *oqxAB-aac(6′)-Ib-cr* genes (GenBank accession number KM877269), but with only 95% identity to that of the prototype R478 (GenBank accession number BX664015) and prototype pAPEC-01-R (GenBank accession number DQ517526) plasmids. Also, among the metal tolerance genes found, our IncHI2 plasmid and pHXY0908 carried only *terF*, contrasting with the prototypes R478 and pAPEC-01-R with multiple metal tolerance genes (e.g. *merA*, *arsB*, *silA* or *pcoD*). The spread of *oqxAB*±*aac(6′)-Ib-cr*-producing bacteria of both human and animal origin in Asia has been linked mostly to IncHI2 MDR plasmids[1,4–6] belonging to ST1/ST2 by pMLST,[4] the two major plasmid variants associated with the dissemination of diverse clinically relevant genes (e.g. bla_{ESBL}).[10] Our MDR IncHI2 plasmid was non-typeable by pMLST (lacking allele smr0199/smr0018=allele number 3), suggesting the occurrence of multiple recombination events and of a new variant, considering those circulating in Europe (http://pubmlst.org/plasmid/).[10]

To the best of our knowledge, we describe for the first time in Europe an MDR *Salmonella* strain harbouring both *oqxAB* and *aac(6′)-Ib-cr* PMQR genes within an IncHI2 plasmid. The spread of these bacteria and/or MDR plasmids, as is occurring in Asia in human/animal settings, is of concern since they may contribute to amplification of *oqxAB-aac(6′)-Ib-cr* among Enterobacteriaceae in Europe, probably under different selective pressures (e.g. antibiotics/biocides/metals). Continuous surveillance to contain further transmission of these PMQR genes for new hosts or different settings, facilitated by international human and animal/food travel, is urgently required to preserve critical compounds with antimicrobial activity, including fluoroquinolones.

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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/).

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Assessment of a phenotypic algorithm to detect plasmid-mediated quinolone resistance in Enterobacteriaceae

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Sir,

Quinolone resistance in Gram-negative bacteria is the result of mutations in the quinolone resistance-determining region (QRDR) in chromosomally located genes encoding type II topoisomerases and, to a lesser extent, altered permeability.[1,2] Plasmid-mediated quinolone resistance (PMQR) has also been reported as encoding different proteins: Qnr proteins, acetyltransferase AAC(6′)-Ib-cr variant and the QepA and OqxAB active efflux pumps.[2]

In Enterobacteriaceae, a single mutation in the QRDR of GyrA (frequently at Ser83) results in high-level resistance to nalidixic acid (MIC >32 mg/L with absence of inhibition zone) and is easily detected. Clinical resistance to fluoroquinolones (ciprofloxacin MIC >2 mg/L according to CLSI)[3] is attained with additional chromosomal mutations or the acquisition of PMQR. In the case of isolates harbouring PMQR, but lacking QRDR mutations, these determinants confer low-level resistance to quinolones and/or fluoroquinolones and molecular tests are needed to detect it. The detection of PMQR usually takes up to six PCRs. There are to date no specific phenotypic tests for the detection of PMQR mechanisms. The current prevalence of PMQR seems to be increasing.[2] The aim of this study was to determine the capacity of phenotypic detection of PMQR in Enterobacteriaceae with low-level quinolone resistance (LLQR) without modifications at the QRDR. This phenotype has been reported mainly in *Salmonella*.[4]

To assess this issue, a sample size of 49 positive cases and 49 negative cases was estimated necessary for a 95% CI, proportion expected of 85%, and estimation accuracy of 0.1, using NQuery Advisor. In our study, 99 (57 *Klebsiella pneumoniae*, 27 *Escherichia coli*, 7 *Enterobacter cloacae*, 2 *Klebsiella oxytoca*, 1 *Enterobacter aerogenes*, 1 *Citrobacter koseri*, 1 *Citrobacter freundii*, 1 *Morganella morganii*, 1 *Proteus mirabilis* and 1 *Serratia marcescens*) isolates (of 10 874 clinical isolates of Enterobacteriaceae) were selected during October 2012 to June 2014. They were susceptible to nalidixic acid, MIC ≤16 mg/L, and showed reduced susceptibility to ciprofloxacin, MIC=1–2 mg/L, on a Wider C095-31WREV1 panel (Soria Melguizo). The panel included the following quinolones and concentrations: nalidixic acid 16 mg/L and ciprofloxacin 0.12–1–2 mg/L. Ten additional enterobacterial isolates (3 *K. pneumoniae* and 7 *E. coli*) fully susceptible to quinolones were included as controls.

Antibiotic susceptibility testing was performed by both microdilution and disc diffusion tests as follows: susceptibility to ciprofloxacin and nalidixic acid was determined by disc diffusion (http://www.eucast.org/) and microdilution;[3] and susceptibility to norfloxacin, ofloxacin, moxifloxacin and levofloxacin was determined by microdilution. After microdilution (Table S1, available as Supplementary data at *JAC* Online), MIC values ranged between 2 and 64 mg/L for nalidixic acid and between 0.03 and 4 mg/L for ciprofloxacin, so that 93% of isolates could be defined as included in this low-level ciprofloxacin-resistant phenotype.