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Subtyping of plasmid-mediated quinolone resistance among *Salmonella* serotypes by whole genome sequencing

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ABSTRACT

Most known plasmids are identified by conferring virulence or antimicrobial resistance phenotypes and such characteristics aid in the success of the dispersion of different plasmid types between bacteria from different sources. This study aimed to perform the subtyping of the plasmid-mediated quinolone resistance, detected in *Salmonella* spp. A total of 34 *Salmonella* strains non-susceptible to ciprofloxacin were evaluated. Strains were selected based on the presence of PMQR determined by Polymerase Chain Reaction and further submitted to Next Generation Sequencing. Most of the strains presented the *qnrB19* in small ColE-like plasmids and *qnrB2* gene associated with IncN/ST5 plasmids also detected. Our results indicated the co-occurrence of PMQR and ESBLs in plasmids that are a lineage of epidemic plasmids circulating in *Salmonella* in which additional resistances were detected, highlighting the potential threat of resistance *Salmonella* to public health, particularly in infections in which antimicrobial therapy is needed.

1. Introduction

Salmonella are frequent causative agents of foodborne diseases, commonly found in developed and developing countries (Hopkins et al., 2005). Recently, the emergence and dissemination of multi-drug resistant *Salmonella* serotypes posed a global public health problem. The indiscriminate use of antimicrobial agents in human and veterinary therapy and prophylaxis has aggravated this situation (Su et al., 2004; Weill et al., 2006). The use of antimicrobial agents in food-producing animals is believed to be the main cause of the emergence and spread of resistant strains of *Salmonella* since food of animal origin represents a major source of *Salmonella* isolates (Piddock, 2002; Threlfall, 2002).

Fluorquinolones (FQ) have become the major antimicrobials used to treat patients with severe *Salmonella* infections, including typhoid fever and chronic salmonellosis. Currently, ciprofloxacin has been recommended as the first drug to treat severe infections in elderly (Hopkins et al., 2005; Su et al., 2004). FQ resistance is primarily associated with accumulation of chromosomal mutations in the targeted topoisomerases. DNA gyrase is encoded by *gyrA* and *gyrB* genes, while topoisomerase IV is encoded by *parC* and *parE* (Hopkins et al., 2005). Additional low-level resistance can be acquired by plasmid-encoded proteins including Qnr proteins that protect the DNA gyrases from antimicrobial action. Moreover, the Aac(6′)-Ib-cr, a variant of aminoglycoside acetyltransferase, and efflux pumps (OqxAB and QepA) also had been found to contribute to resistance to the drugs belonging to this antimicrobial class (Hopkins et al., 2005).

Transfer of antimicrobial resistance among members of the family Enterobacteriaceae may occur by transfer of plasmids. Most known plasmids are identified by conferring virulence or antimicrobial resistance phenotypes and such characteristics aid in the success of the dispersion of different plasmid types between bacteria from different sources or geographic origins. Plasmid typing system (Carattoli et al., 2005) and plasmid multilocus sequence typing are molecular epidemiological tools that allow tracking plasmid dispersion and diversity, including the ones carrying the plasmid-mediated quinolone resistance (PMQR). (Ferreira et al., 2018) In Brazil, the largest country of Latin America, limited data is available on PMQR plasmids of *Salmonella* strains from human (Casas et al., 2016; Ferrari et al., 2011; Pribul et al., 2017) and recent studies have demonstrated the occurrence of plasmids in *Salmonella* isolates mainly from food chain (Casas et al., 2016; Ferrari et al., 2011; Pribul et al., 2017).

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The aim of this study was subtyping the PMQR-carrying plasmids. Plasmid diversity and dispersion, as well as additional resistance genetic markers, were evaluated in order to provide more comprehensive details on the analysis of plasmids carrying ciprofloxacin resistance in *Salmonella* isolates.

2. Methods

2.1. Strains

A total of 34 *Salmonella* strains, isolated between 2009 and 2013 that displayed plasmid-mediated quinolone resistance genes were enrolled in this study. Out of the 34 isolates, most of them (n = 23, 67%) were obtained from human sources, such as blood (15%), urine (6%), stool (47%) and 11 from non-human such as food (6%), poultry (20%) and poultry environment sources (6%).

2.2. Serotyping

The isolates were serotyped based on somatic O and phase 1 and phase 2 of H flagellar antigens by agglutination tests with antisera (prepared in the Laboratory of Enteric Pathogens, Instituto Adolfo Lutz, São Paulo), as specified in the Kauffmann–White-Le Minor scheme for *Salmonella* serotyping.

2.3. Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed by using the disk diffusion method according to the guidelines and interpretation criteria of the CLSI (CLSI, 2016). The following antimicrobial disks were tested: nalidixic acid, amoxicillin/clavulanic acid, ampicillin, amikacin, aztreonam, ceftazidime, cefotaxime, ceftriaxone, cefepime, ciprofloxacin, chloramphenicol, streptomycin, gentamicin, imipenem, trimethoprim/sulfamethoxazole, sulfonamide, and tetracycline.

MIC of nalidixic acid and ciprofloxacin were determined by the E-test® technique according to the manufacturer's recommendations (BioMérieux, Marcy l'Etoile, France). The quality control results for all MIC and disk diffusion tests were within acceptable quality control ranges according to CLSI guidelines (CLSI, 2016). *Escherichia coli* ATCC 25992 and *Pseudomonas aeruginosa* ATCC 27853 were used as control on each test.

2.4. Conjugation and transformation assays

Conjugal transfer of plasmids was carried out in mixed broth cultures with J53 *E. coli* strain (sodium azide-resistant) as the recipient. Transconjugants were selected on MacConkey (Merck, Darmstadt, Germany) agar plates containing ciprofloxacin (0.05 mg/L; Sigma-Aldrich, St. Louis, MO) and sodium azide (100 mg/L; Sigma-Aldrich, St. Louis, MO).

For the strains in which transfer of plasmids encoding ciprofloxacin resistance was not achieved by conjugation, transformation experiments were conducted: total plasmids were extracted using commercial kits (Qiagen, Venlo, Netherlands) according to the manufacturer's instruction. Purified plasmids were transformed using Electromax (Gibco Invitrogen, USA) and *E. coli* DH10B competent cells. Transformants were selected on Trypticase soy agar (Merck, Darmstadt, Germany) plates containing ciprofloxacin (0.05 mg/L; Sigma-Aldrich, St. Louis, MO). The resistance profile of transconjugants and transformants was determined by the disk diffusion method as described above.

2.5. Plasmid analyses

Plasmids from parental and transformant/tranconjugant strains were assigned to Incompatibility (Inc) groups by PCR-based replicon typing (PBRT) to characterize all plasmids, including the non-conjugative ones (Carattoli et al., 2005). Identification of additional plasmid groups were added to the typing schema (García-Fernández et al., 2011; Villa et al., 2010).

2.6. Whole-genome sequencing and de novo assembly

Total DNA was extracted from overnight cultures of the 34 *Salmonella* isolates using the Wizard Genomic DNA purification System (Promega, USA). Whole genome shotgun sequencing was prepared according to the Illumina Nextera XT sample preparation guide, and DNA concentrations were measured using a Qubit fluorometer (Life Technologies, MD). Whole genome sequencing was performed using v2 chemistry with paired-end – 2 by 200 bp reads on a HiSeq platform (Illumina, San Diego, CA). Read trimming and assembly were performed using BioNumerics v. 7.6 software. Genomes were automatically annotated using the PGAP (NCBI Prokaryotic Genome Annotation Pipeline v.4.3). Online tools of Center for Genomic Epidemiology were used to determine in silico serotype, plasmid classification (plasmid-based replicon typing – PBRT, pMLST), and MLST databases (https://cge.cbs.dtu.dk). Mobile genetic elements such Insertion Sequence (IS) and Trasnposon (Tn) were manually curated using ISFinder database (https://isfinder.biotoul.fr).

2.7. Nucleotide accession numbers

The whole-genome shotgun project reported here have been deposited at DDBJ/EMBL/GenBank was retrieved via Bioproject PRJNA482596. The version described in this paper has accession numbers: QRCN01000000 (strain 521/12) and QRCO01000000 (strain 98/15).

3. Results

A total of 34 *Salmonella* strains belonging to 13 different serotypes were identified. *S. heidelberg* (n = 6; 18%) was the most frequent serotype, followed by *S. alachua* serotype (n = 5; 15%), followed by *S. muenchen*, *S. schwarzengrund*, *S. typhimurium* (n = 4; 12%) respectively, three *S. saintpaul*, two *S. braenderup*. The serotypes Corvallis, Derby, Dublin, Enteritidis, Montevideo, and Oranienburg were represented by one isolate (Table 1).

To correlation of susceptibility phenotypes (Disk diffusion test) and genotypes (WGS), disk diffusion tests from susceptibility phenotypes corroborated with the results found in the *silico* analyses. *Silico* analyzes verified that additional resistance genes responsible for resistance to aminoglycosides (*aadA1, aadA5, strA, strB*), sulfonamides (*sul1, sul2),* trimethoprim (*dfrA17, dfrA21, dfrA25*), beta-lactamases (bla_{TEM-1}) extended spectrum beta lactams ($bla_{CTX-M-2}$, $bla_{CTX-M-8}$, bla_{CMY-2}), tetracycline (*tetA, tetB*), chloramphenicol (*floR*) and fosfomycin (fosA7) were detected among the PMQR-positive strains. Of the 34 strains, six had mutations in the *gyrA* genes. Nalidixic acid resistance (MIC ≥32 mg/L) and reduced susceptibility to ciprofloxacin (MIC range: 0.125–0.75 mg/L) were detected in all *Salmonella* strains.

Were analyzed the 34 plasmid-mediated quinolone resistance genes. Thirty-two strains are positive to *qnrB19* gene, that were located in small ColE-like plasmids (Fig. 1B), sizes ranging from 2.744 to 3.119 bp. In these plasmids, *qnrB19* are located downstream the *pspF* gene (Fig. 1).

qnrB2 gene were found in two *Salmonella* serovars (*S.* Alachua and *S.* Dublin) associated with IncN/ST5 plasmids. These ~50 Kb plasmids were transferred by conjugation and an increasing of four fold in the MIC of ciprofloxacin was observed. In both plasmids, *qnrB2* was inserted in a complex class 1 integron (Fig. 1).

Table 1
Characteristics of *Salmonella enterica* strains carrying plasmid-mediated resistance quinolones.

Strain n°.	Serotype	MLST	Source	Plasmids	PMQR gene (location)	Other resistance genes and QRDR mutations
181/09	Corvallis	1541	Environment	colE-like	*qnrB19* (ColE-like)	ND
179/10	Enteritidis	11	Stool	colE-like	*qnrB19* (ColE-like)	*aadA1; aac(6′)-lb, bla*$_{TEM-1}$; *bla*$_{CTX-M-8,}$ *bla*$_{OXA-9}$
556/11	Montevideo	4	Stool	colE-like	*qnrB19* (ColE-like)	*bla*$_{TEM-116}$
138/14	Muenchen	112	Stool	colE-like HI2	*qnrB19* (ColE-like)	*aadA1, aac(6′)-Iaa, aph(6)-ld, aph(3′)-Ia, strA, bla*$_{TEM-1}$, *bla*$_{CTX-M-2}$, *tet(A), sul1, sul2*
275/14	Muenchen	112	Stool	colE-like	*qnrB19* (ColE-like)	*aac(6′)-Iaa*
287/14	Muenchen	112	Blood	colE-like; FII; HI2	*qnrB19* (ColE-like)	*aadA1, aph(3′)-Ia, aac(6′)-Iaa, bla*$_{CTX-M-2}$, *tet(A), sul1*
383/14	Muenchen	112	Urine	colE-like; FII, HI2	*qnrB19* (ColE-like)	*aac(6′)-Iy, aadA1, aph(3′)-Ia, bla*$_{CTX-M-2}$, *tet(A), sul1*
08/13	Saintpaul	50	Stool	colE-like	*qnrB19* (ColE-like)	*aac(6′)-Iy*
87/14	Saintpaul	50	Stool	colE-like	*qnrB19* (ColE-like)	*aac(6′)-Iaa*
521/12	Alachua	1298	Stool	colE-like	*qnrB19* (ColE-like)	ND
523/12	Alachua	1298	Stool	colE-like	*qnrB19* (ColE-like)	ND
524/12	Alachua	1298	Food	colE-like	*qnrB19* (ColE-like)	ND
525/12	Alachua	1298	Food	colE-like	*qnrB19* (ColE-like)	ND
98/15	Alachua	1298	Blood	IncN	*qnrB2* (IncN/ST5)	*tet(A), sul1, dfrA25*
115/15	Braenderup	22	Stool	colE-like	*qnrB19* (ColE-like)	*aac(6′)-Iaa*
127/15	Braenderup	22	Stool	colE-like	*qnrB19* (ColE-like)	*aac(6′)-Iaa*
444/12	Derby	*	Blood	colE-like	*qnrB19* (ColE-like)	*aac(6′)-Iy*
96/15	Dublin	10	Blood	colE-like, N, X1, FIB, FII	*qnrB2* (IncN/ST5)	*aadA1, aph(6)-ld, tet (A), tet(B), sul1, sul2, dfrA25*
277/14	Oranienburg	23	Urine	colE-like	*qnrB19* (ColE-like)	*aac(6′)-Iy*
554/12	Schwarzengrund	96	Stool	colE-like	*qnrB19* (ColE-like)	*aph(6)-ld, aph(3″)-lb, tet(A), sul2*
579/12	Schwarzengrund	96	Blood	colE-like	*qnrB19* (ColE-like)	*aph(6)-ld, aph(3″)-lb, tet(A), sul1, sul2*
347/13	Schwarzengrund	96	Environment	colE-like	*qnrB19* (ColE-like)	*aph(6)-ld, aph(3″)-lb, tet(A), sul2*
06/15	Schwarzengrund	96	Stool	colE-like	*qnrB19* (ColE-like)	*aph(6)-ld, aph(3″)-lb, tet(A), sul2*
242/13	Saintpaul	1654	Poultry	colE-like, FIB	*qnrB19* (ColE-like)	*aac(6′)-Iaa*
154/15	Typhimurium	19	Stool	colE-like, FIB	*qnrB19* (ColE-like)	*aadA5, aac(6′)-Iaa, sul2, dfrA17*
127/13	Typhimurium	19	Stool	colE-like, I1	*qnrB19* (ColE-like)	*aadA5, aac(6′)-Iaa, sul2, dfrA17*
176/13	Typhimurium	19	Stool	colE-like, I1; FII, F1B	*qnrB19* (ColE-like)	*aadA5, aac(6′)-Iaa, sul2, dfrA17*
177/13	Typhimurium	19	Stool	colE-like, I1; F1B	*qnrB19* (ColE-like)	*aadA5, aac(6′)-Iaa, sul2, dfrA17*
443/12	Heidelberg	15	Poultry	colE-like I1; A/C	*qnrB19* (ColE-like)	*fosA7, aac(6′)-Iaa, parC-S80R, gyrA-D87G, gyrA-A119E*
555/12	Heidelberg	15	Poultry	colE-like; I1; A/C	*qnrB19* (ColE-like)	*fosA7, sul2, tet(A), cmy-2, gyrA-S83F*
133/12	Heidelberg	15	Poultry	colE-like; I1; A/C	*qnrB19* (ColE-like)	*fosA7, sul2, tet(A), cmy-2, gyrA-S83F*
306/14	Heidelberg	15	Poultry	colE-like; I1; A/C	*qnrB19* (ColE-like)	*fosA7, sul2, tet(A), cmy-2, gyrA-S83F*
309/14	Heidelberg	15	Poultry	colE-like; I1; A/C	*qnrB19* (ColE-like)	*fosA7, sul2, tet(A), cmy-2, gyrA-S83F*
93/16	Heidelberg	15	Poultry	colE-like; I1; A/C	*qnrB19* (ColE-like)	*fosA7, sul2, tet(A), cmy-2, gyrA-S83F*

ND = No other resistance genes detected.
* : Not determined.

4. Discussion

Rapid dissemination of plasmid-mediated quinolone resistance (PMQR) in Enterobacteriaceae has been described in recent years and information on the prevalence of these resistance determinants and characterization of plasmids in developing countries is scarce. *qnrB19* gene is one the most found variants of *qnr* genes worldwide (Ciesielczuk et al., 2013; García-Fernández et al., 2011). In this study, 32 strains carrying *qnrB19* in small ColE-like plasmids. Studies from Europe and USA have been reporting *qnrB19* present in IncN or not-typed plasmids ranging from 40 to 80 Kb (Cattoir et al., 2008; Garcia-Fernández et al., 2009; Jacoby et al., 2014; Rice et al., 2008; Schink et al., 2012). Previous studies in South America have reported small ColE-like plasmids carrying *qnrB19* (Cunha et al., 2017; Karczmarczyk et al., 2010; Tran et al., 2012). Most of ColE/*qnrB19* plasmids found by us had high nucleotide identity (100–99%) with plasmids pPAB19–2 (GenBank accession n°. JN979787) and pPAB19–3 (JN985534) described by Tran et al. (2012) in Argentina or pFA27–1 (KX452394) described by Cunha et al. (2017) in Brazil. This fact suggests that ColE-like plasmids drive the dissemination of *qnrB19* in South America. Two strains of serovars Muenchen (138/14) and Saintpaul (242/13) presented a plasmid with 99% identity with a pHAD28 (KU674895), a novel variant of ColE-like plasmid bearing *qnrB19* found in *Salmonella* Hadar isolates recovered from chicken abattoirs in Germany (Fiegen et al., 2017).

IncN plasmids are one of the plasmid families most related to resistance genes (García-Fernández et al., 2011). In our study the *qnrB2* gene was inserted into IncN/ST5 plasmids in strains of the serovars Alachua and Dublin. IncN/ST5 plasmids carrying *qnrB2* was previous described by Garcia-Fernández et al. (2009) in *Salmonella* spp. strains from The Nederland's. The complex class 1 integron with *qnrB2* also is present in pBK31551, an IncN/ST6 plasmid that coharbor blaKPC-4 from an isolate of *K. pneumoniae* from USA. Chen et al. 2009 showed that *qnrB2* was mobilized to a class 1 integron via rolling circle transposition and recombination mediated by IS*CR1*.

In summary, this study brings up information regarding the epidemiology of plasmid-mediated quinolone resistance particularly among human *Salmonella* strains. Our findings indicated the co-occurrence of ESBL and PMQR genes into these epidemic plasmids reinforces the need for enhanced continuous surveillance of antimicrobial resistance, since there is a paucity of data is still regarding quinolones resistance in *Salmonella* in developing countries. These findings show the need for enhanced surveillance of the indiscriminate use of fluoroquinolones in human and veterinary medicine and alert to prevent the fail in treatment of severe invasive salmonellosis.

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Transparency declarations

None declared.

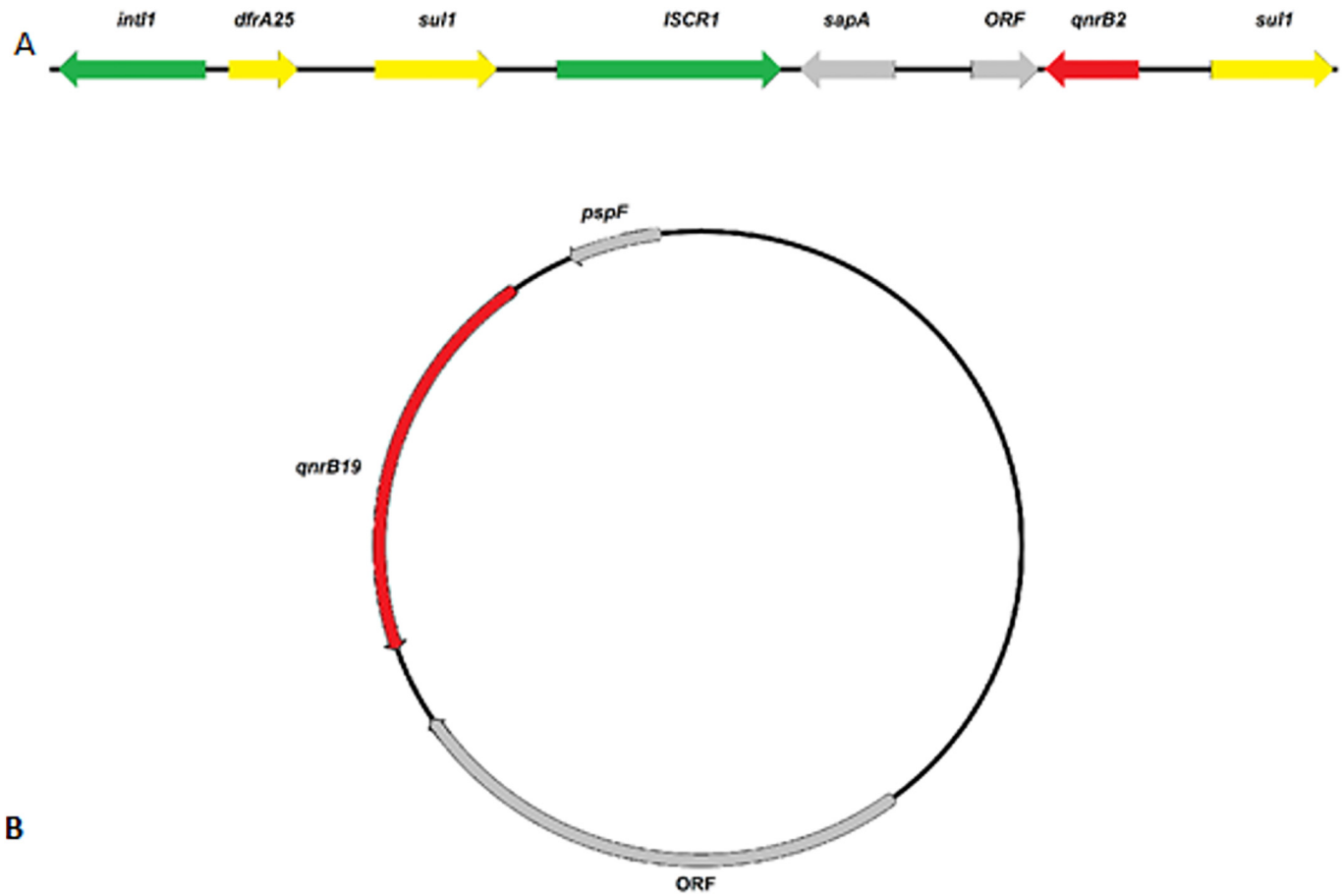

Fig. 1. Representation of the genetic environment for *qnrB2* (A), and ColE/*qnrB19* plasmid (B). Arrows represent genes pointing in the direction of transcription. Red arrows indicate *qnr* genes, yellow indicate other resistance genes, green indicate genes related to mobile genetic elements, and gray indicate ORFs or other functions genes.

Competing interest

None declared.

Ethical approval

Not required.

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