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Characterization of quinolone resistance in *Salmonella* spp. isolates from food products and human samples in Brazil

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ABSTRACT

Non-typhoidal salmonellosis is an important zoonotic disease caused by Salmonella enterica. The aim of this study was to investigate the prevalence of plasmid-mediated quinolone resistance in Salmonella spp. and its association with fluoroquinolone susceptibility in Brazil. A total of 129 NTS isolates (samples from human origin, food from animal origin, environmental, and animal) grouped as from animal ($n = 62$) and human ($n = 67$) food were evaluated between 2009 and 2013. These isolates were investigated through serotyping, antimicrobial susceptibility testing, and the presence of plasmid-mediated quinolone resistance (PMQR) genes (qnr, aac(6′)-Ib) and associated integron genes (integrase, and conserved integron region). Resistance to quinolones and/or fluoroquinolones, from first to third generations, was observed. Fifteen isolates were positive for the presence of qnr genes (8 qnrS, 6 qnrB, and 1 qnrD) and twenty three of aac(6′)-Ib. The conserved integron region was detected in 67 isolates as variable regions, from ±600 to >1000 pb. The spread of NTS involving PMQR carriers is of serious concern and should be carefully monitored.

Introduction

Foodborne diseases caused by non-typhoid *Salmonella* represent not only an important public health problem, but also an economic burden in many parts of the world. It has been estimated that the global incidence of gastroenteritis caused by non-typhoid *Salmonella* is almost 93.8 million cases per year with 155,000 deaths.[1] Non-typhoidal *Salmonella* spp. are zoonotic agents that have been linked to a variety of food

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sources, particularly foods of animal origin, e.g., beef, poultry, eggs, and dairy products as well as raw fruits and vegetables.[2]

The emergence and spread of antimicrobial-resistant *Salmonella* spp. originating from food of animal origin has become a serious health hazard worldwide, especially in developing countries.[3,4] Antimicrobial-resistant bacteria can be selected through the therapeutic treatment of infections caused by susceptible bacterial populations, both in humans and animals; many mechanisms involved in resistance to quinolones have been studied.[5]

Quinolones, particularly fluoroquinolones, are among the most widely used antibiotics for treating salmonellosis in both human and veterinary infections because of their broad spectrum in antimicrobial activity.[6]

Quinolone resistance in *Enterobacteriaceae* is mostly mediated by point mutations in the quinolone resistance-determining regions (QRDR) of the DNA gyrase and topoisomerase IV genes, leading to target modification. Plasmid Mediated Quinolone Resistance (PMQR) has emerged in *Salmonella* spp. and in other *Enterobacteriaceae* with increasing prevalence.[7] This resistance involves efflux pump mechanisms and the more recently discovered target protection mechanisms controlled by the *qnr* genes. Enzymatic modifications encoded by the *aac(6′)Ib-cr* gene have also been found to contribute to the drug resistance of this antimicrobial class.[8]

The aim of this study was to identify the occurrence of some PMQR in *Salmonella* spp. isolated from animal and human origin sin Brazil between 2009 and 2013.

Materials and methods

A total of 129 *Salmonella* spp. isolates with resistance to quinolone and/or fluoroquinolone were evaluated. Of this total, 51.9% (67/129) were from human clinical isolates, 30.2% (39/129) from food products for human consumption (beef, eggs, and milk), 7.1% (9/129) from food of animal origin for human consumption (poultry, swine, and cattle), and 10.8% (14/129) from environmental samples (water, drag swabs). The strains identified were stored in phosphate-buffered agar and sent to the National Reference Laboratory of Enteric Diseases (LRNEB/IOC/RJ) between 2009 and 2013.

Antigenic characterization

Salmonella serotypes were determined by slide agglutination according to the Kauffmann–White scheme using O and H antisera. All antisera used for serological determination were prepared in the LRNEB/IOC/RJ.

Antimicrobial susceptibility

The resistance profiles obtained were confirmed by the disk diffusion test according to CLSI (2013/2014) using representatives of the quinolone class (OXOID) for human and veterinary therapeutic use such as Nalidixic Acid 30 μg, Ciprofloxacin 5 μg, Enrofloxacin 5 μg, Ofloxacin 5 μg, and Levofloxacin 5 μg; bacterial suspensions (0.5 Mac Farland scale) were distributed throughout the surface of plates containing Mueller Hinton agar (OXOID). Discs were deposited on the surface of the culture medium, which already contained the inoculum. After incubation for 24 h at 35 °C, the diameters of inhibition zones formed around the discs were observed and measured in millimeters. The interpretation of results for assignment of the categories of susceptible, intermediate, and resistant was according to CLSI (2013). Quality control was performed in parallel by testing *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853.

MIC determinations were performed in 96-well microplates for Nalidixic Acid (SIGMA), Ciprofloxacin (SIGMA), Enrofloxacin (SIGMA), Levofloxacin (SIGMA), and Ofloxacin (SIGMA) according to the CLSI (2013) broth microdilution assay. MIC was defined as the lowest concentration of drug that inhibits visible growth after 24 h of incubation at 37 °C. Bacterial suspensions grown at 37 °C in BHI broth (OXOID) up to in the concentration at 0.5 of the MacFarland scale that were transferred to BHI broth and plates containing different concentrations of antimicrobials were incubated at 37 °C for 24 h. Quality control was performed for every determination by testing *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and *P. aeruginosa* ATCC 27853.

Detection of PMQR

Total DNA was extracted using the DNEASY Tissue Qiagen® kit and its concentration was measured using the Nanodrop spectrophotometer (ND-1000 Uniscience). The studied genes were detected by PCR amplification using the primer sequences presented in Table 1. The *qnrA*, *qnrB*, and *qnrS* genes were amplified through multiplex PCR reactions; the *rrs* gene was used as the reaction control. The *qnrC, qnrD, aac(6′)-Ib, integrase*, and variable *integron* region genes were amplified by simplex PCR.

Positive and negative controls were included in each PCR reaction. Amplified products were identified by their molecular weights after electrophoresis on 1.0% agarose gels at 180 V for 90 min and staining with ethidium bromide.

Results

Altogether, 26 different *Salmonella* serovars were identified. The predominant serovar was *Salmonella* Typhimurium (48.8%, 63/129) followed by *Salmonella* Enteritidis (19.4%, 25/129). The prevalent serovars associated with resistance to quinolones are presented in Table 2.

The highest incidence of resistant isolates was observed in 2012 (88/129), followed by 2011 (16/129). Most isolates were isolated in 2012.

Among these 129 isolates that were previously resistant to Nalidixic Acid, 5 were sensitive to all tested quinolones (including Nalidixic Acid), 55 (42.6%) were resistant to Ciprofloxacin, 63 (48.8%) to Enrofloxacin, 51 (39.53%) to Ofloxacin, and 48 (37.2%) to Levofloxacin through the disc diffusion test.

The broth microdilution test identified 47 (36.4%) isolates with decreased susceptibility to Ciprofloxacin (MICs between 0.125 mg/mL and 0.5 mg/mL), 26 (20.1%) to Enrofloxacin, 8

Table 1 – Sequence of oligonucleotide primers used in this study.

Primers	Primer sequences (5′-3′)	Target gene	Amplicon size (bp)	Reference
QnrA-F	ATTTCTCACGCCAGGATTTG	*qnrA*	516	Jacoby et al.[9]
QnrA-R	GATCGGCAAAGGTTAGGTCA			
QnrB-F	GATCGTGAAAGCCAGAAAGG	*qnrB*	469	Jacoby et al.[9]
QnrB-R	ACGATGCCTGGTAGTTGTCC			
QnrC-F	GGGTTGTACATTTATTGAATCG	*qnrC*	307	Kim et al.[8]
QnrC-R	CACCTACCCATTTATTTTCA			
QnrD-F	CGAGATCAATTTACGGGGAATA	*qnrD*	582	Cavaco et al.[10]
QnrD-R	AACAAGCTGAAGCGCCTG			
QnrS-F	ACGACATTCGTCAACTGCAA	*qnrS*	417	Jacoby et al.[9]
QnrS-R	TAAATTGGCACCCTGTAGGC			
AAC(6′)-Ib-F	TATGAGTGGCTAAATCGAT	*aac(6′)-Ib*	482	Park et al.[11]
AAC(6′)-Ib-R	CCCGCTTTCTCGTAGCA			
Integrase-F	CCTCCCGCACGATGATC	*Integrase*	250	Peirano et al.[12]
Integrase-R	TCCACGCATCGTCAGGC			
Integron-3	AAGCAGACTTGACCTGA	*Integron*	Variable	Peirano et al.[13]
Integron-5	GGCATCCAAGCAGCAAG			

Table 2 – Distribution of quinolone-resistant *Salmonella* spp. serovars isolated from food chain diseases.

Salmonella Serotype	Year	Number of NTS[a] isolated from				
		Human	Food	Environment	Animal	*Total*
S. Tiphymurium	2013	2	–	–	–	2
	2012	24	14	1	1	40
	2011	7	2	2	–	11
	2010	–	4	–	1	5
	2009	2	2	1	1	6
S. Enteritidis	2013	3	–	–	–	3
	2012	20	1	–	–	21
	2009	1	–	–	–	1
S. Muenchen	2012	2	1	–	–	3
	2011	–	1	–	–	1
S. Saintpaul	2012	1	–	–	2	3
S. Infantis	2012	1	–	1	–	2
	2010	–	1	–	–	1
S. Heidelberg	2012	–	2	–	–	2
	2011	–	–	–	1	1
Others	2013	1	–	–	–	1
	2012	3	8	6	–	17
	2011	–	1	1	1	3
	2010	–	1	1	2	4
	2009	–	1	–	2	3
Total		67	39	14	32	152

[a] Non-typhoid *Salmonella*.

(6.2%) to Levofloxacin, and 12 (9.3%) to Ofloxacin (MICs between 0.5 mg/mL and 1 mg/mL). Seventy-three (56.6%) isolates were resistant to Ciprofloxacin, 83 (64.3%) to Enrofloxacin, 44 (34.1%) to Ofloxacin, and 39(30.2%) to Levofloxacin. A total of 124 (96.1%) were resistant to Nalidixic Acid. The decreased susceptibility breakpoint to Nalidixic Acid is not reported by CLSI (2013).

The resistance profile obtained with the microdilution test showed that 37 (28.7%) isolates were resistant to all tested quinolones; 30 (23.2%) were resistant to Ciprofloxacin, Enrofloxacin, and Nalidixic Acid; 16 (12.4%) were resistant to Enrofloxacin and Nalidixic Acid; 2 (1.5%) were resistant to Ciprofloxacin and Nalidixic Acid; and 39 (30.2%) were resistant to Nalidixic Acid only. The detection of PMQR was showed in the Table 3.

The *qnr* genes were detected in 15 out of the 129 (11.6%) available *Salmonella* spp. isolates. Among these positive isolates, 6 contained the *qnrB* gene, 8 the *qnrS* gene, and 1 the *qnrD* gene (Table 3). These strains were recovered from human samples ($n = 10$), food from animal origin ($n = 3$), environmental samples ($n = 1$), and animal samples ($n = 1$). Seven different serotypes were identified with *qnr* genes. The most *qnr*-positive prevalent serovar was *S.* Typhimurium followed by *S.* Saintpaul and *S.* Livingstone. The *qnrS* was the most frequent *qnr* variant observed. None of the isolates presented the *qnrA* or *qnrC* genes. Three *qnr*-positive isolates presented the

Table 3 – Quinolone susceptibility of qnr-positive isolates.

Isolate	Source	PMQR	Integron[a]	MIC (μg/mL)				
				Cip	Nal	Eno	Lvx	Ofl
S. Typhimurium	H	*qnrB* *aac(6′)-Ib*	–	≥2	≥128	≥4	≥2	≥4
S. Typhimurium	H	*qnrB* *aac(6′)-Ib*	±900 bp	≥2	≥128	≥4	≤0.06	≤0.25
S. Typhimurium	H	*qnrB*	–	≥2	≥128	≥4	≥2	≥4
S. Typhimurium	H	*qnrB*	>1000 bp	≤0.5	≥128	≤1	≤0.06	≤0.06
S. Typhimurium	F	*qnrS*	–	≤0.5	≤64	≤2	≤0.06	≤0.06
S. Typhimurium	F	*qnrD*	–	≤0.03	≤32	≤0.06	≤0.06	≤0.06
S. Saintpaul	A	*qnrS* *aac(6′)-Ib*	±900 bp	≤0.5	≥128	≤0.5	≤0.06	≤0.06
S. Saintpaul	H	*qnrS*	–	≤0.5	≥128	≥4	≤0.06	≤0.12
S. Muenchen2[b]	H	*qnrS*	–	≤0.5	≤32	≤2	≤0.06	≤0.06
S. Livingstone	F	*qnrS*	–	≤0.5	≥128	≥4	≤0.06	≤0.06
S. Orion	E	*qnrS*	–	≤0.5	≥128	≤2	≤0.06	≤0.06
S. Panama	H	*qnrS*	–	≤1	≤64	≤2	≤0.06	≤0.06
S. Enteritidis	H	*qnrB*	±800 bp	≤0.25	≥128	≤1	≤0.06	≤0.06
S. *ent.* subsp*ent.*	H	*qnrB*	>1000 bp	≤1	≥128	≤2	≤0.06	≤0.25
S. Typhimurium2	H	*aac(6′)-Ib*	–	≥2	≥128	≥4	≤2	≤2
S. Typhimurium3	F	*aac(6′)-Ib*	–	≥2	≥128	≥4	≤2	≤2
S. Typhimurium	E	*aac(6′)-Ib*	±900 bp	≥2	≥128	≥4	≤2	≤2
S. Typhimurium	A	*aac(6′)-Ib*	±900 bp	≥2	≥128	≥4	≤2	≤2
S. Typhimurium	H	*aac(6′)-Ib*	±900 bp	≥2	≥128	≥4	≥4	≥4
S. Typhimurium	F	*aac(6′)-Ib*	–	≥2	≥128	≥4	≥4	≥4
S. Typhimurium	F	*aac(6′)-Ib*	±900 bp	≥2	≥128	≥4	≤1	≤1
S. Typhimurium	H	*aac(6′)-Ib*	±800 bp	≤1	≥128	≥4	≥4	≤2
S. Typhimurium	H	*aac(6′)-Ib*	>1000 bp	≤0.25	≥128	≤0.25	≤0.12	≤0.12
S. Enteritidis	H	*aac(6′)-Ib*	–	≤0.5	≥128	≤2	≤0.12	≤0.12
S. Enteritidis	H	*aac(6′)-Ib*	±800 bp	≥2	≥128	≥4	≤1	≤1
S. Give	H	*aac(6′)-Ib*	–	≥2	≤64	≥4	≤1	≤2
S. Hadar	F	*aac(6′)-Ib*	–	≤0.5	≥128	≤2	≤0.06	≤0.06
S. Infantis	E	*aac(6′)-Ib*	–	≤0.25	≤8	≤0.06	≤0.06	≤0.06
S. Muenchen	F	*aac(6′)-Ib*	±600 bp	≤0.5	≤32	≤2	≤0.06	≤0.06
S. Montevideo	E	*aac(6′)-Ib*	–	≤0.5	≤32	≤1	≤0.06	≤0.06
S. Saintpaul	A	*aac(6′)-Ib*	±800 bp	≤0.5	≥128	≤1	≤0.06	≤0.06

H, human; F, food; E, environment; A, animal; CIP, ciprofloxacin; NAL, nalidixic acid; ENO, enrofloxacin; LVX, levofloxacin; OFL, ofloxacin; MIC, minimal inhibitory concentration; DD, diffusion disc.

[a] Variable region.

[b] Isolate quantity.

aac(6′)-Ib in association: two S. Typhimurium and one S. Saintpaul. The two *Salmonella* ser. Typhimurium were resistant to all tested quinolones/fluoroquinolones by the broth microdilution assay at the highest concentration.

The *aac(6′)-Ib* gene was prevalent in 12 out of the 20 *qnr*-negative isolates of S. Typhimurium. The source of isolation was higher in human strains (8/20), followed by foodborne sources (7/20). Eleven isolates were resistant to all tested quinolones (Table 3).

Fourteen isolates that were PMQR positive presented the conserved integron region; two isolates presented both *qnr* and *aac(6′)-Ib* genes (Table 3).

Sixty-seven isolates showed the conserved integron region with only the variable region, from 600 to 1000 bp (data not shown). The S. Typhimurium serovar was the most frequent (39/67) with the conserved region of class 1 integron and the variable regions between ±900 pb and >1000 pb as the most observed. This gene was mostly identified in human samples (38/67) followed by food samples (15/67).

Discussion

The high prevalence of antimicrobial-resistant *Salmonella* has been a serious concern for public health over the past decade.[13,14] Fluoroquinolones are important in treating serious infections caused by *Salmonella* spp. resistance to Nalidixic Acid increased significantly in recent years, but high-levels of resistance to fluoroquinolones are, so far, rare.[15,16]

Mutations in the quinolone resistance-determining region (QRDRs) of the *gyrA* and *parC* genes alter the DNA-gyrase binding sites of these antibiotics and can result in resistance to quinolones. In *Salmonella* spp., these mutations are related to Nalidixic acid (NAL) resistance and reduced susceptibility to FQs such as Ciprofloxacin (Cip).[17,18]

Plasmid-mediated quinolone resistance in *Salmonella* spp. has important public health implications. The acquisition of PMQR genes leads to decreased susceptibility to

fluoroquinolones, accelerating the selection of fluoroquinolone-resistant mutants.[19] Moreover, interactions between mutations in the QRDR and PMQR genes can result in high fluoroquinolones MIC.[20]

In the present study, the phenotypic tests and detection of plasmid-mediated quinolone resistance by molecular basis among strains isolated from food of animal origin ($n = 62$) and human samples ($n = 67$), between 2009 and 2013 (6 in 2013, 88 in 2012, 16 in 2011, 10 in 2010, and 9 in 2009), were investigated. High prevalence of resistance to fluoroquinolone was identified through the disc diffusion and broth microdilution tests (56.6% resistance to Ciprofloxacin, 64.3% to Enrofloxacin, 34.1% to Ofloxacin, and 30.2% to Levofloxacin). Similar results are reported in others studies in Asia, Europe, and North America.[7]

The discrepancies between disk diffusion and broth microdilution results indicate that although the disk diffusion method is an effective screening test, it does not accurately identify all resistant profiles.

A high prevalence of isolates carrying PRQM genes was reported in the present evaluation (23%, 35/152). Although most of the isolates were derived from human samples (51.4%, 18/35), other sample sources also have roles as carriers of quinolone resistance genes in plasmids (10/35 from food of animal origin, 4/35 from environmental samples, and 3/35 from animal samples).

The majority of isolates exhibiting PMQRS genes were obtained in 2012; only one foodborne *S.* Typhimurium isolate presented *aac(6′)-Ib* in 2010. This result is explained not only by the high index of isolates that were resistant to quinolones in this period but also by the storage process (nutrient phosphate agar) that can lead to loss of mobile genetic elements in older strains.

The most prevalent serovar associated with the presence of PMQR genes was *Salmonella* ser. Typhimurium (18/35). The importance of this serovar in the dissemination of these resistance genes is evident in all types of samples. Other serovars such as *Salmonella* ser. Enteritidis, *Salmonella* ser. Muenchen, and *Salmonella* ser. Saintpaul were also important in spreading PMQR genes in the studied period.

The *qnrS*, *qnrB*, and *qnrD* genes were detected in the present study. The incidence of these genes has been reported worldwide.[16,21–23] In Brazil the detection of qnr genes was previously reported by Ferrari et al.[17] where were identified one *S.* Enteritidis qnrA positive and one *S.* Corvalis qnrS positive in chicken sample.

These genes confer low-level resistance to fluoroquinolones and facilitate the development of mutations in its gyrA QRDR region, a region involved in quinolones resistance mechanisms.[18,24] Nevertheless, some authors state that the presence of the *qnr* genes may lead to increased fluoroquinolone resistance (Ruiz et al., 2012).[25] The involvement of plasmid genes in the resistance to fluoroquinolones is not as well-known as that of other resistance mechanisms.[9]

The *aac(6′)-Ib-cr* gene encodes an acetyl transferase that is responsible for phenotype resistance to aminoglycoside and Ciprofloxacin (norfloxacin). It was originally reported in 2003 in an *E. coli* clinical isolate collected in Shanghai, China, and later described among various enterobacteria in several countries from Asia, North America, and Europe.[7] The *aac(6′)-Ib* gene was found in 23 out of the 129 evaluated isolates. Among those 23, two isolates corresponded to *Salmonella* Typhimurium strains with the *qnrB* and *aac(6′)-Ib* genes, isolated from human samples. The *qnrS* and *aac(6′)-Ib* genes were detected in *Salmonella* Saintpaul. Nevertheless, the emergence of the *aac(6′)-Ib-cr* gene among NTS is of serious concern because the decreased susceptibility to fluoroquinolones from the expression of the *aac(6′)-Ib-cr* genes is plasmid-mediated and therefore, mobile, and could spread through horizontal transmission to other susceptible isolates in the community.

Although some authors recognize that the location of the *aac(6′)-Ib* gene is mostly in class1 integrons, the absence of the integron gene in all positive *aac(6′)-Ib* strains is controversial.[19,26] In this study, 11 *aac(6′)-Ib* positive isolates showed the integron region, demonstrating a significant correlation between these genes.

A high prevalence of isolates with integron genes was identified (71/152) with variable regions, from ±600 to >1000 pb. The occurrence of class 1 integrons with gene cassettes of different organizations related to antimicrobial resistance suggests genetic evolution and demonstrates the role of integrons in the dissemination of antimicrobial resistance across microbial communities.[27]

In conclusion, plasmid-mediated quinolone resistance in *Salmonella* spp. has important public health implications. We report 15 *qnr*-positive (8 *qnrS*, 6 *qnrB*, and 1 *qnrD*) and 23 *aac(6′)-Ib* positive *Salmonella* spp. strains. The conserved integron region was detected in 67 isolates with variable regions, from ±600 to >1000 pb. Fluoroquinolones are commonly used to treat adults with invasive illness or other serious infections caused by *Salmonella* species. The surveillance and monitoring over the spread of PMQR in *Salmonella* spp. is essential for the public health control of NTS.

Conflicts of interest

The authors declare no competing interests exist.

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