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Research Paper

Plasmid-mediated quinolone resistance (PMQR) and mutations in the topoisomerase genes of *Salmonella enterica* strains from Brazil

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Abstract

The objective of this study was to identify mutations in the Quinolone Resistance Determining sources Regions (QRDR) of the *gyrA*, *gyrB*, *parC*, and *parE* genes and to determine if any of the *qnr* variants or the *aac(6')-Ib-cr* variant were present in strains of *Salmonella* spp. isolated in Brazil. A total of 126 *Salmonella* spp. strains from epidemic (n = 114) and poultry (n = 12) origin were evaluated. One hundred and twelve strains (88.8%) were resistant to nalidixic acid (NAL) and 29 (23.01%) showed a reduced susceptibility to ciprofloxacin (Cip). The mutations identified were substitutions limited to the QRDR of the *gyrA* gene in the codons for Serine 83, Aspartate 87 and Alanine 131. The sensitivity to NAL seems to be a good phenotypic indication of distinguishing mutated and non-mutated strains in the QRDR, however the double mutation in *gyrA* did not cause resistance to ciprofloxacin. The *qnrA1* and *qnrB19* genes were detected, respectively, in one epidemic strain of S. Enteritidis and one strain of S. Corvallis of poultry origin. Despite previous detection of *qnr* genes in Brazil, this is the first report of *qnr* gene detection in *Salmonella*, and also the first detection of *qnrB19* gene in this country. The results alert for the continuous monitoring of quinolone resistance determinants in order to minimize the emergence and selection of *Salmonella* spp. strains showing reduced susceptibility or resistance to quinolones.

Key words: *Salmonella*, PMQR, QRDR mutation, quinolone, ciprofloxacin.

Introduction

Salmonellosis is a common cause of foodborne diseases and is a serious public health problem (Arkin, 2008). Fluoroquinolones (FQs) are used to treat invasive salmonellosis in humans and animals, but in recent years, studies have reported an increase in the number of clinical isolates resistant or with a reduced susceptibility to these compounds (Ercis *et al.*, 2006; Kownhar *et al.*, 2007; Souza *et al.*, 2010).

The FQs inhibit the activity of the enzymes topoisomerase II (DNA-gyrase) and topoisomerase IV, inducing cell death. Mutations in the quinolone resistance-determining regions (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) (Cavaco and Aarestrup, 2009; Hopkins *et al.*, 2005). Other mechanisms associated with low susceptibility or resis-

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tance to FQs, such as, efflux pump over-expression, modification of porin regulation, and plasmid-mediated quinolone resistance (PMQR) have been reported in *Salmonella* (Sjolund-Karlsson *et al.*, 2010).

The PMQR *qnrA* gene encodes a 218-amino acid protein, a member of a family of pentapeptides that protect DNA-gyrase and topoisomerase IV from the quinolones' activity (Martinez-Martinez *et al.*, 1998; Tran *et al.*, 2005). This pentapeptide blocks the action of Cip, resulting in a low-level quinolone resistance with an increase in the minimal inhibitory concentration of ciprofloxacin (CipMIC) (Robicsek *et al.*, 2006). At present, the *qnr* gene is known to encode six proteins: QnrA, with variants from QnrA1 to QnrA6; QnrB, with variants from QnrB1 to QnrB20; QnrS, with variants from QnrS1 to QnrS3; QnrVC; QnrC; and QnrD (Cavaco and Aarestrup, 2009; Fonseca *et al.*, 2008; Robicsek *et al.*, 2006; Wang *et al.*, 2009). Another PMQR-based resistant mechanism involves the ciprofloxacin resistance (*cr*) variant of the *aac(6')-Ib* gene, which encodes an aminoglycoside acetyltransferase. This variant contains substitutions in codons 102 and 179 conferring a reduced susceptibility to Cip by N-acetylation at the amino nitrogen on its piperazinyl substituent (Robicsek *et al.*, 2006).

In the present study, *Salmonella* spp. strains from epidemic and poultry origins isolated in Brazil were screened for mutations in the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes and for the presence of any of the *qnr* variants and the *aac(6')-Ib-cr* variant.

Materials and Methods

Strains of *Salmonella* spp. A total of 126 *Salmonella* spp. strains from epidemic (*i.e.*, isolates from outbreaks) and poultry origins, isolated between 1999 and 2007 in Parana State, Brazil, were evaluated. The strains were obtained from the Central Laboratory of Parana State (LACEN, Curitiba, Parana) and serotyped by the Osvaldo Cruz Foundation (Rio de Janeiro, Brazil). The strains were kept at -15 °C in brain-heart infusion broth (BHI) (Difco®) containing 15% glycerol. The resistance of these strains to NAL, by disc diffusion and, the CipMIC were previously determined by Souza *et al.* (2010). Among all of the strains, 112 (88.8%) were resistant to NAL, and 29 (23.01%) showed a reduced susceptibility to Cip with a CipMIC from 0.125 μg/mL to 0.5 μg/mL (Souza *et al.*, 2010). Of the tested strains, 114 were of epidemic origin and 12 were from poultry.

Isolation, Amplification and Sequencing of DNA. The strains were cultivated overnight in BHI agar (Difco®) at 37 °C. The DNA was isolated using 10% Chelex-100. To identify the PMQR genes, including the *qnrA*, *qnrB1*, *qnrB5*, *qnrB19*, *qnrS1*, *qnrC*, and *qnrD* alleles as well as *aac(6')-Ib-cr*, the DNA was amplified according the processes described by Gay *et al.* (2006), Cavaco and Aarestrup (2009) and Wang *et al.* (2009). The positive genes *qnrA*, *qnrB1*, *qnrB5*, *qnrS1*, *qnrC*, *qnrD* and *aac*(6)*Ib-cr* were used as positive controls. The QRDR of the *gyrA*, *gyrB*, *parC*, and *parE* genes was amplified following the method described by Eaves *et al.* (2004). The sequence analysis of the PCR products was performed by Macrogen Laboratory (Seoul, Korea).

Sequence analysis. The sequences obtained were compared with those deposited in the database of the National Center for Biotechnology Information (NCBI, Hyperlink http://www.ncbi.nlm.nih.gov/www.ncbi.nlm.nih.gov) using BLAST searches.

Statistic analysis

The results were analyzed using SPSS Statistics 18.0 software. The geometric means (GMM) of the CipMIC, which indicate the central tendency, were calculated using the formula $\sqrt[n]{y1\ y2\ y3 \ldots\ yn}$, where y represents the CipMIC of each individual strain and n is the number of CipMIC used (Ling *et al.*, 2006).

Results and Discussion

Susceptibility to quinolones and mutations in the topoisomerase genes

The 14 *Salmonella* spp. strains (11.1%) that were NAL-susceptible did not contain mutations in the QRDRs of *gyrA, gyrB, parC* and *parE.* Seventy and three strains (57.9%) from the 112 NAL-resistant strains (88.8%) analyzed contained mutations in QRDR *gyrA* gene, with 60 having one and 13 two mutations. No mutations were found in the other genes. These results suggest that NAL susceptibility could indicate QRDR mutation occurrence. According to Cavaco and Aarestrup (2009), the NAL-MIC could clearly differentiate between susceptible strains and strains with one or more QRDR mutations.

Of the 13 NAL-resistant strains with two *gyrA* mutations, only one, belonging to the Enteritidis serovar, was susceptible to Cip with MIC of 0.064 μg/mL. Among the other strains, 11 *S.* Enteritidis strains and one *S.* Johannesburg strain showed a reduced susceptibility to this FQ, with MIC ≥ 0.125 μg/mL. This reduced susceptibility was also observed in 9 of the 39 NAL-resistant strains without a mutation in the *gyrA* gene. Similar results were reported by Cavaco and Aarestrup (2009), who also observed a reduced susceptibility or resistance to Cip in strains without *gyrA* mutations, probably due to other resistance mechanisms.

The geometric mean (GMM) of the CipMIC for the 73 strains with *gyrA* mutations was higher than that of the 53 strains without mutations, with means of 0.10 μg/mL and 0.06 μg/mL, respectively. Although many of the isolates of *Salmonella* spp. are considered susceptible to FQs according to CLSI criteria (Aarestrup *et al.*, 2003; Giraud *et al.*, 2006; Gunell *et al.*, 2009; Souza *et al.*, 2010), there have been an increasing number of reports documenting the

incidence of strains with a reduced susceptibility to these compounds, including cases of therapeutic failure (Aarestrup *et al.*, 2003; Crump *et al.*, 2003; Cui *et al.*, 2009; Piddock, 2002; Ricci and Piddock, 2009; Vashist *et al.*, 2009).

All of the mutations identified in this study were substitutions in the QRDR of the *gyrA* gene; Serine 83 (Ser 83), Aspartate 87 (Asp 87) or Alanine 131 (Ala 131) codons (Table 1). The Ser 83 mutations were found in 41 strains, with substitutions by Tyrosine (Tyr) and Phenylalanine (Phe) in 19 and 22 strains, respectively. The Asp 87 mutations were found in 44 strains, with substitutions by Asparagine (Asn), Tyr, and Glycine (Gly) in 31, 9, and 4 strains, respectively. The Ala 131 mutation was found in only one strain, with a substitution by Gly. The substitutions observed in the present study in *Salmonella* spp. strains from epidemic and poultry origins were previously described in animals and humans by Giraud *et al.* (2006). Although mutations were found in the isolates from epidemic and poultry origins, a direct relationship between the origin of the strain and the type of mutation could not be established.

Both the positions of the mutations and the type of substitution found in the mutated region differed among the serovar type in this study. In the five strains belonging to *S.* Enteritidis, four substitutions were found in Asp 87 → Gly and one in Ala 131 → Gly. In addition, the Asp 87 → Tyr mutation was found in nine strains including seven Enteritidis and two Johannesburg serovars. The Asp 87 → Asn mutation was present in 31 strains belonging to 5 different serovars. The most common mutations were substitutions of Asp 87 in the serovar Enteritidis, which accounted for 50.8% of the analyzed strains. This finding is in agreement with the results reported by Soto *et al.* (2003), who also found a greater frequency of mutations in the codon Asp 87.

Giraud *et al.* (2006) reported that mutations in Ser 83 and Asp 87 were not equally distributed among the serovars of *Salmonella*, with a greater frequency of Ser 83 mutations in the serovars Newport, Virchow and Typhimurium and of Asp 87 mutations in the serovars Hadar and Kottbus. Seminati *et al.* (35) identified Asp 87 → Tyr substitutions only in serovar Enteritidis (2 strains), Ser 83 → Tyr only in serovar Anatum (3 strains), and Ser 83 → Phe only in serovar Virchow (5 strains).

The occurrence of mutations in the Ser 83 codon is considered to be important for the development of FQ resistance (Piddock *et al.*, 1998; Weigel *et al.*, 1998; Weigel *et al.*, 2002), a hypothesis that is supported by the results of this study. The Ser 83-mutant strains had a higher CipMIC (GMM 0.13 μg/mL) than the Asp 87-mutant strains (GMM 0.061 μg/mL). Similar results were described by Ling *et al.* (2003) and Giraud *et al.* (2006) that also showed an associa-

Table 1 - Distribution of the mutations observed in QRDR of the *gyrA* gene in *Salmonella* strains of different serovars with the corresponding minimal inhibitory concentration for ciprofloxacin (CipMIC) and geometric mean (GMM) data.

Serovar	Mutation	N. strains	Strains origin	CipMIC[a] (μg/mL)	GMM[b] (μg/mL)
Enteritidis	Ser 83 → Tyr	12	Poultry (2); Epidemic (10)	0.064 to 0.500	0.102
	Ser 83 → Phe	14	Poultry (1); Epidemic (13)	0.064 to 0.250	0.082
	Asp 87 → Asn	20	Epidemic	0.032 to 0.064	0.052
	Asp 87 → Tyr	4	Epidemic	0.032 to 0.064	0.058
	Asp 87 → Gly	2	Epidemic	0.032	0.032
	Ser 83 → Thy/Asp 87 → Gly	1	Epidemic	0.125	0.125
	Ser 83 → Thy/Asp 87 → Asn	3	Poultry (1); Epidemic (2)	0.125 to 0.250	0.220
	Ser 83 → Phe/Asp 87 → Asn	4	Poultry (1); Epidemic (3)	0.064 to 0.125	0.105
	Ser 83 → Phe/Asp 87 → Gly	1	Epidemic	0.125	0.125
	Ser 83 → Phe/Asp 87 → Tyr	2	Epidemic	0.125	0.125
	Ala 131 → Gly/Asp 87 → Tyr	1	Epidemic	0.125	0.125
Typhimurium	Ser 83 → Tyr	1	Epidemic	0.125	0.125
	Asp 87 → Asn	1	Epidemic	0.125	0.125
Johannesburg	Asp 87 → Tyr	2	Epidemic	0.064	0.064
	Ser 83 → Tyr/Asp 87 → Asn	1	Epidemic	0.125	0.125
Heidelberg	Ser 83 → Tyr	1	Poultry	0.500	0.500
	Asp 87 → Asn	1	Poultry	0.064	0.064
Infantis	Ser 83 → Phe	1	Epidemic	0.064	0.064
Newport	Asp 87 → Asn	1	Epidemic	0.064	0.064

[a]Souza *et al.*, 2010; [b]geometric mean.

tion between the Ser 83 and Asp 87 mutations and the level of FQ resistance. The different substitutions in the same codon could also alter the binding of the quinolones to the DNA-gyrase complex.

Although the relationship between the mutations in the topoisomerase genes and quinolone resistance has been demonstrated, 39 of the NAL-resistant strains in the present study did not have mutations in the QRDRs of the four genes that were analyzed. Currently, it is known that the resistance to quinolones can also occur in the absence of mutations in *gyrA.* Nath and Maurya (2010) isolated ciprofloxacin-resistant *Salmonella* spp. strains without mutations in the QRDR of *gyr*A. Similarly, Gunell *et al.* (2009) observed a CipMIC of 0.5 μg/mL for a strain of *S. Typhimurium* without a mutation in the QRDR of *gyrA.*

The differences in antimicrobial susceptibility could be associated to additional mechanisms of resistance, such as efflux pumps over-expression and modification of porin regulation. Efflux pumps are transporter proteins that expel toxic substrates (including antibiotics) from inside cells to the outside. Efflux pumps over-expression can be caused by chromosomal mutations or in response to bacterial stress and could play an important role in resistance, especially when fluoroquinolones are involved (Eaves *et al.*, 2004; Giraud *et al.*, 2006; Piddock, 2002). Changes in permeability of the membrane with decreasing in intracellular penetration of the antimicrobial agent could also occur by a mechanism in which porins intervene (Hopkins *et al.*, 2005).

In recent years, some research groups (Capoor *et al.*, 2009; Crump *et al.*, 2003; Friedman *et al.*, 2001) have suggested that the QRDR of the gene *gyrA* could be amplified because mutations in the regions outside the QRDR domain that are associated with quinolone resistance had already been observed.

PMQR

In the present study, the *qnrA1* gene was detected in a strain of the serovar Enteritidis that was resistant to NAL with a CipMIC of 0.062 μg/mL without mutation in the QRDRs of the four genes analyzed (GenBank accession number GU731067). Similarly, Cui *et al.* (2009) found the *qnr* genes *S2*, *A,* and *B6* in *Salmonella* spp. that were susceptible to this fluoroquinolone. In contrast, Cavaco *et al.* (2007) found *qnrA* or *qnrS* in *Salmonella* spp. strains that were resistant or had an intermediate sensitivity to NAL without mutations but showed a reduced susceptibility to Cip. Gay *et al.* (2006) also related the presence of *qnr* genes, including *B2*, *B5*, *S1,* and *S2*, in *Salmonella* spp. strains with a reduced susceptibility to Cip.

The present study the gene *qnrB19* was found in a strain of *S.* Corvallis from poultry origin with CipMIC of 0.5 μg/mL and without mutations in the QRDRs of the four genes that were analyzed (GenBank accession number GU731069). This gene was also identified in strains of *S.* Typhimurium with a reduced susceptibility to Cip in France (Cattoir *et al.*, 2007) and in the Netherlands (Garcia-Fernandez *et al.*, 2009).

The *qnr* gene, always associated with plasmids genes of quinolones resistance in *Enterobacteriaceae* (Garcia-Fernandez *et al.*, 2009; Hopkins *et al.*, 2005; Jacoby *et al.*, 2003), has already been found in *Salmonella* strains with different levels of susceptibility to Cip. However, in this work the presence of plasmids in the strains was not evaluated. According to previous studies (Castanheira *et al.*, 2007; Cavaco and Aarestrup, 2009; Jacoby *et al.*, 2003; Li, 2005; Minarini *et al.*, 2008; Zhao *et al.*, 2008) the presence of this gene confers a low level of resistance to Cip while also facilitating the development of mutations in the QRDR of the *gyr*A gene. Chong *et al.* (2010) and Jacoby *et al.* (2003) reported that the increase in FQ resistance resulting from the presence of *qnr* genes could reduce the clinical effectiveness of this class of antibiotics. However, according Jacoby *et al.* (2009), the precise level of the involvement of plasmid genes in the resistance to FQs is still poorly understood when compared with our understanding of other mechanisms of resistance.

In conclusion, despite previous detection of *qnrA1*, *qnrB2*, *qnrB8*, *qnrVC1*, *qnrVC2* genes in other bacterial species in Brazil (28), it is the first report of the *qnr* gene in *Salmonella*, and also the first detection of the *qnrB19* gene in this country. The results suggest that an integrated approach between the medical and veterinary communities is required to effectively control drug resistance. It was also shown that the sensitivity to NAL seems to be a good phenotypic indication to differentiate between strains with and without a mutation in the QRDR, however the double mutation in *gyrA* did not cause resistance to ciprofloxacin. The mechanisms by which enteric zoonotic bacteria become resistant need to be continuously monitored in order to prevent the selection of strains with a reduced susceptibility and resistance to quinolones. Only through these measures may correct preventive decisions be taken to continue the effective therapeutic use of this class of antibiotics.

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