DETECTION OF QUINOLONE-RESISTANCE MUTATIONS IN *SALMONELLA* SPP. STRAINS OF EPIDEMIC AND POULTRY ORIGIN

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ABSTRACT

Mutations into codons Aspartate-87 (62%) and Serine-83 (38%) in QRDR of *gyr*A were identified in 105 *Salmonella* strains resistant to nalidixic acid (94 epidemic and 11 of poultry origin). The results show a high incidence of mutations associated to quinolone resistance but suggest association with others mechanisms of resistance.

Key words: Quinolones, *gyr*A, Quinolone Resistance Determining Region (QRDR), AS-PCR-RFLP.

Resistance to quinolones in *Salmonella* spp. is mainly due to mutations that alter the binding site of the antimicrobial with DNA-gyrase, target of action of these antimicrobials. Generally, the mutations occur in a specific region of *gyr*A gene, between amino acids 67 and 106, called quinolone resistance determining region (QRDR) (11). Point mutations in the QRDR of *gyr*A gene may be sufficient to generate high levels of resistance of *Salmonella* spp. to non-fluorine quinolones such as nalidixic acid (NAL) (9). Additional mutations are necessary for resistance to fluoroquinolones (14). Fluoroquinolones are usually the antimicrobials of choice for treatment of severe or systemic human salmonellosis (12). An increase in isolation of *Salmonella* strains with reduced susceptibility to fluoroquinolones such as ciprofloxacin is a public health problem, since it may cause failure of clinical treatment, limiting the therapeutic options for treatment (1).

Resistance to fluoroquinolones is relatively uncommon in *Salmonella* spp., when compared to other genera of the *Enterobacteriaceae* family. However, for changes in their susceptibility NAL-resistant *Salmonella* strains require lower exposure to fluoroquinolones than NAL-susceptible ones (3). An increase in NAL-resistant strains with reduced susceptibility to fluoroquinolones has been observed (7). The most frequent changes in QRDR of *gyr*A gene in NAL-resistant *Salmonella* spp. occurs in codons corresponding to Serine-83 (Ser83) and Aspartate-87 (Asp87) (6). Strains that carry substitutions in these codons have different levels of reduced susceptibility to fluoroquinolones (10).

Aim of study was to assess mutations in the QRDR of the *gyr*A gene associated with resistance to the nalidixic acid and reduced susceptibility to ciprofloxacin through allele specific (AS) polymerase chain reaction (PCR) restriction fragment

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length polymorphisms (RFLP) (8).

A total of 123 *Salmonella* strains of poultry origin or associated to human salmonellosis outbreaks, resistant and susceptible to nalidixic acid, were evaluated (Table 1). The strains belonging to the nine different serovars isolated in the State of Parana, Brazil, between 1999 and 2006. The epidemic strains were provided by the Central Laboratory of the State of

Parana (LACEN, Curitiba, Parana, Brazil) and those of poultry origin were obtained from Avian Health Laboratory accredited by the Brazilian Ministry of Agriculture (MAPA, Brazil). Serotyping was performed at the Laboratory of Enterobacteria, Department of Bacteriology, Osvaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil.

Table 1. Distribution of *Salmonella* spp strains isolated in the period between 1999 to 2006 in the state of Parana, Brazil, according to serovar, resistance to nalidixic acid (NAL) and isolation source.

Origin** NAL*- resistant	Serovar	N***	Origin NAL- sensitive	Serovar	N
Epidemic	Enteritidis	85	Epidemic[a]	Enteritidis	5
Epidemic	Typhimurium	3	Epidemic	Typhimurium	1
Epidemic	Joannesburg	3	Epidemic	Oraniemburg	1
Epidemic	O9:12	1	Epidemic	Saintpaul	1
Epidemic	Albany	1	Epidemic	Infantis	2
Epidemic	Newport	1	____	____	__
Poultry	Enteritidis	9	Biological material[b] discarded eggs[c]	Enteritidis	2
Poultry	Heidelberg	2	Biological material	Panama	2
Poultry	Bredeney	1	Biological material	Ohio	1
Poultry	Corvallis	1	One day old chicken	Anatum	1
Total NAL-resistant strains		107	Total NAL-sensitive strains		16

*Nal= nalidixic acid, **origin= isolament source, ***N= strain number of each serovar, a= strains isolated from patients and foods including egg and meat products, b= biological material: cloacae swab and organs of chickens hens, c= fertilized discarded eggs of poultry.

The strains were grown in BHI broth (Acumedia [TM]) at 37°C for 18h before extraction of the genomic DNA, according to Swanenburg *et al.* (17). The PCR reactions were performed in final volume of 25 µL, containing dNTPs (200 mM), MgCl$_2$ (10 mM), buffer for Taq (10 mM), Taq-polymerase (0.5 U) (Invitrogen [TM]) and DNA (5 ng). The primers forward used were STGYRA1 and AS-81 at concentration 25 pMol each one. The STGYRA-*Hin*fI/87 was the reverse primer for the reactions, used at concentration of 50 pMol (7). For the RFLP reactions, 5 µL of the PCR product was digested with 0.5 µL of *Hin*fI (Invitrogen [TM]) in final volume of 7.5 µL, at 37°C for 2h. Ladder of molecular weight of 100 bp and 25 bp were used (Invitrogen [TM]). After electrophoresis in agarose gel 3%, the gels were treated with ethidium bromide, viewed under ultraviolet light (UV) and documented. The RFLP profile

obtained for each strain was evaluated for the occurrence of mutations as per the size of fragments generated according to Giraud *et al.* (8).

In order to verify mutations associated with resistance to NAL in two resistant strains that had susceptible genotype in AS-PCR-RFLP, sequencing of part of the *gyr*A gene was performed. The fragments generated in PCR were purified with Concert TM TRapid PCR Purification System [TM] (GIBCO BRL, UK) and subsequently, the cloning was performed with the TOPO TA for sequencing kit (Invitrogen[TM]), according to manufacturer's instructions. The sequencing was conducted with the DYEnamic TM ET dye Terminator Cycle Sequencing kit (Amersham, GE HealthCare) in MegaBace 1000 (Amersham – GE HealthCare). Quality of the sequences was evaluated in PhredPhrap-Consed. The sequences obtained were

compared to those deposited in the database of the National Center for Biotechnology Information (NCBI). The bioinformatics analyses were performed with the ClustalW and Bioedit softwares, and with analysis in the databases of NCBI.

Salmonella spp. strains evaluated in this study showed, after PCR, a fragment of 195bp and another of 80bp. These results demonstrate no mutations in the Gly81 codon, since the PCR generates only a fragment of 195bp when this mutation is present (8). A genetic profile corresponding to the absence of mutations in QRDR of *gyr*A gene was obtained in RFLP for all susceptible strains tested. Among 107 NAL-resistant strains analyzed 98% (n = 105) showed mutations in the QRDR of *gyr*A gene (94 epidemic strains and 11 of poultry origin). Mutations in Asp87 and Ser83, respectively, occurred in 62% and 38% of these strains. Interestingly, two of the NAL-resistant strains of poultry origin, serovars Bredeney (AL 285/03) and Corvallis (AL 297/06), showed restriction pattern corresponding to the absence of mutations. Figure 1 shows the fragments observed in the PCR for strains and RFLP profiles corresponding to the mutations detected, or absence of those in AS-PCR-RFLP.
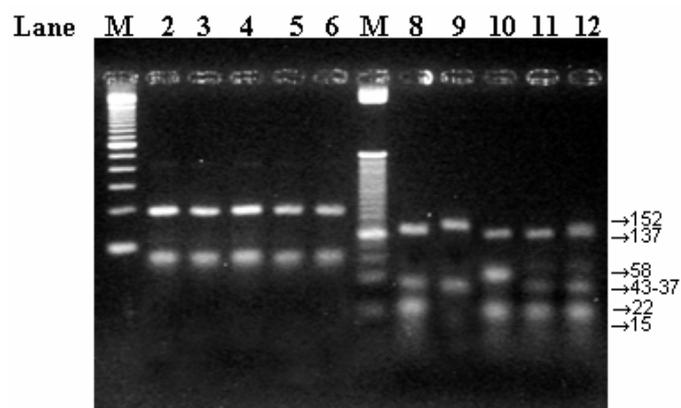

Figure 1. Considering reading from left to right: Lane 2 to 6: fragments resulting from PCR observed for all strains (195-80bp). Lane 8 to 12: fragments obtained after RFLP of the product of PCR with *Hinf*I enzyme. Profile I (137-43-22-15 bp), lane 8, 11 and 12 correspond to the absence of mutations in the QRDR of *gyr*A. Profiles II (152-43-37 bp), lane 9, and III (137-58-22 bp) lane 10, represent mutations in codons Serine 83 and Aspartate 87, respectively. Lane 1 and 7 correspond to markers of molecular weight of 100 bp and 25 bp, respectively.

The results observed in AS-PCR-RFLP are in agreement with reports that in NAL-resistant strains, the highest frequency of change occurs in codons Ser83 and Asp87 of the QRDR of *gyr*A gene (13). It is known that mutation in *gyr*A gene has been related to increased level of resistance to fluoroquinolones (7) and that concomitant alterations in these two residues are usually present in strains resistant to fluoroquinolones such as ciprofloxacin (18). In this study, two mutations were not found in any of the strains evaluated and all strains were susceptible to ciprofloxacin, according to the Clinical Laboratory Standards Institute (4, 15). However, 24 NAL-resistant strains, including the two strains that showed sensitivity profile in the RFLP, showed reduced susceptibility in the minimum inhibitory concentration of ciprofloxacin (CipMIC), with CipMICs between 0.125 g / mL and 0.250 g / mL (15).

The main source of *Salmonella* infection for humans is chicken meat, pork, eggs and egg products (5, 16). The non-therapeutic use of fluroquinolones in veterinary medicine, as in prophylactic supplements or growth-promoting agents, can facilitate the selection of resistant bacteria or reduce susceptibility to these antimicrobials. Thus, the use of antimicrobial agents in animals raised for human consumption is, probably, the main cause for the increase and spread of resistant *Salmonella* strains (7, 9).

In the present study, two serovars of poultry origin, Corvallis (AL 297/06) and Bredeney (AL 285/03), showed CipMIC values of 0.250 g/mL and 0.125 g/mL, respectively. In spite of these CipMIC values, the *in silico* analysis of AL 285/03 and AL297/06 strains revealed susceptible profile in the AS-PCR-RFLP. The reduced ciprofloxacin susceptibility, probably, could be related to other mechanisms like an over-expression of efflux pumps, porin down-regulation or plasmid-mediated (2, 8). Another possibility are mutations out of the sequenced region like *gyr*B, *par*E or *par*C that have been identified with a lower frequency than *gyr*A mutations (7).

The sequenced fragment of NAL-resistant serovars *S.* Bredeney (GenBank accession n°.GQ358014) and *S.* Corvallis (GenBank accession n°.GQ358015) strains, which showed

genetic profile corresponding to the absence of mutation in RFLP and resistance of NAL, showed homology with a portion of the *gyr*A gene of *S.* Typhimurium deposited in NCBI. The alignment of sequences obtained with two sequences of *S.* Typhimurium (GenBank accession n^o AAB60062.1 and n^o CAA55580.1) revealed 100% of identity without nucleotide deletion, addition or substitution, excluding the nucleotide substitution of *Hinf*I restriction site in the primer described by Giraud *et al.* (8). The sequenced region corresponds to partial portion of the *gyr*A gene located between amino acids 63 to 96, of which 30 amino acids correspond to the likely QRDR partial region. Indeed, it is reported that the QRDR region with higher frequency of mutations is between amino acids 67 and 106 in *Salmonella* spp. (7).

The AS-PCR-RFLP methodology used in this study was useful in detection of mutations that occur more frequently in *Salmonella* spp. The data presented here show a high incidence of point mutations in *Salmonella* strains belonging to different serovars and isolated from different origins and warn for the judicious use of quinolones in treatment of infections by *Salmonella* spp.

ACKNOWLEDGMENTS

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