Pontifícia Universidade Católica do Rio Grande do Sul

Faculdade de Biociências

Programa de Pós-Graduação em Biologia Celular e Molecular

Guilherme Drescher

Caracterização de resistência a quinolonas em *Salmonella enterica* isoladas de materiais de origem avícola do sul do Brasil

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular, da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientadora: Profa. Dra. Sílvia Dias de Oliveira

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Aprovado em_____de_____

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RESUMO

A *Salmonella enterica* é considerada um importante patógeno zoonótico que pode ser responsável por perdas na produção animal, especialmente na criação de aves. Diferentes classes de antimicrobianos têm sido utilizadas de forma profilática e/ou terapêutica na produção avícola, entre elas destacam-se as quinolonas, que também têm indicação para uso humano. A ampla utilização destes antimicrobianos pode contribuir para a seleção de microrganismos resistentes a estes fármacos. A resistência a quinolonas tem sido atribuída a mutações nos genes da DNA girase e da topoisomerase IV, além de estar associada à presença de determinantes de resistência carreados por plasmídeos (PMQR), especialmente aqueles codificados pelos genes *qnr* e *aac(6')-Ib-cr*. Desta forma, o objetivo do presente trabalho foi avaliar a presença de sistemas de bombas de efluxo envolvidos na resistência a quinolonas utilizando o inibidor Cianeto de Carbonila Clorofenilhidrazona (CCCP), bem como identificar determinantes de resistência a quinolonas em isolados de *S. enterica* de origem avícola. Para tanto, foram utilizados 36 isolados de *S. enterica* resistentes quinolonas. A redução na atividade dos sistemas de bombas de efluxo foi observada em 66,7% dos isolados testados para o ácido nalidíxico e para a ciprofloxacina com adição do CCCP. Mutações na DNA girase foram determinadas por sequenciamento e análise do gene *gyr*A, e os genes *qnr* (A, B, D e S) e *aac(6')-Ib-cr* foram detectados através de PCR. A análise das sequências do gene *gyr*A nos isolados fenotipicamente resistentes a quinolonas identificou a presença de mutação levando à alteração Ser83→Phe e Asp87→Gly, Asn ou Tyr em 38,9% e 22,2%, respectivamente. A análise do perfil plasmidial revelou nove perfis com plasmídeos que variaram de ~2 kb até ~50 kb e os genes PMQR foram encontrados em 22,2% dos isolados de *S. enterica*. O gene *qnr*A e o

*qnr*B foram detectados em 11,1% e 5,5% dos isolados, respectivamente, e o gene *qnr*S em 2,7%. O gene *qnr*D não foi encontrado em nenhum dos isolados testados. O gene *aac(6')-Ib-cr* foi detectado em 8,3% dos isolados. Até onde se sabe, este trabalho relata pela primeira vez mutações no gene *gyr*A em *S.* Worthington de origem aviária, bem como, este é o primeiro relato da presença dos genes *qnr*A, *qnr*S e *aac(6')-Ib-cr* em cepas de *S. enterica* isoladas de amostras relacionadas com a cadeia produtiva de frangos no Brasil. A presença de determinantes de resistência a quinolonas em isolados de *S. enterica* de origem aviária alerta para a possível seleção de resistência pelo uso destes antimicrobianos na produção animal.

Palavras-chaves: *Salmonella enterica*; resistência a quinolonas; DNA girase; genes PMQR; aves.

ABSTRACT

Salmonella enterica is considered an important zoonotic pathogen that can be responsible by losses in animal production, especially in poultry husbandry. Different classes of antimicrobials have been used as a prophylactic and/or therapeutic in poultry production, highlighting the quinolones, which also are indicated for human use. The wide use of these antimicrobials may contribute to the selection of microorganisms resistant to these drugs. Resistance to quinolones has been assigned to mutations in genes encoding DNA gyrase and topoisomerase IV, in addition to being associated with the presence of plasmid-mediated quinolone resistance (PMQR), especially encoded by *qnr* and *aac(6')-Ib-cr* genes. Thus, the aim of this study was to evaluate the presence of efflux pump systems involved in the resistance to quinolones using the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and identify the presence of resistance determinants to quinolones in isolates of *S. enterica* from poultry-related material. For this, 36 *S. enterica* strains resistant to quinolones were used. The reduction of the activity of efflux pump systems was detected in 66.7% of isolates tested for nalidixic acid and ciprofloxacin with addition of CCCP. Mutation in DNA gyrase was determined by sequencing and analysis of the *gyr*A gene, and *qnr* (A, B, D and S) and *aac(6')-Ib-cr* genes were detected by PCR. Analysis of the *gyr*A gene sequences in isolates phenotypically resistant to quinolones identify the presence of mutation leading to the alteration Ser83→Phe and Asp87→Gly, Asn or Tyr in 38,9% and 22,2%, respectively. Plasmid profile analysis showed nine profiles with plasmids that ranged from ~2 kb to ~50 kb, and PMQR genes were found in 22.2% of *S. enterica* isolates. *qnr*A and *qnr*B genes were detected in 11.1% and 5.5% of isolates, respectively, and *qnr*S was found in 2.7%. None *qnr*D gene was found in the

isolates tested. *aac(6')-Ib-cr* gene was detected in 8.3% of the isolates. To our knowledge, this study reports for the first time mutations in *gyr*A in *S*. Worthington from poultry, as well as this is the first report of the presence of *qnr*A, *qnr*S and *aac(6')-Ib-cr* in *S. enterica* isolated from samples related to poultry production chain in Brazil. The presence of resistance determinants to quinolones in *S. enterica* isolates from poultry leads to concern regarding to potential resistance selection due to the use of these antimicrobials in animal production.

Keywords: *Salmonella enterica,* quinolone and fluoroquinolone resistance, mutations in QRDRs, PMQR genes, poultry meal.

LISTA DE ABREVIAÇÕES

ABC – ATP-*binding cassete*

Arg – *Arginine* (Arginina)

Asp – *Aspartic acid* (Ácido aspártico)

ATCC – *American Type Culture Collection*

ATP – *Adenosine Triphosphate* (Trifosfato de adenosina)

bp – *Basis pair* (Pares de bases)

BLAST – *Basic Local Alignment Search Tool*

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CCCP – *Carbonyl Cyanide m-Chlorophenylhydrazone* (Cianeto de Carbonila Clorofenilhidrazona)

CDC – *Center of Disease Control*

CIP – Ciprofloxacina

CLSI – *Clinical Laboratory Standards Institute*

DMT – *Drug Metabolite Transporter*

DNA – *Deoxyribonucleic acid* (Ácido desoxirribonucleico)

dNTP – *Deoxynucleotide triphosphates* (Desoxinucleosídeos trifosfatados)

DTA – Doenças Transmitidas por Alimentos

EDTA – *Ethylenediaminetetraacetic acid* (Ácido etilenodiamino tetra-acético)

F – Antígeno flagelar

Kb – *Kilobase* (Quilobase)

MATE – *Multi-drug and Toxic Compound Extrusion*

MDR – *Multiple-Drug-Resistente*

MFS – *Major Facilitador Superfamily*

MIC – *Minimum Inhibitory Concentration* (Concentração Inibitória Mínima)

µg – Micrograma

µM – Micromolar

mL – Mililitro

NAL – Ácido nalidíxico

O – Antígeno somático

PCR – *Polymerase Chain Reaction* (Reação em Cadeia pela Polimerase)

pH – Potencial hidrogeniônico

PMQR – *Plasmid Mediated Quinolone Resistance*

PUCRS – Pontifícia Universidade Católica do Rio Grande do Sul

Qnr – *Quinolone resistance*

RND – *Resistance-Nodulation-Division*

SGI-1 – *Salmonella Genomic Island-1*

SMR – *Small Multi-drug Resistance*

TE – Tris-tris (hidroximetil) aminometano/EDTA – Ácido etileno diamino tetracético

Trp – *Tryptophan* (Triptofano)

Tyr – *Tyrosine* (Tirosina)

U – Unidades

UV – Ultravioleta

Vi – Antígeno de virulência

SUMÁRIO

Capítulo 1

Introdução

Objetivos

1.Introdução

O gênero *Salmonella* é composto pelas espécies *Salmonella bongori* e *Salmonella enterica*. A *S. bongori* normalmente é isolada em animais de sangue frio, e a *S. enterica* está associada a animais de sangue quente, incluindo o homem, sendo dividida em seis subespécies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* e *S. enterica* subsp. *indica* (1,2). A diferenciação de *Salmonella* spp. em sorovares é realizada através da caracterização de antígenos somáticos (O), flagelares (F) e capsulares (Vi) (3), tendo sido descritos 2.610 sorovares compondo este gênero (2). *Salmonella* spp. são caracterizadas como bacilos Gram negativos, anaeróbios facultativos, não formadores de esporos e móveis, com exceção de *Salmonella enterica* subsp. *enterica* sorovar Pullorum (*S.* Pullorum) e *S.* Gallinarum (4).

Além de estarem presentes no ambiente, *Salmonella* spp. são habitantes do trato digestório de várias espécies animais (5), tais como bovinos (6), suínos (7) e aves (8,9). A infecção de humanos por *Salmonella* spp. normalmente ocorre pela ingestão de alimentos contaminados, principalmente os de procedência animal (7,8,10), onde se destaca especialmente produtos de origem aviária (11,12,13,14,15). Desta forma, a salmonelose é considerada uma das mais importantes zoonoses, com especial relevância para a saúde pública (16,17,18). *S.* Enteritidis e *S.* Typhimurium têm sido os principais patógenos causadores de doenças alimentares em humanos (11,19,20), porém surtos envolvendo outros sorovares têm sido reportados em todo o mundo (21,22,23).

A infecção por *Salmonella* spp. em humanos pode causar febre, cefaleia, diarreia e dor abdominal, sendo que alguns indivíduos podem apresentar bacteremia, o que pode contribuir para o agravamento da enfermidade (24). No ano de 2010, ocorreram aproximadamente 93,8 milhões de casos de gastrenterite, com estimativa de 155.000 mortes em decorrência de infecções por *Salmonella* spp. neste ano em todo o mundo (25). Nos países em desenvolvimento, estima-se que um terço da população é acometida por doenças de origem alimentar, sendo *Salmonella* spp. os principais microrganismos envolvidos nestas enfermidades (26,27,28). No Brasil, durante o período de 2000 até 2011 foram registrados 8.663 surtos de Doenças Transmitidas por Alimentos (DTA), sendo que 41,85% deles estavam relacionados com *Salmonella* spp. (29).

Além da importância da infecção por *Salmonella* spp. em humanos, estes microrganismos são de grande relevância na produção animal, tanto por constituir uma barreira sanitária no comércio de animais e alimentos entre diferentes países, como pela possibilidade de acometer animais e levar à diminuição de produtividade, resultando, em última análise, em prejuízo econômico. A salmonelose aviária gera preocupação para a indústria avícola devido aos inúmeros prejuízos gerados na diminuição do ganho de peso, bem como pela possibilidade de mortalidade de aves durante o desenvolvimento do lote (30,31). Esse microrganismo pode ser introduzido nas granjas por trabalhadores, pela água fornecida aos animais e por produtos utilizados durante a criação das aves (32,33), sendo a água, a ração e os roedores as mais importantes portas de entrada desse patógeno nas granjas avícolas durante o ciclo de criação (34,35,36).

Inúmeras práticas de controle de infecções bacterianas, causadas não só por *Salmonella* spp., mas por outros microrganismos, como *Escherichia coli*, vêm sendo utilizadas na produção avícola (37,38,39,40,41,42). Cabe destacar a utilização de antimicrobianos como método de profilaxia, metafilaxia e como promotores de crescimento inseridos na alimentação animal. Entretanto, esta prática pode levar à seleção de bactérias resistentes a antimicrobianos, que podem ser veiculadas por alimentos para seres humanos, dificultando o tratamento de infecções causadas por estas bactérias (32,43,44,45). A ocorrência de *Salmonella* spp. multirresistentes (MDR), com resistência a três ou mais classes de diferentes antimicrobianos (46), especialmente *S*. Typhimurium, *S*. Hadar, *S*. Choleraesuis e *S*. Schwarzengrund (47,48), tornou-se um sério problema mundial, sendo que, nas últimas décadas, houve aumento considerável na descrição de *Salmonella* spp. MDR isoladas de humanos e animais na Europa, Ásia, África e Américas do Norte e do Sul (44,45,48,49,50,51,52,53).

O fenótipo MDR pode estar relacionado com a capacidade de aquisição e disseminação de genes de resistência entre bactérias patogênicas e/ou comensais de humanos e animais (8,54,55,56,57) através de elementos genéticos móveis, tais como plasmídeos, transposons e também por meio da ilha genômica 1 em *Salmonella* (SGI-1) (54,58,59). A mobilidade dos elementos genéticos constitui forma importante de microrganismos adquirirem resistência contra uma variada gama de antimicrobianos, incluindo fármacos amplamente utilizados na produção animal, como quinolonas, aminoglicosídeos e tetraciclina (60,61,62).

A resistência à ciprofloxacina, ampicilina, sulfonamidas e ácido nalidíxico pode ser atribuída a sistemas de bombas de efluxo, que podem ou não ser substrato-específicos. As bombas de efluxo são compostas por canais formados por proteínas de membrana, que agem expulsando ativamente as drogas antimicrobianas para o meio extracelular, impedindo a sua ação no interior da bactéria (63,64,65,66). O decréscimo da quantidade do antimicrobiano no interior da bactéria provocado pelo efluxo pode agir de forma sinérgica com outros mecanismos de resistência e facilitar a seleção de mutantes, o que aumenta o risco do aparecimento de microrganismos multirresistentes (63,67).

Além do sistema *Major Facilitador Superfamily* (MFS), são descritas outras quatro famílias de sistemas de bombas de efluxo em bactérias que atuam utilizando prótons como força motriz: *Resistance-Nodulation-Division* (RND) (65), *Small Multi-drug Resistance* (SMR), *Multi-drug and Toxic Compound Extrusion* (MATE) (68) e *Drug Metabolite Transporter* (DMT) (63,65,69,70,71). Há ainda o sistema ATP-*binding cassete* (ABC), que utiliza ATP como fonte de energia para o efluxo (72), transportando ativamente o substrato.

A identificação de bombas de efluxo em microrganismos pode ser realizada de forma direta ou indireta, utilizando inibidores específicos e inespecíficos. A forma direta de detecção de bombas de efluxo é realizada através de PCR, onde um gene que codifica para uma porção do sistema de efluxo é identificado (73,74). A presença de sistemas de efluxo também pode ser determinada através de inibidores que interagem especificamente com um tipo de bomba de efluxo, ou por inibidores inespecíficos que atuam sobre vários

sistemas de efluxo, como, por exemplo, o Cianeto de Carbonila Clorofenilhidrazona (CCCP) (75).

O CCCP tem ação especialmente em sistemas de efluxo que utilizam prótons como força motriz (71,76), sendo que ele penetra na bactéria na forma protonada, descarrega sua carga elétrica que atua no desacoplamento de alguns sistemas de efluxo. Então, torna-se um ânion que irá sequestrar os prótons necessários para que outros sistemas de efluxo funcionem corretamente. Este processo compromete a função das proteínas localizadas na membrana, consequentemente causa o acúmulo do antimicrobiano no interior da bactéria e possibilita restabelecer a função dele contra o microrganismo (75,77). Por ser próton dependente, ele inibe processos diretos que são dependentes de prótons como força motriz, ou indiretos via utilização de ATP, que aumentam o acúmulo de substratos no interior da célula bacteriana (78). O CCCP tem sido utilizado para detectar a atividade de bombas de efluxo em diversos microrganismos (79,80), incluindo *Salmonella* spp. (47,81).

Embora os sistemas de efluxo possam ser responsabilizados pela resistência às quinolonas, que, atualmente, constituem o tratamento de eleição para os casos de salmonelose humana com indicação de terapia antimicrobiana, estas bombas não têm sido o principal mecanismo associado à resistência a esta classe de drogas. Quinolonas e fluoroquinolonas possuem o mesmo mecanismo de ação baseado na interferência na síntese de DNA bacteriano. Estas drogas estabilizam as rupturas no DNA realizadas pela DNA girase, que tem suas subunidades codificadas pelos genes *gyr*A e *gyr*B, ou topoisomerase IV, codificada pelos genes *par*C e *par*E. Assim, a interação destas drogas com DNA girase e/ou topoisomerase IV e DNA inibe a progressão da polimerase e,

consequentemente, a replicação do DNA (82). Entretanto, tem sido relatada a incidência de resistência às quinolonas em *Salmonella* spp. isoladas de alimentos de origem aviária, o que constitui um importante problema de saúde pública (83). Isto talvez possa dever-se, pelo menos parcialmente, ao intenso uso de enrofloxacina, fluoroquinolona de uso veterinário, de forma profilática em animais, especialmente em aves, uma vez que foi demonstrado que o emprego desta droga seleciona salmonelas mutantes resistentes ao ácido nalidíxico e às fluoroquinolonas *in vivo* (84). Primeiramente, a resistência a quinolonas foi atribuída a mutações cromossômicas nas Regiões Determinantes de Resistência a Quinolonas (QRDR) (85). As mutações levam a alterações no sítio de ligação do antimicrobiano na DNA girase em regiões específicas do gene *gyr*A e *gyr*B, e na topoisomerase IV, nos genes *par*C e *par*E, sendo as alterações em *gyr*B e *par*E menos frequentes em *Salmonella* spp. (49,86,87). Alterações na *gyr*A têm sido apontadas como a principal causa do aparecimento cada vez maior de cepas de *Salmonella* spp. com altos níveis de resistência a quinolonas (88) e as principais mutações reportadas têm sido nos aminoácidos Asp87 e Ser83 (49,84,89). Também têm sido descrita a existência de isolados carreando mutações em ambos os genes, *gyr*A e *par*C (90,91,92,93).

O fenótipo de resistência a quinolonas também tem sido associado a determinantes de resistência carreados por plasmídeos (PMQR), que ainda pode proporcionar a transferência horizontal de genes de resistência entre isolados (94,95,96,97). A PMQR é mediada por três mecanismos: sistemas de efluxo QepA e OqxAB, modificação enzimática de ciprofloxacina e norfloxacina pela acetiltransferase Aac(6')-Ib-cr (98) e proteção das topoisomerases mediada pelos genes *qnr* (*qnr*A, *qnr*B, *qnr*S, *qnr*C, *qnr*D e *qnr*VC) (99,100,101,102,103,104). Os sistemas de efluxo de quinolonas QepA (102,105) e OqxAB

(102,106) são mecanismos de resistência que podem, eventualmente, ser encontrados em plasmídeos, mas normalmente são identificados no cromossomo bacteriano (105,106,107,108). A presença dos genes *qnr*, *aac(6')-Ib-cr* e *qep*A no mesmo plasmídeo já foi descrita (109), contudo, existe maior número de relatos de concomitância de genes *qnr* e *aac(6')-Ib-cr* no mesmo plasmídeo em patógenos de interesse veterinário e humano (97,110,111).

A Aac(6')-Ib-cr é uma variante da acetiltransferase codificada pelo gene *aac(6')-Ib*, que confere resistência à canamicina, amicacina e tobramicina. Esta variante possui duas substituições de aminoácidos, sendo uma no códon 102 (Trp → Arg) e outra no códon 179 (Asp → Tyr), e é capaz de modificar não só aminoglicosídeos, mas também fluoroquinolonas com um grupo piperazinil pela acetilação de aminoácidos nitrogenados neste grupo químico (98). O gene *aac(6')-Ib-cr* tem sido descrito em diversos microrganismos (112), incluindo *Salmonella* spp. isoladas de produtos de origem aviária (97,113).

Os genes *qnr* codificam para uma pentaproteína denominada Qnr, responsável pela proteção da DNA girase e da topoisomerase IV contra a ação das quinolonas e fluoroquinolonas (102). A Qnr foi descrita primeiramente em *Klebsiella pneumoniae*, sendo denominada QnrA (95). Posteriormente, a QnrS foi descrita em *Shigella flexneri*, apresentando elevada similaridade com a QnrA (100). Em 2006, na Índia, foi descrita a proteína QnrB em *K. pneumoniae*, com semelhança com QnrA e QnrS (101). O gene *qnr*VC foi descrito em *Vibrio cholerae* no Brasil em 2008 (114), sendo que até agora só existem relatos da presença dele na família *Vibrionaceae* (115). A QnrC foi identificada

em *Proteus mirabilis* e a QnrD foi reportada em *Salmonella* spp., sendo descritas em 2008 e 2009, respectivamente (99,103).

Os genes *qnr*A, *qnr*B, *qnr*D e *qnr*S têm sido encontrados em *S. enterica* isolada de amostras clínicas humanas e também em isolados clínicos de aves e em produtos de origem aviária (62,111,116), sendo os mais importantes e prevalentes até o momento. O gene *qnr*C foi descrito somente em isolados clínicos de *P. mirabilis* provenientes de infecções em humanos (103). A presença dos genes *qnr* em plasmídeos em *Salmonella* spp. foi descrita em isolados provenientes da África, Europa e Américas do Norte e do Sul (116,117,118), inclusive no Brasil com uma prevalência baixa em isolados de origem aviária (116).

1.2 Objetivos

1.2.1 Objetivo Geral

Verificar a presença de determinantes de resistência a quinolonas em isolados de *S. enterica* oriundos materiais de origem avícola, principalmente de farinhas de origem aviária e do ambiente de aviários, bem como determinar o papel de sistemas de bombas de efluxo em isolados que apresentarem resistência a quinolonas.

1.2.2 Objetivos Específicos

1.2.2.1 Determinar a presença de mutações no gene *gyr*A em isolados de *S. enterica*;

1.2.2.2 Determinar o perfil plamidial de isolados de *S. enterica* com suscetibilidade reduzida às quinolonas;

1.2.2.3 Detectar a presença dos determinantes de resistência *qnr*A, *qnr*B, *qnr*D, *qnr*S e *aac(6')-Ib-cr* em isolados de *S. enterica* fenotipicamente resistentes a quinolonas;

1.2.2.4 Avaliar a possibilidade de redução de suscetibilidade ao ácido nalidíxico e à ciprofloxacina de isolados de *S. enterica* ser devida a sistemas de efluxo através da utilização de CCCP como inibidor;

Capítulo 2

Artigo Científico

Characterization of quinolone resistance in Salmonella enterica from poultry-related samples

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Original Article

Characterization of quinolone resistance in *Salmonella enterica* from poultry-related samples

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Short title: Quinolone resistance in *Salmonella enterica* from poultry

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Abstract

This study aimed to characterize quinolone resistance mechanisms in *Salmonella enterica* from Brazilian poultry samples. Minimal inhibitory concentrations (MICs) of nalidixic (NAL) and ciprofloxacin (CIP) were determined by microdilution, and ranged from 32 to 4,096 µg/mL for NAL, and from 0.12 to 1 µg/mL for CIP. The addition of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) decreased MIC values by one to five dilution steps in 66.7% of isolates. A single-point mutation in *gyr*A leading to a Ser83→Phe alteration was detected in 39% of isolates (respective MICs ranged from 256 to 4,096 µg/mL), including the two CIP-resistant isolates. Other single alterations (Asp87→Tyr, Asn or Gly) were identified in 22% of isolates that presented MICs ranging from 256 to 1,024 µg/mL). Nine different plasmid profiles were detected, and 22.2% of isolates presented at least one of the following plasmid-mediated quinolone resistance (PMQR) genes: *qnr*A, *qnr*B, *qnr*S, and *aac(6')-Ib-cr*. All PMQR genes were found in plasmids ranging from ~2.0 to ~7.0 kb. To our knowledge, this is the first report of the *qnr*A, *qnr*S and *aac(6')-Ib-cr* in *S. enterica* from Brazilian poultry. The presence of resistance determinants to quinolones in *S. enterica* from poultry leads to a special concern regarding the potential resistance selection derived from the use of these antimicrobials in animal production.

Keywords: *Salmonella enterica*, quinolone resistance, *gyr*A, PMQR, poultry.

1. Introduction

Salmonella enterica is an important zoonotic pathogen (1), which can colonize or infect humans as well as a variety of domesticated and wild animals ranging from mammals to birds (2). Food derived from animals has been an important transmission source of transmission of *S. enterica* to humans (3), which has led to a special concern due to presence of antibiotic resistance (4–6). The wide antibiotic chemotherapy treatment in humans, and the antimicrobial use to prevent diseases in food animal husbandry, as well as growth promoters, may have contributed to increase the bacterial resistance to antimicrobials (7).

In the last decades, resistance to quinolones in *S. enterica* isolated from poultry products has increased considerably (8,9,10). Resistance to these antimicrobials can be assigned to chromosomal mutations in quinolone resistance-determining regions (QRDR), which are present in the DNA gyrase subunit A and B, as well as in the topoisomerase IV subunits encoded by *par*C and *par*E genes. Indeed, mutations in *gyr*A have been associated to high levels of resistance to quinolones (11), and have been the most important resistance mechanism to these antimicrobials identified in *S. enterica* from poultry around the world (12,13,14).

The plasmid-mediated quinolone resistance (PMQR) are other important resistance mechanisms to quinolones in *S. enterica* strains from animal (15), that it is conferred by *qnr* genes (A, B, C, D, S, and VC) (16), *aac(6')-Ib-cr* (17), and genes that codify to quinolone extrusion, *qep*A and *oqx*AB (1). Qnr protects DNA topoisomerase (16), being QnrA, QnrB and QnrS the most found in *S. enterica* strains isolated from poultry products (14,18,19).

aac(6')-Ib-cr encodes a variant aminoglycoside acetyltransferase that reduces the activity of quinolones by N-acetylation (17), which have been detected in *S. enterica* strains from poultry products around the world (19,20).

Therefore, the aim of this study was to evaluate the contribution of efflux pump systems in antimicrobial resistance phenotypes using the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), as well as detect determinants involved in quinolone resistance in *S. enterica* isolated from poultry-related samples.

2. Materials and methods

2.1. Bacterial strains

Thirty-six non-duplicate *S. enterica* isolates collected between 2003 and 2011 from poultry breeding in Southern Brazil were included in this study. Twenty-three isolates were from environment of broiler houses, seven from poultry by-product meals, three from poultry organs, two from cloacal swab, and one from pipped eggs (Table 1). In a previous study (21), all isolates showed to be resistant to nalidixic acid, and seventeen presented intermediate or full resistance to ciprofloxacin. All isolates were cultured in trypticase soy broth (TSB) (BioBras, Brazil) at 37 °C for 24 h and stored with 20% glycerol at -80 °C.

2.2. Antimicrobial susceptibility testing and efflux pump systems inhibition

The strains previously characterized as resistant or intermediate resistant to nalidixic acid and ciprofloxacin by disk diffusion test were submitted to determination of minimum inhibitory concentration (MIC) to these antimicrobials using the microdilution on Mueller-

Hinton broth (Himedia, India) method (CLSI, 2013) (22). Afterwards, the influence of efflux pumps in the resistance to nalidixic acid and ciprofloxacin was evaluated using the inhibitor CCCP (Sigma-Aldrich, USA) in strains resistant to these drugs. To analyze the antibiotic susceptibility were repeated the MIC test containing CCCP at final concentrations of 10, 5, and 2.5 µM and the influence of an efflux system in the effectiveness of the antimicrobial tested was inferred when the MIC showed to be lower in the presence of CCCP. All assays were performed in duplicate and the evaluation of the two tests was made visually and by spectrophotometry at 620 nm. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control.

2.3. Detection of antimicrobial resistance determinants

2.3.1. Resistance genes

Mutations in *gyr*A gene were determined in all isolates resistant to nalidixic acid independent of the MIC value to ciprofloxacin. PCR was used to amplify the *gyr*A gene (23) and detect the presence of the PMQR genes *qnr*A (24), *qnr*B (25), *qnr*D (26), *qnr*S (25) and *aac(6')-Ib* (27). Primers used for each target gene are shown in Table 2.

2.3.2. Genomic DNA extraction

Bacterial genomic DNA was extracted as described by Rademaker & de Bruijn (1997) (28) and eluted in 100 µL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The DNA obtained was quantified and evaluated spectrophotometrically at A260 nm and by the A260nm/A280nm ratio, respectively. The DNA concentration was standardized to 100 ng/µL and stored at -20°C.

2.3.3. PCR amplification

PCR amplifications were performed in a total volume of 25 µL containing 0.2 mM of each deoxynucleoside triphosphate (dNTP) (Invitrogen, USA), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 U *Taq* DNA polymerase (Invitrogen, USA), 0.8 µM of each primer (IDT, USA) and 4 ng/µL template DNA. *S. enterica* strains carrying the target genes were used as positive control, while negative controls comprise reactions containing a mix without template DNA. Fragments from *gyr*A were amplified by initial denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, annealing for 1 min, an extension at 72ºC for 1 min, and a final extension at 72ºC for 7 min. The detection of 16S rRNA gene, *qnr* and *aac(6')-Ib* were performed with initial denaturation at 94ºC for 5 min, 30 cycles of denaturation at 94ºC for 1 min, annealing for 30 s, an extension at 72ºC for 45 s, and a final extension at 72ºC for 7 min, all reactions were performed in duplicate and controls were always included in each analysis. $MgCl_2$ concentration and annealing temperature used for each primer pair are shown in Table 2. Amplifications were carried out in a thermocycler (Veriti Thermal Cycler, Applied Biosystems, USA). The amplicons were visualized by electrophoresis on agarose gel stained with ethidium bromide (0.5 µg/mL) and analyzed using a Gel Doc L-Pix image system (Loccus Biotecnologia, Brazil). A 100 bp DNA ladder (Ludwig Biotecnologia, Brazil) was used as molecular mass marker.

2.3.4. DNA sequencing

Amplicons from *gyr*A and *aac(6')-Ib* were purified using ammonium acetate and sequenced in an automated DNA sequencer ABI 3130 XL Genetic Analyzer XL (Applied Biosystems). The sequences were edited, aligned and analyzed using the software BLAST

2.0 (http://www.ncbi.nlm.nih.gov/BLAST) available at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST), and compared with the reference sequences deposited in GenBank for *gyr*A (accession number NC003197, NC011083, NC017046, 011294) and *aac(6')-Ib-cr* (accession number NC019133, NC019089 and EU195449). The presence of mutations in *gyr*A as well as presence of the variant *aac(6')-Ib-cr* were determined by the complete similarity with references sequences deposited in GenBank.

2.4. Plasmid profile analysis

Plasmid profile analyses were performed in all strains tested. Plasmid DNA was obtained using the alkaline lysis protocol described by Kado & Liu (1981) (29), with an additional chloroform treatment. The plasmid DNA was analyzed by electrophoresis on 0.8% agarose gel stained with 0.5 μg/mL ethidium bromide and visualized under ultraviolet radiation. *E. coli* V517 (30) was used as reference strain for the evaluation of plasmid size . Afterwards, strains carrying PMQR genes were submitted to a new plasmid extraction using the kit Pure Yield™ Plasmid Miniprep System (Promega, USA), according manufacture's protocol. The plasmids were then purified using the Wizard® SV Gel and PCR Clean-up System (Promega, USA) kit and submitted to new amplifications targeting the *qnr* and *aac(6')-Ib-cr* genes. Amplification of the 16S rRNA gene (31) (Table 2) was performed using the purified plasmid DNA in order to confirm the absence of chromosomal DNA.

2.5. Statistical analysis

Fisher's exact and Student's *t* tests were used to evaluate MIC values between replicates. The analyses were performed using SPSS software version 18.0 (IBM) and *P* value <0.05 was considered as statistically significant for all tests (95% confidence or 5% significance).

3. Results

3.1. MIC determination

MIC determination for the antimicrobials tested is shown in Table 1. In the presence of CCCP, 24 (66.7%) *S. enterica* isolates resistant to nalidixic acid presented reduced MIC values ranging from one to five fold. The *S.* Senftenberg and *S.* Heidelberg resistant to ciprofloxacin presented reduction of three and two fold in MIC values, respectively, when tested with the addition of CCCP.

3.2. Mutation in gyrA

Mutations in *gyr*A were detected by sequencing only in isolates presenting MIC values for nalidixic acid ≥ 256 µg/mL (Table 1). Among the 36 *S. enterica* isolates resistant to nalidixic acid or ciprofloxacin, 22 (61.1%) presented mutation in *gyr*A (Table 1) (Genbank accession numbers KM384713 to KM384734). Three (8.3%) isolates with the change Asp87-Tyr presented MIC values of 1,024 µg/mL for nalidixic acid. The Asp87-Gly and Asp87-Asn alterations were presented by three (8.3%) and two (5.5%) isolates, respectively. All *S.* Worthington presenting nalidixic acid MIC values ranged between 1,024 and 4,096 µg/mL showed the alteration Ser83-Phe, and only one was positive for

PMQR genes. Five (13.8%) isolates that presented a mutation in *gyr*A harbored one or more PMQR genes.

3.3. Plasmid profile

All except six (83.4%) quinolone-resistant *S. enterica* isolates carried one to three plasmids with molecular sizes ranging from ~2 to ~50 kb (Table 1). Nine different plasmid profiles (A to I) were detected among 28 strains. Profile A, presenting a single ~7 kb plasmid, was found in 13 *S. enterica* isolates. Plasmid profiles B (~7, ~5 and ~3 kb), C (~50 and ~7 kb) and D (~7 and ~2 kb) were observed in six, four and two *S. enterica* isolates, respectively. Other profiles (E – I) were found only in one isolate each.

3.4. Presence of PMQR genes

Eight (22.2%) isolates resistant to quinolone carried one or more PMQR genes, which were found in plasmids ranging from ~2.0 to ~7.0 kb (Table 1). Four (11.1%), two (5.5%) and one (2.7%) isolates harbored *qnr*A, *qnr*B and *qnr*S, respectively. Five *S. enterica* isolates harbored *aac(6')-Ib*. However, after sequence comparison with the reference sequences deposited in GenBank (EU195449 and KJ716225), only three (8.3%) presented *aac(6')-Ib-cr*, and the respective sequences were deposited in GenBank (accession numbers KM387720, KM387721 and KM387722). The *S.* Montevideo (S35) isolate from meat meal and one *S.* Senfentenberg (S166) isolate from drag swab harbored both *qnr*A and *aac(6')-Ib-cr* in the same plasmid. *qnr*D gene was not detected in the isolates studied. The presence of PMQR genes was not necessarily implicated in higher MIC values for quinolones.

4. Discussion

The increase in the quinolones resistance spread among *S. enterica* isolates from poultry-origin has been described in many countries (3,5,6,32). Quinolone resistance in *S. enterica* is mainly associated with alterations in GyrA subunit, however plasmid genes also are found in this microorganism (11,33). To address this aspect, mutations in *gyr*A, presence of PMQR genes and the role of efflux pump systems were investigated, and 77.7 % of isolates presented at least one of these resistance mechanisms.

Extrusion of antimicrobials by efflux pumps is an important mechanism of resistance in bacteria (34). The CCCP inhibitory action allowed us to infer that efflux pumps can be, at least partially, responsible to nalidixic acid and ciprofloxacin resistance detected in the most *S. enterica* isolates evaluated, as previously described (35,36). After addition of CCCP, only 8.3% of quinolone-resistant isolates showed efflux pump as single mechanism among those studied, while 58.3% presented an overlapping of resistance mechanisms.

Single mutations at codons for amino acids Ser83 or Asp87 in *gyr*A were found in 61.1% of quinolone-resistant isolates, similarly to those found in different countries (Asp87→ Asn, Gly, or Tyr; Ser83→ Phe) (13,14,15). All mutations were detected in isolates presenting nalidixic acid MIC values ≥ 256 μg/mL. However, MIC values even higher were not associated to *gyr*A mutations, indicating that other important quinolone resistance mechanisms are present in this isolates. Therefore, among the mechanisms investigated, efflux pump and *qnr*A were found in isolates with MIC values ≥ 256 μg/mL in the absence of *gyr*A mutation.

To our knowledge, only *qnr*B gene has been reported in *S. enterica* strains from poultry-origin isolated in Brazil (37), so this is the first identification of *qnr*A, *qnr*S and *aac(6')-Ib-cr* genes in *S. enterica* strains isolated from poultry in this country. PMQR genes were found in 22.2% of isolates, findings similar those reported by other studies analyzing *Salmonella* strains (14,18,38). PMQR genes have been associated to plasmids of different sizes (15,39,40), but those detected in this study were found only in plasmids of ≤7 kb. Even though these genes have been detected in a small number of isolates, the fact that they are carried by plasmids leads to a great concern due to the possible spread to other bacteria, which may be selected by the intense use of quinolones for treatment of various diseases in both veterinary and human medicine.

The presence of more than one PMQR gene, or the association of PMQR genes with mutations in *gyr*A in the same strain were found in 13.8% of strains resistant to quinolones. However, the MIC values to nalidixic acid and/or ciprofloxacin were not associated with the presence of different quinolone resistance determinants in a same strain.

The characterization of quinolone resistance determinants in *S. enterica* isolates from Brazilian poultry samples contributes to the understanding of the dissemination pattern of these mechanisms in the poultry production chain and may help to define strategies of antimicrobials use in animals and humans.

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Competing interests: None declared.

Ethical approval: Not required.

Table 1

Minimum inhibitory concentration (MIC) values to nalidixic acid (NAL) and ciprofloxacin (CIP), action of CCCP, mutation in *gyr*A, presence of plasmid-mediated quinolone resistance (PMQR) and plasmid profile of *Salmonella enterica* strains from poultry-origin.

Isolate (identification number)	Origin	MIC (µg/mL)		MIC (µg/mL)	*gyr*A		PMQR		Plasmids (kb)
		NAL	CIP	NAL/CIP**	N. change	A. change	*qnr*	*aac(6')-Ib-cr*	
S. Schwarzengrund (S98)	Viscera meal	512		128					7[A]
S. Schwarzengrund (S140)	Drag swab	32	0.12	32					N
S. Montevideo (S35)	Meat meal	32		32			*qnr*A	+	5*,2[G]
S. Cerro (S91)	Flesh and bones meal	32	0.12	32			*qnr*A		7[A]
S. Mbandaka (S23)	Meat meal	1,024		512	GAC-TAC	Asp87-Tyr	*qnr*B		7*,5,3[B]
S. Mbandaka (S08)	Feathers meal	1,024		512	GAC-TAC	Asp87-Tyr			7,5,3[B]
S. Braenderup (S18)	Meat meal	1,024	0.12	256	GAC-TAC	Asp87-Tyr			7,5,3[B]
S. Senftenberg (S153)	Poultry organs	256		32	TCC-TTC	Ser83-Phe			7[A]
S. Senftenberg (S148)	Drag swab	1,024		128	TCC-TTC	Ser83-Phe			N
S. Senftenberg (S166)	Drag swab	2,048	1	512/0.12	TCC-TTC	Ser83-Phe	*qnr*A	+	7,5*,3[B]
S. Heidelberg (S109)	Drag swab	1,024	0.50	512	TCC-TTC	Ser83-Phe			7,2[D]
S. Heidelberg (S110)	Drag swab	1,024	1	128/0.25	TCC-TTC	Ser83-Phe			10,7,2[F]
S. Worthington (S204)	Drag swab	4,096	0.25	1,024	TCC-TTC	Ser83-Phe			50,7[C]
S. Worthington (S113)	Drag swab	1,024	0.50	256	TCC-TTC	Ser83-Phe			7[A]
S. Worthington (S146)	Drag swab	1,024	0.25	256	TCC-TTC	Ser83-Phe		+	7,5,2*[E]
S. Worthington (S147)	Drag swab	2,048	0.25	256	TCC-TTC	Ser83-Phe			7[A]
S. Worthington (S170)	Drag swab	2,048	0.25	1,024	TCC-TTC	Ser83-Phe			50,7[C]
S. Worthington (S185)	Drag swab	2,048	0.25	1,024	TCC-TTC	Ser83-Phe			50,7[C]
S. Worthington (S192)	Drag swab	2,048		64	TCC-TTC	Ser83-Phe			50,7[C]
S. Worthington (S194)	Drag swab	4,096	0.25	256	TCC-TTC	Ser83-Phe	*qnr*S		10,3*,2[I]
S. enterica (O:4,5:l,v:-) (S169)	Disposable shoes covers	1,024	0.25	512			*qnr*A		7[A]
S. enterica (O:4,5:l,v:-) (S187)	Drag swab	1,024		1,024	GAC-AAC	Asp87-Asn			7[A]
S. enterica (O:4,5:l,v:-) (S210)	Poultry organs	256		64	GAC-AAC	Asp87-Asn	*qnr*B		7*,5[H]
S. enterica (O:4,5) (S108)	Drag swab	1,024		128					7,2[D]
S. enterica (S197)	Drag swab	256		32					7[A]
S. enterica (S200)	Drag swab	256		256					7[A]
S. enterica (S101)	Blood meal	128		128					7[A]
S. enterica (S181)	Pipped eggs	512		512					N
S. enterica (S172)	Cloacal swab	512		64	GAC-GGC	Asp87-Gly			7[A]
S. enterica (S173)	Cloacal swab	1,024		128	GAC-GGC	Asp87-Gly			7[A]
S. enterica (S174)	Poultry organs	512	0.12	512	TCC-TTC	Ser83-Phe			N
S. enterica (S160)	Disposable shoes covers	1,024		256	GAC-GGC	Asp87-Gly			N
S. enterica (S161)	Disposable shoes covers	512		512					N
S. enterica (S119)	Drag swab	256	0.12	256					7,5,3[B]
S. enterica (S120)	Drag swab	256	0.12	256					7,5,3[B]
S. enterica (S125)	Drag swab	256		256					7[A]

N. change = nucleotide change. A. change = amino acid change. Letters (A to I) represent plasmid profile. N = not found.

* Plasmid in which the PMQR genes has been found. ** After addition of CCCP.

Table 2

Primers and PCR conditions for the detection of quinolones resistance determinants.

Target gene	Primer sequence (5'→3')	$MgCl_2$ (mM)	Annealing temperature (°C)	Amplicon size (bp)	Reference
*qnr*A	F ATTTCTCACGCCAGGATTTG R GATCGGCAAAGGTTAGGTCA	2.5	52	516	Robicsek et al. (2005)
*qnr*B	F GATCGTGAAAGCCAGAAAGG R ACGATGCCTGGTAGTTGTCC	2.0	52	469	Robicsek et al. (2006)
*qnr*D	F CGGGGAATAGAGTTAAAAAT R TATCGGTGAACAATAACACC	1.5	52	614	Veldman et al. (2011)
*qnr*S	F ACGACATTCGTCAACTGCAA R TAAATTGGCACCCTGTAGGC	2.5	59	417	Robicsek et al. (2006)
aac(6')-Ib	F TTGCGATGCTCTATGAGTGGCTA R CTCGAATGCCTGGCGTGTTT	1.5	59	482	Park et al. (2006)
*gyr*A	F CGAGAGAAATTACACCGGTCA R AGCCCTTCAATGCTGATGTC	2.5	57	660	Chuanchuen et al (2009)
*tet*A	F GTAATTCTGAGCACTGTCGC R CTGCCTGGACAACATTGCTT	1.0	60	956	Aarestrup et al. (2003)
*tet*B	F CTCAGTATTCCAAGCCTTTG R ACTCCCCTGAGCTTGAGGGG	2.0	53	414	Aarestrup et al. (2003)
*tet*C	F GGTTGAAGGCTCTCAAGGGC R CCTCTTGCGGGAATCGTCC	2.0	62	505	Aarestrup et al. (2003)
16S rRNA	F GCGGCAGGCCTAACACAT R GCAAGAGGCCCGAACGTC	2.0	59	182	O'Regan et al. (2009)

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Capítulo 3

Considerações Finais

3.1 Considerações finais

A *S. enterica* é um dos principais patógenos zoonóticos relacionados com perdas significativas na avicultura mundial, e o aumento crescente de cepas de *S. enterica* resistentes a diversos antimicrobianos tem elevado a preocupação relacionada com a utilização dessas drogas na produção animal (58,91,97,113,119). Dentro deste cenário, o presente trabalho procurou investigar determinantes de resistência a quinolonas, que é o antimicrobiano de eleição para o tratamento de casos graves de salmonelose em humanos, em isolados de *S. enterica* de origem aviária.

Os resultados obtidos mostraram a presença de determinantes de resistência a quinolonas em isolados de *S. enterica* provenientes de origem aviária, especialmente ambiente avícola e farinhas de produtos resultantes da criação de aves. Mutações na enzima DNA girase consistem no principal determinante de resistência a estas drogas (82), os dados encontrados no presente trabalho corroboram com a literatura, sendo importante manter as informações atualizadas frente à resistência a quinolonas em *S. enterica*. Além disso, este estudo relata a primeira descrição dos genes *qnr*A, *qnr*S e *aac(6')-Ib-cr* em isolados de *S. enterica* com procedência aviária no Brasil. A presença de genes plasmidiais relacionados com a resistência a quinolonas em isolados de *S. enterica* de origem aviária pode contribuir para a transmissão horizontal de genes envolvidos na resistência a estas drogas para outras cepas de *S. enterica*, ou ainda para outros microrganismos de interesse veterinário e humano, que pode levar ao insucesso terapêutico de infecções causadas por estas bactérias.

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