Pontifícia Universidade Católica do Rio Grande do Sul

Faculdade de Biociências

Programa de Pós-Graduação em Biologia Celular e Molecular

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Caracterização da resistência a antimicrobianos em isolados de *Salmonella enterica* provenientes de materiais de origem avícola

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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_____

de_____

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Porto Alegre

2013

AGRADECIMENTOS

À Professora Dra. Sílvia Dias de Oliveira, se faz excepcional na arte de ser mestre, agradeço pelo carinho, amizade e, principalmente, por ter acreditado e me concedido a oportunidade para a realização deste trabalho, que sem sua importante ajuda não teria sido concretizado. Também agradeço ao Professor Dr. Carlos Alexandre Sanchez Ferreira que com suas experiências e conhecimentos, auxiliaram na contribuição para meu crescimento profissional e pessoal.

As professoras Dra. Marjo Cadó Bessa e a Dra. Renata Medina pelo apoio, conselhos e compartilhamento de ideias, os quais também contribuíram de maneira significativa para que esta pesquisa fosse finalizada com maior êxito.

Aos meus pais, Valdir Antônio Mattiello e Marli Hermes Fontana Mattiello, pelo apoio incondicional e pelos ensinamentos que me fizeram não temer desafios e a superar os obstáculos com confiança e principalmente com humildade. À minha irmã Shaiana Paula Mattiello, que se tornou uma fiel seguidora dos meus sonhos, e a todas as pessoas da minha família que de uma forma ou de outra sempre me impulsionaram para que continuasse essa jornada.

Não poderia de forma alguma me esquecer de agradecer ao meu noivo Guilherme Drescher, que abraçou com todas as forças esse sonho para que ele pudesse ser realizado com êxito, com todo seu amor, carinho, companheirismo, paciência e extrema dedicação.

Aos pós-graduandos do Laboratório de Imunologia e Microbiologia, Valdir Cristovão Barth Jr., pelos socorros prestados, a Anelise Baptista pelos desabafos e

consolos, a Stephanie Wagner Gallo, a Julia Puffal, Fernanda Cattani e a Bruna Ferreira Leal pela amizade.

Aos alunos de Iniciação Científica do Laboratório de Imunologia e Microbiologia pela ajuda nas atividades, em especial ao Cid Vaz e ao Wagner Jardim que atuaram de forma direta na execução desse trabalho.

Aos meus amigos de longa jornada Daniel Bruno Momoli, Lilian Kolling, Andreia Inês Ferronato, Crislaine Botesini, Juceli Negri, Suzana Salvadego e Suelen de Araujo pelo incentivo e apoio durante a realização do trabalho.

A DEUS, que me deu vida e inteligência, e que me dá força para continuar a caminhada em busca dos meus objetivos.

Às pessoas que direta ou indiretamente auxiliaram o desenvolvimento e conclusão desse trabalho.

Algumas pessoas marcam a nossa vida para sempre, umas porque nos vão ajudando na construção, outras porque nos apresentam projetos de sonho e outras ainda porque nos desafiam a construí-los.

RESUMO

A *Salmonella enterica* é um importante patógeno causador de gastrenterite transmitido para humanos através do consumo de alimentos contaminados, principalmente os de origem animal. O uso de antimicrobianos para fins terapêuticos na medicina veterinária e como promotores de crescimento em animais destinados à produção de alimentos tem sido apontado como uma das causas do surgimento e disseminação de *S. enterica* multi-resistentes (MDR), constituindo um grande risco para a saúde publica. Dessa forma, o objetivo do presente trabalho foi determinar o perfil de resistência a antimicrobianos, bem como caracterizar os principais determinantes envolvidos nos fenótipos de resistência em isolados de *S. enterica* provenientes de farinhas de aves e de outras amostras oriundas da cadeia produtiva do frango, especialmente do ambiente de criação. Um total de 203 isolados de *S. enterica* foi analisado, sendo 106 oriundos de farinhas de aves e 97 provenientes, principalmente, de suabes de arrasto. Percentuais mais elevados de resistência foram detectados em *S. enterica* isoladas de suabe de arrasto, quando comparadas com isolados de farinhas de aves. Os maiores percentuais de resistência foram encontrados para sulfonamida, seguida por tetraciclina, trimetoprim-sulfametoxazol, ácido nalidíxico, estreptomicina e espectinomicina. A maioria dos isolados foi sensível à ciprofloxacina e à enrofloxacina. Fenótipos de MDR foram observados em 37 (18,2%) isolados, sendo que o perfil penta-resistente (ampicilina, cloranfenicol, estreptomicina, sulfametoxazol e tetraciclina) foi detectado em *S.* Heidelberg, *S.* Cerro e em duas *S.* Senftenberg. Integrons de classe 1 foram detectados em 26 isolados (12,7%), e não foi observada a presença de integron de classe 2. Uma *S.* Senftenberg isolada a partir do ambiente apresentou dois integrons de classe 1: um com um 3'CS típico e outro com 3′CS atípico ligado a *qacH–sul3*. Os genes *sul*1, *sul*2 e *sul*3 foram detectados, respectivamente, em 18,7%, 32,5% e 31,2% dos isolados de *S. enterica* fenotipicamente resistentes à sulfonamida. Os genes bla_{CMY}, $bla_{\text{CTX-M}}$ e bla_{TEM} foram detectados 23,8%, 9,5% e 85,7% dos isolados resistentes aos β-lactâmicos, respectivamente. Os determinantes de resistência *tet*A, *tet*B e *tet*C foram observados em 70%, 10% e 10% dos isolados resistentes à tetraciclina, respectivamente. Os genes *aad*A e *aad*B foram encontrados em 26,1% e 32,1 % dos isolados resistentes aos aminoglicosídeos, assim como a presença dos genes

*str*A e *str*B foi detectada em 44,4% e 34,9% dos isolados de *S. enterica* fenotipicamente resistentes à estreptomicina. A presença de um perfil heterogêneo de determinantes de resistência e de elementos genéticos móveis nos isolados analisados indica o potencial risco que estas bactérias representam para a saúde humana.

Palavras-chave: *S. enterica*; Farinhas de aves; Integrons; Genes *sul*; Genes *tet*; Aminoglicosídeos; β-lactamases.

ABSTRACT

Salmonella enterica is an important pathogen that causes gastroenteritis, and is transmitted to human through the consumption of contaminated food, especially from animal origin. The use of antimicrobials for therapeutic purposes in veterinary medicine and as growth promoters in animals used for food production has been considered one of the causes of emergence and spread of multi-drug resistant (MDR) *S. enterica*, representing a major risk to public health. Thus, the aim of this study was to determine antimicrobial resistance profiles as well as to characterize the main determinants involved on phenotypes of resistance in *S. enterica* isolates from poultry by-product meal and from other samples derived of poultry production chain, especially from environment of broiler houses. A total of 203 *S. enterica* isolates was analyzed, being 106 from poultry by-product meal and 97 isolated mainly from drag swabs. Higher percentages of resistance were detected in *S. enterica* isolated from drag swab when compared with isolates from poultry by-product meal. The highest percentages of resistance were found to sulfonamides followed by tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, streptomycin and spectinomycin. The majority of isolates was sensitive to ciprofloxacin and enrofloxacin. MDR phenotypes were detected in 37 (18.2%) isolates and the profile penta-resistant (ampicillin, chloramphenicol, streptomycin, tetracycline and sulphamethoxazole) was detected in *S.* Heidelberg, *S.* Cerro and two *S.* Senftenberg. Class 1 integrons was found in 26 isolates (12.7 %), and did not detect the presence of class 2 integron. A *S.* Senftenberg isolated from environment was found to harbor two class 1 integrons: one integron with a typical 3'CS, and the other with an atypical 3′CS linked to the *qacH–sul3*. The *sul*1, *sul*2 and *sul*3 genes were detected in 18.7%, 32.5% and 31.2% *S. enterica* phenotypically

resistant to sulfonamide, respectively. *bla*$_{CMY}$, *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes were detected in 23.8%, 9.5% and 85.7% of isolates resistant to β -lactams, respectively. Resistance determinants *tet*A, *tet*B and *tet*C were observed in 70%, 10% and 10% of isolates resistant to tetracycline, respectively. *aad*A and *aad*B genes were detected in 26.1% and 32.1% of isolates resistant to aminoglycosides, as well as the presence of *str*A and *str*B genes in 44.4% and 34.9% of *S. enterica* isolates phenotypically resistant to streptomycin. The presence of a heterogeneous profile of antimicrobial determinants and mobile genetic elements in the isolates analyzed indicates the potential risk that these bacteria represent to human health.

Keywords: *S. enterica*; Poultry by-product meal; Integrons; *sul* genes; *tet* genes; Aminoglycosides; β-lactamase.

LISTA DE ABREVIAÇÕES

AMI - Amicacina

AMP – Ampicilina

AmpC – β-lactamase cromossômica

Asp – Ácido aspártico

ATCC - American Type Culture Collection

BLAST - Basic Local Alignment Search Tool

BPLS - Brilliant Green Phenol Red Lactose Sucrose Agar (Agar Verde Brilhante Vermelho de Fenol, Sacarose e Lactose)

CDC – Center of Disease Control

CEC - Cefaclor

CFC - Ceftiofur

CIP - Ciprofloxacina

CLO - Cloranfenicol

CLSI - *Clinical Laboratory Standards Institute*

CMY – Cefalosporinase

CS – Segmento conservado

CTX-M – cefotaximase

DHPS - Diidropteroato sintetase

dNTP - desoxinucleosídeos trifosfatados

DTA - Doenças transmitidas por alimentos

EDTA - Ácido etilenodiamino tetra-acético

ENO - Enrofloxacina

ESBL - β-lactamases de espectro estendido

ESP - Espectinomicina

EST - Estreptomicina

F - Antígenos flagelares

FLF - Florfenicol

GEN - Gentamicina

KPC – *Klebsiella pneumoniae* carbapenemase

LIA – Agar lisina

MAPA - Ministério da Agricultura, Pecuária e Abastecimento

MDR – Multi-resistente

MIC – Concentração inibitória mínima

NA – Agar nutriente

NAL – Ácido nalidíxico

NEO - Neomicina

O - Antígenos somáticos

PABA – Ácido paraminobenzóico

PBPs - Proteínas ligadoras de penicilina

PCR - Polymerase Chain Reaction (Reação em Cadeia pela Polimerase)

pH - Potencial hidrogeniônico

PNSA - Programa Nacional de Sanidade Avícola

Qnr – Quinolona resistente

QRDR – Regiões determinantes de resistência a quinolonas

Ser - Serina

SIM – Indol, sulfato, motilidade

SUL - Sulfonamida

SUT – Sulfametoxazol/trimetoprim

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

TET - Tetraciclina

Tn7 - Tranposon 7

TOB - Tobramicina

TSB – Caldo de soja

TSI – Três açúcares e ferro

U - Unidades

Vi - Antígeno de virulência

SUMÁRIO

Capítulo 1

Introdução

Objetivos

1.1 Introdução

Bactérias do gênero *Salmonella* pertencem à família *Enterobacteriaceae*, sendo caracterizadas como bacilos Gram negativos, anaeróbias facultativas, não formadoras de esporos e capazes de se mover, com exceção da *Salmonella* Pullorum e da *Salmonella* Gallinarum. Normalmente este microrganismo é produtor de H_2S, oxidase negativo e catalase positivo, não fermentador de lactose, além de utilizar citrato de sódio como única fonte de carbono e descarboxilar a lisina e a ornitina (1). A diferenciação das salmonelas em sorovares é realizada através do esquema de Kaufmann-White (1981) (2), no qual são caracterizados os antígenos somáticos (O), os antígenos flagelares (F) e o antígeno de virulência (Vi), tendo sido descritos 2.610 sorovares (3). A classificação inclui apenas duas espécies: *Salmonella enterica*, mais comumente isolada do homem e de outros animais de sangue quente, e *Salmonella bongori*, geralmente isolada de animais de sangue frio. A *S. enterica* é dividida em seis subespécies *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) e *S. enterica* subsp. *indica* (VI) (4). Os sorovares pertencentes à *S. enterica* subsp. *enterica* têm sido designados pelo local onde foram primeiramente isolados, e usualmente são escritos sem a inclusão do epíteto específico e da subespécie, como por exemplo *Salmonella* Typhimurium. Os sorovares pertencentes a outras subespécies são referidos pela sua fórmula antigênica, seguindo o nome da subespécie (3). Clinicamente, esses patógenos são classificados em tifoides e não tifoides. *S.* Typhi e *S.* Paratyphi pertencem ao grupo das tifoides e têm o ser humano como reservatório, causando a febre tifoide. O grupo das não tifoides é composto por diferentes

sorovares de *S. enterica* encontrados em animais, sendo responsáveis por surtos de doença gastrintestinal em humanos (1).

As *Salmonella* spp. estão amplamente distribuídas na natureza e podem ser isoladas de uma variedade de animais, podendo ser encontradas no trato digestório de diversas espécies, mas mais frequentemente em aves, bovinos e suínos (5,6,7). Estes microrganismos são considerados os principais patógenos envolvidos em doenças transmitidas por alimentos (DTA) (8), pois apesar de todo o desenvolvimento tecnológico e da adoção de medidas de higiene adequadas, a salmonelose humana é uma das DTAs mais prevalentes em todo o mundo (9,10). A infecção humana por *S. enterica* normalmente ocorre pela ingestão de alimentos contaminados, principalmente os de origem animal, tendo sido associada mais frequentemente com alimentos de origem avícola (11,12).

No Brasil, nem todas as unidades federativas dispõem de dados precisos de vigilância epidemiológica quanto às DTAs; no entanto, estima-se que no período de 1999 a 2008, foram registrados 6.602 surtos de DTAs, sendo 42,9% relacionados com *S. enterica* (10). Nos Estados Unidos, segundo dados do CDC, aproximadamente 42.000 casos de salmonelose humana são reportados todos os anos, sendo alguns desses de origem conhecida. Apesar da diversidade de sorovares encontrados em surtos alimentares, em nível mundial os principais sorovares encontrados são *S.* Enteritidis e *S.* Typhimurium (9).

A partir de 1993, no Brasil, a *S.* Enteritidis emergiu como um importante problema para a indústria avícola e para a saúde pública (13). A avicultura é uma atividade de extrema importância no Brasil, que é o maior exportador mundial de carne de frango e um dos maiores produtores desta fonte de proteína animal. Segundo dados da Associação Brasileira de Produtores e Exportadores de Frango (ABEF), em 2011, a produção brasileira

atingiu uma marca histórica de 13 milhões de toneladas de carne de frango, garantindo ao Brasil uma posição entre os três maiores produtores mundiais de carne de frango, juntamente com Estados Unidos e China. Além disso, o Brasil mantém a posição de maior exportador mundial desde 2004, tendo chegado em 2011 com a marca de 3,9 milhões de toneladas (14). Desta forma, para garantir a qualidade na produção de aves, o Ministério da Agricultura, Pecuária e Abastecimento (MAPA) instituiu o Programa Nacional de Sanidade Avícola (PNSA), que objetiva o controle e a erradicação das principais doenças aviárias importantes para a saúde animal, que inclui algumas zoonoses, entre elas, a salmonelose (15).

A salmonelose aviária é considerada a doença bacteriana de maior impacto na indústria avícola, decorrente do elevado prejuízo relacionado à queda na produção de ovos, à perda de peso devido à baixa conversão alimentar e à mortalidade dos lotes, bem como à necessidade de adequação às exigências do mercado externo (16,17,18). As salmoneloses aviárias são divididas em três grupos: (1) pulorose, causada pela *S.* Pullorum; (2) tifo aviário, causado pela *S.* Gallinarum; (3) paratifo aviário, causado por outros sorovares de *Salmonella enterica* (19). Muitas vezes, a infecção por *S. enterica* não está associada a manifestações clínicas, o que leva as aves portadoras assintomáticas a serem consideradas fontes de contaminação entre os lotes, pois estas podem eliminar estes microrganismos nas fezes, contaminando a cama do aviário. A detecção de *S.* Enteritidis em amostras de *swab* de arrasto indicaram a manutenção desse sorovar durante toda a vida da ave mesmo após o vazio sanitário (13). Além disso, a presença de *S. enterica* em pele, penas, pés e trato digestório de aves é um fator agravante para a indústria avícola, pois esse patógeno pode

ser transferido para carcaças de frango dentro do abatedouro, ainda no processamento, tornando-se um risco para a saúde pública (20).

Além da contaminação horizontal dos lotes, a *S. enterica* pode ser introduzida nas granjas por produtos utilizados durante a criação do frango. As rações das aves são compostas por diferentes subprodutos, incluindo os próprios resíduos gerados pela produção avícola, como farinhas de vísceras, ossos, sangue, penas e farinhas mistas. Tais subprodutos retornam ao ciclo de produção do frango, pois são uma fonte adequada de gordura, aminoácidos, minerais, principalmente cálcio, fósforo e vitaminas (21); porém, podem constituir uma forma de re-introdução de *S. enterica* em granjas avícolas, uma vez que níveis elevados de contaminação por *S. enterica* foram detectados nestes subprodutos (22,23), além de possibilitar a disseminação de cepas resistentes a antimicrobianos (24,25). Esta re-contaminação, provavelmente, é derivada do processamento inadequado, uma vez que *S. enterica* não apresenta resistência ao calor e nem ao tratamento químico das rações (22,26).

A contaminação por *S. enterica* pode ocorrer em muitos estágios ao longo da cadeia de alimentos para consumo humano. O acometimento de humanos via consumo de produtos avícolas contaminados com *S. enterica* poderá causar gastrenterite, que pode ser severa, sendo caracterizada por diarreia, febre, dor abdominal e desidratação, mas pode agravar-se levando à infecção sistêmica (27). Em geral, a salmonelose é uma infecção auto-limitante, necessitando de tratamento apenas quando pacientes imunodeprimidos são acometidos. No entanto, *S. enterica* veiculadas por alimentos podem carrear resistência a drogas antimicrobianas, dificultando não só o tratamento de infecções causadas por esta bactéria, mas especialmente pela possibilidade destes microrganismos transferirem os

genes de resistência a outras bactérias (25,28). A resistência a antibióticos entre bactérias patogênicas veiculadas por alimentos, como *S. enterica*, não é incomum, sendo, muitas vezes, associada com o uso de antimicrobianos na alimentação animal (25,28,29).

Para obtenção da alta produtividade e qualidade dos produtos finais na criação do frango, agentes antimicrobianos em pequenas dosagens vêm sendo empregados como promotores de crescimento de modo contínuo junto à ração, agindo através da redução das bactérias patogênicas normalmente presentes no trato digestório (30). No entanto, esta prática pode levar à seleção de bactérias resistentes, que podem atuar como contaminantes do produto final, podendo constituir um considerável problema de saúde pública. Além disso, o incremento na exportação brasileira de carne de frango vem acompanhado de uma exigência cada vez maior por parte dos importadores, principalmente europeus, em relação à qualidade dos produtos, inclusive excluindo a possibilidade de importar carne de frango oriunda de um ciclo produtivo que empregue a adição de antibióticos como promotores de crescimento na ração (14,31). Tal fato desperta a preocupação de uma possível adequação ao mercado importador, bem como aumento de qualidade de produto disponibilizado para o mercado interno.

Desta forma, a contínua vigilância da suscetibilidade a antimicrobianos de patógenos de origem alimentar tem sido fortemente recomendada para identificar a emergência de resistência a antimicrobianos na produção de alimentos (32). A incidência aumentada de microrganismos multi-resistentes tem levado a um grande interesse nos mecanismos genéticos de resistência apresentados por essas bactérias, uma vez que o principal fator no desenvolvimento de cepas multi-resistentes é a capacidade da bactéria

em adquirir e disseminar genes exógenos através de elementos genéticos móveis, tais como plasmídeos e transposons (33,34,35).

Os genes de resistência a antimicrobianos presentes em plasmídeos e transposons podem estar inseridos em integrons, que são capazes de capturar genes por recombinação sítio-específica, desempenhando um papel importante na propagação e disseminação de genes de resistência a antibióticos, bem como sendo capazes de integrar-se ao cromossomo (36,37). Integrons das classes 1, 2 e 3 transportam cassetes gênicos contendo genes de resistência e são encontrados em uma grande variedade de espécies bacterianas. O integron de classe 1 é o mais encontrado em isolados clínicos de *S. enterica* (38,39) e frequentemente é relacionado com o fenótipo de multi-resistência (MDR) (40). O integron de classe 2 é menos prevalente em isolados de *S. enterica* e é comumente associado ao Tn7 (41); no entanto, até o momento, não foi reportada a presença do integron de classe 3 em isolados de *S. enterica* (42,43)

Estruturalmente, integrons de classe 1 podem ser divididos em três regiões caracterizadas pela presença de um segmento conservado próximo à extremidade 5' (5'CS), um segmento conservado na extremidade 3' (3'CS) e cassetes de genes entre esses segmentos (44,45). O segmento conservado 5' inclui o gene que codifica para a integrase *int*I e um sítio de recombinação *att*I, onde os cassetes são inseridos (46). O segmento conservado 3' geralmente contém o gene *qacE*ΔI, que confere resistência a compostos de amônio quaternário, e o gene *sul*I ou, menos frequentemente, o gene *sul*3, que codificam resistência às sulfonamidas. Os cassetes gênicos inseridos no integron de classe 1 contêm um elemento denominado "elemento de 59 pares de base" ou sítio *att*C, que é reconhecido pela integrase, que medeia a integração e a excisão de cassetes (43,47,48). Diversos genes

de resistência podem estar inseridos na região variável entre 5'CS-3'CS do integron de classe 1, como os genes *aad*A e *aad*B que codificam enzimas aminoglicosídeo adeniltransferases, responsáveis pela resistência aos aminoglicosídeos (49,50). No entanto, a resistência aos aminoglicosídeos especialmente estreptomicina, também pode ser codificada pelos genes *str*A e *str*B, que não tem sido relatado inserido na região variável do integron de classe 1 (39,51).

Existe uma associação entre integron de classe 1 e genes que codificam resistência às sulfonamidas (*sul*1 e *sul*3) (52,53), classe de drogas bastante empregada na avicultura devido ao baixo custo e à relativa eficácia para várias doenças bacterianas (30). As sulfonamidas são análogos estruturais do ácido paraminobenzóico (PABA), o qual está diretamente envolvido na biossíntese do acido fólico, inibindo competitivamente a enzima diidropteroato sintetase (DHPS) (54). A resistência às sulfonamidas foi descoberta na década de 1960, mas mecanismos genéticos responsáveis por esta resistência foram caracterizados mais tarde, na década de 1980, com a identificação dos genes *sul*1 e *sul*2 (55). O gene *sul*1 é frequentemente encontrado próximo da extremidade 3' do integron de classe 1, o que não é observado no integron de classe 2 (38). O gene *sul*2 tem sido associado à plasmídeos, e sua presença em integrons de classe 1 não tem sido descrita (56). Posteriormente, foi identificado um terceiro gene denominado *sul*3 (52), que também foi associado ao integron de classe 1 na ausência do gene *sul*1, o que sugere que a detecção do gene *sul*1 como marcador da presença de integron pode levar a conclusões errôneas (43). Entre esses três genes que codificam resistência às sulfonamidas, o gene *sul*1 é o mais frequentemente encontrado em isolados de *S. enterica* resistentes a estas drogas (57,58,59,60,61), enquanto o gene *sul*3 tem sido observado com menor frequência

(43,58,62). Contudo, a presença do gene *sul*2 sozinho ou concomitante com a presença dos genes *sul*1 e/ou *sul*3 tem sido reportada em isolados de *S. enterica*, sendo mais comumente indicado como responsável por resistência às sulfonamidas do que *sul*3 (51,58,63).

A tetraciclina tem sido utilizada em animais de produção de forma terapêutica, especialmente em aves, por apresentar baixo custo e pelo fato de ser solúvel em água (64). Essa classe de droga atua no microrganismo por meio da difusão passiva e age ligando-se na subunidade 30S do ribossomo, evitando a associação do aminoacil-tRNA a esta organela, o que resulta na inibição da síntese proteica (65). A tetraciclina e seus análogos exibem atividade contra bactérias Gram positivas, Gram negativas e também contra outros microrganismos (66) por terem facilidade de penetrar no alvo e chegar ao local de ação. O amplo emprego da tetraciclina nas últimas décadas pode ter contribuído para aumentar a resistência bacteriana a esta classe de drogas (49,67,68). Mais de 45 diferentes determinantes de resistência à tetraciclina têm sido identificados (64), conferindo resistência através de extrusão da droga por sistemas de efluxo, inativação enzimática do fármaco e proteção do ribossomo (66). A proteção do ribossomo é atribuída às proteínas TetM, TetO e o TetW (69), entre outras, mas elas não têm sido detectadas com frequência em *S. enterica* resistentes à tetraciclina (49,68). Por outro lado, os genes *tet*A, *tet*B, *tet*C e *tet*G, que codificam para sistemas de efluxo, têm sido mais comumente associados à resistência à tetraciclina em isolados de *S. enterica*. provenientes de aves e humanos (49,68,70).

Durante muitos anos, ampicilina, cloranfenicol e trimetoprim associado ao sulfametoxazol foram às drogas mais utilizadas para o tratamento de salmoneloses graves em humanos. Porém, o aumento na resistência a estes agentes reduziu significativamente o

seu uso na clínica médica e, consequentemente, as fluoroquinolonas, especialmente ciprofloxacina e norfloxacina, passaram a ser os principais antimicrobianos empregados para o tratamento de infecções humanas, sendo, sobretudo, indicadas para pacientes adultos e/ou imunocomprometidos (71,72).

Com o surgimento de cepas de *S. enterica* resistentes às quinolonas e fluoroquinolonas e, tendo seu uso contraindicado para crianças, fez-se necessária a utilização das cefalosporinas, que pertencem à classe dos β-lactâmicos, como tratamento de escolha para casos de salmonelose nestes casos (73). Entretanto, resistência aos β-lactâmicos em *S. enterica* tem sido descrita (74,75,76,77), sendo atribuída a inúmeros mecanismos, tais como produção de β-lactamases, diminuição da permeabilidade de membranas externas, provavelmente devido à perda ou modificação das porinas, alteração da afinidade de proteínas ligadoras de penicilina (PBPs) e pela hiper-expressão de bombas de efluxo (78).

As β-lactamases são codificadas por genes que podem estar inseridos no cromossomo, bem como por genes carreados por plasmídeos ou transposons, o que facilita a rápida disseminação deste importante mecanismo de resistência a β-lactâmicos entre os microrganismos (79). De acordo com a classificação de Ambler, estas enzimas são divididas em quatro classes (A, B, C e D), baseando-se nas suas sequências de nucleotídeos e de aminoácidos (80). As β-lactamases das classes A, C e D possuem serina no sítio ativo, enquanto a classe B é composta por metalo-β-lactamases que requerem zinco para a sua atividade catalítica (79). As β-lactamases de espectro estendido (ESBL) TEM, SHV e CTX-M, que pertencem à classe A, estão entre as principais responsáveis pela resistência de *S. enterica* às cefalosporinas. A CTX é uma cefotaximase codificada pelo

gene *bla*$_{\text{CTX-M}}$, que apresenta uma potente atividade hidrolítica contra esta cefalosporina (81). Nos últimos anos, a presença desta enzima tem sido reportada em várias espécies de *Enterobacteriaceae* isoladas de humanos e animais (82), incluindo *S. enterica* não tifoides (83). As enzimas SHV e TEM codificadas pelos genes *bla*$_{\text{SHV}}$ e *bla*$_{\text{TEM}}$, respectivamente, têm sido relatadas em isolados de *Klebsiella pneumoniae*, *Escherichia coli* e *Pseudomonas aeruginosa* envolvidas em surtos de infecção hospitalar (84,85,86,87,88), e reportadas com menos frequência em isolados de *S. enterica* com suscetibilidade reduzida às cefalosporinas (83,89,90,91).

Recentemente, as β-lactamases AmpC, pertencentes à classe C de Ambler, como a CMY, também têm sido descritas em muitos membros da família *Enterobacteriaceae*, sendo prevalente entre isolados de *S. enterica* (83,92,93). A CMY é uma cefalosporinase codificada pelo gene *bla*$_{\text{CMY}}$, que, frequentemente, tem sido associado ao cromossomo. Porém, este gene já foi encontrado em plasmídeos conferindo resistência aos β-lactâmicos, com exceção das cefalosporinas de quarta geração (cefepime) e dos carbapenêmicos (79).

1.2 Objetivo

1.2.1 Objetivo Geral

Avaliar a resistência a drogas antimicrobianas, bem como aocorrência de integrons e de genes relacionados à resistência a drogas antimicrobianas de *S. enterica* isoladas de materiais de origem avícola.

1.2.2 Objetivos Específicos

1.2.2.1 Caracterizar fenotipicamente a resistência de *S. enterica* isoladas de farinhas de origem animal, amostras ambientais de aviários e de vísceras de aves frente a diversas drogas antimicrobianas através da difusão do disco em agar;

1.2.2.2 Determinar a concentração inibitória mínima (CIM) à ciprofloxacina, ácido nalidíxico, sulfonamida, sulfonamida associada ao trimetoprim, cloranfenicol, ceftazidima e ampicilina em isolados de *S. enterica* fenotipicamente resistentes a estas classes de drogas no teste da difusão do disco em agar;

1.2.2.3 Determinar a presença de integrons das classes 1, 2 e 3 através de PCR, tendo os genes *int*I1, *int*I2 e *int*I3 como alvo;

1.2.2.4 Determinar a presença dos genes *sul*1, *sul*2 e *sul*3 através de PCR em isolados de *S. enterica* fenotipicamente resistentes à sulfonamida;

1.2.2.5 Detectar determinantes de resistência em isolados de *S. enterica* fenotipicamente resistentes aos beta-lactâmicos através de PCR, tendo como alvos os genes $bla_{\text{CTX-M}}$, bla_{CMY} e $bla_{\text{TEM;}}$

1.2.2.6 Determinar a presença dos genes *aad*A, *aad*B, *str*A e *str*B através de PCR em isolados de *S. enterica* fenotipicamente resistentes aos aminoglicosídeos;

1.2.2.7 Detectar a presença dos determinantes gênicos *tet*A, *tet*B e *tet*C em isolados de *S. enterica* fenotipicamente resistentes à tetraciclina.

Capítulo 2

Artigo Científico

Characterization of antimicrobial resistance in *Salmonella enterica* isolated from Brazilian poultry samples

Artigo científico submetido ao periódico científico *The Veterinary Journal*, publicado pela Elsevier.

Fator de impacto: 2.424

Submission Confirmation for The Veterinary Journal

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Original Article

Characterization of antimicrobial resistance in *Salmonella enterica* isolated from Brazilian poultry samples

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Abstract

The antimicrobial resistance and the presence of integron were evaluated in 203 *Salmonella enterica* isolates derived from poultry breeding in Southern Brazil during the period 2002-2012. Isolates from poultry environment showed to be significantly more resistant to antimicrobials than the remaining isolates, especially those isolated from poultry by-product meal. Thirty-seven isolates showed to be resistant to at least three antimicrobial classes. Integrons were detected in 26 isolates, all characterized as class 1. The analysis of the variable region between 5'CS and 3'CS of each class 1 integron positive isolate showed 13 with a typical 3'CS and 14 containing an atypical 3′CS. A *S.* Senftenberg isolate harbored two class 1 integrons. The highest percentage of resistance was found to sulfonamides, and *sul*genes were detected in most resistant isolates. Thirty and 37 isolates resistant to at least one aminoglycoside presented *aad*A and *aad*B, respectively. Among isolates resistant to streptomycin, *str*A and *str*B were detected in 44.4% and 34.9%, respectively. Twenty-one isolates presented reduced susceptibility to β-lactams and harbored bla_{TEM}, bla_{CMY} and/or $bla_{\text{CTX-M}}$. Forty isolates showed reduced susceptibility to tetracycline, which most isolates presented *tet* genes. Surveillance of antimicrobial resistance in *Salmonella* contributes to the debate on the impact that antimicrobial use in the animal production and the consequent selection of resistant strains may exert in the human health. Additionally, we highlighted the importance of environment as reservoir of resistant *Salmonella*, which may enable the persistence of resistance determinants in poultry production.

Keywords: *Salmonella enterica*; poultry by-product meal; poultry environment; antimicrobial resistance; poultry production chain.

Introduction

Salmonella enterica is an important pathogen involved in foodborne diseases that are mostly derived from consumption of food from animal origin, especially poultry products. This microorganism is responsible for 1.2 million illnesses and 450 estimated deaths annually worldwide (CDC, 2014), and has become a major concern due the emergence of *S. enterica* strains that are resistant to antimicrobials (Crum-Cianofle, 2008; Majowicz et al., 2010; Van et al., 2012).

The emergence and dissemination of multidrug-resistant (MDR) *Salmonella* have been associated to the broad use of antimicrobials, especially as growth promoters in food-producing animals, which can enhance the positive selection of resistance determinants in bacteria (Threlfall et al., 2000; Molbak, 2005; Vo et al., 2006). In this context, Brazil, which is the main exporter and the third producer country of chicken meat (ABEF, 2011), has adopted restrictive practices in the use of antimicrobials as feed additives. The use of avoparcin was forbidden in 1998 (MAPA, 1998), followed by the banishment of chloramphenicol and nitrofurantoin in 2003 (MAPA, 2003); tetracycline, β-lactams, quinolones, and systemic sulfonamides in 2009 (MAPA, 2009); and spiramycin and erythromycin in 2012 (MAPA, 2012).

Antimicrobial resistance has been usually determined by the presence of resistance genes in plasmids and/or in the bacterial chromosome (Bush and Jacoby, 2010; Dierikx et al., 2010; Sjölund-Karlsson et al., 2010; Folster et al., 2011). Several resistance gene cassettes are additionally harbored in integrons, and therefore can be spread by lateral genetic transfer via conjugative transposons and/or plasmids (Rodriguez et al., 2006; Hall, 2012). Class 1 integron is the most commonly found in *S. enterica* and has been often associated with MDR phenotypes (Kim et al., 2011). Class 1 integron contains a recombination site (*att*I) and an integrase gene (*int*I) in the 5' conserved segment (CS). The 3' CS end possesses the *qacE*Δ1 gene, which encodes a semi-

functional derivative of the quaternary ammonium resistance gene *qac*E, and frequently presents the *sul*1 gene (*sul* genes encode resistance to sulfonamides). However, an atypical 3'CS from class 1 integron has showed *sul*3 replacing *sul*1 (Toro et al., 2011; Wannaprasat et al., 2011). Another *sul* gene, *sul*2, has been found in plasmids carried by *S. enterica* (Hur et al., 2011), not inserted in integrons, and usually associated to *str*AB genes, which confer resistance to aminoglycosides (Yau et al., 2010). The presence of different resistance gene cassettes has been described in the variable region of class 1 integron located between the 5'CS and 3'CS, including the *aad*, *dfr* and *bla* genes, which encode aminoglycoside adenyltransferases (resistance to aminoglycosides), dihydrofolate reductases (resistance to trimethoprim), and β-lactamases (resistance to β-lactams), respectively (Firoozeh et al., 2012; Glenn et al., 2013). A complex class 1 integron has been found to be located on the chromosomal *Salmonella* Genomic Islands 1, which usually carry genes encoding resistance to β-lactams, tetracycline, sulfonamides, aminoglycosides, and chloramphenicol (Glenn et al., 2011; Hur et al., 2011; Brunelle et al., 2013). Additionally, several determinants of resistance to these classes of antimicrobials may also be present outside of integrons (Bush and Jacoby, 2010; Dierikx et al., 2010; Sjölund-Karlsson et al., 2010; Folster et al., 2011).

Considering that *Salmonella enterica* is a zoonotic pathogen that presents an important economic impact to poultry production chain, this study aims to contribute to the surveillance of the antimicrobial resistance profiles and the investigation of genetic determinants involved in the resistance phenotype found in Brazilian poultry isolates.

Materials and methods

Bacterial isolates

A total of 203 *S. enterica* isolates derived from poultry breeding in Southern Brazil was analyzed in this study. One hundred-six isolates were from several poultry by-product meals as follow: meat (n=38), feathers (n=21), meat and bones (n=9), viscera (n=25), blood (n=9), and mixed poultry by-product meals (n=4). Eighty-eight isolates from poultry environment samples included drag swab from broiler houses (n=76), disposable shoe covers (n=11), and swab from feed factory environment (n=1). Nine *S. enterica* isolates from pipped egg (n=1), cloacal swab (n=2), poultry carcass (n=1) and poultry organs (n=5) were grouped as poultry samples. Samples were collected from 2002 to 2012. 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.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of *S. enterica* isolates was evaluated by disk diffusion method following the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2013). Antimicrobial drugs tested were: nalidixic acid (NAL) - 30 µg, amikacin (AMI) - 30 µg, ampicillin (AMP) - 10 µg, cefaclor (CEC) - 30 µg, ciprofloxacin (CIP) - 5 µg, chloramphenicol (CLO) - 30 µg, streptomycin (EST) - 10 µg, gentamicin (GEN) - 10 µg, spectinomicyn (ESP) - 100 µg, sulfonamides (SUL) - 300 µg, trimethoprim/sulfamethoxazole (SUT) - 25 µg, tetracycline (TET) - 30 µg, and tobramycin (TOB) - 10 µg (Sensifar, Brazil). The inhibition zones were measured and scored as sensitive, intermediate resistant and resistant according to the CLSI guidelines (CLSI, 2008; CLSI, 2013). Additionally, antimicrobial susceptibility to ceftiofur (CFC) - 30 µg,

enrofloxacin (ENO) - 5 µg, florfenicol (FLF) - 30 µg, and neomycin (NEO) - 30 µg was determined by agar disk diffusion and interpreted following the manufacturer's instructions (Cefar, Brazil).

Isolates presenting reduced susceptibility to ciprofloxacin, sulfamethoxazole, trimethoprim/sulfamethoxazole, chloramphenicol, nalidixic acid, ampicillin and tetracycline on disk diffusion were evaluated regarding the minimal inhibitory concentration (MIC) to these drugs using the microdilution method (CLSI, 2008; CLSI, 2013). All tests were performed in duplicate for each antibiotic tested. MIC results were analyzed visually and by spectrophotometry at 620 nm.

Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference cultures for the antibiotic quality control in all antimicrobial resistance tests.

Molecular determinants of resistance

The presence of integrons and genes encoding resistance determinants to sulfonamides, β-lactams, tetracycline, and aminoglycosides were evaluated. Initially, it was performed a screening in order to determine the presence of integrons using a degenerate primer pair targeting the integrases 1, 2 and 3 (White et al., 2000). Integron-positive isolates were then analyzed to detect specifically class 1 and 2 integrons (White et al., 2001; Su et al., 2006). The variable region of the class 1 integron-carrying isolates was amplified using primers annealing within the 5' and 3' CS that flank it (White et al., 2000). To determine the presence of the atypical 3'CS of class 1 integron, it was performed a PCR targeting *qac*H (Chuanchuen et al., 2008a) and *sul*3 genes (Chuanchuen and Padungtod, 2009). All isolates phenotypically resistant to sulfamethoxazole were evaluated

regarding the presence of *sul*1 (Grape et al., 2003), *sul*2 (Kerrn et al., 2002), and *sul*3 (Chuanchuen and Padungtod, 2009). The presence of the resistant determinants to β-lactams bla_{CTX-M} (Edelstein et al., 2003), bla_{CMY} (Winokur et al., 2001), and bla_{TEM} (Carlson et al., 1999) was verified in isolates presenting reduced susceptibility to this class of drugs. Isolates with reduced susceptibility to aminoglycosides and only to streptomycin were analyzed regarding the presence of *aad*A (Madsen et al., 2000) and *aad*B (Frana et al., 2001, and *str*A and *str*B (Gebreyes and Thakur, 2005), respectively. The resistance determinants *tet*A, *tet*B and *tet*C (Aarestrup et al., 2003) were investigated in isolates with reduced susceptibility to tetracycline. All primers used in this study are shown in Supplementary Table 1 (see Appendix A).

Genomic DNA extraction

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

PCR amplification

The amplification conditions for each PCR assay were performed in a total volume of 25 µL containing: 0.2 mM of each deoxynucleoside triphosphate (dNTP) (Invitrogen, Brazil), 50 mM potassium chloride (KCl), 10 mM Tris-HCl (pH 8.3), 0.2 U *Taq* DNA polymerase (Invitrogen), 0.8 µM of each primer (IDT, Brazil) and 4 ng/µL DNA template. Amplifications were carried out in a thermocycler (Veriti Thermal Cycler, Applied Biosystems, USA) with $MgCl_2$ concentration and the annealing temperature as specified for each primer in Supplementary Table 1 (see Appendix A).

The cycling parameters were 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 1 min, annealing for 1 min, an extension of 72 ºC for 1 min, and a final extension at 72 ºC for 7 min. All reactions were performed in duplicate and positive and negative controls were used for all reactions. 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 base pairs (bp) DNA ladder (Ludwig Biotecnologia, Brazil) was used as the molecular mass marker.

DNA sequencing

To determine the content of class 1 integron in *S. enterica* isolates that presented a typical 3'CS, the amplicons were purified using ammonium acetate and sequenced in an automated DNA sequencer ABI 3130 XL Genetic Analyzer XL (Applied Biosystems, USA). The sequences of the isolates obtained were edited, aligned, analyzed, and compared with sequence databases using the MEGA software version 5.0 (www.megasoftware.net) and the BLAST software version 2.0 (http://www.ncbi.nlm.nih.gov/BLAST).

At least one amplicon from each resistance gene was also submitted to sequencing to evaluate the specificity of the primers.

Statistical analysis

The results from disk diffusion and correlation between resistance phenotypes and resistance genes were compared by Cochran and Chi-Square tests. 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 or <0.001 were considered as statistically significant for all tests (95% confidence or 5% significance).

Results

Resistance percentages found to 17 different antimicrobials in the 203 *S. enterica* isolates tested are summarized in Table 1. The MIC values for chloramphenicol, ampicillin, ceftazidime, ciprofloxacin, nalidixic acid, tetracycline, sulfamethoxazole and trimethoprim/sulfamethoxazole are shown in Fig. 1. Taking together the results from MIC and disk diffusion tests, 40 (19.7%) isolates presented susceptibility to all antimicrobials tested, and isolates from poultry environment showed to be significantly more resistant to antimicrobials than the remaining isolates ($P<0.05$), especially those isolated from poultry by-product meal ($P<0.001$). The highest percentage of resistance was found to sulfonamides, although isolates from different poultry samples, not including meal and environment, presented a higher percentage of resistance to nalidixic acid. Sixty different patterns of antimicrobial resistance were found (Table 2), 163 (80.3%) isolates showed reduced susceptibility to at least one antimicrobial, and 37 (18.2%) isolates showed to be MDR (resistant to three or more classes of drugs). Among the MDR isolates, four (*S.* Heidelberg, *S.* Cerro, and two *S.* Senftenberg strains) showed the penta-resistant phenotype ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline), and the *S.* Heidelberg ACSSuT strain showed to be also resistant to other seven antimicrobials.

Class 1 integron was present in 26 isolates (12.7%), 21 (80.8%) of them displaying the MDR phenotype, while class 2 was not detected. None of the isolates from poultry by-product meal presented integrons, and 25 integron-carrying isolates (96.1%) were from the poultry environment.

The variable region between 5'CS and 3'CS of each class 1 integron positive isolate was analyzed by PCR, showing 13 isolates presenting the typical 3'CS and 14 containing an atypical 3′CS linked to the *qacH–sul3* domain in the absence of the *sul*1 gene. The amplification and sequencing of the variable region between 5'CS and the typical 3'CS showed 10 isolates with 1.7 kb-fragments, presenting the *aad*A1 and *dfr*A1 genes (GenBank accession numbers KJ848440, KJ848441, KJ848442, KJ848443, KJ848444, KJ848445, KJ848446, KJ848447, KJ848448, and KJ848449), while 3 isolates showed fragments of approximately 1 kb, presenting only *aad*A1 (GenBank accession numbers KJ756515, KJ756516, and KJ756517) (Table 2 and Fig. 2). A *S.* Senftenberg isolated from environment was found to harbor two class 1 integrons: one integron with a typical 3'CS and a variable region 1.7 kb-long, and the other with an atypical 3′CS linked to the *qacH–sul3*.

Fifty-two (25.6%) isolates were resistant to aminoglycosides, and 45 (86.5%) of these harbored at least one gene encoding resistance to this antimicrobial class. *aad*A and *aad*B were detected in 30 (26.1%) and 37 (32.1%) *S. enterica* isolates resistant to at least one aminoglycoside, respectively, while both genes were observed in 17 (14.7%) (Table 2). Among *S. enterica* isolates resistant to streptomycin, *str*A and *str*B were detected in 28 (44.4%) and 22 (34.9%) isolates, respectively, and both genes were present in 17 (26.9%) (Table 2).

As can be seen in Tables 2 and Supplementary Table 2 (see Appendix A), resistance to sulfonamide was significantly associated to the presence of *sul* genes ($P<0.05$), since 60% of the sulfonamide-resistant isolates presented at least one *sul* gene. *sul*1, *sul*2 and *sul*3 were detected in 15 (18.7%), 26 (32.5%) and 25 (31.2%) isolates, respectively. *sul*2 was detected in concomitance with *sul*1 and *sul*3 in 11 (13.7%) and 7 (10%) isolates, respectively. *sul*1 and *sul*3 were found

concomitantly in the *S.* Senftenberg isolate that harbored two class 1 integrons. The three *sul* genes were not detected in a same isolate. Comparing the MIC values presented by the isolates tested with the presence of *sul* genes, it was verified that 20 (80%) isolates with MIC of 2,048 μg/mL presented *sul*3 (see Appendix A: Supplementary Table 2). The majority of isolates carrying *sul*1 (86.7%) harbored class 1 integron. However, two *sul*1 positive and 11 *sul*3 positive isolates did not carry integrons.

Among the 21 isolates with reduced susceptibility to β-lactams, 85.7% presented bla_{TEM}, which was significantly associated with the phenotype of resistance to this antimicrobial ($P<0.05$). bla_{CMY} was detected in 5 (23.8%) isolates, while 2 (9.5%) showed to harbor $bla_{\text{CTX-M}}$ (Table 2 and see Appendix A: Supplementary Table 3).

Forty (19.7%) *S. enterica* isolates showed reduced susceptibility to tetracycline. The *tet*A gene was detected in 28 (70%) isolates, whereas *tet*B or *tet*C were found in 4 (10%). The concomitant presence of *tet*A with *tet*B or with *tet*C was detected in 2 (5%) isolates. The three *tet* genes were not detected simultaneously in any isolate (Table 2 and see Appendix A: Supplementary Table 4).

Discussion

S. enterica is an important pathogen involved in foodborne diseases that is usually transmitted by poultry-derived products (Van et al., 2012). Moreover, the presence of resistance determinants to antimicrobials used in human medicine may turn this microorganism into a major threat to public health (Collignon et al., 2009). In this context, the characterization of antimicrobial

resistance in *Salmonella* isolated from poultry samples can help in the understanding of the role of practices, supplies, devices and/or outdoor and indoor environments in the re-introduction and maintenance of resistant strains in poultry farms. Many studies have been performed with the purpose to determine the antimicrobial resistance in *Salmonella* isolates from poultry (Hur et al., 2011; Campioni et al., 2014), including poultry-derived food (Wouafo et al., 2010; Lai et al., 2014) and even from animal feed (Li et al., 2012), but the environment of poultry houses and components of poultry feed have been poorly investigated (Hofacre et al., 2001; Thakur et al., 2013; Campioni et al., 2014; Sapkota et al., 2014). Therefore, in order to help in filling this gap, this study focused special concern in isolates from poultry by-product meal and drag swab from poultry houses.

Feed has been considered a potential source of *Salmonella* contamination in poultry farms (Maciorowski et al., 2006; Ge et al., 2013; Saptoka et al., 2014), whose origin may be derived from its ingredients (Sapkota et al., 2007). Although poultry meal has been described as an important feed ingredient that may present bacteria resistant to five or more antibiotics (Hofacre et al., 2001), the majority of the *S. enterica* isolates from poultry by-product meal analyzed here showed to be sensitive to all antimicrobials tested. On the other hand, isolates presenting reduced susceptibility to ceftiofur, a third generation cephalosporin used in day-old chicks to control infections and reduce the mortality, were detected in poultry by-product meal. So, the use of ceftiofur in poultry production can result in the selection of cephalosporin-resistant isolates, which leads to a special concern since cephalosporins are the drugs of choice for treatment of invasive and severe salmonellosis in children and pregnant women (CDC, 2013).

A great number of MDR isolates was also found in the poultry house environment, including resistance to the drugs of choice for the treatment of salmonellosis in humans (CDC,

2013). Moreover, the penta-resistant phenotype (ACSSuT), usually associated to *S*. Typhimurium (Yu et al., 2008), was found in the serovars *S*. Senftenberg, *S*. Heidelberg, and *S*. Cerro, highlighting the horizontal spread of the resistance determinants responsible for this phenotype, which are often carried by mobile genetic elements (Dionisi et al., 2011). This may be even more troubling since resistance can be spread to commensal bacteria, which can act as reservoir of resistance genes. Therefore, an appropriate sanitization of the indoor environment and equipment is mandatory in order to avoid the persistence of MDR *Salmonella*, and a possible contamination between poultry lots. Furthermore, the improvement of biosecurity can be a way to decrease the antimicrobial use in animal production systems, as described by Laanen et al. (2013), which found a negative association between biosecurity in pig's production and the prophylactic use of antimicrobials. The use of antimicrobials as growth promoters in animal feed or even for therapeutic purposes in veterinary medicine exerts a selective pressure favoring resistant isolates (Kempf et al., 2013), which do not seem to be overcome rapidly. Indeed, some isolates presented high MIC values to chloramphenicol, which is no longer used in Brazilian animal production for over 10 years (MAPA, 2003). This resistance was possibly co-selected with other antimicrobials under use in animal production, whose determinants of resistance would be carried by the same mobile genetic elements. Alternatively, the maintenance of chloramphenicol resistance can be due to the cross-resistance with other antibiotics and biocides (Braoudaki and Hilton, 2004; Chuanchuen et al., 2008b).

A high proportion of isolates resistant to sulfonamide was found, even in isolates from poultry by-product meal, which possibly are due to the wide use of sulfonamide in poultry production over many years. The resistance to this antimicrobial was associated to the presence of *sul*1, *sul*2 and/or *sul*3 genes in the majority of isolates, as already described for *S. enterica* (Grape et al., 2003; Machado et al., 2013; Soufi et al., 2012). Although *sul*1 is usually reported as the most

prevalent *sul* gene in *S. enterica* (Douadi et al., 2010; Dionisi et al., 2011), the isolates evaluated in this study harbored predominately *sul*2 and/or *sul*3, which have also been described for *Salmonella* isolated from poultry (Chuanchuen and Padungtod, 2009; Anjum et al., 2011; Hur et al., 2011). The low prevalence of *sul*1 is in accordance with the presence of class 1 integron in only 12.3% of isolates, since this gene has been associated to the typical 3'CS position of class 1 integron. However, two *sul*1-positive isolates did not carry integrons. Therefore, *sul*1 can be inserted into plasmids lacking integrons, as previously described (Wu et al., 2010; Han et al., 2012). *sul*3 has been associated with an atypical 3'CS in the absence of the *sul*1 (Wannaprasat et al., 2011; Machado et al., 2013), which was observed in half of our isolates that harbored class 1 integron. Additionally, a *S.* Senftenberg presented two distinct class 1 integrons and both *sul1* and *sul3*, associated to the presence of the typical and atypical 3'CS, respectively. The concomitant presence of two class 1 integrons in *Salmonella* have already been described in poultry and human isolates (Lee et al., 2009; Firoozeh et al., 2012). However, 44% of the *sul*3-positive isolates did not carry integrons, being probably inserted into plasmids outside integrons, as previously described in *Salmonella* spp. (Curiao et al., 2011; Han et al., 2012). *sul*2, in contrast with the other *sul* genes, showed a lower association with integrons, what is in accordance with its integron-independent plasmid origin (Antunes et al., 2005). *sul*2 has been usually located in plasmids carrying *str*AB genes (Yau et al., 2010), which was not found in the isolates analyzed in this study, since most of isolates carrying *str* genes did not harbor *sul*2, and some isolates harbored *sul*2 in the absence of *str* genes.

str genes were found in the majority of isolates resistant to streptomycin, which were most prevalent in our isolates when comparing with other studies performed in isolates from animals (Anjum et al., 2011; Glenn et al., 2011; Soufi et al., 2012; Glenn et al., 2013). Aminoglycosides have been widely used in veterinary medicine to treat and prevent infections by Gram-negative

bacteria (Schwarz et al., 2001; Schwarz and Chaslus-Dancla, 2001), which probably may have led to a large selection of isolates resistant to this antimicrobial class in animals. Although the highest percentage of resistance between aminoglycosides has been found for streptomycin, resistance to other members of this class was also observed, as well as *aad*A and *aad*B genes were detected in isolates with reduced susceptibility to aminoglycosides. *aad* genes have been usually found inserted in the variable region between 5'CS and 3'CS (Hsu et al., 2006; Firoozeh et al., 2012; Kim et al., 2011), as was also observed in the isolates from this study. Also, the variable regions 1.7 kb-long showed the *dhfr*A1 gene associated to *aad*A1, as previously described in *S. enterica*, exhibiting resistance to trimethoprim and aminoglycosides (White et al., 2000; Kim et al., 2011).

Many environmental isolates showed to be resistant to tetracycline, which was expected due to the wide use of this antimicrobial in veterinary medicine. Resistance to tetracycline in *Salmonella* isolated from animals is usually conferred by specific efflux pump systems coded by *tet* genes (Bacci et al., 2012; Frye and Jackson, 2013; Glenn et al., 2013), which we found in most isolates that presented reduced susceptibility to tetracycline. *tet*A was detected in the majority of isolates, as has also been described for *S. enterica* isolated from poultry-origin (Aslam et al., 2012; Bacci et al., 2012; Glenn et al., 2013).

Resistance to β-lactams was found in only few isolates compared to other classes of drugs evaluated, which has also been described in other studies, including Brazilian isolates (Costa et al., 2013; Jong et al., 2014), but the presence of isolates resistant to third-generation cephalosporins *per se* is already a cause for concern by limiting the therapeutic options to treat human salmonellosis. All β-lactam-resistant isolates showed at least one of the *bla* genes investigated, which corroborates

that the β-lactamase production is the main mechanism of resistance to β-lactams in *Salmonella* (Arlet et al., 2006; Li et al., 2007; Bush and Jacoby, 2010; Folster et al., 2011).

Conclusion

Surveillance of antimicrobial resistance in *Salmonella* contributes to the debate on the impact that antimicrobial use in the animal production and the consequent selection of resistant strains may exert in the human health. Additionally, we highlighted the importance of environment as reservoir of resistant *Salmonella*, which may enable the persistence of resistance determinants in poultry production, reinforcing the need for other strategies to prevent infectious diseases that may compensate, at least partially, the loss of productivity of avian industry due to a possible banning of antimicrobials as growth promoters.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgments

The authors thank the Laboratório Porto Belo for supplying the majority of *Salmonella* spp. used in this study. S. P. Mattiello received a scholarship from Probolsas/PUCRS.

Funding: Probolsas, PUCRS, Brazil

Competing interests: None declared.

Ethical approval: Not required

Appendix A: Supplementary material

Supplementary data associated with this article can be found, in the online version, at

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Table 1

Antimicrobial resistance in *Salmonella enterica* isolated from poultry-related samples.

Sample (number of strains)		Resistance to antimicrobial drugs (%)																
		AMI	**ESP**	**EST**	**GEN**	**NEO**	**TOB**	**AMP**	**CFC**	**CTF**	**NAL**	**CIP**	**ENO**	**SUL**	**SUT**	**CLO**	**FLF**	**TET**
Poultry meal	R	1.8	4.7	5.6	1.8	1.8	0	0	0	0	6.6	0	0	28.3	0	0	0	0
(n=106)	IR	0.9	12.2	19.9	2.8	76.4	2.8	0	1.8	1.8	0	1.8	1.8	0	0	0	0	0
Poultry (n=9)	R	0	11.1	11.1	0	0	0	11.1	0	0	55.5	0	0	44.4	11.1	11.1	11.1	11.1
	IR	11.1	0	0	0	77.7	0	0	0	0	0	11.1	11.1	0	0	0	0	11.1
Poultry environment (n=88)	R	6.8	19.3	28.4	4.5	1.1	13.6	13.6	12.5	12.5	27.2	1.1	1.1	51.1	38.6	7.9	7.9	43.1
	IR	4.5	9.1	11.3	1.1	0	2.8	1.1	0	0	0	13.6	13.6	0	0	4.5	4.5	2.2

R, Resistant; IR, intermediate resistance; NAL, nalidixic acid; AMI, amikacin; AMP, ampicillin; CFC, cefaclor; CTF, ceftiofur; CIP, ciprofloxacin; CLO, chloramphenicol; ENO, enrofloxacin; ESP, spectinomycin; EST, streptomycin; FLF, florfenicol; GEN, gentamicin; NEO, neomycin; SUL, sulfonamides; SUT, trimethoprim/sulfamethoxazole; TET, tetracycline and TOB, tobramycin.

Poultry by-product meal includes viscera meal; feathers meal; flesh and bones meal; meat meal; mixed meal and blood meal. Poultry samples include piped egg; cloacal swab; poultry carcass and poultry organs. Environment samples include drag swab from broiler house; disposable shoe covers and swab from feed factory environment.

Table 2

Antimicrobial resistance pattern, presence of integron and resistance genes in *Salmonella enterica* isolated from poultry-related samples.

Isolate (identification number)	Origin	Resistance pattern	Integron and resistance gene
S. enterica (S107)	Drag swab	EST	*str*A
S. Schwarzengrund (S141)	Drag swab	AMP	bla_{CMY}, bla_{TEM}
S. enterica (S134)	Drag swab	TET	*tet*C
S. enterica (S122)	Drag swab	ESP, EST	*aad*B, *str*A, *str*B
S. Gafsa (S97)	Viscera Meal	ESP, NEO	*aad*B
S. enterica (O:4,5) (S163)	Blood meal	ESP, SUT	*sul*2, *aad*A
S. enterica (S143)	Drag swab	EST, SUL	*sul*1, *aad*B
S. Anatum (S83)	Meat Meal	EST, NEO	*aad*B, *str*B
S. Anatum (S76)	Meat Meal	EST, NEO	*aad*B
S. Mbandaka (S93)	Meat Meal	EST, NEO	*aad*B
S. Cerro (S82)	Meat Meal	EST, NEO	*aad*B
S. Infantis (S100)	Blood Meal	EST, NEO	*str*B
S. Adelaide (S39)	Viscera Meal	GEN, ESP	*aadB*
S. enterica (S158)	Drag swab	TOB, SUL	*sul*2, *aad*B
S. enterica (S142)	Drag swab	CFC, CTF	bla_{TEM}
S. Senftenberg (S153)	Poultry organs	NAL, SUL	*sul*1
S. enterica (S174)	Poultry organs	NAL, SUL	*sul*2
S. enterica (S200)	Drag swab	NAL, SUL	*sul*2, *sul*3
S. Senftenberg (S128)	Drag swab	ESP, SUL, SUT	*int*I1[d], *sul*3, *aad*A, *aad*B
S. Senftenberg (S10)	Meat Meal	EST, NEO, SUL	*aad*B, *str*B
S. Senftenberg (S124)	Drag swab	EST, SUL, TET	*int*I1[d], *sul*3, *str*A, *str*B, *tet*A
S. enterica (S182)	Drag swab	EST, AMP, TET	*aad*B, *str*A, *str*B, bla_{TEM}, *tet*B
S. Infantis (S156)	Drag swab	AMP, CFC, CTF	bla_{CMY}
S. enterica (S127)	Drag swab	AMP, CFC, CTF	bla_{TEM}
S. enterica (S103)	Feather Meal	CFC, CTF, SUL	bla_{TEM}
S. Senftenberg (S184)	Drag swab	SUL, SUT, TET	*sul*3, *tet*A
S. Gallinarum (S193)	Poultry organs	SUL, SUT, TET	*int*I1[d], *sul*2, *sul*3, *tet*A
S. enterica (S195)	Drag swab	SUL, SUT, TET	*sul*3, *tet*A
S. enterica (S202)	Drag swab	SUL, SUT, TET	*int*I1[d], *sul*3, *tet*A
S. enterica (S102)	Blood Meal	AMI, ESP, EST, NEO	*aad*A, *aad*B
S. Infantis (S179)	Drag swab	ESP, EST, SUL, TET	*sul*2, *aad*B, *str*A, *tet*A
S. Worthington (S139)	Drag swab	ESP, SUL, SUT, TET	*int*I1[d], *sul*3, *aad*B, *tet*A
S. enterica (S203)	Drag swab	ESP, SUL, SUT, TET	*sul*3, *aad*B, *tet*A
S. Senftenberg (S164)	Drag swab	EST, GEN, SUL, TET	*sul*2, *aad*A, *aad*B, *str*B, *tet*A
S. Senftenberg (S148)	Drag swab	AMP, CFC, CTF, NAL	bla_{CMY}, bla_{TEM}
S. Adelaide (S40)	Viscera Meal	AMI, ESP, NEO, GEN, SUL	*aad*B
S. Senftenberg (S54)	Viscera Meal	ESP, NEO, CFC, CTF, SUL	*aad*A, bla_{TEM}
S. Senftenberg (S177)	Drag swab	ESP, EST, SUL, SUT, TET	*int*I1[d], *sul*3, *aad*B, *str*A, *tet*A
S. enterica (S118)	Drag swab	ESP, EST, SUL, SUT, TET	*sul*3, *aad*A, *str*A
S. enterica (S196)	Drag swab	ESP, EST, SUL, SUT, TET	*int*I1[d], *sul*3, *aad*B, *str*A, *tet*A
S. enterica (O:13,23) (S180)	Drag swab	EST, AMP, SUL, SUT, TET	*sul*2, *aad*B, *str*A, *str*B, bla_{TEM}, *tet*B
S. enterica (O:4,5:l,v:-) (S169)	Disposable shoes covers	NAL, CIP, ENO, SUL	*sul*3
S. enterica (O:4,5) (S108)	Drag swab	AMP, CFC, CTF, NAL, SUL	*sul*2, bla_{CMY}

S. enterica (S120)	Drag swab	AMI, NEO, NAL, CIP, ENO, SUL	*aad*B
S. Senftenberg (S167)	Drag swab	AMI, ESP, EST, SUL, SUT, TET	*int*I1[d], *sul*2, *sul*3, *aad*A, *aad*B, *str*A, *tet*A
S. Senftenberg (S129)	Drag swab	ESP, EST, NEO, SUL, SUT, TET	*sul*3, *aad*A, *aad*B, *str*A, *str*B
S. Infantis (S130)	Drag swab	ESP, EST, NEO, SUL, SUT, TET	*int*I1[d], *sul*3, *aad*A, *aad*B, *str*A, *str*B, *tet*A
S. Senftenberg (S112)	Drag swab	ESP, EST, TOB, SUL, SUT, TET	*int*I1[d], *sul*3, *aad*A, *str*A, *tet*A
S. Montevideo (S205)	Drag swab	ESP, EST, AMP, SUL, SUT, TET	*sul*2, *sul*3, *aad*A, *aad*B, *str*A, *str*B, bla_{CTX-M}
S. Senftenberg (S114)	Drag swab	AMI, ESP, EST, GEN, SUL, SUT, TET	*int*I1[d], *sul*3, *aad*A, *aad*B, *str*A, *tet*A
S. Senftenberg (S171)	Drag swab	AMI, ESP, EST, NEO, SUL, SUT, TET	*sul*3, *aad*A, *aad*B, *tet*A
S. enterica (S119)	Drag swab	ESP,TOB, CFC, CTF,CIP, ENO, NAL	*aad*A, *aad*B, bla_{TEM}
S. Senftenberg (S138)	Disposable shoes covers	ESP, EST, SUL, SUT, CLO, FLF, TET	*int*I1[a,c,d], *sul*1, *sul*3, *aad*A1, *dfr*A1, *str*A
S. enterica (O:4,5:l,v:-) (S187)	Drag swab	ESP, NAL, SUL, SUT, CLO, FLF	*sul*3
S. Cerro (S176)*	Drag swab	EST, AMP, SUL, SUT, CLO, FLF, TET	*sul*2, *aad*A, *aad*B, *str*A, *str*B, bla_{TEM}
S. Worthington (S192)	Drag swab	EST, NAL, SUL, SUT, CLO, FLF, TET	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, *aad*B, *str*A, *str*B, *tet*A
S. Worthington (S194)	Drag swab	EST, NAL, CIP, ENO, SUL, SUT, TET	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, *aad*B, *str*A, *str*B
S. Worthington (S204)	Drag swab	EST, NAL, CIP, ENO, SUL, SUT, TET	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, *aad*B, *str*A, *str*B
S. Heidelberg (S109)	Drag swab	AMI, ESP, NEO, NAL, CIP, ENO, SUL, TET	*sul*2, *tet*C
S. Senftenberg (S201)	Poultry carcass	AMI, ESP, EST, AMP, SUL, CLO, FLF, TET	*sul*2, *sul*3, *aad*B, *str*B, bla_{CMY}, bla_{TEM}, *tet*A, *tet*B
S. Senftenberg (S123)*	Drag swab	AMI, ESP, AMP, SUL, SUT, CLO, FLF, TET	*int*I1[d], *sul*2, *sul*3, *aad*A, *aad*B, bla_{TEM}, *tet*A
S. Worthington (S170)	Drag swab	AMI, EST, NAL, CIP, ENO, SUL, SUT, TET	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, *aad*B, *str*A, *tet*A
S. Worthington (S113)	Drag swab	EST, TOB, NAL, CIP, ENO, SUL, SUT, TET	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, *str*A, *str*B, *tet*A
S. Worthington (S146)	Drag swab	ESP, CFC, CTF, NAL, CIP, ENO, SUL, SUT, TET	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, bla_{TEM}, *tet*A
S. Worthington (S185)	Drag swab	EST, NAL, CIP, ENO, SUL, SUT, CLO, FLF, TET	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, *str*A, *str*B, *tet*A
S. Senftenberg (S166)	Drag swab	EST, NAL, CIP, ENO, SUL, SUT, CLO, FLF	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, *str*A
S. Schwarzengrund (S140)	Drag swab	ESP, EST, CFC, CTF, NAL, CIP, ENO, SUL, SUT, TET	*int*I1[a,b], *sul*1, *sul*2, *aad*A1, *str*A, *str*B, bla_{TEM}, *tet*A, *tet*B
S. Heidelberg (S111)	Drag swab	ESP, EST, GEN, NEO, TOB, AMP, CFC, CTF, SUL, TET	*int*I1[a,b], *sul*1, *aad*A1, *str*A, *str*B, bla_{CTX-M}, bla_{TEM}, *tet*A, *tet*C
S. Senftenberg (S165)*	Drag swab	ESP, EST, NEO, AMP, CFC, CTF, SUL, SUT, CLO, FLF, TET	*int*I1[d], *sul*2, *sul*3, *aad*A, *aad*B, *str*A, *str*B, bla_{TEM}, *tet*A
S. Worthington (S147)	Drag swab	AMI, ESP, CFC, CTF, NAL, CIP, ENO, SUL, SUT, CLO, FLF, TET	*int*I1[a,c], *sul*1, *sul*2, *aad*A1, *dfr*A1, bla_{TEM}, *tet*A
S. Heidelberg (S110)*	Drag swab	ESP, EST, GEN, NEO, TOB, AMP, CFC, CTF, NAL, CIP, ENO, SUL, CLO, FLF, TET	*int*I1[a,b], *sul*1, *sul*2, *aad*A1, *str*A, *str*B, bla_{TEM}, *tet*A, *tet*C

NAL, nalidixic acid; AMI, amikacin; AMP, ampicillin; CFC, cefaclor; CTF, ceftiofur; CIP, ciprofloxacin; CLO, chloramphenicol; ENO, enrofloxacin; ESP, spectinomycin; EST, streptomycin; FLF, florfenicol; GEN, gentamicin; NEO, neomycin; SUL, sulfonamides; SUT, trimethoprim/sulfamethoxazole; TET, tetracycline, and TOB, tobramycin.

The underlined drugs showed intermediate resistance.

[a] indicates 3' conserved segment of class 1 integron. [b] indicates approximately amplicon size for 5'CS-3'CS region of 1.0 kb, and [c] 1.7 kb. [d] indicates atypical 3' conserved segment of class 1 integron with *sul*3 gene. * indicates the presence of penta-resistant phenotype (ACSSuT).

Appendix A

Supplementary Table 1

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

Target	Primer sequence (5'→3')	$MgCl_2$ concentration	Annealing temperature (°C)	Amplicon size (bp)	Reference
*int*I	F TGCGGGTYAARGATBTKGATTT R CARCACATGCGTRTARAT	2.5	55	491	(White et al., 2000)
*int*I1	F ACGAGCGCAAGGTTTCGGT R GAAAGGTCTGGTCATACATG	2.0	59	565	(Su et al., 2006)
5'CS 3'CS	F TCATGGCTTGTTATGACTGT R GTAGGGCTTATTATGCACGC	2.5	57	variable	(White et al., 2000)
*int*I2	F CGGGATCCCGGACGGCATGCACGATTTGTA R GATGCCATCGCAAGTACGAG	2.5	57	2200	(White et al., 2001)
*qac*H	F CTCGCACTCAAGTCCATCC R CTAACGATAAGTCCCATGCC	2.0	55	140	(Chuanchuen et al., 2008a)
*sul*1	F ATGGTGACGGTGTTCGGCATTCTGA R CTAGGCATGATCTAACCCTCGGTCT	2.5	64	839	(Grape et al., 2003)
*sul*2	F GCGCTCAAGGCAGATGGCATT R GCGTTTGATACCGGCACCCGT	1.5	67	293	(Kerrn et al., 2002)
*sul*3	F GGGAGCCGCTTCCAGTAAT R TCCGTGACACTGCAATCATTA	1.5	58	500	(Chuanchuen and Padungtod, 2009)
bla_{CMY}	F ATGATGAAAAAATCGTTATGC R TTGCAGCTTTTCAAGAATGCGC	2.0	56	1143	(Winokur et al., 2001)
bla_{CTX-M}	F TTTGCGATGTGCAGTACCAGTAA R CGATATCGTTGGTGGTGCCATA	2.0	59	544	(Edelstein et al., 2003)
bla_{TEM}	F GCACGAGTGGGTTACATCGA R GGTCCTCCGATCGTTGTCAG	2.5	55	300	(Carlson et al., 1999)
*str*A	F CTTGGTGATAACGGCAATTC R CCAATCGCAGATAGAAGGC	2.0	54	546	(Gebreyes and Thakur, 2005)
*str*B	F ATCGTCAAGGGATTGAAACC R GGATCGTAGAACATATTGGC	2.0	53	509	(Gebreyes and Thakur, 2005)
*aad*A	F GTGGATGGCGGCCTGAAGCC R AATGCCCAGTCGGCAGCG	1.5	66	525	(Madsen et al., 2000)
*aad*B	F GAGCGAAATCTGCCGCTCTGG R CTGTTACAACGGACTGGCCGC	2.5	59	320	(Frana et al., 2001)
*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)
*qac*H *sul*3	R CTAACGATAAGTCCCATGCC F GGGAGCCGCTTCCAGTAAT	2.0	55	2000	(Chuanchuen et al., 2008; Chuanchuen and Padungtod, 2009)

Supplementary Table 2

Presence of *sul* genes and minimal inhibitory concentration (MIC) values to sulfamethoxazole and sulfamethoxazole associated to trimethoprim in *Salmonella enterica*.

Isolate (identification number)	MIC (µg/mL)		*sul*1	*sul*2	*sul*3
	SUL	SUT			
S. Cerro (S176)	1,024	64/1,216	-	+	-
S. Gallinarum (S193)	512	32/608	-	+	+
S. Heidelberg (S109)	256	0.5/9.5	-	+	-
S. Heidelberg (S110)	1,024	NR	+	+	-
S. Heidelberg (S111)	1,024	0.5/9.5	+	-	-
S. Infantis (S179)	512	0.5/9.5	-	+	-
S. Infantis (S130)	2,048	64/1,216	-	-	+
S. Montevideo (S205)	2,048	32/608	-	+	+
S. Schwarzenbrund (S140)	1,024	0.5/9.5	+	+	-
S. Senftenberg (S114)	2,048	32/608	-	-	+
S. Senftenberg (S123)	2,048	8/152	-	+	+
S. Senftenberg (S124)	1,024	64/1,216	-	-	+
S. Senftenberg (S128)	2,048	16/304	-	-	+
S. Senftenberg (S129)	2,048	16/304	-	-	+
S. Senftenberg (S138)	2,048	32/608	+	-	+
S. Senftenberg (S112)	2,048	16/304	-	-	+
S. Senftenberg (S153)	1,024	NR	+	-	-
S. Senftenberg (S164)	2,048	NR	-	+	-
S. Senftenberg (S165)	2,048	16/304	-	+	+
S. Senftenberg (S166)	1,024	8/152	+	-	-
S. Senftenberg (S167)	2,048	8/152	-	+	+
S. Senftenberg (S171)	2,048	32/608	-	-	+
S. Senftenberg (S177)	2,048	8/152	-	-	+
S. Senftenberg (S184)	2,048	16/304	-	-	+
S. Senftenberg (S201)	2,048	0.5/9.5	-	+	+
S. Worthington (S113)	2,048	64/1,216	+	+	-
S. Worthington (S192)	2,048	64/1,216	+	+	-
S. Worthington (S194)	1,024	32/608	+	+	-
S. Worthington (S204)	2,048	8/152	+	+	-
S. Worthington (S139)	2,048	32/608	-	-	+
S. Worthington (S146)	2,048	8/152	+	+	-
S. Worthington (S147)	2,048	64/1,216	+	+	-
S. Worthington (S170)	2,048	32/608	+	+	-
S. Worthington (S185)	1,024	4/76	+	+	-
S. *enterica* (O:4,5) (S163)	512	NR	-	+	-
S. *enterica* (O:4,5) (S108)	512	NR	-	+	-
S. *enterica* (O:4,5) (S187)	2,048	64/1,216	-	-	+
S. *enterica* (O:4,5,l,v:-) (S169)	512	NR	-	-	+
S. *enterica* (O:13,23) (S180)	2,048	32/608	-	+	-
S. *enterica* (S118)	1,024	4/76	-	-	+
S. *enterica* (S143)	512	NR	+	-	-
S. *enterica* (S158)	512	NR	-	+	-
S. *enterica* (S174)	512	NR	-	+	-
S. *enterica* (S195)	2,048	16/304	-	-	+
S. *enterica* (S196)	2,048	32/608	-	-	+
S. *enterica* (S200)	2,048	NR	-	+	+
S. *enterica* (S202)	1,024	64/1,216	-	-	+
S. *enterica* (S203)	2,048	16/304	-	-	+

SUL, sulfamethoxazole; SUT, trimethoprim/sulfamethoxazole; NR, non-resistant; +, present; -, absent

Supplementary Table 3

Presence of *bla* genes and minimal inhibitory concentration (MIC) values to beta-lactams in *Salmonella enterica*.

Isolate (identification number)	MIC (µg/mL)		*bla* $_{CMY}$	*bla* $_{CTX-M}$	*bla* $_{TEM}$
	CAZ	AMP			
S. Cerro (S176)	NR	1,024	-	-	+
S. Heidelberg (S110)	512	128	-	-	+
S. Heidelberg (S111)	8	1,024	-	+	+
S. Infantis (S156)	64	64	+	-	-
S. Montevideo (S205)	NR	256	-	+	-
S. Senftenberg (S123)	NR	512	-	-	+
S. Senftenberg (S54)	8	NR	-	-	+
S. Senftenberg (S148)	32	32	+	-	+
S. Senftenberg (S165)	32	512	-	-	+
S. Senftenberg (S201)	NR	64	+	-	+
S. Schwarzengrund (S140)	128	NR	-	-	+
S. Schwarzengrund (S141)	NR	16	+	-	+
S. Worthington (S146)	32	NR	-	-	+
S. Worthington (S147)	64	NR	-	-	+
S. *enterica* (S103)	8	NR	-	-	+
S. *enterica* (O:4,5) (S108)	32	64	+	-	-
S. *enterica* (S119)	16	NR	-	-	+
S. *enterica* (S127)	512	32	-	-	+
S. *enterica* (S142)	64	NR	-	-	+
S. *enterica* (O:13,23) (S180)	NR	128	-	-	+
S. *enterica* (S182)	NR	256	-	-	+

AMP, ampicillin; CAZ, ceftazidime; NR, non-resistant; +, present; -, absent

Supplementary Table 4

Minimum inhibitory concentration (MIC) values to tetracycline and presence of *tet* genes in *Salmonella enterica*.

Isolate (identification number)	MIC (µg/mL)	*tet*A	*tet*B	*tet*C
S. Gallinarum (S193)	8*	+	-	-
S. Infantis (S130)	64	+	-	-
S. Infantis (S179)	64	+	-	-
S. Cerro (S176)	64	-	-	-
S. Montevideo (S205)	64	-	-	-
S. Schwarzengrund (S140)	8*	+	+	-
S. enterica (O:13,23) (S180)	64	-	+	-
S. Heidelberg (S109)	64	-	-	+
S. Heidelberg (S110)	64	+	-	+
S. Heidelberg (S111)	64	+	-	+
S. Worthington (S146)	64	+	-	-
S. Worthington (S139)	64	+	-	-
S. Worthington (S147)	64	+	-	-
S. Worthington (S170)	128	+	-	-
S. Worthington (S113)	128	+	-	-
S. Worthington (S185)	64	+	-	-
S. Worthington (S192)	64	+	-	-
S. Worthington (S194)	64	-	-	-
S. Worthington (S204)	128	-	-	-
S. Senftenberg (S201)	256	+	+	-
S. Senftenberg (S138)	128	-	-	-
S. Senftenberg (S112)	64	+	-	-
S. Senftenberg (S129)	64	-	-	-
S. Senftenberg (S114)	64	+	-	-
S. Senftenberg (S165)	32	+	-	-
S. Senftenberg (S123)	64	+	-	-
S. Senftenberg (S124)	64	+	-	-
S. Senftenberg (S164)	64	+	-	-
S. Senftenberg (S167)	256	+	-	-
S. Senftenberg (S171)	64	+	-	-
S. Senftenberg (S177)	64	+	-	-
S. Senftenberg (S184)	64	+	-	-
S. enterica (S182)	128	-	+	-
S. enterica (S134)	8*	-	-	+
S. enterica (S195)	128	+	-	-
S. enterica (S196)	64	+	-	-
S. enterica (S202)	64	+	-	-
S. enterica (S203)	64	+	-	-
S. enterica (S118)	64	-	-	-
S. enterica (S125)	8*	-	-	-

* Intermediate resistance to tetracycline; +, present; -, absent

Antimicrobials	Drug concentration (μg/mL)																	
	0.03	0.06	0.12	0.25	0.5 (0.5/9.5)	1 (1/19)	2 (2/38)	4 (4/76)	8 (8/152)	16 (16/304)	32 (32/608)	64 (64/1,216)	128 (128/2,432)	256	512	1,024	2,048	4,096
Ampicillin								1	1	1	2	3	2	2	2	2		
Ceftazidime						34		1	3	1	4	3	1		2			
Chloramphenicol						2	1	1	1	4	5	3						
Ciprofloxacin	4		6	7	2	2												
Nalidixic acid							12	14	2	4	4		1	7	6	11	4	2
Sulfamethoxazole									21			6	19	24	16	11	28	
Trimethoprim/ sulfamethoxazole					10			2	6	10	11	8						
Tetracycline							4	1	4		1	27	6	2				

Fig. 1. Minimum inhibitory concentration (MIC) to antimicrobial agents tested against intermediate resistant or resistant *Salmonella enterica* isolates previously evaluated by disk diffusion test. The concentration of trimethoprim/sulfamethoxazole is shown in parentheses, and the concentration range used for each antimicrobial is shown in gray. Solid lines represent breakpoints established by CLSI (2013).

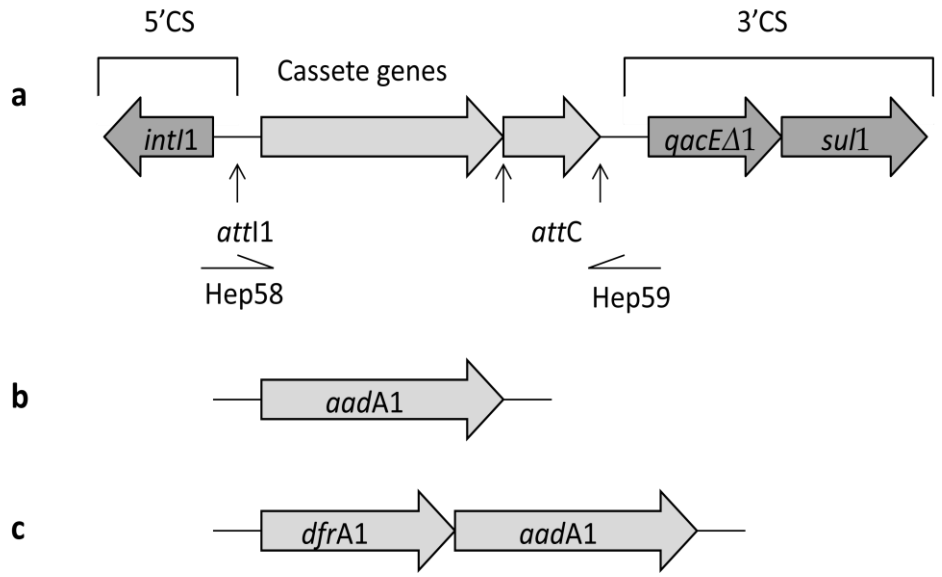

Fig. 2. Structures of class 1 integrons. **a**. Schematic structure of a class 1 integron with the 5'CS (Conserved Segments) with the class 1 integrase gene *intI*1 and *attI*1 site, and typical 3'CS with *qacEΔ*1 and *sul*1. Location and direction of gene transcription are indicated. Inserted gene cassettes are represented by unfilled arrows and their associated *att*C sites are indicated. Hep58 and Hep59 primer annealing sites are indicated. **b**. Structure of the variable region between 5'CS and 3'CS of a typical class 1 integron presenting the insertion of *aad*A1. **c**. Structure of the variable region between 5'CS and 3'CS of a typical class 1 integrons presenting the insertion of *dfr*A1 and *aad*A1.

Capítulo 3

Considerações Finais

3.1 Considerações finais

Sorovares de *Salmonella enterica* estão entre os principais patógenos causadores de gastrenterites em humanos, estando envolvidos em surtos alimentares relacionados com o consumo de alimentos contaminados, principalmente os de origem avícola, o que pode constituir um grande problema de saúde pública (9,10,11,12). Além disso, este patógeno pode ser responsável por perdas econômicas significativas na produção de frango, especialmente por representar uma barreira para a exportação (16,17,18). A contaminação de produtos avícolas por *S. enterica* pode ser ainda mais preocupante quando estas bactérias apresentarem resistência a antimicrobianos, o que tem sido associado com o uso frequente de antimicrobianos em doses terapêuticas e especialmente sub-terapêuticas inseridas na alimentação animal como promotores de crescimento (25,28,29). Desta forma, nos últimos anos, várias medidas de controle relacionadas ao uso de antimicrobianos na produção animal têm sido adotadas, desde a retirada de promotores de crescimento que utilizam antimicrobianos na alimentação animal até a restrição no uso terapêutico de algumas drogas em alguns países (94). Dentro deste contexto, este trabalho procurou investigar padrões e determinantes de resistência em isolados de *S. enterica*, com especial atenção aos subprodutos avícolas que poderiam constituir uma forma de reintrodução de isolados resistentes na cadeia de produção. Entretanto, os resultados obtidos neste trabalho indicam que, provavelmente, as farinhas de aves possam não representar um reservatório de genes de resistência tão importante quanto o próprio ambiente de criação das aves, uma vez que foram observadas taxas de resistência significativamente maiores entre os isolados de amostras ambientais dos aviários, inclusive com fenótipo de multi-resistência, quando

comparadas àquelas obtidas nos isolados de farinhas de aves. Desta forma, aliada à preocupação com a intensa utilização de antimicrobianos na produção avícola, deve-se enfatizar o emprego de medidas adequadas de higienização e desinfecção dos aviários para evitar que o ambiente atue como reservatório de *S. enterica* resistentes a antimicrobianos, o que poderia implicar na possível disseminação de genes de resistência entre bactérias de diferentes lotes.

Consistente com o contexto descrito observou-se a detecção de um maior percentual de isolados resistentes à sulfonamida em relação aos demais antimicrobianos testados, uma vez que esta droga têm sido uma das mais utilizadas ao longo do tempo na produção animal. Esta observação reforça a preocupação de que a utilização de antimicrobianos como profilaxia, promoção de crescimento, ou até mesmo com objetivos terapêuticos na produção animal, pode selecionar isolados resistentes à droga empregada e a outros antimicrobianos cujos determinantes de resistência sejam co-transportados com os genes de resistência para a droga utilizada. A pressão de seleção de isolados resistentes associada ao fato de que o fenótipo de resistência é frequentemente conferido por determinantes que podem ser carreados por elementos genéticos móveis, como foi demonstrado nos isolados analisados neste trabalho, indica um potencial de disseminação de genes de resistência entre *S. enterica* ao longo da cadeia produtiva, bem como para outras bactérias patogênicas, diminuindo as opções terapêuticas, e para bactérias pertencentes à microbiota normal, que podem atuar como reservatórios de genes de resistência.

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