Pontifícia Universidade Católica do Rio Grande do Sul
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Júlia Puffal

Investigação de genes de resistência a antimicrobianos e da capacidade de formação de biofilme em isolados de *Salmonella* Enteritidis

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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

Co-orientador: Prof. Dr. Carlos Alexandre Sanchez Ferreira

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Aprovado em_____de_____de_____

BANCA EXAMINADORA:

Fernanda Bueno Morrone

Adriano Sakai Okamoto

Wladimir Padilha da Silva

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Resumo

A *Salmonella* Enteritidis tem sido o sorotipo mais prevalente no Brasil, principalmente associado a produtos de origem avícola, que têm sido prioritariamente envolvidos em surtos de doenças transmitidas por alimentos. A alta prevalência de suscetibilidade reduzida a antimicrobianos em diversos sorotipos de *Salmonella* isolados de amostras relacionadas a animais de produção, a humanos e a alimentos de origem animal vem sendo relatada no mundo inteiro. Com isto, tem aumentado o interesse em investigar os mecanismos genéticos envolvidos na resistência a antimicrobianos, especialmente elementos genéticos capazes de carrear cassetes de genes de resistência, o que pode constituir a origem de cepas multi-resistentes. Além da resistência a antimicrobianos determinada geneticamente, as bactérias também podem apresentar resistência pela habilidade de formar biofilme, protegendo-as de estresses ambientais, favorecendo a colonização e persistência desses microrganismos no ambiente. Deste modo, o objetivo deste estudo foi determinar a suscetibilidade a antimicrobianos de cepas de *S.* Enteritidis e investigar os genes envolvidos nas principais resistências determinadas, bem como avaliar a capacidade de formação de biofilme destas cepas. Para tanto, foram analisadas 47 cepas de *S.* Enteritidis isoladas de humanos, aves, suínos e alimentos. Dezesseis isolados (34%) se mostraram fenotipicamente resistentes a pelo menos um antimicrobiano testado. Destes, quatro apresentaram integron de classe 1. Todas as cepas resistentes à sulfonamida apresentaram concomitantemente os genes *sul*1 e *sul*2. Os genes *str*A, *str*B, *aad*A e *aad*B foram identificados na maioria dos isolados que apresentaram resistência a aminoglicosídeos, sendo que 92,9% apresentaram o gene *str*A, 71,4% *str*B, 7,1% *aad*A e 50% *aad*B. O gene *tet*B foi detectado em duas das três cepas resistentes à tetraciclina e o *tet*C em uma. Já as três cepas resistentes à ampicilina apresentaram o gene bla_{TEM}. No total, dentre as 47 cepas de *S.* Enteritidis testadas, 89,4% foram capazes de formar biofilme em placas de poliestireno. Dentre estas, 42,4% foram consideradas fracas produtoras de biofilme, 14,9% produtoras moderadas e 34% fortes produtoras. Foi demonstrado que a maioria das cepas que mostraram resistência a pelo menos um

antimicrobiano foram capazes de formar biofilme, o que aumenta a preocupação a respeito da contaminação de alimentos, especialmente pela possibilidade de persistência de microrganismos resistentes a antimicrobianos no ambiente e a subsequente disseminação destas cepas para humanos.

Palavras-chave: *Salmonella* Enteritidis; resistência a antimicrobianos; genes de resistência; biofilme.

Abstract

Salmonella Enteritidis is the most prevalent serotype isolated in Brazil, mainly associated with poultry products, which have been primarily involved in foodborne disease outbreaks. The high prevalence of reduced susceptibility to antimicrobial agents in various *Salmonella* serotypes isolated from samples related to livestock animal, animal foods and human has been reported worldwide. Therefore, has increased the interest in investigating the genetic mechanisms involved in resistance to antimicrobial agents, especially genetic elements capable of carrying resistance genes cassettes, which could be the origin of multi-resistant strains. Besides the genetically determined antimicrobial resistance, bacteria can also exhibit resistance by the ability to form biofilm, which protects bacteria from environmental stresses, favoring the colonization and persistence of these microorganisms in the environment. Thus, the aim of this study was to determine the antimicrobial susceptibility of *S.* Enteritidis strains and investigate the genes involved in the main resistance determined, as well as evaluate the ability of these strains in to produce biofilm. Forty-seven *S.* Enteritidis strains isolated from human, poultry, swine, and food were analyzed. Sixteen isolates (34%) were phenotypically resistant to at least one antibiotic tested. Of these, four isolates harbored class 1 integron. All strains resistant to sulfonamide had concomitantly genes *sul*1 and *sul*2. The genes *str*A, *str*B, *aad*A and *aad*B were identified in the majority of the aminoglycosides resistant isolates, whereas 92.9% showed *str*A, 71.4% *str*B, 7.1% *aad*A and 50% *aad*B. The *tet*B gene was detected in two of the three strains resistant to tetracycline, and *tet*C in one. In the three strains resistant to ampicillin the bla_{TEM} gene was detected. Overall, among the 47 *S.* Enteritidis tested, 89.4% strains were able to form biofilm on polystyrene plates. Among these, 42.4% were considered weak biofilm producers, 14.9% moderate producers and 34% strong producers. It has been demonstrated that the majority of the *S.* Enteritidis strains that showed resistance to at least one antimicrobial agent were able to form biofilm, which increases concerns about food contamination, especially by the possibility of persistence of bacteria resistant to antibiotics on the environment and the subsequently dissemination of these strains to human.

Keywords: *Salmonella* Enteritidis; antimicrobial resistance; resistance genes; biofilm.

Lista de abreviações

AMI – Amicacina

AMP – Ampicilina

ATCC – *American Type Culture Collection*

BGA – *Bright Green Agar*

BHI – *Brain Heart Infusion*

CDC – *Centers for Disease Control and Prevention*

CFC – Cefaclor

CIP – Ciprofloxacina

CHL – *Chloramphenicol* (Cloranfenicol)

CLSI - *Clinical Laboratory Standards Institute*

CMY – Cefalosporinase

CTX-M – *Cephalosporinase* (Cefotaximase)

DNA – Ácido desoxirribonucleico

DTA – Doenças Transmitidas por Alimentos

ENO – Enrofloxacina

FLO – Florfenicol

GEN – Gentamicina

LB – *Lysogeny Broth*

MAPA - Ministério da Agricultura, Pecuária e Abastecimento

MDR – *Multidrug Resistance*

MIC – *Minimum Inhibitory Concentration* (Concentração inibitória mínima)

NAL – Ácido Nalidíxico

NEO – Neomicina

OD – *Optical Density* (Densidade Ótica)

ORF – *Open reading frame* (Fase de leitura aberta)

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

PT – *Phage type* (Fagotipo)

SPT – Espectinomicina

STR – Estreptomicina

SUL – Sulfonamida

SXT – Sulfonamida/Trimetoprim

TIO – Ceftiofur

TET – Tetraciclina

TOB – Tobramicina

TSB – *Trypticase Soy Broth*

UBABEF – União Brasileira de Avicultura

Sumário

Capítulo 1

Introdução

Objetivos

1. 1 Introdução

As bactérias do gênero *Salmonella* são caracterizadas como bacilos Gram negativos, móveis em sua maioria, incapazes de fermentar lactose e formar esporos, produtores de H_2S e capazes de descarboxilar a lisina e a ornitina. Este gênero é composto por duas espécies: *Salmonella bongori* e *Salmonella enterica*; sendo a *S. enterica* 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) (1,2). O esquema de Kauffmann-White (1981) (3) classifica as subespécies de *Salmonella* em sorotipos de acordo com a caracterização dos antígenos somáticos (O), flagelares (H) e de virulência (Vi). Atualmente, existem 2.610 sorotipos descritos, sendo a maioria destes pertencentes à *S. enterica* subsp. *enterica* (4). Os isolados pertencentes a um mesmo sorotipo ainda podem ser caracterizados quanto à suscetibilidade a bacteriófagos líticos, determinando o seu fagotipo, o que pode ser importante epidemiologicamente na medida em que pode auxiliar na diferenciação da origem de isolados (5–7).

Os sorotipos de *S. enterica* são amplamente distribuídos na natureza, sendo que alguns podem causar doença tanto em humanos quanto em animais (8–16), dependendo do sorotipo, dose infectante e condições do indivíduo infectado (17–19). Dentre estes, alguns dos mais frequentes são *S.* Enteritidis, *S.* Typhimurium, *S.* Newport, *S.* Heidelberg, *S.* Infantis, *S.* Virchow, *S.* Hadar e *S.* Agona, distribuídos de maneiras distintas no mundo (18,20–22). No Brasil, o sorotipo mais prevalente é a *S.* Enteritidis (13,21,23,24), sendo o PT4 (fagotipo 4) o fagotipo predominantemente encontrado (25–29). A *S.* Enteritidis tem sido bastante associada a produtos de origem avícola (9–11,20,30,31), mas também, em menor escala, a amostras derivadas de suínos (8,12). Estes produtos têm sido prioritariamente envolvidos em surtos de doenças transmitidas por alimentos, embora uma grande variedade de animais seja considerada reservatório de *Salmonella* spp. do grupo não-tifóide (10,22,32,33).

De acordo com os dados epidemiológicos de doenças transmitidas por alimentos (DTA) apresentados pelo Ministério da Saúde, na última década, dos agentes etiológicos identificados ocasionando surtos notificados no Brasil, cerca de 40% foram identificados como *Salmonella* spp., demonstrando a importância desta bactéria durante o processamento do alimento, bem como a sua detecção no produto final (34). No Brasil, este dado é especialmente preocupante devido à associação de *Salmonella* spp. com a cadeia de produção avícola, uma vez que segundo a União Brasileira de Avicultura, o Brasil é o terceiro maior produtor mundial de carne de frango, e líder em sua exportação, sendo a região Sul juntamente com o estado de São Paulo, os principais produtores (35).

Desta forma, a constante vigilância da presença de *Salmonella* spp. em produtos de origem animal, bem como a sua caracterização quanto à suscetibilidade a antimicrobianos, são de grande importância para o fornecimento de produtos microbiologicamente seguros para o mercado interno e para a adequação às exigências dos exportadores.

A resistência a antimicrobianos em bactérias isoladas de animais de produção é um fator preocupante por constituir um risco de infecção de difícil tratamento, quando este é preconizado, em humanos após o consumo do alimento contaminado (36,37), bem como pela possibilidade de disseminação de determinantes de resistência a outras bactérias (38–40).

A utilização de antibióticos como promotores de crescimento, profilaxia e tratamento de doenças bacterianas em animais de produção, como aves e suínos, vem sendo discutida como uma possível origem de resistência bacteriana a antimicrobianos. Diversos antibióticos, como sulfonamidas, tetraciclinas e aminoglicosídeos, têm sido empregados com estas finalidades, e sua utilização, principalmente em doses subclínicas, pode contribuir para a emergência da resistência bacteriana a antimicrobianos, tornando estes animais possíveis reservatórios de microrganismos resistentes (36,41–46).

A alta prevalência de suscetibilidade reduzida a antimicrobianos em diversos sorotipos de *Salmonella* isolados de amostras relacionadas a animais de produção,

alimentos de origem animal e humanos vem sendo relatada no mundo inteiro (23,24,47–53), inclusive com relatos de isolados multi-resistentes (24,48,51,53,54). Em relação à *S*. Enteritidis no Brasil, foi observado que há uma prevalência de resistência a antimicrobianos relativamente alta entre os isolados testados (24,28,54–56).

A detecção de resistência a antimicrobianos em microrganismos resistentes a antimicrobianos leva ao interesse de investigar os mecanismos genéticos envolvidos na resistência, sendo que o principal fator da origem de cepas multi-resistentes é a capacidade da bactéria adquirir e disseminar genes através de elementos genéticos móveis, como plasmídeos e transposons (57–64).

Transposons e plasmídeos podem carrear integrons, que são elementos gênicos que incorporam sequências exógenas através de recombinação sítio-específica, podendo estar envolvidos na disseminação de genes de resistência a antimicrobianos (59,60,65,66). Os integrons das classes 1, 2 e 3 são encontrados em diversas espécies bacterianas, sendo que todos os integrons descritos até o momento possuem três elementos chave necessários para a captura desses genes: o gene *intI*, que codifica para a integrase pertencente à família da tirosina-recombinase; um sítio de recombinação primário (*attI*) e um promotor (P_c) que direciona a transcrição dos genes capturados (66,67). Cada cassete gênico inserido no integron geralmente possui um gene, ou uma *open reading frame* (ORF) cuja função é desconhecida, e uma sequência de repetição invertida denominada *att*C, ou "elemento de 59 bases", necessária para a integração do cassete no integron (68,69). Em *Salmonella* spp., os integrons de classe 1 são os mais encontrados, geralmente associados a plasmídeos ou transposons, e estão frequentemente relacionados a microrganismos multi-resistentes (57,58,60,61,70,71). Os integrons de classe 2 e 3 são normalmente encontrados em transposons, sendo o integron de classe 2 menos reportado nas bactérias pertencentes a este gênero do que o de classe 1, não existindo relato, até o momento, de integron de classe 3 em *Salmonella* spp. (67,72). Os integrons de classe 1 apresentam uma região conservada 5' (5'CS), onde está localizado o gene *intI*1, bem como o sítio de recombinação *attI*, e outra região conservada na porção 3' (3'CS), onde geralmente são encontrados os genes *qacE*Δ1, que codificam para a resistência a compostos de quaternário de amônio, e *sul*1, que confere resistência às sulfonamidas (59,73). Entre estas duas regiões conservadas pode

ocorrer a inserção de cassetes gênicos, que, comumente, contêm genes que conferem resistência a antimicrobianos.

Em *Salmonella* spp., a resistência às sulfonamidas tem sido associada principalmente ao gene *sul*1. Entretanto, os genes *sul*2 e *sul*3 também podem mediar esta resistência, embora sejam menos prevalentes entre bactérias deste gênero (39,57,59,63,74–76). O *sul*2 está geralmente localizado em plasmídeos e não tem sido descrito em integrons (57,77), enquanto o gene *sul*3 vem sendo associado a integrons de classe 1, quando há a ausência de *sul*1 (61).

Além de se observar uma frequente resistência às sulfonamidas em diversos sorotipos de *Salmonella* spp., principalmente em isolados de origem avícola (24,28,55), tem-se encontrado uma alta prevalência de isolados de *Salmonella* spp. provenientes de animais de produção resistentes à tetraciclina (61,78–81). São diversos os genes que conferem resistência a este antimicrobiano especialmente relacionados a bombas de efluxo, sendo os mais encontrados em *Salmonella* spp.: *tet*A, *tet*B, *tet*C, *tet*D, *tet*E e *tet*G, principalmente os dois primeiros (59,61,63,74,75,78,79,82–84). Nenhum destes genes parecem estar associados a integrons (58,59,61,70,71,85), estando geralmente presentes em cromossomos, na Ilha Genômica de *Salmonella* 1 (SGI-1), ou ainda em plasmídeos (38,86,87).

Outra classe de antimicrobianos a qual *Salmonella* spp. têm se mostrado resistentes são os aminoglicosídeos. Uma alta prevalência de resistência à estreptomicina tem sido relatada (24,48,49,76,79,81), sendo esta resistência geralmente atribuída à presença dos genes *str*A e *str*B, que não têm sido associados a integrons (40,53,64,88–91), ou ainda à presença de *aad*A, que tem sido encontrado inserido na região variável entre 5’CS e 3’CS do integron de classe 1 (58,88,92). Além disto, a resistência à gentamicina pode estar presente em *Salmonella* spp., podendo ser atribuída à presença do gene *aad*B, que está fortemente relacionado a integrons (24,28,38,58,61,82,92,93).

Uma vez que os β-lactâmicos estão entre os antibióticos de escolha no tratamento da salmonelose, a investigação da resistência a esta classe de antimicrobianos em isolados de *Salmonella* spp. tem sido amplamente relatada. Alguns

artigos trazem uma porcentagem considerável de resistência a essa classe de antibióticos em diversos sorovares de *Salmonella* (48,51,53,94), entretanto, esta porcentagem cai em isolados de *S.* Enteritidis (28,53). A resistência a β-lactâmicos é determinada principalmente pela produção de β-lactamases, que podem ser de espectro estendido (ESBL), como a CTX-M. Esta enzima está entre as principais responsáveis pela resistência de *Salmonella* spp. às cefalosporinas. A CTX, abreviatura de cefotaximase, tem potente atividade hidrolítica contra esta cefalosporina (95) e é codificada pelo gene $bla_{\text{CTX-M}}$, podendo ser encontrado inserido em estruturas genéticas como integrons de classe 1 e transposons, sendo frequentemente associado a plasmídeos (96). A β-lactamase CMY, uma cefalosporinase codificada pelo gene bla_{CMY}, também tem sido descrita em isolados de *Salmonella* spp. (97–100). Já a resistência de *Salmonella* spp. às penicilinas tem sido associada com a presença do gene bla_{TEM} (89,101). Além destas, outras enzimas responsáveis pela resistência a β-lactâmicos também já foram descritas em *Salmonella* spp., como SHV e PSE (53,94,100).

A resistência a antimicrobianos pode ainda ser agravada pela capacidade das bactérias em formar biofilme, tendo sido observada uma maior resistência a antimicrobianos em bactérias presentes nesta estrutura, quando comparadas a células planctônicas (102). Além disso, o biofilme protege as bactérias de estresses ambientais, tais como a ação de desinfetantes, favorecendo a colonização e persistência desses microrganismos no ambiente (103,104). Os biofilmes são comunidades microbianas complexas que se aderem a superfícies bióticas ou abióticas, envoltas por matriz extracelular polimérica (105). Acredita-se que as bactérias, inclusive *Salmonella* spp., possuem uma alta capacidade de adesão e formação de biofilmes em superfícies de materiais hidrofóbicos, como plástico, material utilizado amplamente em uma planta de processamento de alimentos em equipamentos e utensílios (105,106). Assim, pode-se formar um reservatório de patógenos nestas estruturas, aumentando o risco de contaminação na indústria alimentícia, levando a problemas de saúde pública e potencial impacto econômico (103–105,107–109).

1. 2 Objetivos

1. 2. 1 Objetivo Geral

Este trabalho teve como objetivo determinar a capacidade de formação de biofilme e avaliar a presença de integrons e de genes de resistência a antimicrobianos em cepas de *S*. Enteritidis isoladas de humanos, aves, suínos e alimentos.

1. 2. 2 Objetivos Específicos

- Determinar a resistência de *S*. Enteritidis através do método de disco-difusão frente a quinolonas, fluoroquinolonas, aminoglicosídeos, β-lactâmicos, fenicóis, tetraciclina, sulfonamida e trimetoprim;
- Determinar a concentração inibitória mínima para ácido nalidíxico, ampicilina, cloranfenicol, sulfonamida, sulfonamida/trimetoprim e tetraciclina nos isolados que apresentaram resistência a estas drogas no método de disco-difusão;
- Determinar a presença de integrons das classes 1, 2 e 3 em isolados de *S*. Enteritidis resistentes a pelo menos um antimicrobiano;
- Determinar a presença dos genes *sul*1, *sul*2 e *sul*3 em isolados de *S*. Enteritidis fenotipicamente resistentes à sulfonamida;
- Detectar os genes $bla_{\text{CTX-M}}$, bla_{CMY} e bla_{TEM} em isolados de *S*. Enteritidis fenotipicamente resistentes a β -lactâmicos;
- Determinar a presença dos genes *str*A, *str*B, *aad*A e *aad*B em isolados de *S*. Enteritidis fenotipicamente resistentes a aminoglicosídeos;
- Determinar a presença dos genes *tet*A, *tet*B e *tet*C em isolados de *S*. Enteritidis fenotipicamente resistentes à tetraciclina;
- Avaliar a capacidade de formação de biofilme das cepas de *S*. Enteritidis.

Capítulo 2

2. 1 Artigo Científico

Investigation of antimicrobial resistance genes and biofilm formation capacity in *Salmonella* Enteritidis

Artigo científico submetido ao periódico científico *Food Research International*, publicado pela Elsevier.

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Investigation of antimicrobial resistance genes and biofilm formation capacity in *Salmonella* Enteritidis

Júlia Puffal, Valdir C. Barth Jr., Carlos A.S. Ferreira, Sílvia D. Oliveira*

Laboratório de Imunologia e Microbiologia, Faculdade de Biociências, PUCRS, Porto Alegre, Brazil

*Corresponding author:

Sílvia Dias de Oliveira

Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS)

Av. Ipiranga 6681, 90619-900, Porto Alegre, Brasil

E-mail address: silviadias@pucrs.br

Tel.: +55-51-33534953; fax: +55-51-33203568

ABSTRACT

The development of antimicrobial resistance in *Salmonella* Enteritidis constitutes a risk for human infection following the consumption of contaminated food. Besides the genetically mediated antimicrobial resistance, bacteria can also present phenotypic resistance due to the ability to form biofilm. Therefore, the aim of this study was to determine the antimicrobial susceptibility of *S*. Enteritidis strains and investigate the genes involved in the main antimicrobial resistance found, as well as evaluate the ability of these strains to form biofilm. Forty-seven Southern Brazil *S*. Enteritidis strains isolated from humans, poultry, swine, broiler carcasses and food involved in outbreaks, as well as strains from other countries were used in this study. Overall, 16 (34%) isolates were resistant to at least one antimicrobial, and four harbored class 1 integron. Sulfonamide-resistant strains presented *sul*1 and *sul*2 genes. *str*A, *str*B, *aad*A and *aad*B were identified in the isolates presenting resistance to aminoglycosides, and *tet*B and *tet*C were detected when tetracycline resistance was observed. All ampicillin-resistant isolates harbored the bla_{TEM} gene. In total, among the 47 *S*. Enteritidis tested, 89.4% strains were able to produce biofilm in polystyrene microplates. We demonstrated that the majority of the *S*. Enteritidis strains that showed some antimicrobial resistance were able to form biofilm, which increases the concerns about food contamination, especially because of the possibility of the persistence of antimicrobial resistant bacteria in the environment and the subsequently spread to human.

Keywords:

Salmonella; antimicrobial resistance; resistance genes; biofilm

1. Introduction

Salmonella spp. are widely spread in nature (Arguello, Carvajal, Collazos, García-Feliz, & Rubio, 2012; Dunkley et al., 2009; Rostagno & Callaway, 2012), being *Salmonella* Enteritidis one of the most prevalent serovars isolated worldwide (Finstad, O'Bryan, Marcy, Crandall, & Ricke, 2012; Hendriksen et al., 2011; Jones et al., 2008; Medeiros, Oliveira, Rodrigues, & Freitas, 2011). A variety of producing animals are considered reservoirs of non-typhoid *Salmonella*, although *S*. Enteritidis has been especially associated to poultry (Braden, 2006; Dunkley et al., 2009; Finstad et al., 2012; Howard, O'Bryan, Crandall, & Ricke, 2012; Martelli & Davies, 2012; Silva & Duarte, 2002), and less extensively to swine (Chuanchuen & Padungtod, 2009; Gomes-Neves et al., 2012; Shinohara et al., 2008), since products derived from these animals have been involved in foodborne outbreaks in human (Chen, Wang, Su, & Chiu, 2013; Foley & Lynne, 2008; Gormley et al., 2011).

The use of antimicrobial agents as growth promoters, prophylaxis and treatment of bacterial diseases in livestock is widespread throughout the world. Many antimicrobial agents have been employed, and their extensive use in subclinical doses can contribute to the emergence of antimicrobial resistance among bacteria, making these animals possible reservoirs of resistant microorganisms (Emborg et al., 2007; Schwarz, Kehrenberg, & Walsh, 2001; Smith, Harris, Johnson, Silbergeld, & Morris, 2002). This scenario can constitute a risk for a raise in the difficulties for the treatment of human infections following the consumption of contaminated food, since antibiotic therapy may be hampered when needed (Aarestrup, 1999; Smith et al., 2002).

Besides the genetically mediated antimicrobial resistance, bacteria can also present phenotypic resistance due to the production of biofilms, being observed that the antimicrobial resistance is greater in the microorganisms embedded in this structure when compared to planktonic cells (Capita & Alonso-Calleja, 2013). Biofilm protects bacteria from external agents of stress, favoring the colonization and persistence of these microorganisms in the environment (Shi & Zhu, 2009; Vestby, Møretrø, Langsrud, Heir, & Nesse, 2009). It is believed that *Salmonella* spp. possess great affinity for adhering and forming biofilm in hydrophobic material surfaces, such as plastic, which is widely used in equipments and utensils in food processing plants

(Steenackers, Hermans, Vanderleyden, & de Keersmaecker, 2012; Tondo et al., 2010). Thus, a reservoir of pathogens can be formed in these structures, increasing the contamination risk in the food industry, leading to public health problems and potential economic impact (Manijeh, Mohammad, & Roha, 2008; Shi & Zhu, 2009; Steenackers et al., 2012; Vestby et al., 2009).

Therefore, the purpose of this study was to determinate the antimicrobial susceptibility of *S*. Enteritidis isolated from poultry, swine, broiler carcasses, food, and human, and to investigate the genes involved in the main antimicrobial resistance phenotypes found, as well as to evaluate the ability of these strains to form biofilm in a polystyrene surface.

2. Materials and methods

2.1. Bacterial strains

Forty-seven *S*. Enteritidis strains phage types 4, 4a, 6, 6a, 7, 7a, 9 and 11 were analyzed in this study. Thirty-eight of these strains were previously isolated from human (n = 7), poultry (n = 7), swine (n = 10), broiler carcasses (n = 7) and food involved in outbreaks (n = 7) in Southern Brazil, and nine epidemiologically unrelated strains were obtained from other countries (Zimbabwe, Egypt, Italy, Albania and Tanzania). The strains were stored in trypticase soy broth (TSB) (Biobrás, Brazil) with 20% glycerol at -80ºC for long-time storage. The strains were grown on Brilliant Green Agar (Himedia, India), and one colony was cultivated in TSB at 37ºC for 24 h.

2.2. Antimicrobial susceptibility test

The antimicrobial susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI, 2012) for the agar disk diffusion. The antimicrobial agents tested were: nalidixic acid (NAL; 30 µg), amikacin (AMI; 30 µg), ampicillin (AMP; 10 µg), cefaclor (CFC; 30 µg), ciprofloxacin (CIP; 5 µg), chloramphenicol (CHL; 30 µg), streptomicyn (STR; 10 µg), gentamicin (GEN; 10 µg), sulfonamide (SUL; 300 µg), sulfonamide/trimethoprim (SXT; 25 µg), tetracycline (TET; 30 µg) and tobramicyn (TOB; 10 µg). The inhibition zones were measured and interpreted according to the CLSI, M100-S22 (2012). Additionally, the antimicrobial

susceptibility to ceftiofur (TIO; 30 µg), enrofloxacin (ENO; 5 µg), spectinomicyn (SPT; 100 µg), florphenicol (FLO; 30 µg), and neomicyn (NEO; 30 µg) was determined by agar disk diffusion and interpreted following the manufacturer instructions (Cefar, Brazil). Strains presenting resistance or intermediate resistance to nalidixic acid, ampicillin, chloramphenicol, sulfonamide, sulfonamide/trimethoprim and tetracycline were submitted to broth microdilution method according to CLSI (2012) for determination of the minimum inhibitory concentration (MIC). The methods were performed using the reference culture *Escherichia coli* ATCC 25922 as quality control.

2.3. Resistance genes detection

2.3.1. Resistance genes

All strains were screened for the presence of integrons using degenerated oligonucleotide primer sequences targeting the *intI*1, *intl*2 and *intl*3 genes (White, McIver, Deng, & Rawlinson, 2000). The integron-positive strains were then submitted to detection of class 1 and class 2 integrons using specific oligonucleotide primer pairs targeting *intI*1 and *intI*2 genes, respectively (Lévesque, Piché, Larose, & Roy, 1995; White et al., 2001). The variable region of the class 1 integron of *intI*1-positive strains was amplified using primers annealing within the 5' and 3' conserved sequences that flank it (White et al., 2000).

The strains phenotypically resistant to sulfonamide were evaluated for the presence of the genes *sul*1 (Grape, Sundström, & Kronvall, 2003), *sul*2 (Kerrn, Klemmensen, Frimodt-Moller, & Espersen, 2002), and *sul*3 (Chuanchuen & Padungtod, 2009). Detection of the genes $bla_{\text{CTX-M}}$ (Edelstein, Pimkin, Palagin, Edelstein, & Stratchounski, 2003), bla_{CMY} (Winokur, Vonstein, Hoffman, Uhlenhopp, & Doern, 2001), and bla_{TEM} (Carlson et al., 1999) were performed in the isolates phenotypically resistant to β-lactams. When showed to be resistant to aminoglycosides, the strains were tested for the presence of the genes *str*A, *str*B (Gebreyes & Altier, 2002), *aad*A (Madsen, Aarestrup, & Olsen, 2000), and *aad*B (Frana, Carlson, & Griffith, 2001). The strains phenotypically resistant to tetracycline were assessed for the detection of *tet*A, *tet*B, and *tet*C genes (Aarestrup et al., 2003). All oligonucleotide primer sequences used in PCR assays were previously described (Table 1).

2.3.2. DNA amplification

Bacterial genomic DNA was extracted by a method using guanidine isothiocyanate (Rademaker & de Bruijn, 1997). The PCR assays were performed, in duplicate, in a Veriti® Thermal Cycler (Applied Biosystems, USA) within a reaction mixture of 25 µL final volume, comprising 100 ng of DNA template, 1 U of *Taq* DNA polymerase (Invitrogen, Brazil), 1 X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl] (Invitrogen), 200 µM of each deoxyribonuceotide (Ludwig Biotecnologia, Brazil), and 0.8 µM of each primer (IDT, USA or Invitrogen). The $MgCl_2$ (Invitrogen) concentration for each reaction is shown in Table 1. Appropriate negative and positive controls were used in each analysis. The amplifications were performed using an initial denaturation step of 94 ºC for 5 min, followed by 30 to 35 cycles of denaturation at 94 ºC, annealing temperature depending on the primer set used (Table 1) and extension at 72 ºC, with a final extension at 72ºC for 7 min. PCR products were analyzed by electrophoresis on agarose gel stained with 0.5 µg/µL of ethidium bromide.

2.4 DNA sequencing

The amplified variable regions of class 1 integron were sequenced using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequences were then analyzed and compared to sequences in GenBank database using the software MEGA 5.1.

2.5 Biofilm formation

All isolates were tested for the ability to form biofilm on polystyrene microplates of 96 wells, in triplicate. Initially, a pre-cultivation was performed in TSB for 18 h at 37 ºC. One microliter from each bacterial suspension, comprising of approximately 10^5 colony-forming units (CFU), was inoculated in a well containing 200 µL of Lysogeny Broth (LB) and incubated at 37 ºC for 72 h. The negative control used consisted of only 200 µL of LB. Afterwards, the content of the microplates was discarded and each well was washed twice with 200 µL of phosphate buffered saline (PBS). The bacteria adhered to the polystyrene plate were then fixed at 60 ºC for 15 min. After, 250 µL of 0.1% crystal violet was added to each well and incubated for 5

min. The excess of dye was removed by running water and the plates were air dried. Then, the dye bound to the adhered cells was resolubilized with 250 µL of ethanol at 96º per well and after 15 min, the optical density of each well was measured at 570 nm (OD_{570}) with 5 seconds of agitation using a Spectra Max 190 (Molecular Devices, USA) microplate reader. The cut-off OD (OD_c) was defined as the mean OD plus three standard deviations of the negative control. Strains were classified as no biofilm producer when $OD \leq OD_c$, weak biofilm producer when $OD_c < OD \leq (2 \times OD_c)$, moderate biofilm producer when $(2 \times OD_c) < OD \leq (4 \times OD_c)$ and strong biofilm producer when $(4 \times OD_c) < OD$. All tests were carried out in triplicate and the results were averaged. A Student's *t* test ($p < 0.05$) was used to confirm the biofilm formation comparing to the negative control.

3. Results and discussion

Several *Salmonella* serovars, including *S.* Enteritidis, isolated from human, livestock animals, and animal derived food have been reported to be resistant to various antimicrobial agents (Hur, Jawale, & Lee, 2012; Kusumaningrum, Suliantari, & Dewanti-Hariyadi, 2012; Medeiros et al., 2011; Tajbakhsh et al., 2012; Yildirim, Gonulalan, Pamuk, & Ertas, 2011). In addition, antimicrobial-resistant pathogens, especially those multi-drug resistant, can be even more harmful when also present the ability to persist in the environment. Therefore, this study aimed to evaluate the biofilm production and investigate some resistance genes possibly involved in the phenotype of resistance detected in *S.* Enteritidis isolated from different sources in Southern Brazil and other countries. Overall, it was observed that 34% of the *S.* Enteritidis isolates were resistant to at least one antimicrobial agent tested, and seven antimicrobial resistance patterns were identified in the strains studied. The penta-resistance (ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline - ACSSuT) pattern was detected in two strains (Table 2), of which one was isolated from food involved in outbreak. It is known that this resistance pattern is increasingly prevalent in distinct serovars of *S. enterica*, and has been commonly found in strains that harbor class 1 integron (Gebreyes & Thakur, 2005; Hsu et al., 2013; Krauland, Marsh, Paterson, & Harrison, 2009), although not all determinants that characterize this phenotype are carried within it (Glenn et al, 2011; Firoozeh, Zahraei-Salehi, Shahcheraghi, Karimi, &

191 Aslani, 2012; Hsu et al., 2013). Here, class 1 integron was detected in these two strains,
192 as well as in other two strains that also showed resistance to sulfonamide. Class 2
193 integron was not identified, in agreement with the findings of other studies, which
194 demonstrate that class 1 integron is the most frequent in *Salmonella* spp. (Antunes,
195 Machado, Sousa, & Peixe, 2005; Jin & Ling, 2009; Kim et al., 2011; Naghoni et al.,
196 2010).

197 Gene cassettes that encode antimicrobial resistance determinants can be inserted
198 in a variable region between the 5' and 3' conserved sequences within class 1 integron
199 (Hall, Brown, Brookes, & Stokes, 1994; Lévesque et al., 1995). In this study, the
200 amplification of this variable region generated an amplicon of 800 bp in two
201 trimethoprim-resistant strains (Fig. 1; Table 2). In both of these integron fragments,
202 sequence analysis showed the presence of the trimethoprim resistance gene *dfr*A7
203 (AY245101.1; HM769861.1; HQ132376.1), which is in accordance with authors that
204 have already associated this amplicon size with the presence of *dfr*A (Antunes et al.,
205 2005; Kim et al., 2011). In another isolate, the amplification of the class 1 integron
206 variable region produced an amplicon of approximately 1500 bp. The sequence analysis
207 of this fragment showed *dfr*A5, encoding resistance to trimethoprim, and two open
208 reading frames, *orf*2 and *orf*D, which have unknown function (Peirano, Agersø,
209 Aarestrup, dos Reis, & Rodrigues, 2006). Although this variable region size can be
210 associated with the presence of *aad*A and *aad*B, in the absence of *dfr*A (Kim et al.,
211 2011), we could not detect these aminoglycosides resistance genes in this isolate, which
212 is consistent with the phenotypical antimicrobial resistance analysis (Table 2). A third
213 variable region size of approximately 1100 bp was identified in a strain that harbored
214 both *aad*A and *aad*B genes. The presence of *aad*A is commonly associated with this
215 variable region size (Gebreyes & Altier, 2002; Krauland et al., 2009; Wannaprasat,
216 Padungtod, & Chuanchuen, 2011) and sequencing the amplicon revealed the presence
217 of the *aad*A1 gene (HQ874651.1; EU200458.1; EF204551.1).

218 Our results showed that the four strains that harbored class 1 integron and were
219 phenotypically resistant to sulfonamide (8.5%), also carried *sul*1 gene, which is known
220 to be highly associated with class 1 integron (Antunes et al., 2005; Firoozeh et al., 2012;
221 Jin & Ling, 2009). All strains that harbored *sul*1 also carried *sul*2, which may be located

outside of integrons, and is often found within plasmids (Hoa, Nonaka, Viet, & Suzuki, 2008). The presence of the gene *sul*3 was not detected. These findings corroborate that *sul*1 and *sul*2 are the most frequently detected genes encoding resistance to sulfonamides in *Salmonella* spp. (Antunes et al., 2005; Glenn et al., 2011; Louden, Haarmann, Han, Foley, & Lynne, 2012).

Resistance to chloramphenicol was found in 4.3% of the strains, while6.4% were resistant to tetracycline and ampicillin. All isolates were susceptible to nalidixic acid, ciprofloxacin, enrofloxacin, amikacin, neomycin, cefaclor, ceftiofur, and florfenicol. The reduced susceptibility to aminoglycosides found in this study (29.8% to streptomycin, 19.1% to tobramycin and gentamicin, and 2.1% to spectinomycin) is consistent with other studies that found a greater percentage of resistance to this class of antimicrobials, especially to streptomycin, in *Salmonella* serovars, including *S.* Enteritidis (Firoozeh et al., 2012; Turki et al., 2012; Van Boxstael et al., 2012; Yildirim et al., 2011). We detected only one strain resistant to spectinomycin, which showed the four aminoglycoside resistance genes investigated. All strains but one that were phenotypically resistant to streptomycin harbored at least one gene responsible for the most important mechanisms of streptomycin resistance in *S. enterica* (Soufi et al., 2012). The *str*A was the most frequent in this group of isolates, being present in 92.9% of these strains, followed by the *str*B gene in 71.4%. The *aad*A gene was detected only in one strain. The same resistance genes were previously described as the most prevalent in *S.* Enteritidis by Zou et al. (2012) as well as in other *Salmonella* serovars (Srinivasan et al., 2008; Tajbakhsh et al., 2012; Glenn 2011). The *aad*B gene, which encodes resistance to gentamicin, was detected in two of the three strains that presented phenotypically this resistance, and three strains that showed intermediate resistance to gentamicin also harbored this gene. Although *aad*B is a gene encoding resistance to gentamicin frequently found in *Salmonella* spp., other genes responsible for this resistance that were not investigated here can be probably found in the *aad*B-negative strain phenotypically resistant to this antimicrobial (Bacci et al., 2012; Gebreyes & Altier, 2002; Louden et al., 2012; Smith et al., 2002; Wannaprasat et al., 2011). Two strains that presented resistance to streptomycin but no resistance to gentamicin were also positive for the presence of *aad*B, suggesting that this gene can be responsible for the resistance against other aminoglycoside, as also described by other authors (Glenn et

al., 2013; Hsu et al., 2013; Marrero-Ortiz et al., 2012). Furthermore, it was observed that the tobramycin resistance had a relation with the gentamicin resistance, since all the strains that demonstrated resistance to tobramycin also showed an intermediate resistance or resistance to gentamicin, which corroborates the findings indicating that these resistance phenotypes could be encoded by the same genes (Kozak, Boerlin, Janecko, Reid-Smith, & Jardine, 2009; Lévesque et al., 1995). Only one strain showed resistance to streptomycin and intermediate resistance to gentamicin and did not present any gene investigated. This finding was also described previously (Srinivasan et al., 2008), and the phenotype of resistance found may be due to several other less common genes found in *Salmonella*, such as *aac*, *aph* and *ant*, which have a much lower prevalence in this microorganism (Bacci et al., 2012; Gebreyes & Altier, 2002; Louden et al., 2012; Srinivasan et al., 2008).

All three strains that showed phenotypical resistance to ampicillin harbored bla_{TEM}. The prevalence of this gene encoding β-lactam resistance is well reported among *Salmonella* (Aslam et al., 2012; Chuanchuen & Padungtod, 2009; Glenn et al., 2013; Hur, Kim, Park, Lee, & Lee, 2011; Zou, Keelara, & Thakur, 2012). Although genes bla_{CMY} or $bla_{\text{CTX-M}}$ are also among the most common resistant determinants in β-lactams-resistant *Salmonella* spp., especially bla_{CMY} (Aslam et al., 2012; Hur, Kim, Park, Lee, & Lee, 2011; Marrero-Ortiz et al., 2012; Sjölund-Karlsson et al., 2010), they were not present in any of the three isolates resistant to ampicillin tested in this study. Our results are in agreement with other authors, which describe that resistance to penicillin class of antimicrobial in *S.* Enteritidis may be primarily associated with the production of TEM enzymes (Hur et al., 2011; Zou et al., 2012).

It can be noted that the tetracycline resistance has a close relation to the resistance to ampicillin, chloramphenicol, streptomycin and sulfonamide. This resistance phenotype is emerging among the *Salmonella* serovars, especially *S.* Typhimurium (Gebreyes & Altier, 2002; Hsu et al., 2013; Krauland et al., 2009), and the tetracycline resistance can be assigned to the presence of *tet* genes (Douadi, Thong, Watanabe, & Puthucheary, 2010; Glenn et al., 2011). In this study, we observed two isolates with this multi-resistant phenotype, which presented *tet*B or *tet*C. Other tetracycline-resistant strain presented *tet*B, while *tet*A was detected in none of these

285 strains. Conversely, *tet*A is the most prevalent tetracycline resistance encoding gene
286 found in *Salmonella* spp. in several studies, although other *tet* genes have also been
287 widely detected (Glenn et al., 2013; Soufi et al., 2012; Wannaprasat et al., 2011; Zou et
288 al., 2012).

289 We were able to detect genetic determinants responsible for the majority of the
290 main antimicrobial resistance phenotypes found in these isolates. The fact that the same
291 genes were identified in unrelated strains isolated from different sources and geographic
292 areas, and even in strains from distinct phage types, corroborates with other authors that
293 also detected these genes in different *Salmonella* serovars isolates from several origins
294 (Aslam et al., 2012; Gebreyes & Thakur, 2005; Hsu et al., 2013), as well as other
295 studies that detected some of these genes in other bacteria species (Frye et al., 2011;
296 Srinivasan et al., 2008). These findings must be analyzed with special concern since
297 they indicate that these genes encoding antimicrobial resistance are widespread among
298 *Salmonella*, and can be disseminated to other bacteria, enabling the propagation of
299 resistant microorganisms from livestock animals to human, which can result in
300 infections difficult to treat when antibiotic therapy is needed.

301 All *S.* Enteritidis strains that were resistant to at least one antimicrobial agent,
302 except for one strain, were able to produce biofilm at some degree. Among the 47 *S.*
303 Enteritidis tested, 89.4% strains were able to form biofilm in polystyrene microplates,
304 with all p values < 0.04. A total of 34% of strains were classified as strong biofilm
305 producers (OD_{570} 1.566±0.443), 14.9% were moderate producers (OD_{570} 0.529±0.090),
306 and 42.4% weak biofilm producers (OD_{570} 0.306±0.069). This ability was also
307 described by other authors, which evaluated the biofilm formation in different
308 *Salmonella* serovars, including *S.* Enteritidis (Díez-García et al., 2012; Marin,
309 Hernandiz, & Lainez, 2009; Stepanović, Cirković, Ranin, & Svabić-Vlahović, 2004;
310 Vestby et al., 2009). It is known that the biofilm protects bacteria against environmental
311 harm and there are evidences that bacteria within biofilm are more resistant to
312 disinfectants and antibiotics (Capita & Alonso-Calleja, 2013; Shi & Zhu, 2009; Vestby
313 et al., 2009). Biofilm production is an important characteristic for bacterial persistence
314 phenotype, especially in food factory environments (Vestby et al., 2009). Plastic
315 materials are widely used in this environment (Pompermayer & Gaylarde, 2000;

316 Stepanović et al., 2004), and since *Salmonella*, along with other bacteria, has been
317 shown to adhere in a large number of this kind of hydrophobic surfaces (Steenackers et
318 al., 2012; Tondo et al., 2010), this could become a source of contamination for food
319 passing through a processing line (Manijeh et al., 2008).

320 A better understanding of the factors that potentially contribute to the
321 development and dissemination of resistant *Salmonella* can improve the control of
322 antimicrobial resistance and allow an appropriate treatment to salmonellosis. Our results
323 highlight that *S.* Enteritidis can be embedded in biofilms and the combination with
324 antimicrobial resistance can be a great concern regarding human health. Once the
325 biofilm is formed in a food processing plant, for instance, bacteria in this structure can
326 become more resistant to sanitization, contributing to the persistence of the
327 microorganism in the environment and increases the risk of food contamination, which
328 can be further more dangerous when the biofilm producer bacteria is also resistant to
329 antimicrobials.

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Table 1
Oligonucleotide primer sequences and conditions used in the PCR assays targeting integrons, sulfonamides, β-lactamics, aminoglycosides and tetracycline resistance genes.

Gene	Oligonucleotide primer sequences (5'→3')	$MgCl_2$ (mM)	Annealing temperature (ºC)	Amplicon size (pb)	Reference
int	Hep35 TGCGGGTYAARGATBTKGATTT Hep36 CARCACATGCGTRTARAT	2.0	54	491	White et al., 2000
*intI*1	F ACGAGCGCAGGAAGGTTTCGGT R GAAAGGTCTGGTCATACATG	2.0	59	549	Lévesque et al., 1995
*intI*2	Hep74 CGGGATCCCGGACGGCATGCACGATTTGTA Hep51 GATGCCATCGCAAGTACGAG	2.0	57	2.200	White et al., 2001
5'CS-3'CS[a]	Hep58 TCATGGCTTGTTATGACTGT Hep59 GTAGGGCTTATTATGCACGC	2.0	57	Variable	White et al., 2000
*sul*1	F ATGGTGACGGTGTTCGGCATTGTGA R CTAGGCATGATCTAACCCTCGGTCT	2.0	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 & Padungtod, 2009
bla_{CTX-M}	F TTTGCGATGTGCAGTACCAGTAA R CGATATCGTTGGTGGTGCCATA	2.0	58	544	Edelstein et al., 2003
bla_{CMY}	F ATGATGAAAAAATCGTTATGC R TTGCAGCTTTTCAAGAATGCGC	2.5	56	1.143	Winokur et al., 2001
bla_{TEM}	F GCACGAGTGGGTTACATCGA R GGTCCTCCGATCGTTGTCAG	2.5	55	310	Carlson et al., 1999
*str*A	F CTTGGTGATAACGGCAATTC R CCAATCGCAGATAGAAGGC	2.0	54	549	Gebreyes and Altier, 2002
*str*B	F ATCGTCAAGGGATTGAAACC R GGATCGTAGAACATATTGGC	2.0	53	509	Gebreyes and Altier, 2002
*aad*A	F GTGGATGGCGGCCTGAAGCC R AATGCCCAGTCGGCAGCG	2.0	64	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, 2003
*tet*B	F CTCAGTATTCCAAGCCTTTG R ACTCCCCTGAGCTTGAGGGG	2.0	53	414	Aarestrup, 2003
*tet*C	F GGTTGAAGGCTCTCAAGGGC R CCTCTTGCGGGAATCGTCC	2.5	57	505	Aarestrup, 2003

[a]5'Conserved Segment-3'Conserved Segment

Table 2
Phenotypic antimicrobial resistance patterns and resistance genes detected in different phage types of *Salmonella* Enteritidis.

Isolate	Origin	Phage type	Antimicrobial resistance pattern	Resistance genes
1	Food	PT4	AMP, CHL, STR, SUL, SXT, TET	*int*1[b], *dfr*A7[e], *sul*1, *sul*2, *str*A, *str*B, *aad*B, *tet*B, bla_{TEM}
2	Tanzania	PT9	AMP, CHL, STR, SUL, SXT, TET	*int*1[b], *dfr*A7[e], *sul*1, *sul*2, *str*A, *str*B, *tet*C, bla_{TEM}
3	Human	PT4	SUL, SXT	*int*1[c], *dfr*A5[e], *sul*1, *sul*2
4	Egypt	PT4	SPT, STR, SUL	*int*1[d], *sul*1, *sul*2, *str*A, *str*B, *aad*A1[e], *aad*B
5	Swine	ND	AMP, TET	*tet*B, bla_{TEM}
6	Human	PT4	STR	*str*A
7	Human	PT4a	STR	*str*A
8	Swine	ND	STR, GEN[a], TOB	*str*A, *str*B, *aad*B
9	Poultry	PT4	STR, GEN[a], TOB	*str*A, *str*B
10	Poultry	PT7	STR, GEN[a], TOB	*str*A, *aad*B
11	Poultry	PT4a	STR, GEN[a], TOB	*str*A, *str*B
12	Swine	PT6a	STR, GEN[a], TOB	---[f]
13	Swine	PT6a	STR, GEN[a], TOB	*str*A, *str*B, *aad*B
14	Swine	PT6	STR, GEN, TOB	*str*A, *str*B, *aad*B
15	Swine	PT6a	STR, GEN, TOB	*str*A, *str*B, *aad*B
16	Swine	PT6a	STR, GEN, TOB	*str*A, *str*B

ND: not determined. [a]Intermediate resistance. Approximately amplicon size for 5'CS-3'CS region: [b]800 bp; [c]1500 bp; [d]1100 bp. [e]Genes detected by sequencing of integron variable region. [f]None resistance gene detected. AMP: ampicillin; CHL: chloramphenicol; GEN: gentamicin; SPT: spectinomycin; STR: streptomycin; SUL: sulfonamide; SXT: sulfonamide/trimethoprim; TET: tetracycline; TOB: tobramycin.

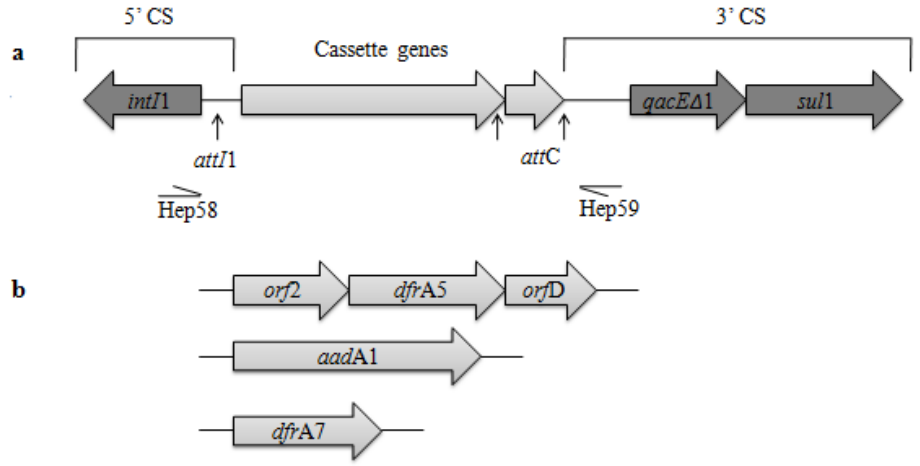

Fig. 1. Structure and characterization of class 1 integrons. **a**. Schematic structure of a class 1 integron showing the 5'- and 3'-Conserved Segments. The location and the direction of transcription of genes are indicated. The class 1 integrase gene *intI*1 and *attI*1 site are located in the 5′-CS. *qacEΔ*1 gene and *sul*1 gene are located in the 3′-CS. 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**. The genetic structure of the gene cassettes amplified using PCR primers that target the 5'- and 3'-CS of typical class 1 integrons (cassette genes detected: *orf*2-*dfr*A5-*orf*D, *aad*A1, and *dfr*A17).

Capítulo 3

Considerações Finais

3.1 Considerações finais

A resistência bacteriana a antimicrobianos pode ser atribuída a diversos mecanismos que impedem a ação do antimicrobiano sobre o microrganismo, como inativação enzimática e alteração de proteínas (1). Estes mecanismos são codificados por genes que podem estar disseminados entre bactérias de mesma espécie, ou ainda entre espécies distintas. Neste estudo, foi observado que os determinantes de resistência encontrados foram condizentes com a resistência fenotípica apresentada pelos isolados. Além disto, detectamos os mesmos genes que conferem resistência a determinados antimicrobianos em cepas que não possuíam relação conhecida, isoladas de diferentes locais, incluindo animais de produção, alimentos e humanos, o que demonstra que estes genes estão disseminados entre cepas de *S.* Enteritidis, que podem chegar aos humanos através da cadeia alimentar.

A propagação de resistência entre os isolados se deve à capacidade de troca de genes de resistência entre os microrganismos mediada por plasmídeos ou transposons, que podem carrear integrons (28,110). Os integrons são elementos genéticos capazes de adquirir e disseminar genes através da integração de cassetes em sua região variável (59,73). A investigação destes elementos torna-se importante para o entendimento da disseminação da resistência antimicrobiana entre os microrganismos. No presente estudo, foi detectada a presença de integrons em quatro cepas, que continham em sua região variável genes condizentes com a resistência fenotípica observada.

Sabe-se que a utilização de antimicrobianos em aves e suínos como promotores de crescimento e profilaxia de infecções bacterianas pode selecionar cepas resistentes (36,37,41,43,46). Assim, a diminuição da administração destes antibióticos deve ser considerada para reduzir a pressão seletiva sobre os microrganismos encontrados na cadeia de produção, especialmente em relação àqueles antibióticos aos quais mecanismos de resistência sejam compartilhados com drogas empregadas para o tratamento de infecções humanas. A utilização de probióticos, modulando a microbiota intestinal dos animais, pode servir como uma alternativa aos antibióticos utilizados, minimizando a colonização de patógenos, portanto promovendo uma melhor produção animal, bem como evitando a seleção de microrganismos resistentes a antimicrobianos.

Consequentemente, o emprego destes probióticos diminui a contaminação do ambiente de criação e posteriormente do abatedouro, evitando a contaminação do produto final (111,112).

A formação de biofilme nos ambientes de criação e, especialmente, no abatedouro pode proporcionar um reservatório de microrganismos, uma vez que a estrutura do biofilme protege as bactérias da ação de agentes químicos empregados na desinfecção de equipamentos e utensílios ao longo da cadeia produtiva (103,104). A capacidade de formação de biofilmes em placas de poliestireno de cepas resistentes a antibióticos demonstrada neste estudo nos indica a possibilidade de persistência de um microrganismo resistente a antimicrobianos no ambiente. Além disto, nossos resultados alertam para a capacidade destes microrganismos de formarem biofilme no ambiente da indústria, já que o plástico é frequentemente utilizado em ambientes de produção de alimentos (105,113). Deste modo, a formação de biofilme nestes locais merece especial atenção por seu potencial como uma fonte de contaminação microbiana aos alimentos, podendo transmitir doenças, além de aumentar a resistência à limpeza e sanitização. Estas observações corroboram com a discussão de que a capacidade de formação de biofilme deve ser levada em consideração no momento de escolha do tipo de material mais apropriado para ser utilizado na cadeia produtiva, bem como de quais produtos químicos seriam mais adequados para a desinfecção.

Como perspectivas deste trabalho, pretende-se averiguar a presença de outros genes que determinam as resistências fenotípicas encontradas. Será analisada a presença de genes que determinam a resistência ao cloranfenicol, além de outros genes que conferem resistência a aminoglicosídeos, para melhor caracterização dos isolados. O entendimento de como a resistência aos antimicrobianos é determinada, além de como estes genes podem ser passados de uma bactéria para outra é de especial importância para alertar para a disseminação de microrganismos resistentes que podem vir a causar doenças em humanos.

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