UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

ESTUDO DOS GENES DE RESISTÊNCIA E INTEGRONS EM CEPAS DE *SALMONELLA* MULTIDROGAS RESISTENTES (MDR) ISOLADAS EM LIFONODOS DE SUÍNOS

FÁBIO SOSSAI POSSEBON

Botucatu – SP

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Resumo

A segurança dos alimentos é essencial para o desenvolvimento da sociedade e as doenças transmitidas por alimentos (DTAs) acometem milhares de pessoas todos os anos. Dentre os agentes de DTA, *Salmonella* se destaca como a principal causa de hospitalizações e mortes. A carne suína é uma importante fonte de nutrientes, sendo a mais consumida no mundo. Vários relatos apontaram o isolamento de *Salmonella* multidrogas resistentes (MDR) associados com a cadeia produtiva dos suínos, e a ocorrência de elementos que possibilitam a transferência de genes de resistência entre populações bacterianas estão diretamente relacionados a essa característica. O presente trabalho objetivou determinar os genes associados com resistência a antimicrobianos de 9 isolados de *Salmonella* MDR provenientes de linfonodos mesentéricos de suínos, além de aplicar um protocolo *in silico* para predizer a localização de cada gene (plasmidial ou cromossomal) e detectar e caracterizar a presença de *Integrons* de resistência. Diversos genes de resistência para a mesma classe de antimicrobianos foram encontrados em cada cepa, e a localização presumida dos genes foi heterogênica, com exceção para os genes de resistência a sulfonamidas, todos classificados como plasmidiais. Cinco cepas apresentaram *integrons* classe 1, agrupados em três diferentes perfis. A comparação destes perfis com sequências do *GenBank* revelou que bactérias de diversas espécies, isoladas em diversos países e proveniente de diferentes fontes, inclusive amostras clínicas de humanos, abrigam *integrons* semelhantes. A vasta diversidade de elementos de resistência para a mesma classe de antimicrobianos pode ser reflexo da grande pressão de seleção a qual estas linhagens bacterianas são submetidas durante a cadeia produtiva

dos suínos, e a presença destes genes em elementos de motilidade, como plasmídeos e integrons, os quais já foram isolados em bactérias de diversas origens, demonstram a importância que estas cepas possuem do ponto de vista de disseminação de resistência a antimicrobianos e consequentemente a saúde pública.

Palavras chave: *Salmonella*, perfil de resistência, WGS, plasmídeos, *integrons*

Abstract

Food safety is essential for society development, and foodborne diseases affect thousands of people every year. Among the main foodborne disease agents, *Salmonella* stands out as the most associated with hospitalizations and deaths. Pork products are an important source of nutrients for population and the most consumed meat in the world. Several reports reveal the presence of Multidrug Resistant (MDR) *Salmonella* strains in the pork production chain, and the occurrence of elements that allows transference of resistance genes between bacterial populations is related to this characteristic. The present study aimed to determinate the genetic bases associated with Antimicrobial Resistance (AMR) in nine MDR *Salmonella* strains isolated from swine mesenteric lymph-nodes, beside applying an *in-silico* protocol to determinate location of the AMR genes (chromosomal or plasmidial) and characterize resistance integrons. Different resistance genes for the same antimicrobial class were present in each strain, and gene localization was heterogenous, except for sulphonamides resistance genes, where all were classified as plasmidial. Five isolates harbored Class 1

integrons, which were classified in three distinct Integron Profiles (IPs). Comparison of IPs with sequences of *GenBank* database revealed that different species of bacteria, from different countries and isolation sources, including human clinical isolates had the same integrons. The diversity of resistance mechanisms for the same antimicrobial class in each strain can be a reflect of the high selection pressure that these bacterial lineages face during the pork production chain, and the presence of gene motility elements, like plasmids and integrons, that have already been isolated in different situations demonstrate the importance of these strains for AMR maintenance and dissemination, and consequently public health.

Key words: *Salmonella*, resistance profile, WGS, plasmids, integrons.

Capítulo 1

Introdução

A inocuidade dos alimentos tem sido um tema recorrente nos debates sobre saúde pública, estando entre os principais tópicos de discussão da Organização Mundial da Saúde (OMS) e o fornecimento de alimentos livres de perigos constitui um dos pilares para o desenvolvimento das sociedades. Dentre os possíveis perigos veiculados, bactérias se destacaram como a maior causa de hospitalizações e mortes decorrentes das Doenças Transmitidas por Alimentos (DTAs), afetando aproximadamente 2 bilhões de pessoas anualmente no mundo (WHO, 2015).

A carne suína representa uma importante fonte de nutrientes para a população, sendo a mais consumida no mundo e apresentando elevados níveis de proteínas de alto valor nutricional, aminoácidos essenciais, vitaminas do complexo B e minerais (KAUFFMAN, 2001). O Brasil é uma peça importante no mercado mundial deste alimento, exportando mais de setecentas mil toneladas anualmente, e gerando um lucro de mais de 1,5 bilhão de dólares (ABPA, 2018).

O gênero *Salmonella* tem se destacado como um importante patógeno no contexto da inocuidade da carne suína. A literatura apontou o isolamento em várias etapas da cadeia produtiva, da fazenda até o produto final, de sorovares que são frequentemente envolvidos em surtos de salmonelose em humanos (SANCHEZ et al., 2007; ZHENG; BONDE; SØRENSEN, 2007; LITTLE et al., 2008; YANG et al., 2010). Além do potencial patogênico, a resistência aos antibióticos foi outra característica das cepas isoladas na cadeia produtiva dos suínos, onde diversos trabalhos trouxeram uma alta porcentagem de cepas resistentes a diversos compostos simultaneamente,

chamadas de Multidrogas Resistentes (MDR) (CASTAGNA et al., 2001; WEISS et al., 2002; GOMEZ-LAGUNA et al., 2011; THAI et al., 2012).

As técnicas de biologia molecular se mostraram importantes ferramentas para o estudo dos patógenos veiculados por alimentos. Tais técnicas possibilitaram não só a detecção, mas também uma maior compreensão dos mecanismos de patogenicidade, de resistência aos antibióticos e da sua epidemiologia. O desenvolvimento de técnicas de sequenciamento massivo de DNA possibilitou o estudo aprofundado dos elementos genéticos presentes nos patógenos, bem como a filogenia e a caracterização evolutiva das diferentes cepas, agregando informações importantes do ponto de vista da epidemiologia das DTAs.

Revisão de Literatura

-*Salmonella* nas DTAs

No Brasil, foram notificados 12.503 surtos de DTAs entre 2000 e 2017, com mais de 200 mil pessoas doentes e 182 óbitos confirmados. Deste total, somente 2.593 tiveram o agente etiológico identificado, sendo que as bactérias foram apontadas como responsáveis em 92,2% dos casos (BRASIL, 2018). Ressalta-se que muitos casos não foram notificados aos órgãos de saúde pública por diversos fatores, como a falta de procura pelo serviço de saúde quando o indivíduo acometido apresenta sintomas de DTAs, ou a falta de empenho ou condição dos envolvidos em diagnosticar os agentes e notificar os órgãos de vigilância epidemiológica. Dentre os surtos com agente etiológico confirmado laboratorialmente, *Salmonella* esteve presente em 30% dos casos, sendo o agente mais prevalente (Brasil, 2018).

Outros trabalhos também apontaram a importância deste patógeno dentro do contexto da saúde pública. Nos Estados Unidos, *Salmonella* foi o patógeno de maior incidência e associado ao maior número de hospitalizações e mortes dentre as DTAs (CRIM et al., 2015) e estima-se que anualmente são acometidas 1,4 milhões de pessoas e os gastos terapêuticos decorrentes da infecção ultrapassam a ordem dos 2,6 bilhões de dólares (MEAD et al., 2010). Na União Europeia, este agente é o segundo mais prevalente e responsável pela morte de aproximadamente 2.000 pessoas por ano, sendo o mais associado a casos letais de DTAs (WHO, 2015). Em países em desenvolvimento e com menos recursos, estima-se que as DTAs, incluindo a salmonelose, tenham uma importância ainda mais expressiva, levando-se em conta as dificuldades encontradas em algumas regiões com relação ao acesso e conservação do alimento e água, saneamento básico, serviços médicos e vigilância epidemiológica.

O gênero *Salmonella* compreende bacilos Gram-negativos, não formadores de esporos, pertencentes à família Enterobacteriaceae. São ainda micro-organismos anaeróbios facultativos, capazes de utilizar citrato como única fonte de carbono, quase sempre produzem gás a partir da glicose e raramente fermentam lactose ou sacarose. Em sua maioria, são móveis, pois possuem flagelos peritríquios (FRANCO; LANDGRAF, 1996). Atualmente, o gênero é dividido em duas espécies (*Salmonella bongori* e *Salmonella enterica*) e seis subespécies (*Salmonella enterica subespécie enterica, Salmonella enterica subespécie salamae, Salmonella enterica subespécie arizonae, Salmonella enterica subespécie diarizonae, Salmonella enterica subespécie houtenae* e *Salmonella enterica subespécie indica*) (AGBAJE et al., 2011),

porém a classificação mais importante sob o ponto de vista epidemiológico consiste na determinação do sorovar, através da fórmula antigênica proposta por Kauffmann e White (POPOFF; LE MINOR, 2001).

A salmonelose é a doença causada por sorovares não tifoidais de *Salmonella* e pode ocorrer em animais e humanos. Acomete primariamente o trato gastrintestinal e pode acarretar febre, diarreia, náuseas e desconforto abdominal. Em humanos, os sintomas se iniciam geralmente entre 12 e 36 horas após a exposição ao patógeno, e podem durar entre dois e sete dias. Em casos mais graves, principalmente em crianças, idosos e pessoas imunocomprometidas, os quadros entéricos podem evoluir para desidratação severa e sepse. O diagnóstico é feito através da coprocultura e o tratamento consiste em terapia suporte para os casos leves e moderados, sem acometimento sistêmico e intervenção com antibióticos em casos graves, geralmente utilizando-se cefalosporinas. O prognóstico é favorável na maioria dos casos, porém casos de hospitalização e óbito são reportados com frequência, principalmente para indivíduos pertencentes aos grupos de risco (WHO, 2018).

-*Salmonella* na cadeia produtiva da carne suína

Vários trabalhos reportaram a prevalência de *Salmonella* na cadeia produtiva da carne suína, com diferentes metodologias e resultados variados. Na Itália, Piras et al. (2011) relataram uma prevalência de animais positivos na ordem de 30,5% quando eram analisados os linfonodos mesentéricos, 16,4% quando a pesquisa era realizada em fezes e conteúdo intestinal, e 14,1% quando foram utilizados *swabs* de carcaças e fígado. Foi realizada ainda a pesquisa do patógeno nas superfícies do abatedouro, sendo positivas 38,0%

das que não estavam em contato direto com a carne, e 35% das que tinham contato direto. Foram determinados oito diferentes sorovares no trabalho, sendo *S.* Derby e *S.* Typhimurium os mais prevalentes (42,7% e 23,3%, respectivamente).

Sanchez et al. (2007) realizaram uma metanálise de artigos que relatam a prevalência da bactéria em diversas unidades de produção de suínos na Europa e encontraram 59% de propriedades positivas e 17% de animais positivos. Foram também determinados no trabalho os principais pontos responsáveis pelos resultados de prevalências divergentes dentre as diversas pesquisas, sendo destacados: o método microbiológico utilizado, o número de amostras analisadas e o país onde o estudo foi realizado como os fatores mais significativamente envolvidos em tal variação.

Pesquisas brasileiras também reportaram a presença do patógeno nos animais abatidos. Silva et al. (2009) relataram que no estado do Mato Grosso foram detectados 16,6% dos animais positivos para o patógeno quando pesquisados linfonodos mesentéricos e tonsilas. Os principais sorovares isolados foram novamente *S.* Derby (16%) e *S.* Typhimurium (14%), seguidos por *S.* London e *S.*Give (ambos 12%).

Em trabalho semelhante realizado no Rio Grande do Sul, Bessa et al. (2004) encontraram uma maior prevalência do patógeno, da ordem de 55,6% de positivos. Tal trabalho também se utilizou da análise de linfonodos mesentéricos e conteúdo intestinal para a pesquisa de *Salmonella*. Os sorovares que se destacaram como mais isolados foram *S.* Typhimurium (24,3%), *S.* Agona (19,9%), *S.* Derby (13,2%) e *S.* Bredeney (12,0%).

A pesquisa de *Salmonella* nos linfonodos mesentéricos é comumente utilizada para indicar o *status* do animal como portador deste patógeno (BAHNSON et al., 2006) e quando a amostragem é realizada a partir do conteúdo intestinal, pode demonstrar seu caráter excretor (DAVIES et al., 1998). Autores relataram que a infecção dos animais pela bactéria ocorre predominantemente por via oral e os linfonodos mesentéricos atuam primariamente como uma barreira e posteriormente se tornam reservatórios do patógeno, que pode sobreviver intracelularmente nos macrófagos e neutrófilos até que alterações fisiológicas desencadeadas pelo stress, como a liberação de catecolaminas e alterações da motilidade intestinal, propiciem sua liberação nas fezes em poucas horas. Ressalta-se que em muitos casos animais positivos para o patógeno não apresentam sintomatologia clínica (STRAW et al., 2012).

- Resistência a antibióticos de cepas de *Salmonella* isoladas em suínos

Os alimentos de origem animal desempenham papel de destaque na veiculação de *Salmonella*, estando relacionados a maioria dos casos e surtos por este agente. A ocorrência de micro-organismos resistentes a antimicrobianos nos produtos alimentícios originários de tais fontes pode ser considerada um grave problema de saúde pública, ainda mais se consideradas as drogas que são concomitantemente utilizadas no tratamento dos animais e dos seres humanos (THAI et al., 2012). A alta tecnificação da suinocultura moderna possibilita que a crescente demanda pela carne e seus derivados seja suprida com eficiência e racionalização dos recursos. Porém, como em grande parte da produção animal, os antibióticos desempenham importante papel dentro da cadeia produtiva, seja como promotores de crescimento, melhorando

índices zootécnicos, ou como medidas profiláticas ou terapêuticas. Tal utilização resulta na seleção de micro-organismos resistentes a diversos compostos, e o uso indiscriminado ou sem orientação técnica adequada agrava ainda mais este quadro (ANGULO et al., 2000; STRAHILEVITZ et al., 2009).

Gomez-Laguna et al. (2011) determinaram o perfil de resistência a antimicrobianos em cepas de Salmonella isoladas em suínos que foram criados em sistema *free-range* na Espanha e destacaram a resistência aos seguintes antimicrobianos: estreptomicina (46%), tetraciclina (30%), sulfonamidas (25%) e ampicilina (23%). Revelaram ainda que 36% das cepas que apresentaram algum tipo de resistência foram concomitantemente resistentes a 4 ou mais compostos, sendo consideradas pelos autores como multidrogas resistentes (MDR).

Em estudo similar realizado no Vietnã, porém contemplando carne de origem avícola e suína, Thai et al. (2012) determinaram que 78,4% dos isolados eram resistentes a pelo menos um antimicrobiano. Os compostos que apresentaram menor efetividade foram tetraciclina (58,5%), sulfonamidas (58,1%), estreptomicina (47,3%) ampicilina (39,8%), cloranfenicol (37,3%), trimetoprim (34,0%), e ácido nalidíxico (27,8%). Um total de 23,2% dos isolados foi classificado como MDRs, sendo que 8,3% foram resistentes a mais de 9 antibióticos.

Dados brasileiros revelaram perfis semelhantes de resistência. Castagna et al. (2001) ao analisarem isolados de suínos provenientes do Rio Grande do Sul, destacaram como principais compostos aos quais as cepas do patógeno apresentaram resistência: sulfonamidas (83,9%), tetraciclina (37,4%),

cotrimoxazol (25,2%), ampicilina (20,2%), cloranfenicol (16,1%), estreptomicina (14,1%) e ácido nalidíxico (10,1%). As amostras MDR representaram 24,2% do total de isolados.

Weiss et al. (2002), também em estudos no Rio Grande do Sul, utilizando-se de fezes, *swabs* retais e linfonodos mesentéricos, isolaram cepas de Salmonella resistentes a sulfonamidas (97,8%), estreptomicina (82,6%), tetraciclina (36,9%) e sulfazotrim (15,2%).

Possebon (2016) avaliou os linfonodos mesentéricos de 250 animais abatidos em diferentes frigoríficos do Estado de São Paulo e reportou uma prevalência de 36,4% de amostras positivas para *Salmonella*. Destas amostras, mais que 70% foram resistentes a quatro ou mais antibióticos simultaneamente (MDR), sendo que a maior parte dos isolados foram resistentes a tetraciclina, estreptomicina, ampicilina, sulfonamidas e cloranfenicol.

Ressalta-se que os isolados de *Salmonella* provenientes da cadeia produtiva dos suínos apresentam alta taxa de resistência aos principais compostos utilizados na terapêutica animal e humana e representam potencial risco para casos onde a intervenção terapêutica é necessária.

- Mecanismos de resistência a antibióticos da *Salmonella*

A frequência de isolamento de cepas de *Salmonella* resistentes a antibióticos aumentou ao longo dos anos, estimulando a comunidade científica a avançar nos estudos referentes aos mecanismos envolvidos neste evento. Métodos fenotípicos de determinação de resistência, como os que avaliam gradientes de concentração dos fármacos ou a mensuração de zonas de inibição de crescimento foram aliados a novos avanços da biologia molecular,

como a Reação em Cadeia da Polimerase (PCR), o sequenciamento de DNA e RNA e técnicas de proteômica para aprofundar a compreensão dos componentes que atuam no processo de resistência.

Os mecanismos de resistência aos antibióticos ocorrem em variadas formas, dependendo do mecanismo de ação da droga envolvida (Tabela 1). A modificação dos alvos, a produção de enzimas que lizam os compostos e a utilização de bombas que eliminam ativamente o fármaco do ambiente celular são exemplos do vasto repertório presente nas bactérias para reduzir ou inibir os efeitos dos antimicrobianos, porém todos estes mecanismos possuem uma origem genética, podendo ser um gene adquirido ou uma mutação específica. Desta forma, o estudo dos elementos genéticos que conferem resistência aos antibióticos auxilia na predição ou confirmação de tal efeito, além de possibilitar o estudo da evolução, a classificação e comparação entre cepas e até mesmo espécies.

Outro importante enfoque dentro deste campo de estudo é o dos elementos de transferência de resistência. Além da transferência clonal dos genes e mutações adquiridos, plasmídeos e *integrons* desempenham papel importante na aquisição de novos genes, onde uma combinação de diversos genes conferindo resistência a diferentes compostos pode ser adquirida simultaneamente (ALCAINE; WARNICK; WIEDMANN, 2007; SEKYERE; ASANTE, 2018).

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Tabela 1. Mecanismos de ação das classes de antibióticos e genes de resistência mais frequentemente encontrados em *Salmonella*

Classe de Antibiótico	Mecanismo de ação do fármaco	Mecanismo de resistência da bactéria	Genes de resistência mais frequentes
Aminoglicosídeos	Inibição da síntese proteica	Diminuição da absorção e inibição enzimática	*aac(3)-IV, aac(3)-IVa, aacC2, strA, strB, aph(3')-IIA, aadA1, aadA2, aadB*
Beta-lactâmicos	Inibição da formação da membrana celular	Inibição enzimática	bla_{CMY-2}, bla_{CTX-M9}, bla_{TEM-1}, bla_{TEM-53}, bla_{CARB2}, bla_{OXA-30}
Cloranfenicol	Inibição da síntese proteica	Inibição enzimática e bomba de efluxo	*cat1, cat2, cmlA, floR*
Quinolonas	Inibição da Topoisomerase	Modificação do alvo e bomba de efluxo	*gyrA, gyrB, parC**
Tetraciclinas	Inibição da síntese proteica	Bomba de efluxo	*tet(A), tet(B)*
Sulfonamidas	Inibição da síntese do ácido tetrahidrofolico	Modificação do alvo	*sul1, sul2, sul3*
Trimetoprim	Inibição da síntese do ácido tetrahidrofolico	Modificação do alvo	*dhfr1, dfrA1, dhfr12*

*Resistência conferida por mutações pontuais nestes genes. Adaptado de (ALCAINE; WARNICK; WIEDMANN, 2007)

- Integrons

Integrons são elementos de DNA que podem transferir genes de resistência a antibióticos entre diferentes bactérias, participando da disseminação horizontal de resistência (ASGHARPOUR et al., 2018). A estrutura básica do *integron* é composta por três elementos principais, que possibilitam a expressão de genes exógenos (Figura 1). A primeira estrutura é o gene que codifica a integrase, uma proteína que catalisa a recombinação entre os genes adquiridos. A segunda estrutura é o sítio de recombinação *attI,* onde os novos genes irão se inserir. Uma vez incorporados, os genes adquiridos são expressos com o auxílio da terceira estrutura, que é o promotor do *Integron* (GILLINGS, 2014).

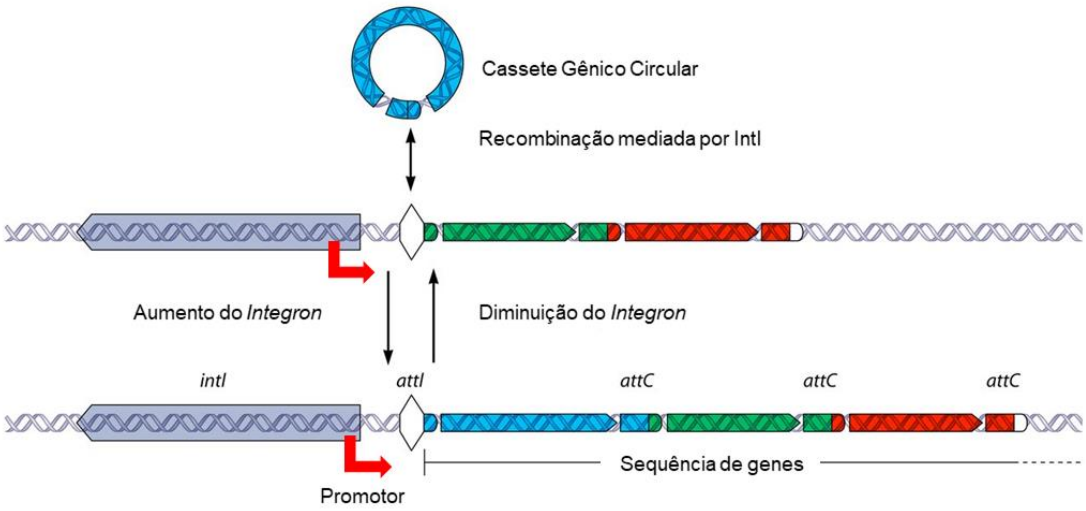

Figura 1 – Estrutura do *integron* e mecanismo de aquisição de cassetes gênicos.
IntI - Região codificadora da integrase; setas vermelhas representam os promotores; *attI* - sítio de recombinação do *integron*; *attC* - área de recombinação dos cassetes gênicos.
Adaptado de Michael R. Gillings Microbiol. Mol. Biol. Rev. 2014; doi:10.1128/MMBR.00056-1313

Os *integrons* adquirem novos genes incorporando elementos chamados cassetes gênicos, que são estruturas de DNA circular, compostas normalmente por um gene com uma fase de leitura aberta (ORF), e uma região de

recombinação, chamada de *attC.* Os cassetes gênicos são integrados pela recombinação entre a região *attC* e *attI* do *integron,* mediada pela enzima integrasse (Figura 1). Este é um processo reversível, de modo que os mesmos podem ser excisados na forma de elementos circulares livres novamente. A inserção junto ao *attI* permite a expressão deste gene com o auxílio do promotor do *integron* e a dinâmica de incorporação e excisão dos genes, permitem ainda um rearranjo na estrutura do *integron*, possibilitando a seleção de células com diferentes genes de resistência mais próximos ao promotor, e consequentemente a maior expressão destes genes, selecionando resistentes frente aos desafios que os micro-organismos são submetidos (GILLINGS, 2014).

Com o sistema de *integrons* novos genes podem ser incorporados em regiões específicas de recombinação (*attI)* sem modificar outros genes existentes. Além disso, os novos genes incorporados são expressos através do promotor do próprio *integron,* estando prontamente aptos a serem utilizados e expostos a seleção natural. Desta forma, em populações de células que apresentam *integrons,* cada uma com arranjos de cassetes gênicos diferentes, qualquer nova variante que apresente uma vantagem evolutiva irá ser expressa imediatamente, conferindo um novo fenótipo adaptado as condições em que está inserido. Os *integrons* possuem papel ativo na adaptação e seleção de bactérias resistentes a antibióticos (GILLINGS, 2014).

Os *integrons* podem ser classificados de acordo com a estrutura do gene que codifica a enzima integrase. Atualmente existem mais de 90 tipos de *integrons* catalogados, sendo a maioria dos tipos presentes em regiões cromossomais de bactérias. Estima-se que estas estruturas desempenharam

papel importante na evolução e adaptação destes micro-organismos, e que aproximadamente 10% de todo genoma bacteriano carrega estes elementos (MAZEL, 2006; BOUCHER et al., 2007). Porém, do ponto de vista de resistência a antimicrobianos, as classes 1, 2 e 3 se destacam como as mais importantes, também chamados de *"integrons* de resistência". Destes, os *integrons* classe 1 são os mais ubiquitários, estando presentes em 40 a 70% dos isolados de origem em amostras de pacientes e de alimentos de origem animal (GILLINGS et al., 2008).

Devido à importância para a saúde pública, *integrons* classe 1 são mais reportados em bactérias da família *Enterobacteriacea*, onde os estudos até então foram mais frequentes, porém outros gêneros de Gram-negativas e em menor frequência Gram-positivas também albergam estas estruturas (DENG et al., 2015). Estudos reportaram a presença de Integrons classe 1 em cepas de *Salmonella* isoladas a partir de diversas fontes, como ambiente de processamento de alimentos, alimentos prontos para o consumo de origem vegetal e animal, animais destinados ao abate e amostras de humanos (WATURANGI; GUSTIANI, 2008; WANNAPRASAT, W.; PADUNGTOD, P.; CHUANCHUEN, 2011; DENG et al., 2015; ABATCHA; EFFARIZAH; RUSUL, 2018).

Trabalhos brasileiros detectaram *integrons* em cepas de *Salmonellla* MDR e determinaram a capacidade de transferência de genes de resistência entre diferentes enterobactérias (OKAMOTO et al., 2009; RIBEIRO et al., 2011), porém o avanço das técnicas de sequenciamento de DNA permitem uma melhor caracterização dos elementos que os compõem, possibilitando o

estudo de sua estrutura, bem como sua localização no genoma (cromossomal ou plasmidial, por exemplo).

- Banco de dados e as pesquisas genômicas

O desenvolvimento das técnicas de bioinformática aliados ao incremento substancial da capacidade de processamento e armazenamento de dados e à facilidade na disseminação da informação pela internet possibilitaram a criação de bancos de dados genômicos. Mecanismos para armazenar, comparar e estudar de forma rápida e eficiente os dados abrem várias possibilidades de análises e colaborações entre pesquisadores e institutos. (CHAN, 2016).

O *National Center for Biotechnology Information – NCBI* é o órgão veiculado ao governo dos Estados Unidos que é curador e coordenador do maior banco de dados sobre biotecnologia e genômica disponível online, o *GenBank* (BENSON et al., 2011). Neste são depositados por usuários do mundo todo sequências de nucleotídeos dos mais diversos organismos, além de elementos como genes e proteínas codificadas, quando possível. Em 2019, o número de sequências depositadas ultrapassou os 3 bilhões. Outra ferramenta disponível na plataforma do *NCBI* é o BLAST (*Basic Local AlignmentSearch Tool),* que realiza uma análise onde uma sequência de nucleotídeos ou proteínas é comparada ao banco de dados, de forma que estatísticas quantitativas são atribuídas aos resultados, demonstrando o grau de similaridade (ALTSCHUL et al., 1990). Com a popularização das tecnologias de sequenciamento e estudo dos nucleotídeos e proteínas, o número de sequências depositadas no *GenBank* tem crescido exponencialmente, dobrando a cada 18 meses, e abordagens analíticas que levem em

consideração esse grande volume de informação são necessárias para obtenção de resultados representativos e confiáveis.

Objetivo

O presente trabalho objetivou caracterizar os elementos genéticos envolvidos na resistência a antibióticos de cepas de Salmonella MDR isoladas em linfonodos de suínos e comparar com dados obtidos em testes fenotípicos (Capítulo 2), bem como caracterizar geneticamente os integrons presentes e suas semelhanças com os dados disponíveis no *GenBank* (Capítulo 3).

Capítulo 2

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Running Title: Resistance genes in MDR *Salmonella* strains

Antimicrobial resistance genes in MDR *Salmonella* strains isolated from swine lymph nodes

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Abstract

Food safety is essential to the development of a society, and foodborne diseases affect thousands of people every year. Among the main foodborne disease agents, *Salmonella* stands out as the most often associated with hospitalizations and deaths. Pork products are an important source of nutrients for the population, being the most consumed meat in the world. Several reports reveal the presence of Multidrug-Resistant (MDR) *Salmonella* strains along the pork production chain, and the occurrence of elements that allow for the transference of resistance genes between bacterial populations is a public health concern. The present work aimed to determinate the genetic bases associated with Antimicrobial Resistance (AMR) in MDR *Salmonella* strains isolated from swine mesenteric lymph nodes. Nine strains were submitted to whole-genome sequencing with the Illumina *NextSeq* system. The results were submitted to the *Resfinder* server for evaluation of acquired genes and point mutations related to AMR, and an *in silico* protocol to predict gene locations (plasmid or chromosomal) was applied. Different resistance genes for the same antimicrobial class were present in each strain, and gene localization was heterogenous, except for that sulphonamide resistance genes, where all were classified as plasmidial. In two samples, resistance genes for olaquindox, a growth promoter outlawed in Brazil since 2004, were found. All observed phenotypic resistance had a genetic basis. The diversity of resistance genes for the same antimicrobial class in each strain can be a reflection of the high selection pressure that these bacterial linages face along the pork production chain.

Key words: *Salmonella*, resistance profile, WGS, plasmids.

Introduction

Food safety is key concern the World Health Organization, and suppling pathogen-free food is essential to the development of a society. Among the foodborne hazards, bacteria stands out as the most frequent, affecting more than 2 billion people every year.[1] *Salmonella* is one of the main etiological agents of foodborne diseases, being the most prevalent cause of outbreaks in many countries and the main cause of hospitalizations and deaths due to this kind of illness.[1,2]

Pork is an important nutrient source and the most consumed meat in the world.[3] *Salmonella* is present in several steps of its production chain from farm to fork, and serovars that have been associated with human salmonellosis outbreaks are often reported in pork as well.[4–6] Besides its pathogenic potential, these strains are in many cases resistant to several antibiotics, worsening the public health issue. Although *Salmonella* promotes self-limiting infection in most cases, sometimes it can lead to sepsis and other systematic impairment, especially in young, elderly, or immunocompromised patients, and in these situations, antibiotic use is essential to safeguard life.[7,8] Reports of antimicrobial resistance – AMR in *Salmonella* isolates from the pork production chain have increased over the years, highlighting the occurrence of Multidrug Resistance (MDR), which occurs when four or more compounds are ineffective for the same strain.[9–13] The expressive use of antimicrobials in animal breeding, either for therapeutic or zootechnical purposes, can be pointed as one of the main causes of this.[7,14]

The genetic mechanisms of resistance for the different antibiotic classes have being a point of interest for researchers in recent years. Acquired genes or

mutations involved in such mechanisms have being studied and compared, as have the structures of gene transference, such as integrons, plasmids, and transposons, in order to understand its evolution and peculiarities. Available data has increased substantially in recent years, with the development of new DNA sequencing techniques and bioinformatics analysis. Several databases are now available online, in a way that new resistance patterns and emergent strains with public health importance can be detected and evaluated with celerity.[15]

The aim of this study was to evaluate the presence of AMR genes associated with the phenotypic test of inhibition zone measurement (Kirby-Bauer), in MDR *Salmonella* strains isolated from mesenteric lymph nodes of pigs and to determine the location of the genes (plasmid or chromosome) with an *in silico* method.

Materials and Methods

Nine MDR *Salmonella* strains belonging to the serovars *S.* Typhimurium (n = 3), *S.* Panama (n = 3), *S*.I.4,5,12:i:- (n = 2) and *S.* Infantis (n = 1), isolated from pigs slaughtered in three different slaughterhouses in São Paulo state, Brazil between August/2014 and September/2015, were submitted to genome sequencing with the *Illumina's NextSeq Platform*. The strains were collected from five different slaughtering batches, and each batch came from a different farm. All batches in the same slaughterhouse were collected on the same day. Slaughterhouse and batch distributions are shown in Table 2. All strains were resistant to at least nine antimicrobial compounds (and five antimicrobial classes). The strains were isolated and characterized phenotypically as AMR in a previous study[13] and were stored in nutrient agar in the Biotechnology

Institute of São Paulo State University – UNESP. AMR data were obtained in agreement with the CLSI guidelines,[16] using the disk diffusion method.

- Strain re-isolation

An inoculum was obtained with a sterile needle and transferred to 9 mL of Tryptic Soy Broth – TSB (Oxoid, Hampshire, UK), which was incubated overnight at 36 ºC. After bacterial growth visualization, a 0.5 µL aliquot was strained in Xylose Lysin Deoxycholate – XLD (Oxoid, Hampshire, UK) agar, followed by a 24-hour incubation at 36 ºC, to determine the viability and discard contaminants. A typical colony was chosen and transferred to 9 mL of sterile TSB followed by another overnight incubation at 36 ºC. After this, the bacterial concentration was adjusted to the optical density that represents 10^6 cells/mL with the aid of Densichek plus equipment (bioMeriaux S.A, Marcy-l'Étoile, France.).

- DNA Extraction and Purification

The PureLink™ Genomic DNA Mini Kit (Thermo-Fisher, Waltham, MA, USA) was used to purify a 1 mL aliquot of the standardized bacterial solution. The manufacturer's protocol for bacterial isolates was followed.

- Library Preparation

The purified DNA fragmentation and the attachment of the adaptors and indexes were made with the SureSelect^{QXT} library preparation kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. This resulted in a normalized library of 2 nM mean fragment concentration with a size ranging from 600 to 800 bp.

- DNA sequencing

The libraries were pooled, diluted, and denatured following the Illumina guidelines and sequenced with the NextSeq platform (Illumina, San Diego, CA, USA), using a 500-cycle V2 reagent kit, with a paired-end run (2 x 251 cycles).

- Data assemblage and AMR gene analysis

All data manipulation and analysis were carried out with the aid of *RStudio.*[17] After de-multiplexing, the FASTQ reads were evaluated with the *FastQC* program[18] and then trimmed with *Trimmomatic.*[19] For each sample, a *de novo* assembly of reads was made with the *A5-MiSeq pipeline,*[20] and ORFs were found with the *ORFfinder*. Annotation was carried out with the BLAST program[21] and the *UniProtKB* database.[22] When the *BLASTx* report revealed an *E value* $>1.10^{-20}$ and *pident* <0.9, the protein results were discarded. All samples had at least 100x the genome sequenced. The contigs were then submitted to the *ResFinder* program for a BLAST search of the resistance genes and mutations, with a threshold of at least 90% identity and 60% of the reference length[23]

- AMR gene location prediction

Contigs in which at least one AMR gene was detected were submitted to BLASTn in the NCBI *GenBank*, including results for taxid: 2 – bacteria. The *E value* $>1.10^{-20}$ and coverage >0.9 were stipulated. Contigs when 100% of the hits matched plasmid references were classified as plasmids. Results when 100% of the hits matched chromosomal references were classified as chromosomes. In cases when the BLASTn results showed both plasmidial and chromosomal references, the contig and, consequently, the resistance gene were classified as undetermined.

Results and Discussion

Data about phenotypic and genetic resistance (acquired genes or mutations) are shown in Table 2. An expressive variation of resistance genes was observed, and more than one gene or mutation for the same antimicrobial class was present in all the strains.

For beta-lactam resistance, all strains presented a variation of the class A *bla*$_{tem-1}$ gene group (*bla*$_{tem-1a}$ or *bla*$_{tem-1b}$), which is the most common in *Salmonella* isolates. These genes encode the beta-lactamase enzyme, which hydrolyzes the beta-lactam ring, resulting in beta-amino acids with no antimicrobial activity.[24] All the strains were resistant to ampicillin, but resistance to other beta-lactams was heterogeneous. This can be associated with gene expression regulators or other undetected resistance mechanisms. Resistance to cephalosporins, which are the first choice in salmonellosis therapeutics is an important concern. In all samples, the beta-lactams resistance genes were classified as plasmidial, except for sample 4, in which *bla*$_{TEM-1B}$ was classified as chromosomal.

Aminoglycoside resistance in *Salmonella* is also related to the expression of drug modifying enzymes. These enzymes are classified into three main groups, according to the type of reaction they catalyze: acetyltransferases, encoded by the genes *aac,* phosphotransferases encoded by the genes *aph*, and nucleotidyltransferases, encoded by genes *aadA*. Many variations of genes in each class are described.[25] In this study, all the strains had at least two of the three classes of described genes. Strain 1 did not show phenotypic resistance to any aminoglycoside but had the *aadA2* and *aac(6')-Iaa* silent genes. Strain number 7 revealed 10 different genes related to aminoglycoside resistance,

while strain numbers 2, 3, 8, and 9 had more than five. The predicted location of the aminoglycosides resistance genes revealed that all genes from *aph* group that the protocol was able to identify were from plasmids, while two genes from sample 7 were classified as undetermined. *Aac* and *aad* genes had both plasmidial and chromosomal predicted locations.

Fluoroquinolone resistance can be mediated by mutations in the DNA gyrase-coding regions (*gyrA* and *gyrB*) or topoisomerase-coding region (*parC)*, which are the target molecules of this antimicrobial class.[24] Qnr proteins can also be associated with the protection of the binding sites and are being reported as emergent quinolone resistance mechanism.[26] Strains 3, 7, 8, and 9 presented qnr complex genes. Another important finding is the presence of *oqx*-group genes in strains 8 and 9. These genes are related to olaquindox resistance, which was a popular growth promoter used in swine breeding but has been banned from animal production in Brazil since 2014 due to possible hazardous effects on humans.[27] All *qnr*-group genes were classified as plasmidial, except for the gene *qnRE1*, which was classified as undetermined. All *oqx* genes were classified as chromosomal.

Sulphonamides inhibit dihyrdropteroate synthetase (DHPS), and resistance is mediated by the expression of *sul* genes, which encode resistant enzymes. *Sul1* genes are often associated with class 1 integrons, and *Sul2* genes are often present in plasmids, but not in integrons. *Sul3* genes are associated with plasmids and integrons.[24] All three variants were present in the evaluated strains, and sometimes the three genes were present simultaneously. This can indicate that the lineage had contact with several gene acquisition

mechanisms throughout its evolution. All *sul* genes were classified as plasmidial in the prediction model.

A similar mechanism occurs with trimethoprim. This class of antimicrobial acts in the inhibition of dihydrofolate reductase (DHFR), and genes encoding drug-resistant DHFR are observed. The main classes are *dhfr* and *dfr* genes.[24] In the present study, all evaluated strains were resistant to trimethoprim and presented one or two *dfrA* genes, with some genes classified as plasmidial and others as chromosomal, but never with both classifications in the same isolate. For sample 9, the prediction model could not classify the gene *dfrA1*.

Resistance to phenicol in *Salmonella* is mediated by the expression of an efflux pump encoded by the genes *floR* and *cmlA.* The first one is often present in isolates from different serovars, while the second one is less frequent. In this research, both genes were present. Resistance to this class of antimicrobial is frequent in MDR strains, and those genes are often present in many genomic islands and plasmids.[24] All but one strain (number 2) were resistant to this class of antimicrobials and presented one or both genes. All the genes that the prediction model could classify were plasmidial, but for three samples (4, 5, and 6) the location of the *floR* gene could not be predicted.

Tetracycline resistance is observed when *tet* genes*,* which encode an efflux pump for this drug, are expressed in bacteria. Although several variants of this gene are known, few have been reported in *Salmonella* isolates.[28] In this study, all strains were resistant to tetracycline and had the genes *tet(A), tet(B),* or both. This class of genes was associated with the lowest prediction success

by the model, with 6 genes classified as undetermined of a total of 12 genes found, considering all strains.

Macrolide resistance genes were detected in three samples, (numbers 7, 8, and 9). Sample 7 had the genes *Inu(G)* and *mph(A)*, both classified as plasmidial. Samples 8 and 9 had the genes *mph(A), mdf(A),* and *Inu(G),* whose predicted locations were plasmidial, chromosomal, and undetermined. Macrolide is not a drug of choice for *Salmonella* infection treatment, so no phenotypic tests were performed for this class of antimicrobial, but the widespread use of drugs like Tylosin with zootechnical purposes along the pork production chain may be associated with the occurrence of these genes. All these three gene groups are responsible for enzymatic inactivation of the drug.[29]

A high diversity of resistance genes to the same antimicrobial class in *Salmonella* strains has been reported in other studies,[30,31] but the number of phenotypic resistance and resistance genes for different drug classes of the evaluated strains in this work surpasses what is frequently observed. This may indicate that the pork production chain may be impaired in the selection and grouping of resistance genes. The co-habitation of several bacterial species in a livestock environment is also an important concern for AMR resistance dissemination, as many genes are in plasmids that can be transferred through different populations and reach more pathogenic groups of micro-organisms. The constant selective pressure caused by antimicrobial use in animal production can be associated with not only the selection but also the maintenance of resistance genes in the bacterial population. The determination

of exclusively human antimicrobials can be an important strategy to mitigate the AMR phenomenon.

The gene location prediction method could classify all beta-lactam and sulphonamide resistance genes, and most of the genes for the other antimicrobial classes, except for tetracycline, when 50% of the genes were classified as undetermined. Phenicol resistance genes also had a high rate of undetermined location (42%). This is related to the diversity of available references reporting these sequences in both plasmids and chromosomal genomes, indicating the variability of evolutionary pathways of antimicrobial resistance. Samples 8 and 9 had an identical AMR gene profile, although they were collected in different batches and belonged to different serovars. The only difference between the two is that for sample 8, the trimethoprim resistance gene *dfrA1* was classified as plasmidial, and in sample 9 the same gene was undetermined.

This prediction model brings high confidence, since the whole contig with the resistance gene was used to classify its location, and only results with 100% similar hits were classified, leaving undetermined cases when chromosomal and plasmidial hits were present simultaneously.

Conclusions

Besides resistance to a high number of antimicrobial classes, the evaluated strains presented a large variety of genetic mechanisms, with heterogenous locations within the bacterial genome, which may indicate an intensive selection pressure during the colonization of swine gastrointestinal tracts. The presence of the resistance gene for olaquindox, 15 years after the

prohibition of this compound, corroborates the importance of efficient control of antimicrobial usage in agriculture, and efforts must be spent on AMR awareness and the control of antimicrobial resistance, in order to prevent worse public health problems in the future.

Conflict of interests

The authors declare no conflict of interests

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Table 2 – Serovar, Phenotypic AMR and resistance genes with predicted location for Salmonella isolates from swine mesenteric lymph-node

ID	Serovar	SH*/Batch	Beta-lactam	Aminoglycoside	Fluroquinolone	Sulphonamides	Trimethoprim	Phenicol	Tetracycline	Total N
1	Typhimurium	A/1	**AMP, CTX, MER** *bla*$_{TEM-1B}$ P	*aadA2* P, *aac(6')-Iaa* C	**NAL, CIP** *GyrA*Mutation C	**SUL** *sul3* P	**SUT** *dfrA12* P	**CLO** *floR* P	**TET** *tet(A)* P	**9** 8
2	S.I.4,5,12:i:-	A/1	**AMP, CPM, CTX, CAZ, CRO** *Bla*$_{TEM-1A}$ P	**EST** *aph(3'')-Ib* P, *aadA2* P, *aph(6)-Id* P, *aadA1* P, *aac(6')-Iaa* C		**SUL** *sul2* P, *sul3* P	**SUT** *dfrA8* P	**CLO** *cmlA1* P	**TET** *tet(B)* P	**10** 11
3	S.I.4,5,12:i:-	B/2	**AMP, CPM, MER** *bla*$_{TEM-1B}$ P	**GEN, EST** *aadA1* P, *aph(6)-Id* P, *aph(3'')-Ib* P, *aac(3)-IIa* P, *aac(6')-Iaa* C	**NAL, CIP** *GyrA*Mutatio C, *qnRE1* U	**SUL** *sul1* P	**SUT** *dfrA1* P		**TET** *tet(A)* P	**10** 11
4	Panama	B/3	**AMP, MER** *bla*$_{TEM-1B}$ C	**AMI, EST** *aac(6')-Iaa* C, *aadA1* C	**NAL, CIP** *gyrB and parC*Mutations C	**SUL** *sul2* P	**SUT** *dfrA* C	**CLO** *floR* U	**TET** *tet(B)* U	**10** 9
5	Panama	B/3	**AMP, MER** *bla*$_{TEM-1B}$ P	**AMI, EST** *aac(6')-Iaa* C, *aadA1* C	**NAL, CIP** *gyrB and parC*Mutations C	**SUL** *sul2* P	**SUT** *dfrA1* C	**CLO** *floR* U	**TET** *tet(B)* U	**10** 9
6	Panama	B/3	**AMP** *bla*$_{TEM-1B}$ P	**AMI, EST** *aac(6')-Iaa* C, *aadA1* C	**NAL, CIP** *gyrB and parC*Mutations C	**SUL** *sul2* P	**SUT** *dfrA1* C	**CLO** *floR* U	**TET** *tet(B)* U	**9** 9
7	Typhimurium	C/4	**AMP, CAZ** *bla*$_{TEM-1B}$ P	**GEN, EST** *aph(4)-Ia* P, *aadA1* P, *aac(3)-IV* P, *aadA2* P, *aac(6')-Iaa* C, *aac(6')-Iak* C, *aph(3')-Ia* U, *aph(6)-Id* U, *aph(3'')-Ib* U	**NAL, CIP** *qnrB19* P	**SUL** *sul1* P, *sul2* P	**SUT** *dfrA1* P, *dfrA12* P	**CLO** *floR* P	**TET** *tet(B)* P, *tet(A)* U	**10** 20*
8	Infantis	C/4	**AMP, MER** *bla*$_{TEM-1}$ P	**AMI, GEN, EST** *aph(4)-Ia* P, *aac(3)-IV* P, *aph(6)-Id* P, *aadA2b* P, *aph(3'')-Ib* P, *aac(6')-Iaa* C	**NAL, CIP** *qnrB19* P, *oqxA* C, *oqxB* C	**SUL** *sul1* P, *sul2* P, *sul3* P	**SUT** *dfrA1* P, *dfrA12* P	**CLO** *cmlA1* P, *floR* P	**TET** *tet(A)* P, *tet(B)* U	**11** 22**
9	Typhimurium	C/5	**AMP, CTX** *bla*$_{TEM-1}$ P	**GEN, EST** *aph(4)-Ia* P, *aac(3)-IV* P, *aph(6)-Id* P, *aadA2b* P, *aph(3'')-Ib* P, *aac(6')-Iaa* C	**NAL, CIP** *qnrB19* P, *oqxA* C, *oqxB* C	**SUL** *sul1* P, *sul2* P, *sul3* P	**SUT** *dfrA12* P, *dfrA1* U	**CLO** *cmlA1* P, *floR* P	**TET** *tet(A)* P, *tet(B)* U	**10** 22**
	Total genes (P/C/U)		9 (8/1/0)	39 (23/13/3)	16(3/12/1)	15 (15/0/0)	12 (8/3/1)	10 (7/0/3)	12 (6/0/6)	121 (74/31/16)

First lane of each ID indicates phenotypic resistance pattern, second lane of each ID indicates acquired resistance genes or mutations. Capital letters indicates predicted gene region (P = plasmid, C = chromosome and U = undetermined)

amp = ampicillin (10 µg), cpm = cefepime (30 µg), ctx = cefotaxime (30 µg), caz = ceftazidime (30 µg), cro = ceftriaxone (30 µg), mer = meropenem (10 µg), ami = amikacin (30 µg), gen = gentamicin (10 µg), est = streptomycin (10 µg), nal = nalidixic acid (30 µg), sul = sulfonamides (300 µg), sut = trimethoprim-sulfamethoxazole (01.25 / 23.75 µg), clo = chloramphenicol (30 µg), tet = tetracycline (30 µ)

* SH = Slaughterhouse

** Macrolide resistance genes were detected in sample 7 - genes *Inu(G) and mph(A)*, and samples 8 and 9 – genes *mdf(A), Inu(G) and mph(A)*, but no phenotypical tests were made, according to CLSI guidelines

Capítulo 3

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Characterization of Class 1 Integrons in MDR Salmonella strains isolated from swine lymph-nodes

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Abstract

The emergence of Multi-drug Resistant (MDR) *Salmonella* in livestock has been a public health concern in the past years, and pork production chain has been often impaired in salmonellosis outbreaks. The presence of integrons in these strains are closely related to acquisition and horizontal transfer of Antimicrobial Resistance (AMR) genes, and this exchange can also occur among different species, allowing the perpetuation of these genes not only in livestock ambient, but also on human infections. The aim of this study was to detect and characterize the genetic sequence of integrons in nine MDR *Salmonella* strains isolated from swine mesenteric lymph nodes. The strains had their DNA sequenced, and the presence of integron elements and resistance genes was evaluated. Five isolates harbored class 1 integrons in plasmids, which were classified in three distinct Integron Profiles (IPs). The size of the sequenced IPs ranged from 1.5 to 8 kb, and the gene cassettes were identified. Isolates from different slaughterhouses had the same IP, and isolates from the same farm had different IPs. The IPs sequences were also subjected to a BLAST search with the *GenBank* nr bacteria database, and data about the 100 first matches of similar sequences to each IP was evaluated. At least 20 countries have sequenced strains with similar integron sequences than IP1, 10 and 6 countries with IP2 and IP3 respectively. For IP1, 22 different species from 15 genus were pointed, while 5 genus for IP2 and 3 genus for IP3. All IPs were already reported in animal origin food and human clinical isolates. The presence of the class 1 integrons in isolates from pork production chain, with the same sequences of isolates from human clinical samples reinforces the role of animal production as a reservoir and disseminator of AMR genes.

Keywords: *Salmonella,* MDR, Integrons, livestock, antimicrobials resistance

Introduction

Salmonella stands as an important foodborne disease agent, being lead cause of hospitalizations and deaths in several countries [1,2]. Pork products are frequently related with outbreaks, since this pathogen is present in the productive chain of this protein, reaching final consumer when good manufacturing practices and pathogen control programs fails [3,4]. Other concern about *Salmonella* is antimicrobials resistance (AMR), and occurrence of Multidrug Resistant (MDR) strains, when the bacteria is resistant to more than four antibiotics compounds simultaneously [5], thus becoming a challenge when antibiotics are needed to safeguard patients life. Studies reported increasing prevalence of MDR *Salmonella* in pork production chain [6–8].

Integrons are important genetic elements impaired in the AMR mechanisms, especially in multidrug resistance. They are DNA structures that allow genes transfer, including resistance ones, between different bacterial cells, playing a major role in horizontal transference [9]. The basic structure of integrons are an integrase coding gene, recombination sites and gene cassettes. Gene cassettes are incorporated by the interaction of the recombination sites, catalyzed by the integrase enzyme. This process can lead to different outcomes, like the incorporation of a new gene cassette from the environment, the excision of a gene cassette already incorporated or the reassortment of resistance genes, modulating their expression. A promoter is also frequently present in the integron structure, allowing an efficient expression of its genes. This mechanism also allow that new genes are incorporated in site specific regions of the genome, reducing the interference in other genes and being promptly subjected to selection pressure [10].

The classification of integrons can be based on the integrase gene sequence. Although many variants of integrons are described, mainly in chromosomal DNA of several bacteria species, and playing an important role in prokaryotic evolution, integrons type 1, 2, and 3 are associated with AMR gene transfer, also known as “resistance integrons”, the most frequently observed in pathogenic bacteria [11]. Class 1 integrons are the most prevalent, being present in 40 to 70% of Gram-negative isolates from clinical context and livestock origin [12], and also in some Gram-positive isolates [11].

Most of the class 1 integrons are in mobile DNA elements, such as transposons and plasmids, and the mobility of the integrons has been considered a major concern of AMR. This characteristic allows resistance gene cassettes transference between bacterial cells of the same species or different ones, when colonizing a common environment [13].

Due to its importance in public health, the *Enterobacteriaceae* family stands out as the most reported carriers of class 1 integrons, but the presence of these structures in other families has also been reported [11]. There are reports of *Salmonella* strains from different isolation sources, such as ready-to-eat food, meat, vegetables, livestock, environment and humans, harboring class 1 integrons [11,14–16].

The aim of this study was to characterize the genetic sequences of class 1 integrons found in MDR *Salmonella* strains isolated from swine mesenteric lymph nodes and the similarity of the Integrons Profiles (IPs) with the available data from *GenBank.*

Materials and Methods

Nine MDR *Salmonella* strains isolated from swine mesenteric lymph node slaughtered in three different slaughterhouses in São Paulo State, Brazil, between August/2014 and September/2015, were submitted to genome sequence with the *Illumina's NextSeq Plataform,* and had the resistance genes and presence of class 1 integrons evaluated. The strains were collected from five different slaughtering batches. All batches in the same slaughterhouse were collected in the same day. All strains were resistant to at least 9 antimicrobials compounds (and 5 antimicrobial classes). The strains were isolated and had the phenotypic characterization of the AMR in a previous study (POSSEBON, 2016) and were stored in nutrient agar in the Biotechnology Institute of São Paulo State University – UNESP.

- Strain re-isolation

An inoculum was obtained with a needle and transferred to 9 mL of Triptone Soy Broth – TSB, which was incubated overnight at 36ºC. After bacterial growth visualization, a 0.5 µL aliquot was strained in Xylose Lysin Desoxicolate (XLD) agar, followed by 24 hours incubation at 36ºC, to determinate the viability and discard contaminants. A typical colony was chosen and transferred to 9 mL of sterile TSB followed by another incubation overnight at 36 ºC. After this, bacterial concentration was adjusted to the optical density that represents 10^6 cells/mL with the aid of the Densichek plus equipment (bioMeriaux S.A. Marcy-l'Étoile, France).

- DNA Extraction and Purification

The PureLink™ Genomic DNA Mini Kit (Thermo-Fisher, Waltham, MA, USA) was used to purify a 1 mL aliquot of the standardized bacterial solution. The manufacturer protocol for bacterial isolates was followed.

- Library Preparation

The purified DNA fragmentation and the attachment of the adaptors and indexes were made with the SureSelect^{QXT} library preparation kit (Agilent, Santa Clara, CA, USA) following the manufacturer protocol. This resulted in a normalized library of 2 nM mean fragment concentration.

- DNA sequencing

The libraries were pooled, diluted and denatured following the Illumina's guidelines and sequenced in the NextSeq platform (Illumina, San Diego, CA, USA), using a 500 cycles V2 reagent kit, with a paired end run (2 x 251 cycles).

- Data assemblance

After de-multiplexing, the FASTQ reads were evaluated with *FastQC* program [17] and then trimmed with *Trimmomatic* [18]. For each sample, De Novo assemblance of reads was made with the *A5-MiSeq pipeline* [19]. All samples had at least 100x the genome sequenced.

- Resistance genes annotation and class 1 integrons characterization

Contigs were scanned for the presence of integrase elements (*IntI* genes, *attI* and *attC* regions) with the aid of *IntegronFinder* program [20]. When these elements were detected, *ResFinder* program was used to BLAST search the resistance genes and mutations, with a threshold of at least 90% of identity and 60% of the reference length (ZANKARI et al., 2012). Regions of the

predicted integron when *ResFinder* could not find any gene, were compared with the *GenBank* database using the BLAST program [21]. Similar integrons were called Integron Profile (IP), and these IPs were blasted again with the *GenBank* nr Bacteria (taxid:2) database, considering 99% of similarity, in order to evaluate other reports of the same integrons configuration and obtain metadata about these reports. Results were limited up to 100 matches of each IP.

All data manipulation and analysis were made with the aid of *RStudio* [22], and the plots and graphic gene visualization schemes were made with the *ggplot2* [23] and *gggenes* (https://CRAN.R-project.org/package=gggenes).

Results and Discussion

Of the nine MDR *Salmonella* strains sequenced, five had integrons elements. All integrons observed were classified as class 1 integrons, and this result corroborates other findings with *Salmonella* isolates from swine [15]. Sample 1 was from slaughterhouse A, samples 2 and 3 were from slaughterhouse B and were raised in the same farm, and sample 4 and 5 were from slaughterhouse C but raised in different farms. All isolates had different phenotypical resistance profiles. Three different IPs were observed among the samples: Sample 1, 4 and 5 shared the same IP, although integrons in sample 4 and 5 were identical, but represented a smaller sequence of integron in sample 1. The integron from sample 1 was called IP1a, (approximately 2.5 kb) and integron from samples 4 and 5 were called IP1b (approximately 1.5 kb) (Figure 2). Samples two and three had different integrons profile (IP2 – 4.0 kb and IP3 – 8.0 kb respectively). Serovars, resistance patterns, slaughterhouse,

farm city and IP are show in Table 3**.** Alignment of IP1a and IP1b is shown in supplemental material.

Considering the origin of the isolates, samples from two different slaughterhouses and isolated from animals raised in 3 different farms (and cities) harbored the same IP (IP1a from sample 1 and IP1b from samples 4 and 5). This can be related to the dispersion of the integrons in *Salmonella* isolates from the pork production chain. Two samples for the same farm had two different IPs (sample 1 – IP1a and sample 2 – IP2), demonstrating the variability of IP and resistance patterns in the same livestock.

In all IPs , the integrase gene was reverse orientated with the adjacent gene cassettes, (Figure 2) what is the most frequent for class 1 integrons [10]. All sequences had *attI* regions after the *IntI* and *attC* regions flanking the gene cassettes. IP2 and IP3 finished in *sul* resistance genes, like observed in most of the class 1 integrons. The sequences in IP1a and IP1b were similar among the both, but *sul* gene at the end of the integron was not observed.

For IP1, the first gene cassette after *IntI* was *dfrA12*, a trimethoprim resistance coding gene [24], followed by gene *Duf1010*, which encodes a protein with unknown function. The last identified structure was gene *aadA*, which encodes nucleotidyltransferase enzyme, responsible for aminoglycosides inactivation [25].

The first gene after *Int1*, in IP2 was also a trimethoprim resistance gene, *dhfrI*, which encodes Dihydrofolate reductase, that catalyzes antibiotic degradation. Next, an *aadA* gene was also present, followed by *ebr* gene, which

encodes ethidium bromide and quaternary ammonium resistance [26]. The last structure of IP2 was *sul1*

IP3 was the biggest integron sequenced. The first gene cassette after *IntI* was *rdmC*. This gene encodes an enzyme that modifies anthracyclines, an antibiotic compound produced by actinomycetes and their synthetic analogs [27]. The next gene cassette was *ppax*, which encodes a pyrophosphatase, that acts within the cell metabolism, but with not known antimicrobial function [28]. Forward, two *aadA* aminoglycoside resistance genes (*aadA* and *aadA1*) flanked a phenicol resistance gene *cmlA1.* This gene encodes an efflux pump [24], and was followed by a *qacF* gene, which encodes quaternary ammonium resistance [29]. In the end of the integron sequence, two reverse orientated genes were present: *Bmul_0189,* which represents a transposase element, involved in DNA sequence insertions [30], and the *sul3* gene.

The BLAST results for the IPs reveals the vast distribution of these structures among different countries, hosts and bacterial species. IP1a was chosen as the representative IP1 profile, for being the largest one. The blast parameters were set to detect virtually identical sequences, with 99% or higher similarity of the hole integron sequence.

Table 4 shows the country of origin distribution of the isolates for reports in the 100 first BLAST results for IP1, and all results from IP2 and IP3. At least 20 countries have sequenced strains with similar integron sequences than IP1, 10 and 6 countries with IP2 and IP3 respectively. Isolation source data was created by the aggrupation of host and isolation origin information. Results for this output were classified as: Human Clinical Sample, Animal Clinical Sample, Ambient, Animal Origin Food, Non-Animal Origin Food. Table 5 shows the

distribution of the isolation source for each IP. In IP1, all the classes were present, while in IP2 ambient samples were absent. For IP3, results were classified as animal origin food and human clinical samples. Data about bacterial species that harbored the integron structure is shown in Table 6**.** For IP1, 22 different species (from 15 different genus) were pointed, while 5 genus for IP2 and 3 genus for IP3. All species were Gram-negative, and most of them belonged to Enterobacteriales order.

The higher number of matches in IP1 when compared to IP2 and IP3 can be explained by the smaller size of the DNA fragment, but the vast variability of countries, isolation sources and bacterial species endorses the importance of the class 1 integrons. The number of isolated from human clinical samples reinforces the public health issue, and the presence of the same sequences in food and environment shows the complex epidemiology and ubiquity of these structures.

All integrons were present in contigs that aligned only with plasmid references, corroborating the statement that this resistance integrons are present in mobile genetic elements. This is a major concern in public health, since AMR is considered one of the hugest challenges in medicine, and horizontal transference of resistance genes is the main mechanism involved in the fast spread of this phenomenon, even more if different species and genus interaction is considered [11].

For all samples, there were also other resistance genes not located in the detected integron. This reinforces that acquisition and maintenance of resistance genes by bacteria can happen in multiple ways, and the integrons are extra players in these mechanisms.

Conclusion

Three Class 1 Integrons Profiles (IPs) were characterized from 5 MDR *Salmonella* strains isolated from swine lymph nodes. The results revealed both similar IP in different origin isolates, and different IPs in same origin isolates, demonstrating the complexity of the integrons distribution among *Salmonella* in pork production chain. Several gene cassettes were identified, some being related to AMR and some not. BLAST search of the IPs revealed a high variability of isolation sources, countries and bacterial species with similar sequences, demonstrating that these integrons are widespread among bacterial cells, and many times involved into human infections. The occurrence of the same integrons profiles both in animal production and human clinical samples corroborates the importance of adequate antimicrobials usage in agriculture, and livestock origin strains can be a reservoir of resistance genes to *Salmonella* and other pathogenic bacteria.

Conflict of interests

The authors declare no conflict of interests

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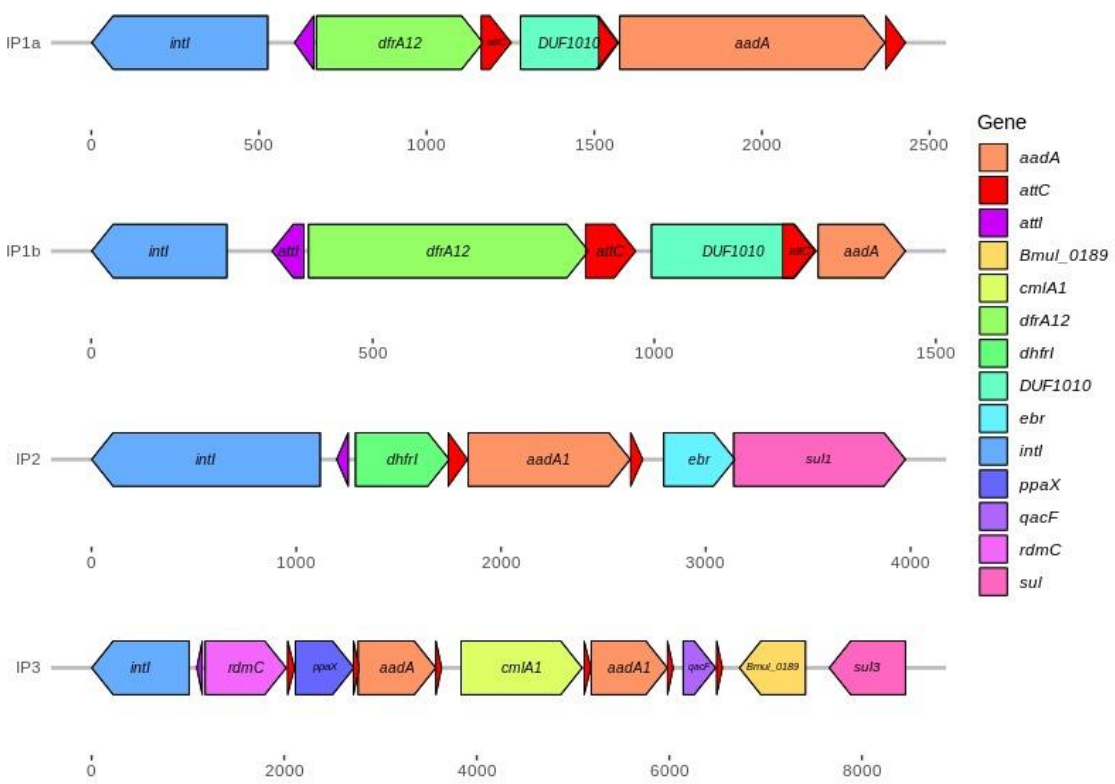

Figure 2. Structure of Class 1 Integrons present in MDR *Salmonella* strains isolated from swine mesenteric lymph nodes
IP = Integron profile. Scale in base pairs.

Table 3 – Serovar, isolation locations, Integrons Profile and phenotypical resistance profile of MDR *Salmonella* isolated from swine mesenteric lymph nodes

ID	Serovar	*Slaughterhouse*	Farm City	Integron Profile (IP)	Phenotypical Resistance Profile	Number of ineffective AM
1	S. Typhimurium	A	a	1a	AMP, CTX, MER, NAL, CIP, SUL, SUT, CLO, TET	9
2	S.I.4,5,12:i:-	A	a	2	AMP, CPM, CTX, CAZ, CRO, EST, SUL, SUT, CLO, TET	10
3	S.I.4,5,12:i:-	B	b	3	AMP, CPM, MER, GEN, EST, NAL, CIP, SUL, SUT, TET	10
4	S. Infantis	C	c	1b	AMP, MER, AMI, GEN, EST, NAL, CIP, SUL, SUT, CLO, TET	11
5	S. Typhimurium	C	d	1b	AMP, CTX, MER, GEN, EST, NAL, CIP, SUL, SUT, CLO, TET	10

amp = ampicillin (10 µg), cpm = cefepime (30 µg), ctx = cefotaxime (30 µg), caz = ceftazidime (30 µg), cro = ceftriaxone (30 µg), mer = meropenem (10 µg), ami = amikacin (30 µg), gen = gentamicin (10 µg), est = streptomycin (10 µg), nal = nalidixic acid (30 µg), sul = sulfonamides (300 µg), sut = trimethoprim-sulfamethoxazole (01.25 / 23.75 µg), clo = chloramphenicol (30 µg), tet = tetracycline (30 µg)

Table 4. Countries of BLAST search matches for three different Integrons Profiles (IPs) obtained from MDR *Salmonella* strains isolated from swine mesenteric lymph nodes

IP1 (20 countries)	n	**IP2** (10 countries)	n	**IP3** (6 countries)	n
China	35	China	24	Switzerland	4
USA	11	Germany	4	Germany	2
Hong Kong	1	USA	4	Denmark	1
Spain	1	Egypt	3	Kenya	1
Taiwan	2	Hong Kong	2	Norway	1
Canada	5	Switzerland	1	United Kingdom	1
Australia	4	United Kingdom	1	**Total Matches**	**10**
Brazil	3	Spain	1		
Greece	2	Taiwan	1		
Mexico	2	Thailand	1		
Singapore	2	**Total Matches**	**42**		
South Korea	2				
Algeria	1				
Argentina	1				
Cambodia	1				
France	1				
India	1				
Korea	1				
Sweden	1				
Vietnam	1				
Total Matches	**78**				

Matches for hits with 99% of similarity with the IPs sequence. For IP1, the first 100 results were considered.

Table 5. Isolation source of BLAST search matches for three different Integrons Profiles (IPs) obtained from MDR *Salmonella* strains isolated from swine mesenteric lymph nodes

IP1	n	**IP2**	n	**IP3**	n
Human Clinical Sample	42	Human Clinical Sample	20	Animal Origin Food	6
Animal Clinical Sample	10	Animal Origin Food	11	Human Clinical Sample	3
Ambient	6	Animal Clinical Sample	10	**Total Matches**	**9**
Animal Origin Food	5	Non-Animal Origin Food	2		
Non-Animal Origin Food	1	**Total Matches**	**43**		
Total Matches	**64**				

Matches for hits with 99% of similarity with the IPs sequence. For IP1, the first 100 results were considered.

Table 6. Bacterial species of BLAST search matches for three different Integrons Profiles (IPs) obtained from MDR *Salmonella* strains isolated from swine mesenteric lymph nodes.

IP1 (22 species)	n	**IP2** (5 species)	n	**IP3** (3 species)	n
Escherichia coli	35	*Escherichia coli*	32	*Escherichia coli*	13
Salmonella enterica	10	*Salmonella enterica*	27	*Salmonella enterica*	8
Klebsiella pneumoniae	30	*Klebsiella pneumoniae*	16	*Yersinia pseudotuberculosis*	1
Proteus mirabilis	1	*Proteus mirabilis*	2	**Total Matches**	22
Citrobacter freundii	2	*Vibrio alginolyticus*	1		
Edwardsiella tarda	2	**Total Matches**	78		
Enterobacter cloacae	2				
Enterobacter hormaechei	2				
Klebsiella aerogenes	2				
Acinetobacter baumannii	1				
Aeromonas sp.	1				
Aeromonas veronii	1				
Citrobacter portucalensis	1				
Cronobacter sakazakii	1				
Edwardsiella piscicida	1				
Escherichia fergusonii	1				
Klebsiella oxytoca	1				
Klebsiella quasipneumoniae	1				
Leclercia adecarboxylata	1				
Morganella morganii	1				
Providencia rettgeri	1				
Shewanella xiamenensis	1				
uncultured bacterium	1				
Total Matches	100				

Matches for hits with 99% of similarity with the IPs sequence. For IP1, the first 100 results were considered.

Capítulo 4

Discussão Geral

O presente trabalho caracterizou os elementos genéticos envolvidos na resistência aos antimicrobianos das cepas de *Salmonella* multirresistentes, isoladas em linfonodos de suínos. Estas cepas se destacaram por apresentarem taxas de resistência maiores do que é usualmente observado (CAPALONGA et al., 2014; GARCÍA-FIERRO et al., 2015; GUERRA FILHO et al., 2016). Uma alta diversidade de genes de resistência foi encontrada, incluindo vários genes para a mesma classes de antibióticos ocorrendo no mesmo isolado ou ainda no mesmo integron. Isto está relacionado com a intensa pressão seletiva a qual estas linhagens foram submetidas ao longo de sua evolução, estando o uso de antimicrobianos de forma inadequada na produção animal associado a este fenômeno.

Para todos os isolados ocorreram genes de resistência que foram classificados como plasmidiais, além de todos os integrons terem sido também classificados como tal. A presença dos elementos de motilidade associados a grande quantidade de genes de resistência agrava ainda mais o potencial destas cepas originarem problemas para a saúde pública. ocorrência das sequencias de integrons em diferentes genomas depositados no GenBank demonstra o quão disseminados estes elementos estão, e os achados pacientes humanos hospitalizados corrobora a importância destes elementos do ponto de vista médico. Podemos destacar o isolamento de cepas de E. coli uropatogênica de pacientes atendidos no Hospital das Clínicas de Botucatu com sequencias virtualmente idênticas aos IP1 avaliados neste trabalho (código de acesso do GenBank CP035337.1, dados ainda não publicados), como um exemplo de ocorrência do mesmo mecanismo de aquisição e

expressão dos mesmos genes de resistência ocorrendo em *Salmonella* isolada na cadeia produtiva dos suínos, e *E. coli* isolada em pacientes hospitalizados. Outro ponto importante, é o fato de que os parâmetros do BLAST com relação aos *integrons* foi ajustado para retornar sequencias virtualmente idênticas, de modo que deleções, adições e rearranjos de genes (que são esperados para estes componentes) não foram detectados. Desta forma, podemos inferir que os resultados obtidos por esta abordagem são somente “a ponta do *iceberg*”, e uma dispersão ainda maior destes elementos deve estar presente nas diferentes cepas bacterianas.

Conclusão geral

As cepas estudadas apresentaram diversos genes de resistência a antimicrobianos, em concordância com a resistência fenotípica observada. Vários genes para a mesma classe da fármacos estavam presentes em cada cepa. A ocorrência de plasmídeos com genes de resistência foi predita em todas as cepas avaliadas, e em 5 cepas foram detectados *integrons* classe 1, também classificados como plasmidiais. Três perfis de *integron* foram determinados baseados em suas semelhanças, e a análise destes perfis junto ao banco de dados do *GenBank* revelou muitos genomas com sequencias virtualmente idênticas, oriundas de vários países, fontes de isolamento e espécies bacterianas. O estudo dos elementos envolvidos na resistência aos antimicrobianos das cepas avaliadas demonstrou a importância das mesmas como reservatórios e potenciais disseminadoras de genes de resistência, não só para outras cepas de *Salmonella*, más também para outras espécies.

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Systematic review articles. There is no length limit for this format. A systematic review, as defined by the Cochrane Handbook, is ‘A review of a clearly formulated question that uses systematic and explicit methods to identify, select, and critically appraise relevant research, and to collect and analyse data from the studies that are included in the review. Statistical methods (meta-analysis) may or may not be used to analyse and summarize the results of the included studies.’ They should include a structured synopsis (with appropriate headings; these may differ from the headings used for Original articles etc.).

Review articles. There is no length limit for this format. These generally aim to give an overview of a field suitable for a wide audience, and they should include a synopsis (250 words maximum). Most reviews are invited. We are pleased to consider unsolicited reviews, but authors are encouraged to consult the Editor-in-Chief in advance of writing to avoid duplicating commissioned material.

Leading articles. These articles are usually in the region of 800-1000 words and may contain the expression of opinion as well as fact. They should address a topical subject, perhaps taking a particular viewpoint and throwing new light on a current debate. A leading article should include a short synopsis (150 words maximum) that should convey the topics and ideas the article covers. Those wishing to contribute a Leading article are encouraged to contact the Editor-in-Chief to discuss their ideas before writing to prevent clashes with any articles already in the pipeline.

For debate. These articles should air contentious issues or discuss controversies so as to stimulate discussion in the Journal on any given topic on antimicrobial chemotherapy. Articles should be as clear and concise as possible, consist of 800-2500 words and must be accompanied by an unstructured synopsis of up to 150 words.

The Editor-in-Chief particularly welcomes pairs of For debate articles offering two opposing viewpoints that aim to persuade readers of their cases. The two resultant articles will be published side by side in the same issue.

Those wishing to contribute a for debate article should first contact the Editor-in-Chief to discuss their ideas and secure a clear agreement before submission. Unsolicited For debate articles will not be considered.

Please note that on publication all Original articles and Brief reports, as well as Antimicrobial practice papers, will be published under the heading of Original research so that articles on similar topics can be grouped together when assigned to an issue. In addition, each piece of Correspondence will be published as either a Research letter or a Letter to the Editor.