Genomic Analysis of Azithromycin-Resistant *Salmonella* Isolated from Food Animals and Retail Meats, 2015-2021, in the U.S.

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Abstract

Introduction: Azithromycin, a 15-membered ring macrolide antibiotic, is critically important to human medicine and can be used to treat *Salmonella* infections. Other macrolides with 14-, 15-, and 16-membered rings are commonly used in veterinary medicine. **Purpose:** We aimed to study the genomic structure of azithromycin resistant *Salmonella* recovered from food animals and retail meats and assess the contribution of macrolide resistance genes to minimum inhibitory concentration (MIC) changes for macrolides with different ring structures **Methods:** Thirty-four *Salmonella* isolated in 2015–2021 that either showed resistance to azithromycin (MIC ≥32 μg/mL) or contained macrolide resistance genes were identified through the National Antimicrobial Resistance Monitoring System (NARMS). Antimicrobial susceptibility testing (AST) was performed using broth microdilution; MiSeq and PacBio assemblies were analyzed with AMRFinder-plus and PlasmidFinder. **Results:** Resistance mechanisms identified included *ere(A), erm(42), erm(B), mef(C), mef(B), mph(A), mph(E), mph(G), and msr(E)*, and a point mutation (acrB_R717L). Among these, *mph(A)* was dominant (56.8%). A macrolide custom AST panel showed that these genes accounted for up to 256-fold increases in MIC against 14- and 15-membered ring macrolides compared with *Salmonella* isolates that lack macrolide resistance genes. The *erm(42)* and acrB_R717L were associated with 4-128-fold higher MICs to the 16-membered ring macrolide tildipirosin. High MICs for most other 16-membered ring macrolides were observed in *Salmonella* with and without the macrolide resistance genes. Macrolide resistance genes were mapped to diverse plasmid replicons, including Col(pHAD28), IncC, IncFIA, IncFIB(K), IncHI1A, IncHI1B, IncHI2, IncN, IncP6, IncQ1, and IncR. Some were hybrid mega-plasmids containing a wide range of genes conferring resistance to multiple drug classes, including critically important antimicrobials such as 3rd generation cephalosporins and fluoroquinolones. **Significance:** Emergence of azithromycin resistant *Salmonella* in food animals and derived meats with co-resistance to critically important antimicrobials is a major public health concern, which warrants continued monitoring and intervention strategies to combat such resistance.

Introduction

The macrolide class of antibiotics represent a large family of protein synthesis inhibitors and have broad-spectrum activity against many bacterial species. They are currently ranked as "critically important" antibiotics on both WHO and FDA's lists of medically important antimicrobials for human medicine. Recently, there has been considerable interest in possibly separating drug products within this drug class based on their different ring structures. However, there is a lack of knowledge whether the various macrolide drug products share any mechanisms of resistance based on their core ring structures. Therefore, understanding these mechanisms would be key to supporting any potential separation and revised ranking of drug products within this important drug class.

Azithromycin, a 15-membered ring macrolide, is one of the most frequently prescribed antimicrobials for various Gram-negative infections, including campylobacteriosis, and salmonellosis due to its favorable permeability, low toxicity, and broad antimicrobial activity. Although the NAMRS surveillance data has showed that the prevalence of Azi^R *Salmonella* has been low in the past thirteen years, it has shown slightly increased in the last few years, especially *Salmonella* isolated from swine and cattle. Azi^R *S.* Newport isolated from food animals has been reported to link to human infections.

Whole genome sequencing (WGS) data showed that various macrolide resistance genes are responsible for resistance to azithromycin, but it is unknown if these genes are also responsible for resistance to 14, 16 member-ring macrolides. NARMS data also showed that many of Azi^R *Salmonella* are co-resistant to critically important antimicrobials which is a major public health concern. The current study is to investigate the resistance mechanisms and genomic structure of MDR plasmids to support FDA's drug ranking and protect public health concern.

Materials and Methods

- **Azithromycin-Resistant *Salmonella* Detection and Controls**

Based on resistance phenotype and genotype, 31 *Salmonella* isolates representing 12 serotypes were selected as the test group (Table 1). In addition, 14 *Salmonella* isolates were selected as controls, including 3 isolates that carried a non-functional macrolide resistance gene.

- **Antimicrobial susceptibility testing using custom macrolide panel**

AST was performed by broth microdilution using a NARMS panel and a custom macrolide panel according to the manufacturer's instructions. The NARMS panel included 14 antimicrobials, and the custom macrolide panel included 8 macrolides that represent 14-, 15-, and 16-member ring macrolide plus clindamycin.

- **Short-Read and Long-Read WGS**

Short-read sequencing was performed on the MiSeq platform, and raw reads were *de novo* assembled using the CLC genomics workbench v.10 (Qiagen). Long-read sequencing was performed on the sequel platform with the sequel sequencing kit 3.0 (PacBio), and the reads were assembled using the hierarchical genome assembly process 4 (HGAP4) pipeline (PacBio).

- **Bioinformatics Analysis and Data Visualization**

AMR genes, including biocide resistance and heavy metal resistance genes, were identified with AMRFinderPlus 3.10. Plasmid replicon typing was conducted using PlasmidFinder 2.1. The plasmid structures from select *Salmonella* isolates were depicted using Angular Plasmid (https://angularplasmid.vixis.com/) and alignment of plasmid sequences with similar structures deposited at the NCBI was generated with Easyfig 2.2.2.

Table 1: AZI^R *Salmonella* Isolates selected in this study

ID#	Serotype	Source	Year	State	Macrolides R Genes	MIC of Azi
N18S0017	Agona	PC	2018	IA	*mef(C),mph(E),mph(G), msr(E)*	1024
FSIS1608447	Schwarzengrund	Chicken	2016	AL	*mph(A)*	128
FSIS1609433	Schwarzengrund	Chicken	2016	GA	*mph(A)*	128
FSIS11704063	Typhimurium	Swine	2017	MN	*mph(A)*	128
FSIS11808786	Ohio	Swine	2018	CA	*mph(A)*	128
FSIS11814458	Newport	Cattle	2018	TX	*mph(A)*	128
FSIS11814474	Mbandaka	Swine	2018	IN	*mph(A)*	128
FSIS12035116	I 4,[5],12:i:-	Swine	2020	SD	*mph(A)*	128
FSIS11922707	I 4,[5],12:i:-	Swine	2019	NC	*mph(E),msr(E)*	128
FSIS11816184	Newport	Beef	2018	TX	*mph(A)*	128
FSIS12027867	Newport	Beef	2020	CA	*mph(A)*	128
FSIS21925668	I 4,[5],12:i:-	GP	2019	MI	*mph(A),mph(E),msr(E)*	128
FSIS1710858	Anatum	Cattle	2017	TX	*erm(42)*	128
FSIS12034723	Newport	Beef	2020	TX	*mph(A)*	128
FSIS12105828	Newport	Beef	2021	TX	*mph(A)*	128
FSIS12106020	Newport	Beef	2021	TN	*mph(A)*	128
FSIS12142912	Newport	Sheep	2021	CA	*mph(A)*	128
FSIS22130757	Newport	Cattle	2021	TX	*mph(A)*	128
FSIS31903059	Newport	Beef	2019	UT	*mph(A)*	128
FSIS32104969	Newport	Beef	2021	WI	*mph(A)*	128
FSIS32105981	Newport	Beef	2021	IL	*mph(A)*	128
FSIS11813680	Senftenberg	Cattle	2018	KS	*erm(42)*	32
N58646	Derby	GT	2015	MN	*acrB_R717L*	32
FSIS1609549	I 4,[5],12:i:-	PC	2016	IN	*mph(E),msr(E)*	512
N17S1465	I 4,[5],12:i:-	Swine	2017	SC	*mph(E),msr(E)*	64
FSIS1702037	Bredeney	Swine	2017	IL	*acrB_R717L*	64
FSIS21821150	Agona	Turkey	2018	NC	*mph(A)*	64
FSIS31901558	I 4,[5],12:i:-	GP	2019	NY	*mph(E),msr(E)*	64
FSIS11920112	I 4,[5],12:i:-	Swine	2019	VA	*mph(E),msr(E)*	64
FSIS1607675	Meleagridis	Beef	2016	KS	*erm(42)*	64
FSIS11809860	I 4,[5],12:i:-	Swine	2018	VA	*mph(E),msr(E)*	64

Results and Discussion

- An array of macrolide resistance genes were identified, including *erm*(42), *mef*(C), *mph*(A), *mph*(E), *mph*(G), and *msr*(E), and a point mutation (*acrB*_R717L), among which *mph*(A) was dominant. Three macrolide resistance genes were confirmed as non-functional macrolide resistance genes [*ere(A), mef(B)*, and *mph(A)*] due to gene truncation and lack of promotor region.
- AST using a macrolide custom panel showed that these genes accounted for several-fold to hundreds-fold increases in MIC against 14- and 15-membered ring macrolides compared with control strains.
- The *erm*(42) and *acrB*_R717L were associated with high MIC to the 16-memeberd ring macrolide tildipirosin.
- 28% Azi^R isolates contained 2≥ macrolide resistance genes; one *S.* Agona isolate from retail pork chop carried 4 resistance genes with highest MIC to Ery (>1024), Gam (>512), Azi (>512).
- High MICs for most 16-membered ring macrolides and lincosamide were observed in *Salmonella* with and without the macrolide resistance genes, suggesting intrinsic resistance.
- Mobile elements-plasmid is the major vehicle to spread macrolide resistance genes.
- Over 50% plasmids were carrying multiple drug class resistance genes, including resistance to some critical antimicrobials, such as 3rd generation cephalosporins and fluoroquinolones.

Table 2. MIC of Different Macrolides

Serotype	Macrolide R Gene	GAM	CLI	AZI	TYL	TIL	TIP	TVN	ERY	SPI	
	mef(C),mph(E),mph(G),										
Agona	*msrE*	>512	>128	>512	>128	>512	16	>128	>1024	>128	
I 4,[5],12:i:-	*mph(E),msrE*	256	>128	256	>128	>512	32	>128	1024	>128	
I 4,[5],12:i:-	*mph(E),msr(E)*	128	>128	128	>128	512	16	>128	1024	>128	
I 4,[5],12:i:-	*mph(A),mph(E),msr(E)*	64	>128	128	>128	256	16	>128	512	>128	
Anatum	*erm(42)*	128	>128	128	>128	>512	>256	>128	>1024	>128	
Typhimurium	*mph(A)*	64	>128	128	>128	512	16	>128	>1024	>128	
Ohio	*mph(A)*	64	>128	128	>128	128	8	>128	>1024	>128	
Newport	*mph(A)*	64	>128	128	>128	256	8	>128	>1024	>128	
Mbandaka	*mph(A)*	64	>128	128	>128	256	16	>128	>1024	>128	Test Group
Schwarzengrund	*mph(A)*	64	>128	128	>128	128	8	>128	>1024	>128	
Meleagridis	*erm(42)*	64	>128	64	>128	>512	>256	>128	>1024	>128	
Bredeney	*acrB_R717L*	128	>128	64	>128	>512	256	>128	512	>128	
I 4,[5],12:i:-	*mph(E),msr(E)*	128	>128	64	>128	>128	16	>128	1024	>128	
Schwarzengrund	*mph(A)*	128	>128	64	>128	128	8	>128	>1024	>128	
Agona	*mph(A)*	32	>128	64	>128	256	8	>128	1024	>128	
I 4,[5],12:i:-	*mph(E),msr(E)*	64	>128	64	>128	128	8	>128	512	>128	
I 4,[5],12:i:-	*mph(E),msr(E)*	128	>128	64	>128	256	16	>128	512	>128	
Senftenberg	*erm(42)*	32	>128	32	>128	>512	256	>128	>1024	>128	
Reading	*acrB_R717L*	64	>128	32	>128	>512	64	>128	256	>128	
I 4,[5],12:i:-	Truncated *ere(A)**	4	>128	4	>128	64	8	>128	64	>128	
Johannesburg	Truncated *mef(B)* *	8	>128	8	>128	128	8	>128	128	>128	
Typhimurium	*mph(A)**	8	>128	4	>128	64	8	>128	64	>128	
Kentucky	No AziR-related genes	16	>128	16	>128	512	16	>128	128	>128	
Johannesburg	No AziR-related genes	32	>128	16	>128	>512	64	>128	256	>128	Control group
Typhimurium	No AziR-related genes	16	>128	16	>128	512	32	>128	128	>128	
I 4,[5],12:i:-	No AziR-related genes	4	>128	8	>128	64	8	>128	64	>128	
Kentucky	No AziR-related genes	4	>128	4	>128	128	8	>128	64	>128	
I 4,[5],12:i:-	No AziR-related genes	8	>128	8	>128	128	8	>128	128	>128	
Newport	No AziR-related genes	8	>128	4	>128	128	16	>128	64	>128	
Newport	No AziR-related genes	8	>128	8	>128	128	8	>128	64	>128	
Newport	No AziR-related genes	8	>128	4	>128	128	4	>128	64	>128	

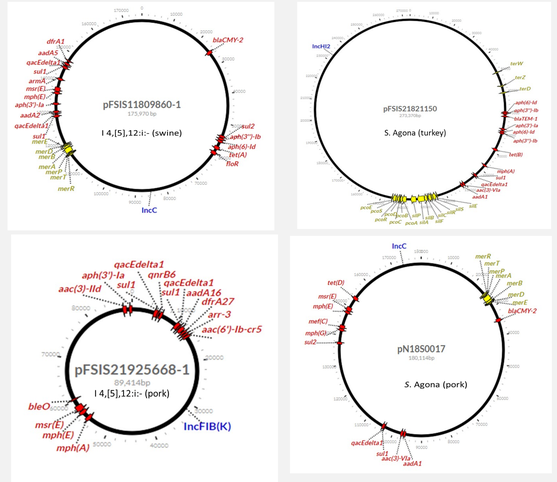
* Non-functional macrolides resistance genes

- **All 31 test strains and 14 controls had high MICs (≥ 128 μg/ml) for spiramycin, tylosin tartrate, and tylvalosin tartrate.**
- **Comparing the test and control groups, up to 256-folds MIC increases against azithromycin and gamithromycin, and the MIC increases were up to 128-, 32-, and 16-folds for tildipirosin, erythromycin, and tilmicosin, respectively.**

Table 3. Resistance Phenotypes and Genotypes in Test Group

Isolates ID	Serotype	Resistance Phenotype	Resistance Genotype
N18S0017	Agona	Amc Amp Azi Axo Fox Gen Fis Tet	*aac(3)-VIa,aadA1,bla_{CMY-2}, fosA, mef(C), mph(E), mph(G), msr(E), sul1, sul2, tet(B), tet(D)*
FSIS1608447	Schwarzengrund	Amp Azi Gen Fis Tet Cot	*aac(3)-IId,aadA2,aph(3'')-Ib,aph(6)-Id,bla_{TEM-1}, dfrA12, mph(A), sul1, sul2, tet(B)*
FSIS11704063	Typhimurium	Amp Azi Chl Gen Fis Tet Cot	*aac(3)-IVa,aadA2,aph(3')-Ia,bla_{CARB-2}, catA1, dfrA12, floR, mph(A), sul1, tet(G)*
FSIS11808786	Ohio	Amp Azi Cip Gen Fis Tet Cot	*aac(3)-IId,aadA2,bla_{TEM-1},dfrA12,mph(A),qnrB19,sul1,tet(B)*
FSIS11814474	Mbandaka	Amc Amp Azi Fox Axo Chl Fis Tet Cot	*aadA2,aph(3'')-Ib,aph(3')-Ia,aph(6)-Id, bla_{CMY-2}, dfrA12, floR, mph(A), sul1 ,sul2, tet(A)*
FSIS11922707	I 4,[5],12:i:-	Amp Azi Cip Gen Nal Fis Tet Cot	*aadA5,aph(3'')-Ib,aph(3')-Ia,aph(6)-Id, armA,bla_{TEM-1}, dfrA1, gyrA_D87N,mph(E), msr(E),sul1,sul2,tet(B)*
FSIS11816184	Newport	Amp Azi Chl Cip Fis Tet Cot	*aadA2,bla_{CARB-2},dfrA1,floR,mph(A), qnrA1, sul1, tet(A)*
FSIS21925668	I 4,[5],12:i:-	Amp Azi Cip Gen Fis Tet Cot	*aac(3)-IId,aac(6')-Ib-cr5,aadA16,aph(3'')-Ib,aph(3')-Ia, aph(6)-Id, arr-3,bla_{TEM-1}, bleO, dfrA27, mph(A), mph(E), msr(E), qnrB6, sul1, sul2, tet(B)*
FSIS1710858	Anatum	Azi	*erm(42)*
FSIS11813680	Senftenberg	Azi	*erm(42)*
N58646	Derby	Azi Fis Tet	*aadA2,fosA,sul1,tet(A) acrB_R717L*
FSIS1702037	Bredeney	Amp Azi Cip Nal Fis Tet	*aac(3)-IVa,aadA7,acrB_R717L,aph(3'')-Ib,aph(4)-Ia,aph(6)-Id,bla_{TEM-1},bleO,gyrA_D87G,qnrB19,sul1,tet(A),tet(B)*
FSIS21821150	Agona	Amp Azi Gen Fis Tet	*aac(3)-VIa,aadA1,aph(3'')-Ib,aph(3')-Ia,aph(6)-Id,bla_{TEM-1}, fosA, mph(A), sul1, tet(B)*
FSIS1607675	Meleagridis	Azi	*erm(42), fosA7.4*
FSIS11809860	I 4,[5],12:i:-	Amc Amp Azi Axo Fox Chl Cip Gen Nal Fis Tet Cot	*aadA2,aadA5,aph(3')-Ia,aph(3'')-Ib,aph(6)-Id,armA,bla_{CMY-2}, bla_{TEM-1}, dfrA1 floR, mphE, msrE, qnrB19, sul1, sul2, tet(A), tet(B)*

Figure 1. Genomic Structure of MDR Plasmids: Red-AMR genes; Yellow-HMR genes; and blue- Plasmid type



Conclusion

- **The emergence of azithromycin-resistant *Salmonella* in food animals and derived meats with co-resistance to critically important antimicrobials in clinical medicine is a major concern, which warrants continued monitoring and intervention strategies to combat such resistance.**
- **Since *Salmonella* is intrinsically resistant to most 16-memebered ring macrolides, it is not possible to evaluate if all AziR genes (except *erm42* and *acrB*_R717L) could cause resistance to 16-membered ring macrolides. More investigations are needed.**
- **Understanding resistance mechanisms provides information needed for risk analysis and ranking the importance of macrolides based on its structure.**