THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

Whole-genome sequence-based molecular characterization of antimicrobial genes in *Salmonella enterica* subsp. Dublin and *Salmonella enterica* subsp. Heidelberg isolated from veal calves

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FALL 2022

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Immunology and Infectious Disease
with honors in Immunology and Infectious Disease

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ABSTRACT

An increase in antimicrobial resistance in *Salmonella* enterica is a serious concern to both animal and public health. In this study, *S.* Dublin and *S.* Heidelberg isolates (2011-2018) in the Penn State Animal Diagnostic Laboratory Culture Repository were selected and subjected to whole-genome sequence analysis. Genomic and bioinformatic tools were used to assess the unique and shared antimicrobial resistance genes and mobile genetic elements in *S.* Dublin and *S.* Heidelberg isolates from veal calf operations in Pennsylvania. *S.* Dublin and *S.* Heidelberg shared antimicrobial genes including *floR, tet(A), sul2, blaCMY-2, aac(6'), aph(6), aph(3'')*, and *aph(3')*. *blaTEM-214, blaTEM-206,* and *blaTEM-1B* were only found in *S.* Dublin isolates while *qnrB19, tet(B), tet(M), tet(O), aac(3), aadA1, aadA5, aadA5, fosA7, mph(A), dfrA17, dfrA12, dfrA34,* and *qacE* were only found in *S.* Heidelberg isolates. It was also observed that both serotypes, *S.* Dublin and *S.* Heidelberg, harbored identical Col(pHAD28), IncC, and IncFII(S) plasmids that likely encoded for antimicrobial genes including *qnrB19, sul2, tet(A), blaCMY-2, floR,* and *aphA*. *S.* Dublin also contained IncX1 plasmid and *S.* Heidelberg contained IncFIB(S), IncHI2, IncHI2A, and IncI1-I(Alpha) plasmids. All three of the *S.* Heidelberg plasmids occur widely in gram-negative bacteria and encode for multiple antimicrobial resistance genes, indicating a broad host range. Based on these observations it can be speculated that co-habitation or sharing the same environment could facilitate sharing of plasmids that encode for antimicrobial-resistant determinants, and this could further promote the dissemination of antimicrobial-resistant genes in food animal agricultural operations.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Bhushan M. Jayarao for his continued guidance through this process. I would also like to thank Dr. Lingling Li and Dr. Manoj Kumar for their assistance during the methodology and analysis portions of this project—without them, I never would have been able to learn the techniques necessary to conduct this research.

To Janel Kolar and Rachel Weininger—thank you for your continued support inside and outside the lab. I gained two great friends from this experience, and you were both so supportive the entire time. I would like to thank Alex Brandis, Allie Giombetti, Maria Smereka, and Sam Doerr for spending countless hours listening to me rambling on about this project. You may not realize it but allowing me to bounce ideas off you was critical for the successful completion of this work.

Of course, I would like to thank my parents, Stacy and Kris Reiss, my brother, Barrett Reiss, and my amazing dogs, Max and Maggie, for their continued support. I know this project was long and I probably talked about it too much, but your continued support and excitement for me lifts me up every day. A part of this project belongs to you as well and I am eternally grateful to your support. For my grandparents, Chuck and Arlene Lawrence, I thank you for your continued investment in my education. I am fortunate to have grandparents so dedicated to my learning and proud of all my accomplishments. I hope to continue to make all of you proud in the future.

Finally, I would like to thank my advisors. To Dr. Pamela Hankey Giblin, you were nothing but supportive of me during my entire time here at Penn State and I will miss your

continued support once I have graduated. Thank you to Dr. Robert Paulson for stepping up during my last semester and guiding me through the final steps before graduation.

This thesis was funded, in part, by a grant from the College of Agricultural Sciences.

Introduction

Salmonella species belong to the family of Enterobacteriaceae. The genus *Salmonella* comprises of two species: *Salmonella bongori* and *Salmonella enterica*. *Salmonella bongori* is associated with cold-blooded animals, however infrequently pet animals and humans can be infected with *S. bongori*. *Salmonella enterica* comprises of six subtypes of which subtype *Salmonella enterica* subsp. enterica is responsible for most of the asymptomatic and symptomatic infections in humans, domestic and wild animals, and birds. The White–Kauffmann–Le Minor scheme [1] listed more than 2600 serotypes of *Salmonella enterica*. Further, subspecies of *Salmonella enterica* subsp. Enterica are grouped as typhoidal (S. typhi and S. paratyphi; host restricted for humans) and non-typhoidal (NTS) serotypes. The NTS affect a wide range of hosts including humans, birds, wild and domestic animals. The severity of clinical symptoms varies according to the serotypes involved and the host species. Almost all NTS can be transmitted between animal species and between animals and humans [1,2].

The distribution of NTS vary considerably with respect to the geographic area and host species, with only a few serotypes tending to be prevalent for a given period and region of the world. Therefore, the knowledge of the prevalent serotypes in each region is critical. Understanding the molecular epidemiology and these serotypes will aid in understanding the overall epidemiology and developing specific prevention and control programs to reduce the burden of disease caused by NTS in animals and humans [2].

In a recent study Luvsansharav et al. [3] examined the top 20 prevalent foodborne illness related NTS and developed a relative ranking of NTS serotypes based on the degree of association of these serotypes with foodborne transmission. They study showed that of the top 20 serotypes, *S.* Saintpaul, Heidelberg, and Berta had the highest association of causing foodborne illness.

Salmonella Heidelberg is a poultry-adapted serotype and can also cause disease in other hosts, including cattle, in particular veal calves. *S.* Heidelberg has been shown to exhibit increased stress tolerance and virulence as compared to other serotypes [4]. Like *S.* Heidelberg, *S.* Dublin is also a nontyphoidal *Salmonella*, however it has been commonly noted as a cattle-adapted serotype [5]. Dublin has been a significant source of salmonellosis within the United States and other countries [6].

In the last two decades, the emergence of antimicrobial resistant bacteria including *Salmonella* pose a significant public health threat both for animals and humans. Of the clinically relevant *Salmonella* serotypes, affecting animals and humans, strains of *Salmonella* Heidelberg encode for several antimicrobial resistance genes, including resistance genes for fourth generation cephalosporins, which are considered as critically important antimicrobials to treat systemic infections in humans.

This study examines the use of comparative genomics to assess the similarities and differences in isolates of *S.* Dublin and *S.* Heidelberg taken from samples from the same geographical area and time, with a primary focus on analyzing the differences and similarities in antimicrobial resistance genes encoded by *S.* Dublin and *S.* Heidelberg isolates from 2011-2018

isolated in the Penn State Animal Diagnostic Laboratory repository from veal calves in Pennsylvania and New York. *S.* Dublin and *S.* Heidelberg samples were matched to compare potential genes and transposon-related elements that could provide an insight to understand the differences and similarities in antimicrobial resistance genes between these two serotypes. The results of this study will provide better understanding on the emergence and transmission of antimicrobial resistance genes in *Salmonella* serotypes. It is anticipated that the findings of the study will allow development of intervention strategies to minimize transfer and adaption of antimicrobial resistance genes among *Salmonella* serotypes identified in the same geographical areas.

The objectives of this study are as follows:

1. To use descriptive epidemiologic analysis of genomic sequences of *S.* Dublin and *S.* Heidelberg isolates from stored meta-data

2. To understand the profiles of antimicrobial resistance genes including transposon-related elements that may contribute to antimicrobial resistance and are found in both Heidelberg isolates and Dublin isolates

3. To propose a new hypothesis on genetic antimicrobial resistance relatedness based on time, veal farm, and location

Review of Literature

Antimicrobial resistance genes in *Salmonella*

The transfer of antimicrobial genes from *S.* Heidelberg to *S.* Dublin poses concerns for both animal and public health. The invasive infections generated from *S.* Dublin would become more difficult to treat than they already are due to a higher degree of antimicrobial resistance [7]. While *S.* Dublin infections are not as common in humans, they are frequently found on both veal and dairy cattle farms [8]. *S.* Dublin presence on veal and dairy cattle farms may become more alarming if it were to become endemic in such locations as it can be costly in terms of animal life and economic output [8].

Another concern of transferring antimicrobial genes among *Salmonella* spp. serotypes is the rise of resistance to antimicrobials. An excellent example of this is quinolones such as fluoroquinolones—for years, resistance had been considered low among *Salmonella* isolates from all sources in the United States [9]. Rising antimicrobial resistance may be attributable to antimicrobial use in some cases such as in Asia where an increase in use of ceftriaxone and ciprofloxacin for febrile illnesses resulted in a higher proportion of pathogens with multiple-drug resistance attributes [10].

Salmonella spp, can encode for several types and classes of genes that encode for antimicrobial resistance. Resistance to sulfonamides is conferred through the genes *sul1* or *sul2* in *Salmonella* serotypes [7]. Beta-lactam resistance is driven by the gene *blaCMY-2* in *Salmonella* isolates that first emerged in 2009 [11]. Related, the *blaTEM-1B* gene contributes to ampicillin resistance in some isolates [11]. For amphenicols such as chloramphenicol, *floR* or

cmlA are known to confer resistance in *Salmonella* spp. [11]. The same study noted that tetracycline resistance was associated with *tet(A)* while aminoglycoside and kanamycin resistance was associated with *aph(3'')* [11]. Trimethoprim resistance may be driven by a variety of genes that are variants of the *dfrA* gene [12]. Aminoglycoside resistance is often conferred through *aadA* gene variants and are carried on gene cassettes or variant *Salmonella* Genomic Islands [12]. Macrolide resistance is typically carried on *ere(A), mph(A), mph(E),* and *msr(E)* and Fosfomycin resistance may be found on *fosA3* and related genes [12].

Often, antimicrobial genes in *Salmonella* spp. are transferred through mobile elements such as plasmids, transposons, and gene cassettes [12]. Certain genes are commonly transferred on specific mobile elements such as aminoglycoside resistance genes that are frequently found in gene cassettes [12]. Plasmids are of special interest in *Salmonella* spp. since they not only encode for virulence genes, but also carry certain antimicrobial resistance genes.

Plasmids in *Salmonella*

Plasmids are mobile genetic elements that participate in conjugation to allow horizontal gene transfer among bacteria [3]. Further, antimicrobial resistance genes that are carried on conjugative plasmids have a greater chance for dissemination to other isolates and serotypes than antimicrobial resistance genes that are carried on non-conjugative plasmids [4]. In *Salmonella* spp., the presence of an extra-chromosomal DNA i.e., plasmid in *Salmonella* spp. is influenced by several factors including serotype, geography, and shared environment with other antimicrobial resistant bacteria. For example, an isolate from the state of Washington may possess different plasmids encoding for different genes bearing resistance to different

antimicrobials than an isolate from the state of New York [13]. The presence of virulence plasmids containing genes encoding for serum resistance has been well-studied in *S.* Dublin [7]. *S.* Heidelberg may also have the capacity to encode for serum resistance in its virulence plasmid [7]. Both *S.* Dublin and *S.* Heidelberg are highly susceptible to acquisition and or loss of plasmids containing antimicrobial resistance genes.

Transfer of plasmids between *S.* Dublin and *S.* Heidelberg has been reported previously, where researchers noted that movement of a hybrid IncA/C2 resistance plasmid that originated in *S.* Heidelberg, was seen in *S.* Dublin [10]. Other plasmids, IncFII(S) and IncX1, in *S.* Dublin are considered as serotype specific virulence plasmids of *S.* Dublin and it is concerning if these plasmids become mobile and transfer to other *Salmonella* spp. [10]. The ability of *Salmonella* serotypes to allow new plasmids to emerge over time is concerning [3]. The movement of plasmids is an important pathway through which *Salmonella* serotypes can disseminate new antimicrobial resistance genes to other *Salmonella* spp. or gram-negative bacteria such as *E. coli* over both time and geographic distance.

Pathogenicity islands in *Salmonella*

Salmonella Pathogenicity Islands (SPIs) in *Salmonella* spp. encode for virulence factors that serve to enhance the overall pathogenesis of *Salmonella* infections in the host [14]. To-date, nearly 23 SPIs have been reported with varying functions (Table 1) [15]. There are a wide range of pathogenicity islands found in *Salmonella* with SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5 being most found in *Salmonella* serotypes [14]. The remainder of pathogenicity islands found in *Salmonella* are distributed across serotypes with some having greater specificity for a specific

serotype over others [14]. With the high number of pathogenicity islands found in *Salmonella* as a whole, it should be noted that some pathogenicity islands play specific roles in pathogenesis itself while others are important for their roles as transporters of antimicrobial resistance elements [14].

The most frequently occurring SPIs encode for a variety of virulence genes allowing them to initiate and establish host-pathogen interactions [14]. Pathogenicity island SPI-2 enables prolonged growth of *S.* Dublin in the intestinal epithelial cells of the host [16]. SPI-2 may also serve to dictate the final fate of infected epithelial cells through the genes encoded in the pathogenicity island [16]. In addition to playing a key role in the growth of *S.* Dublin in epithelial cells, SPI-2 contains several virulence genes that further assist in invasion and replication in the host cells [15]. SPI-1 also contains similar virulence genes to assist in intracellular pathogenesis [15]. SPI-2 is just one of these pathogenicity islands with the capacity to cause considerable risk and subsequent danger to hosts infected by *Salmonella* serotypes containing key pathogenicity islands [15].

Table 1. *Salmonella* Pathogenicity Islands (SPIs), their proposed functions, and common serotypes they have been observed in.

SPI	Proposed function	Common serotypes encoding for the SPI
SPI-1	Encodes T3SS1, a regulatory protein	All *Salmonella* serotypes
SPI-2	Encodes T3SS2, a regulatory protein	All *Salmonella* serotypes
SPI-3	Survival in macrophages	All *Salmonella* serotypes
SPI-4	Encodes for T1SS secretion system	All *Salmonella* serotypes
SPI-5	Involved in enteropathogenicity	All *Salmonella* serotypes
SPI-6	Encodes for T6SS secretion system	Typhi, Typhimurium
SPI-7	Modulates innate immune response	Typhi, Paratyphi, Dublin
SPI-8	Improve bacterial fitness	Typhi, Typhimurium
SPI-9	Modulates bacterial adhesion	Typhi, Typhimurium
SPI-10	Regulates chaperone proteins	Typhi, Typhimurium
SPI-11	Survival in macrophages	Typhi, Typhimurium
SPI-12	Contributes to in vivo adaptability	Typhi, Typhimurium
SPI-13	Affords nutritional fitness	Typhi, Typhimurium
SPI-14	Enhances invasion of host cells	Typhi, Typhimurium
SPI-15	NA*	Typhi
SPI-16	Involved in intestinal persistence	Typhi, Typhimurium
SPI-17	Encodes open reading frames	Typhi, Typhimurium
SPI-18	Encodes open reading frames	Typhi, Typhimurium
SPI-19	Encodes for T6SS secretion system	Dublin, Gallinarum, Derby
SPI-20	Encodes for T6SS secretion system	Dublin, Gallinarum, Derby
SPI-21	Encodes for T6SS secretion system	Dublin, Gallinarum, Derby
SPI-22	Encodes for T6SS secretion system	Dublin, Gallinarum, Derby
SPI-23	Assists in adhesion and invasion	Dublin, Gallinarum, Derby

* Denotes an unclear proposed function in the literature [15, 17, 18, 19]. As indicated, some SPIs are more common in a broad *Salmonella* serotype range than others.

Phages and transposons in *Salmonella*

Phages and transposons are two other important mobile genetic elements encoding for antimicrobial resistance genes in *Salmonella.* Phages may be responsible for proliferating areas of "junk DNA" that seemingly do not code for an understandable function [20]. These same proteins may benefit the *Salmonella* in terms of its virulence and pathogenicity, which may

explain the evolutionary favorability of certain phages [20]. One example of a phage found in *Salmonella* is Gifsy-1 prophage that can introduce genes at a given insertion site [20]. Phages found in certain *Salmonella* serotypes may also vary over time to encode new virulence properties [10]. Transposons are another mobile genetic element responsible for conferring resistance to certain antimicrobials through genes inserted into new *Salmonella* strains [10]. *tet(A)* and *tet(B)* are two examples of antimicrobial resistance genes that are frequently found in transposons to allow *Salmonella* to easily transmit antimicrobial resistance genes to one another [10].

Implications of *Salmonella* infection

The transfer of antimicrobial resistance genes from one *Salmonella* serotype to another poses dangers to animal, human, and environmental health. At present, non-typhoidal *Salmonella* are a common cause of foodborne illness and death for humans across the globe, so an increase in resistance to antimicrobials may make human *Salmonella* infections more difficult to treat in the future [13]. Increases in antimicrobial resistance in *Salmonella* spp. infections have also been associated with higher mortality and morbidity because of limited treatment options [14]. Other negative consequences of antimicrobial resistance in *Salmonella* include longer hospital stays and higher costs of treatment, both of which harm individual finances and the broader economy [21]. The toll on public health from increasing antimicrobial resistance is a considerable concern, yet antimicrobial resistance in *Salmonella* also has an impact in animals as well, and specifically the veal and dairy industries where cattle is involved.

S. Dublin has been a cause for concern in the dairy and veal industry [22]. In cattle, it has been noted for its ability to cause high rates of both morbidity and mortality in cattle across the world [22]. Additionally, the presence of *S.* Dublin in cattle can also cause potential harm in humans if it moves into the food supply. In 2019, there was contamination of *S.* Dublin in ground beef that led to 34,222 pounds of the meat being recalled by the producers [23]. *S.* Heidelberg has caused similar issues in poultry, such as in 2013 when people began to become ill with *S.* Heidelberg from encountering contaminated chicken [24].

The loss of cattle from *S.* Dublin infections has considerable economic effects that harm both cattle owners and the overall agricultural economy [22]. Growing resistance to certain antimicrobials such as cephalosporins is of particular concern because it may make infections more difficult and costly to treat in the future, as it will be with humans [25]. In summary, growing resistance to antimicrobials transported through mobile genetic elements such as plasmids and pathogenicity islands in *Salmonella* is of great concern for human, animal, and environmental health.

Materials and Methods

Isolate collection

A total of 25 isolates of *S.* Dublin (n=12) and *S.* Heidelberg (n=13) from 2011-2018 in the Penn State Animal Diagnostic Laboratory were selected to examine their molecular characteristics with a focus on antimicrobial resistance genes. Metadata on the isolates selected for the study is shown in Table 1.

Library preparation of *Salmonella* isolates

S. Dublin and *S*. Heidelberg isolates were grown overnight at 37 C in a shaker incubator. Following 18h of growth, the growth was centrifuged, pelleted, washed, and used for whole-genome extraction using QIAGEN Genomic-tip 100/G (Qiagen, Germantown, MD) following the manufacturer's instructions.

Next-generation sequencing libraries were prepared with a Nextera XT kit (Illumina, San Diego, CA). Default parameters were primarily used. Extensive polymerase chain reaction procedures were used to prepare the libraries for sequencing. The sizes of the amplified samples were checked to ensure they were large enough for proper sequencing before proceeding to the next generation sequencing of the samples.

Next generation sequencing of *Salmonella* isolates

Libraries were sequenced on an Illumina MiniSeq using 150bp paired-end reads. Default parameters were primarily used. Materials needed for the sequences were used primarily from the Illumina MiniSeq kit and refrigerated accordingly. Polymerase chain reaction was extensively used throughout this process to amplify the DNA in the isolates to prepare the sequencing. Once prepared, the isolates were sequenced for approximately 23 hours. FastqC version 0.11.8 (Andrews, 2010) was used for quality assessment of the Illumina reads after they had been prepared.

Whole-genome assembly and analysis

Genomes of *S.* Dublin and *S.* Heidelberg were assembled with SPAdes v3.13.0 [26]. QUEST [27] was used for quality assessment and evaluating of genome assemblies. Subsequently, Prokka v1.14.6 [28] was used to annotate the genome. Pan-genome analysis was carried out using Roary v3.13.0 [29]. Phandango [30] was used to view the resulting output graphs. Using RAxML v8.2.12 [31], core genome gene sequences were aligned and maximum likelihood trees were produced with the GTRCAT substitution model and 100 iterations for viewing and editing using iTOL [32].

Genome characterization

The assembled genomes were examined for virulence-associated genes using the VirulenceFinder v2.0.3 [33]. Detection of antimicrobial genes was performed by locally installed version of ResFinder [34] available through the Center for Genomic Epidemiology, or CGE.

Multilocus sequence types were determined using a [35] locally installed version of MLST at CGE website (http://www.genomicepidemiology.org/). Sequence types (ST) were determined and compared with those included in the database at CGE server.

Results

12 bovine *S.* Dublin and 13 bovine *S.* Heidelberg isolates were identified in Pennsylvania and New York between 2011 and 2018.

Genomic investigation of *S.* Dublin and *S.* Heidelberg isolates

The assembled genome size ranged from 4,882,693bp to 5,390,588bp. The average of the contigs was 5,067,210.80 bp; the smallest genome of isolate 318 was 4,882,693bp and the largest isolate 66 was 5,390,588bp.

To investigate the molecular epidemiology of animal cases and the possible zoonotic transmission of MDR *S.* Dublin and *S.* Heidelberg to bovine, we selected a convenience sample of isolates for whole-genome sequencing analysis from the bovine isolates collected between 2011 and 2018.

Multilocus sequence typing identified all *S.* Dublin isolates as of sequence type 10 (ST10) (Table 1). The multilocus sequence typing identified all *S.* Heidelberg isolates as of sequence type 15 (ST15). The plasmid replicons detected within all *S.* Dublin isolates included IncFII(S) and IncX1. The only plasmid replicon detected within all *S.* Heidelberg isolates included IncC.

The relatedness of isolates for both *S.* Dublin and *S.* Heidelberg was assessed through building phylogenetic trees that indicated evolutionary relatedness (Fig 1).

Table 2. MLST and antigenic profiles of *S.* Dublin and *S*. Heidelberg.

Identifier	S. Dublin (n=12)	S. Heidelberg (n=13)
Seq MLST	10	15
Antigenic Profile	9:g:p:-	4:r:1,2

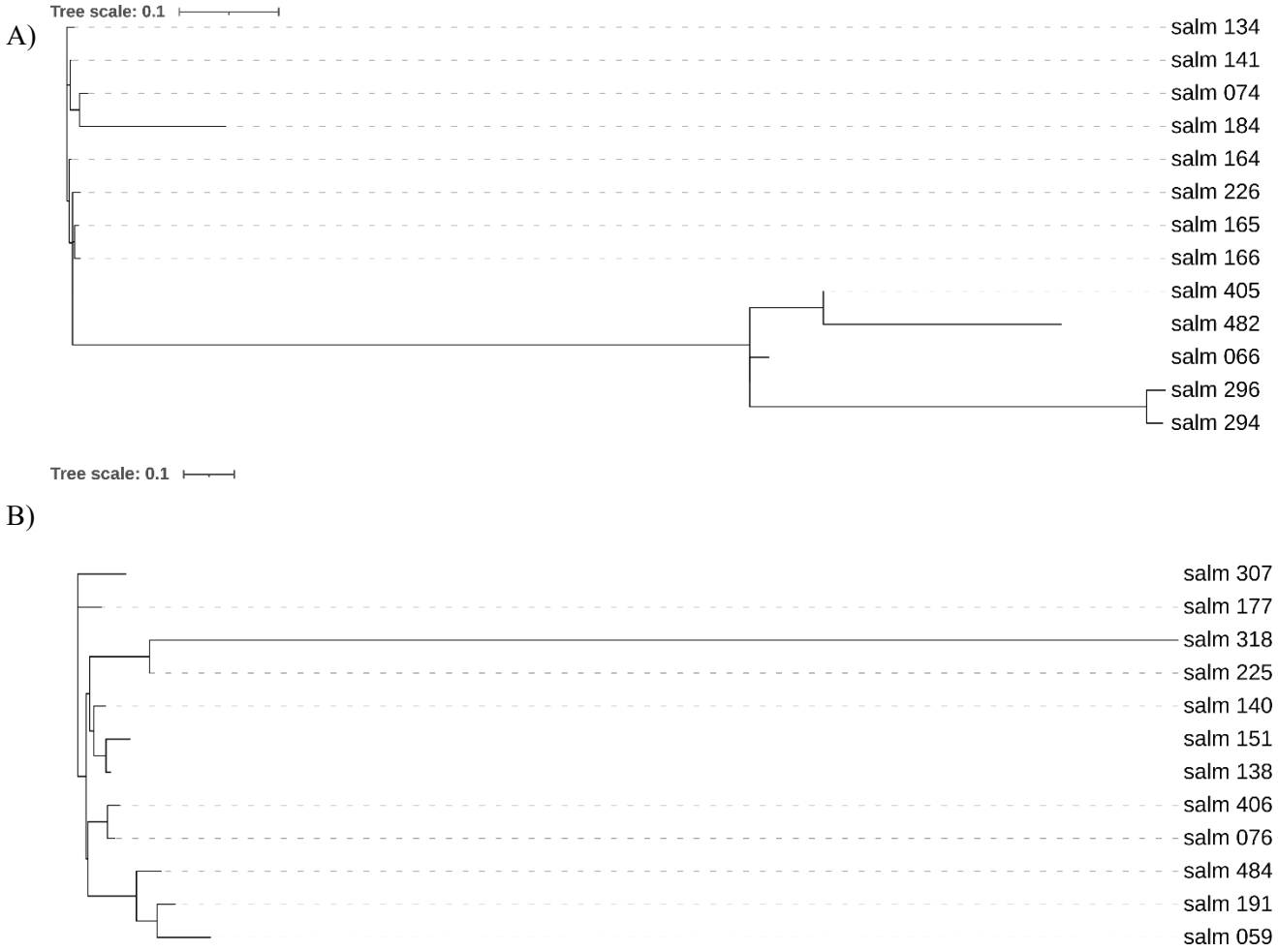

Figure 1. Core genome phylogeny of *S.* Dublin and *S.* Heidelberg. Plate A represents the single-copy core genome phylogeny of 13 isolates of *S.* Heidelberg. Plate B represents the 12 isolates of *S.* Dublin. The RAxML tree is built from core genes identified with Roary [29, 31, 32].

S. Dublin contained resistance to beta-lactam, folate pathway antagonist, tetracycline, and amphenicol antimicrobial classes. Resistance to at least one class of antimicrobials was found in each of the isolates (n=12) (Table 2). Aminoglycoside resistance was driven by *aac(6'), aph(6),*

aph(3''), and *aph(3')*; beta-lactam resistance was driven by *blaCMY-2*, *blaTEM-214, blaTEM-206,* and *blaTEM-1B;* folate pathway antagonist resistance was driven by *sul2*, tetracycline resistance was driven by *tet(A)*, and amphenicol resistance was driven by the presence of *floR.* Isolate 318 contained only one resistance gene, *aac(6')* that conferred resistance to aminoglycosides.

In terms of plasmids, *S.* Dublin isolates contained IncC, IncFII(S), IncX1, and Col(pHAD28) plasmids (Table 3). IncFII(S) and IncX1 were found in all 12 isolates while IncC was found in all but one, 318. Additionally, the Col(pHAD28) plasmid was only found in 1 isolate, 140.

S. Dublin isolates contained SPIs including C63PI, CS54_island, SPI-1, SPI-10, SPI-13, SPI-14, SPI-2, SPI-3, SPI-4, SPI-5, and SPI-9. All 12 *S.* Dublin isolates were positive for C63PI, CS54_island, SPI-1, SPI-10, SPI-13, SPI-14, SPI-2, SPI-3, SPI-5, and SPI-9. For SPI-4, 2 *S.* Dublin isolates did not contain the pathogenicity island, 177 and 225.

S. Heidelberg contained resistance genes to beta-lactam, quinolone, folate pathway antagonist, tetracycline, amphenicol, Fosfomycin, macrolide, trimethoprim, and disinfectant antimicrobial classes. Resistance to at least one class of antimicrobials was found in each of the isolates (n=13) (Table 2). Aminoglycoside resistance was driven by *aac(6'), aph(6), aph(3''), aac(3), aadA1, aph(3'), aadA5,* and *aadA2*; beta-lactam resistance was driven by *blaCMY-2*; quinolone resistance was driven by *qnrB19;* folate pathway antagonist resistance was driven by *sul1* and *sul2*; tetracycline resistance was driven by *tet(A), tet(B), tet(M),* and *tet(O)*; amphenicol resistance was driven by the presence of *floR*; Fosfomycin resistance was driven by *fosA7*; macrolide resistance was driven by *mph(A);* trimethoprim resistance was driven by *dfrA17,*

dfrA12, and *dfrA34.* 1 isolate, 482, was positive for *tet(O)* that conferred resistance to tetracycline as well as *dfrA34* that conferred resistance to trimethoprim.

For plasmids, *S.* Heidelberg contained IncC, IncFII(S), IncFIB(S), IncHI2, IncHI2A, Col(pHAD28), and IncI1-I(Alpha) (Table 3). All 13 *S.* Heidelberg isolates contained the IncC plasmid. For IncFII(S) and IncFIB(S), only 1 isolate was positive for each of these plasmids, 66. 3 isolates possessed the Col(pHAD28) plasmid, 294, 296, and 482. The plasmid first appeared in *S.* Heidelberg with isolate 294 in 2014 and was subsequently found once more in 2014 and once in 2018. The IncI1-I(Alpha) plasmid was also found in very few samples—only 294 and 296 were positive for the plasmid.

The *S.* Heidelberg isolates represented 11 different SPIs. Encoded in the isolates were C63PI, CS54_island, SPI-1, SPI-13, SPI-14, SPI-2, SPI-3, SPI-4, SPI-5, SPI-9, and SGI1. All 13 *S.* Heidelberg isolates were positive for the C63PI, SPI-1, SPI-13, SPI-14, SPI-2, SPI-3, and SPI-5. Only 1 *S.* Heidelberg isolate did not possess the CS54_island or SPI-9, 184. 3 isolates were positive for the SGI, 294, 296, and 405.

All isolates encoded for aminoglycoside resistance gene *aac(6')*. *blaTEM-214, blaTEM-206,* and *blaTEM-1B* were only found in *S.* Dublin samples 59 and 191. Only 3 isolates were positive for *qnrB19* that resulted in quinolone resistance, 294, 296, and 482. All were *S.* Heidelberg samples found in 2014 and later. *tet(B), tet(M),* and *tet(O)* were only found in *S.* Heidelberg isolates. *aac(3), aadA1, aadA5, aadA5, fosA7, mph(A), dfrA17, dfrA12, dfrA34,* and *qacE* were also only found in *S.* Heidelberg isolates.

Multidrug resistance was driven by the presence of plasmids present in both *S.* Dublin isolates and *S.* Heidelberg isolates. The IncX1 plasmid was observed in only *S.* Dublin isolates while the IncFIB(S), IncHI2, IncHI2-A, and IncI1-I(Alpha) plasmids were observed in only *S.*

Heidelberg isolates (Table 3). The Col(pHAD28) plasmid was found in both *S.* Dublin and *S.* Heidelberg samples, but was observed in 3 *S.* Heidelberg isolates, 294, 296, and 482, while it was only observed in 1 *S.* Dublin isolate, 140. IncFII(S) was observed in only 66, an *S.* Heidelberg isolate. IncFIB(S) was also observed in only 1 isolate, 66, an *S.* Heidelberg isolate. IncI1-I(Alpha) was observed in 2 *S.* Heidelberg isolates, 294 and 296, and no *S*. Dublin isolates.

Additionally, BLAST identification indicated that 25% of genes conferring resistance to antimicrobials were found in the original genome of the bacteria while 68% of genes conferring resistance to antimicrobials were found in plasmids acquired by the bacteria.

Pathogenicity islands varied for the samples with only *S.* Dublin isolates carrying the SPI-10 pathogenicity island and only *S.* Heidelberg isolates carrying the SGI1 pathogenicity island. All 25 isolates carried C63PI, SPI-1, SPI-13, SPI-14, SPI-2, SPI-3, and SPI-5 pathogenicity islands (Table 4). The only isolate lacking the CS54_island and SPI-9 pathogenicity islands was *S.* Heidelberg sample, 184.

Table 3. Antimicrobial resistance genes previously reported on plasmids [36, 37, 38, 39].

Antimicrobial resistance gene	*Salmonella* Dublin (n=12) isolates (%)	*Salmonella* Heidelberg (n=13) isolates (%)	Total isolates (%)	Plasmids the gene may be associated with
floR	11 (92)	13 (100)	24 (96)	F, HI1, HI2, N, R
tet(A)	11 (92)	13 (100)	24 (96)	C, F, HI1, HI2, R
tet(B)	0 (0)	9 (69)	9 (36)	C, F, HI1, HI2, R
tet(M)	0 (0)	12 (92)	12 (48)	C, F, HI1, HI2, R
tet(O)	0 (0)	1 (8)	1 (4)	C, F, HI1, HI2, R
sul1	0 (0)	13 (100)	13 (52)	C, F, HI1, HI2, I1, Q1, R
sul2	11 (92)	13 (100)	24 (96)	C, F, HI1, HI2, I1, Q1, R
qnrB19	0 (0)	3 (23)[A]	3 (12)	X
blaCMY-2	10 (83)	12 (92)	22 (88)	C, F, HI1, HI2, I1, R, X
blaTEM-214	2 (17)[A]	0 (0)	2 (8)	C, F, HI1, HI2, I1, R, X
blaTEM-206	2 (17)[A]	0 (0)	2 (8)	C, F, HI1, HI2, I1, R, X
blaTEM-1B	2 (17)[A]	0 (0)	2 (8)	C, F, HI1, HI2, I1, R, X
aac(6')	12 (100)	13 (100)	25 (100)[B]	C, F, Q1, R, X
aph(6)	11 (92)	13 (100)	24 (96)	C, F, Q1, R, X
aph(3'')	11 (92)	13 (100)	24 (96)	C, F, Q1, R, X
aac(3)	0 (0)	4 (31)	4 (16)	C, F, Q1, R, X
aadA1	0 (0)	5 (38)	5 (20)	C, F, Q1, R, X
aph(3')	2 (17)	13 (100)	24 (96)	C, F, Q1, R, X
aadA5	0 (0)	4 (31)	4 (16)	C, F, Q1, R, X
aadA2	0 (0)	8 (62)	8 (32)	C, F, Q1, R, X
fosA7	0 (0)	13 (100)	13 (52)	Chromosome*
mph(A)	0 (0)	8 (62)[B]	8 (32)	HI1, HI2[B]
dfrA17	0 (0)	4 (31)	4 (16)	C, F
dfrA12	0 (0)	7 (54)	7 (28)	C, F
dfrA34	0 (0)	1 (8)	1 (4)	C, F
qacE	0 (0)	13 (100)	13 (52)	Class 1 integron*

Antimicrobial resistance genes were observed in both *S.* Dublin and *S.* Heidelberg. * Denotes a non-plasmid location for the gene in literature, [A] denotes an important finding limited to one or a few isolates, and [B] denotes a finding important for either *S.* Dublin or *S.* Heidelberg in comparison to the other serotype. The plasmid abbreviations are named as such after their replicon type. They are as follows: IncC for the A or C replicon; IncF for the F, FII, FIA, FIB, or FV replicon; IncHI1 and IncHI2 for the HI1 or HI2 replicon; and IncX for the X(1-6) replicon [36].

Table 4. Positive plasmids for *S.* Dublin (n=12) isolates and *S.* Heidelberg (n=13) isolates.

Plasmid	*Salmonella* Dublin (n=12) isolates (%)	*Salmonella* Heidelberg (n=13) isolates (%)	Total isolates (%)
IncC	11 (2)	13 (100)	24 (96)
IncFII(S)	12 (100)	1 (8)	13 (52)
IncX1	12 (100)	0 (0)	12 (48)
IncFIB(S)	0 (0)	1 (8)	1 (4)
IncHI2	0 (0)	8 (62)	8 (32)
IncHI2A	0 (0)	8 (62)	8 (32)
Col(pHAD28)	1 (8)	3 (23)	4 (16)
IncI1-I(Alpha)	0 (0)	2 (15)	2 (8)

Each serotype contained at least one unique plasmid that was not present in any isolate of the other serotype.

Table 5. Positive *Salmonella* Pathogenicity Islands (SPI) for *S*. Dublin (n=12) isolates and *S*. Heidelberg (n=13) isolates.

Salmonella Pathogenicity Island	*Salmonella* Dublin (n=12) isolates (%)	*Salmonella* Heidelberg (n=13) isolates (%)	Total isolates (%)
C63PI	12 (100)	13 (100)	25 (100)
CS54_island	12 (100)	12 (92)	24 (96)
SPI-1	12 (100)	13 (100)	25 (100)
SPI-10	12 (100)	0 (0)	12 (48)
SPI-13	12 (100)	13 (100)	25 (100)
SPI-14	12 (100)	13 (100)	25 (100)
SPI-2	12 (100)	13 (100)	25 (100)
SPI-3	12 (100)	13 (100)	25 (100)
SPI-4	10 (83)	7 (54)	17 (68)
SPI-5	12 (100)	13 (100)	25 (100)
SPI-9	12 (100)	12 (92)	24 (96)
SGI1	0 (0)	3 (23)	3 (12)

S. Dublin and *S.* Heidelberg isolates shared similar SPIs, with multiple SPIs present in all (n=25) isolates.

Discussion

In this study, the genomes of *S.* Dublin and *S.* Heidelberg isolates were compared to evaluate similarities and differences between the serotypes over 8 years. By performing whole-genome analysis of the serotypes, we were able to assess antimicrobial genes, plasmids, and SPIs unique to each serotype and seen in both serotypes. More unique antimicrobial genes were observed in *S.* Heidelberg than in *S.* Dublin such as *qnrB19, tet(B), tet(M), tet(O), aac(3), aadA1, aadA5, aadA5, fosA7, mph(A), dfrA17, dfrA12, dfrA34,* and *qacE.* This was consistent with the plasmid analysis as well where *S.* Heidelberg contained the unique plasmids, IncFIB(S), IncHI2, IncHI2-A, and IncI1-I(Alpha). However, SPIs were consistent across both serotypes, suggesting that antimicrobial resistance genes and plasmids were more diverse in terms of proliferating across serotypes. Over time, more antimicrobial resistance elements were acquired in *S.* Dublin and *S.* Heidelberg samples, even if they did not remain in all subsequent isolates, indicating the importance of considering increasing antimicrobial resistance elements in *Salmonella* found on veal farms.

The 25 isolates came from the Penn State Animal Diagnostic Laboratory repository. They originated in veal calves in Pennsylvania and New York over the years 2011-2018 (Appendix). The majority of the isolates came from Montgomery County, Pennsylvania (n=18), so they had some opportunity for geographic interaction. This study was performed to better understand genomic similarities and differences between *S.* Dublin and *S.* Heidelberg in Pennsylvania and New York. Antimicrobial resistance was a focus of this study to assess how the movement of mobile genetic elements may contribute to increasing resistance in isolates of *S.* Dublin and *S.* Heidelberg in cattle. Through this analysis, we may be able to generate a deeper understanding

of genome characteristics of *S.* Dublin and *S.* Heidelberg within the state of Pennsylvania and make some predictions about certain areas in the state of New York.

S. Dublin and *S.* Heidelberg shared several characteristics in their overall genome characteristics as well as several key differences. Their overall mean genome size was relatively similar—*S.* Dublin reported a mean genome size of 4,966,883 bp and *S.* Heidelberg reported a mean genome size of 5,159,821 bp. *S.* Dublin had a sequence type of 10, which was consistent with the findings of prior literature [10]. Park et al. (2021) used isolates from 2010-2014, which was like those in this study from 2011-2018. *S.* Heidelberg had a sequence type of 15, in contrast. Further, *S.* Dublin and *S.* Heidelberg shared different antigenic profiles of 9:g:p:- and 4:r:1,2, respectively. These characteristics demonstrate the considerable genomic differences between the *S.* Dublin and *S.* Heidelberg isolates used for this study.

For *S.* Dublin, the most common antimicrobial resistance genes were *floR, tet(A), sul2, aac(6'), aph(6), and aph(3'')*, which were observed in 11 of the isolates with the exception of *aac(6')* which was observed in all 12 isolates. These findings are consistent with previous literature, however they do introduce more common genes than have been previously noted as most common. Eyler et al. (2020) reported the most common genes from their study as *aph(3'')-Ib, aph(6)-Id, sul2,* and *tet(A),* which were somewhat consistent with our findings with the addition of *floR* and *aac(6')*. Overall similarities in commonality of genes may be due to geographic distribution since both our study and the previous study evaluated isolates primarily from Pennsylvania, while the slight differences in antimicrobial gene profile could arise because of study duration as their study range was somewhat earlier than ours from 2009-2014 as compared to 2011-2018 [5]. Another related study determined the most common antimicrobial resistance gene groups seen in *S.* Dublin as *aac(6)-Iaa, CMY*, and *sul2* [13]. As with Eyler et al.

(2017), several of these genes were also consistent with our most common genes—in fact, the *CMY* genes were also common in our study with 10 isolates containing *blaCMY-2.* Additional genes observed in our study that were not seen in Carroll et al.'s study may be attributed to geography and time, since the previous study evaluated isolates in Washington and New York from 2008-2012 [13].

For *S.* Heidelberg, the most common antimicrobial resistance genes were *floR, tet(A), tet(M), sul1, sul2, blaCMY-2, aac(6'), aph(6), aph(3''), aph(3'), fosA7*, and *qacE*, which were observed in all 13 *S.* Heidelberg isolates with the exceptions of *tet(M)* and *blaCMY-2* which were observed in 12 *S.* Heidelberg isolates. Compared to *S.* Dublin, *S.* Heidelberg featured antimicrobial resistance to a broader range of antimicrobial classes in addition to containing more genes encoding resistance to a given antimicrobial class except for beta-lactams. For beta-lactams, *S.* Dublin did have greater variety in antimicrobial resistance genes. Over time, greater divergence in antimicrobial resistance gene profiles was observed in several isolates of *S.* Heidelberg, notably, 294 and 296 from 2014. Phylogenetically, these two isolates were genetically like one another as well as different from the other isolates. They also reported more unique antimicrobial resistance gene profiles and plasmids than other *S.* Heidelberg samples such as the *qnrB19* gene and the IncI1-I(Alpha) plasmid. These findings are consistent with prior literature that noted that newer isolates typically contained more antimicrobial resistance genes in addition to a greater variety in antimicrobial resistance genes when compared to older isolates [40]. Similarities between this previous study and ours may be due to overlapping times of isolate collection, since the study's collection period, 2014-2016, fell within our collection period, 2011-2018 [40].

Plasmids commonly observed in *S.* Dublin isolates included IncC (n=11), IncFII(S) (n=12), and IncX1 (n=12). These findings were consistent with prior literature that found common plasmids in *S.* Dublin to be IncX1, IncA/C2, and IncFII [41]. Consistency in results may have been achieved since both studies used isolates that were bovine in origin [41]. The presence of the IncC and IncX1 plasmid are indicative of *S.* Dublin's virulence and antimicrobial resistance [41].

S. Heidelberg experienced greater diversity in plasmids than *S.* Dublin in this study. The most common plasmid noted was IncC, which was observed in all 13 *S.* Heidelberg isolates. The presence of the IncC plasmid has been documented in prior literature, but perhaps not to the same degree as it was in this study. Hoffmann et al. (2014) also reported the presence of an IncC plasmid in *S.* Heidelberg, but it was certainly not in all the isolates as it was in this study [42]. A potential reason for the difference in commonality for the IncC plasmid could be attributed to the fact that this study focused on turkey while our study focused on cattle [42]. *S.* Heidelberg plasmids of note include IncFII(S), IncFIB(S), Col(pHAD28), and IncI1-I(Alpha). While these plasmids were not common to all the isolates, they were present in a few isolates. They provide evidence for transmission of mobile genetic elements that may confer antimicrobial resistance in *S.* Heidelberg. A specific example is the Col(pHAD28) plasmid that was observed along with the *qnrB19* gene, conferring resistance to quinolones as previously studied [43].

S. Dublin and *S.* Heidelberg shared similarities in the presence of their SPIs than in their antimicrobial resistance genes or their plasmids. SPIs they had in common included C63PI, CS54_island, SPI-1, SPI-13, SPI-14, SPI-2, SPI-3, SPI-4, SPI-5, and SPI-9. There were only 2 SPIs that were unique-- SPI-10 for *S.* Dublin and SGI1 for *S.* Heidelberg. These similarities may indicate that certain SPIs are common to both *S.* Dublin and *S.* Heidelberg or that considerable

transmission between the two serotypes has taken place to allow them to have such similarities. For example, prior research has pointed to the importance of SPI-2 in *S.* Dublin for the bacteria to survive in macrophages, which may translate to *S.* Heidelberg as well [16]. This may suggest that harboring this SPI is beneficial to the *Salmonella,* which may explain is high prevalence in both serotypes [16]. SGI1 is a chromosomally encoded genomic island, which may make it more difficult for it to transmit into other bacteria [36]. This may help to explain its low prevalence in all isolates including *S.* Heidelberg where it was observed.

As discussed in the Review of Literature, SPIs frequently contribute to virulence in *Salmonella.* SPI-1, SPI-2, SPI-3, SPI-4, and SPI-5 are reportedly common to all *Salmonella* serotypes, which was consistent with the findings of this study [15]. This same study also noted that *S.* Dublin may contain SPI-19, SPI-20, SPI-21, SPI-22, and SPI-23, however this was not evident in our study [15]. In general, the SPIs assist in the initial invasion and subsequent replication in the host.

An important limitation for this study included the lack of isolates needed to do a proper comparison study for *S.* Heidelberg isolates and *S.* Dublin isolates.

S. Dublin and *S.* Heidelberg presented with unique genomes containing antimicrobial resistance genes, plasmids, and SPIs. Variance between the two serotypes was clear when analyzing antimicrobial resistance genes and plasmids, but SPIs did contain a few differences between the serotypes. The results from this study will help to better understand the virulence and transmission of mobile genetic elements present in *S.* Dublin and *S.* Heidelberg. Further research may involve drawing closer comparisons between *S.* Dublin and *S.* Heidelberg in the same geographic region where the *Salmonella* may have the opportunity to directly interact. This

may help to generate a clearer understanding of direct interaction between the two serotypes that may produce better recommendations for animal and public health officials.

Conclusion

Strains of *Salmonella enterica* serotypes Heidelberg and Dublin encode for a broad range of antimicrobial resistance genes. In Pennsylvania, both serotypes have been known to cause disease and death in dairy cattle. Understanding increasing antimicrobial resistance in *S.* Dublin and *S.* Heidelberg and is essential to better inform both animal health and public health practices in response to rising resistance.

The overall goal of this study was to elucidate the emergence and transmission of genes conferring antimicrobial resistance within these two *Salmonella* serotypes. Three primary objectives were addressed to accomplish this goal. First, descriptive epidemiology was used to analyze the genomic sequences of both *S.* Dublin and *S.* Heidelberg from the stored meta-data. Second, the profiles of the antimicrobial resistance genes were understood including the transposon-related elements that could contribute to this antimicrobial resistance. Third, a new hypothesis was proposed on genetic AMR relatedness based on time, veal farm, and location.

25 total *Salmonella enterica* isolates were analyzed, 12 *S.* Dublin and 13 *S.* Heidelberg. Of these 25 isolates, 24 were in the state of Pennsylvania and the last isolate was in the state of New York. These isolates revealed interesting qualities about antimicrobial resistance genes, plasmids, and pathogenicity islands in both *S.* Dublin and *S.* Heidelberg.

The genomic sequences of the *S.* Dublin and *S.* Heidelberg revealed that the greatest similarities between the two serotypes were in antimicrobial resistance genes and pathogenicity islands. Additionally, phylogenetic trees revealed which *S.* Dublin isolates were like one another and which *S.* Heidelberg isolates were like one another. For *S.* Dublin, 318 was different from

the other isolates. For *S.* Heidelberg, 296 and 294 were different from the other isolates, which was reflected in their antimicrobial profiles.

Antimicrobial genes were assessed for both *S.* Dublin and *S.* Heidelberg and the rise of new genes over time is cause for some concern in terms of both animal health and public health. Furthermore, the connection between antimicrobial genes and certain plasmids was reaffirmed, with a few exceptions where genes that are typically carried on certain plasmids, namely IncC and Col(pHAD28), were not found in the isolates themselves.

The results of this study provide information on the emergence and transmission of antimicrobial resistance genes and elements of *S.* Dublin and *S.* Heidelberg. It can help to advise future decisions made on behalf of animal health and public health.

Appendix

Table 6. Demographic information for *S.* Dublin and *S.* Heidelberg (n=25) from veal farms.

Isolate number	Serovar	Submission date	County*	Source
59	Dublin	7/29/2011	Montgomery	Blood bottles, swab
66	Heidelberg	8/29/2011	Montgomery	Blood culture, swab
74	Heidelberg	10/4/2011	Franklin*	Feces
76	Dublin	10/11/2011	Montgomery	Blood cultures
134	Heidelberg	8/8/2012	Mifflin	Feces
138	Dublin	8/21/2012	Clinton	Calves
140	Dublin	8/22/2012	Montgomery	Swab
141	Heidelberg	8/23/2012	Montgomery	Calves
151	Dublin	9/28/2012	Montgomery	Blood culture, swab
164	Heidelberg	12/18/2012	Montgomery	Swab
165	Heidelberg	12/20/2012	Montgomery	Swab
166	Heidelberg	1/2/2013	Montgomery	Swab
177	Dublin	1/21/2013	Montgomery	Blood cultures, swab
184	Heidelberg	3/13/2013	Montgomery	Swab
191	Dublin	3/28/2013	Crawford	Feces
225	Dublin	8/20/2013	Franklin	Tissues fresh & mixed
226	Heidelberg	8/20/2013	Montgomery	Blood culture, swab, feces
294	Heidelberg	7/25/2014	Schuyler+	Calves
318	Dublin	9/16/2014	Blair	Calf
296	Heidelberg	7/30/2014	Montgomery	Calves
307	Dublin	8/22/2014	Montgomery	Blood, swab
405	Heidelberg	7/1/2016	Montgomery	Blood, feces, fresh tissue
406	Dublin	7/6/2016	Montgomery	Swabs
482	Heidelberg	5/10/2018	Montgomery	Blood culture, swab
484	Dublin	5/16/2018	Montgomery	Blood culture, swab, tissue

*Denotes an estimated county based on available information and + denotes an isolate in New York. All remaining isolates are from confirmed counties in Pennsylvania.

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The Pennsylvania State University, University Park, PA
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Bachelor of Science in Immunology and Infectious Disease
Minor in Art History
Honors in Immunology and Infectious Disease

Experience

Jayarao Lab
Research Assistant
(6/2021-5/2022)

Self
Freelance Content Writer
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Department of Veterinary & Biomedical Sciences
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Awards & Honors

Dreibelbis Endowment for Excellence in Agriculture (2019, 2020, 2021)
R. F. Russell Memorial Scholarship (2019)
College of Agricultural Sciences Alumni Internship Award (2020)
John N. Adam, Jr. Scholarship for Excellence in Agriculture (2021)
Dean's List (Fall 2019, Spring 2020, Fall 2020, Spring 2021, Fall 2021)

Activities

THON (10/2019-3/2022)
Phi Epsilon Kappa (3/2021-Now)
Penn State Fresh START Day of Service (8/2019-9/2021)