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Distribution of Antimicrobial Resistance Genes across *Salmonella enterica* Isolates from Animal and Nonanimal Foods

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Abstract

Antimicrobial-resistant bacteria are a major public health problem. Of particular importance in the context of food safety is the prevalence of antimicrobial resistance (AMR) genes within nontyphoidal *Salmonella*, which is a leading bacterial cause of foodborne disease. We determined the prevalence of AMR genes across a very large number of *Salmonella* genomes (n = 25,647) collected from isolates from 16 common food sources. The average percentage of isolates from nonanimal foods, such as fruit, nuts and seeds, and vegetables, harboring at least one AMR gene was only marginally lower (72%) than that observed in isolates from animal foods such as beef, chicken, turkey, and pork (74%). This high prevalence of AMR genes was primarily driven by the high prevalence of aminoglycoside resistance genes in nearly all food isolates; genes for resistance to tetracycline and sulfonamide also were highly prevalent. However, evaluation of the number of genes per isolate revealed that the prevalence of AMR genes was higher in animal food isolates than in nonanimal food isolates (P = 0.018). A random forest analysis provided evidence that within a given serovar, resistance gene profiles differed according to isolate food source. AMR gene profiles could be used to correctly predict the food of origin for 71% of the isolates, but success differed according to serovar. This information can help inform AMR risk assessments of food commodities and refine processes for targeting interventions to limit the spread of AMR through the food supply.

Keywords

Antimicrobial resistance; Food; Public health; *Salmonella* ; Whole genome sequencing

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SUPPLEMENTAL MATERIAL

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Nontyphoidal *Salmonella* is a leading bacterial cause of foodborne disease worldwide. In the United States, *Salmonella* annually causes approximately 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths (28). From 2004 to 2012, the Centers for Disease Control and Prevention (CDC) estimated the number of culture-confirmed antimicrobial resistant nontyphoidal *Salmonella* infections at ~2 per 100,000 person years (21). Infants, elderly persons, and immunocompromised persons are at greatest risk of infection and severe outcomes from salmonellosis. Most people infected with *Salmonella* recover without treatment, but complicated infections require antibiotic therapy. The CDC (7) has estimated that 100,000 drug-resistant *Salmonella* infections occur each year in the United States. These drug-resistant infections can cause more severe clinical outcomes such as increased rates of hospitalization, sepsis, invasive disease, and death (10, 15, 32, 37).

Humans are exposed to antimicrobial-resistant *Salmonella* strains through food, animals, and the environment (8). Although antimicrobial resistance (AMR) in foodborne pathogens is a complex challenge, the use of antimicrobial agents in humans and food-producing animals is generally considered the main driver of resistance (12, 30). As a result, surveillance programs such as the National Antimicrobial Resistance Monitoring System (NARMS) have been established to track the emergence and spread of AMR that may result from agricultural livestock uses of antibiotics (34). Comparison of phenotypic and genotypic *Salmonella* data in the NARMS isolate collection revealed a high correlation (99.0%) between clinical resistance and the presence of known resistance genes and mutations (18). In a recent study of >5,000 *Salmonella* genomes, MICs were predicted with high confidence (~95%) from the genomic data alone (22). This high predictability of resistance and perhaps MICs from whole genome sequence (WGS) data in the absence of standard susceptibility testing results greatly expands the scope of surveillance to include any genomic sequence available for analysis.

The widespread use of antibiotics in individuals and groups affects the microbiomes of humans, animals, and the environment. As a result, AMR may be viewed within the One Health framework (19); it has been described as “the quintessential One Health issue” (27). An integral part of this One Health approach is understanding the distribution and prevalence of AMR determinants within animal and nonanimal agricultural products. This information can be used to reduce negative impacts on animal, human, and environmental health and for AMR risk assessments of various food commodities to identify the most likely sources of strains of resistant microorganisms that cause zoonotic and foodborne illnesses. This approach permits development of more precise intervention strategies based on the contribution of various sources to the burden of resistance in human infectious diseases.

In this study, we used WGS data to predict resistance and analyzed AMR gene profiles in a large collection of *Salmonella* isolates. The genomic sequences were from numerous food sources and have been deposited in the National Center for Biotechnology Information (NCBI) database by many agencies around the world (e.g., the U.S. Food and Drug Administration, U.S. Department of Agriculture, and Public Health England). We investigated the top 20 most abundant *Salmonella* serovars found in foods for the strength of association between specific AMR profiles and food categories. Our primary objective was

to elucidate the patterns of AMR at the genomic level in *Salmonella* isolates from animal and nonanimal foods to more fully understand the nature and magnitude of AMR within the food supply.

MATERIALS AND METHODS

Data source, AMR gene detection, and serovar prediction.

The *Salmonella* database within the NCBI Pathogen Detection project (https://www.ncbi.nlm.nih.gov/pathogens/) was used as the source of information for this study. The WGS data for each isolate are also accompanied by metadata such as isolation source and collection date. Seventy-five percent of the isolates analyzed here that had an isolation date were collected after 2010. The NCBI also provides AMR gene profiles for each isolate based on the results of the AMRFinder tool (9), which identifies acquired resistance genes in protein, nucleotide, and genomic data. Individual AMR genes identified by AMRFinder were manually sorted into antimicrobial classes also according to AMRFinder (Table 1). The version of NCBI's *Salmonella* Pathogen Detection database analyzed here was PDG000000002.1138.

Distribution of AMR genes differ by origin of isolates and serovar (6, 13); therefore, we stratified resistance gene comparisons across these categories. Although serovar is a field within the metadata, it is not standardized or required. Therefore, we used the SeqSero tool (41) to predict serovar from the WGS data for each isolate in the database. Isolates with ambiguous serovar predictions from SeqSero were removed. Supplemental Table S1 includes the predicted serovar, isolation sources sorted into the food categories, AMR gene, AMR gene class, and BioSample accession for the top 20 serovars.

Categorization of food sources.

Unique isolation sources were sorted into 16 food categories based on a modified version of a hierarchical isolation source categorization scheme (26); we removed data for isolates from nonfood or unknown sources. The 16 categories were beef, chicken, dairy, eggs, fish, fruit, game, grains, nuts and seeds, other meat, other poultry, pork, shellfish, turkey, vegetables, and multi-ingredient foods (Fig. 1). No distinction was made between isolates from beef or dairy cattle; samples from cattle were grouped under "beef." Isolates in NCBI's Pathogen Detection database do not represent a random sample; many isolates were collected as part of risk-based sampling strategies to focus on certain food types because of a long history of public health concerns regarding salmonellosis (e.g., in chicken).

Data analysis.

A paired *t* test was performed in R v.3.3.3 (24) to determine whether there was a difference in the average number of AMR genes present in *Salmonella* isolates from nonanimal foods (fruit, vegetable, nuts and seeds, multi-ingredient, and grains and beans) compared with isolates from animal foods. We also analyzed the distribution of resistance genes by *Salmonella* serovar and food source for the top 20 most abundant serovars found within foods. We focused on the top 20 serovars because AMR genes are known to differ by serovar (6, 13). For each of the top 20 serovars, we also investigated how well AMR gene

profiles of these isolates could be used to predict food category. We used a random forest approach within the R v.3.3.3 package randomForest v.4.6.12 (17). Within the randomForest analysis, the number of trees was 1,000, type was classification, and prediction accuracy was evaluated with type set to response using both out-of-bag error rates and via cross-validation (75% of data for training and 25% for testing); out-of-bag error rates are presented. Plots were generated with the R package ggplot2 v.2.2.1 (39).

RESULTS

Distribution of *Salmonella* isolates among food categories.

The 106,470 *Salmonella* isolates and associated metadata (e.g., AMR gene profiles and isolation source) represented 4,963 isolation sources and 552 predicted serovars. After removing isolates from nonfood or unknown sources, the data set was reduced to 25,647 isolates. To discern relationships between AMR and serovar, we focused on the top 20 most abundant serovars among isolates collected from a food.

Further subsetting the data to the top 20 serovars reduced the number of isolates by 37% to 16,095. Animal food categories (e.g., chicken, pork, turkey, and beef) had the greatest number of isolates in the full and the top 20 serovar data sets (Table 2), with the majority of isolates belonging to *Salmonella* serovars Kentucky, Enteritidis, and Typhimurium (O5–). Abundance of each of the top 20 serovars differed by food category, which is to be expected based on previous work (14); for example, the majority of *Salmonella* Saintpaul isolates were from turkey, the majority of *Salmonella* Enteritidis isolates were recovered from chicken, and *Salmonella* Newport isolates were found in nearly equal numbers in both beef and vegetables (Fig. 1 and Table 2).

Distribution of AMR genes.

We found 279 unique AMR alleles across the entire data set, of which 139 were also found in isolates from foods; genes coding for sulfonamide, tetracycline, and aminoglycoside resistance were the most abundant (Table 1). The number of AMR genes within food isolates differed by serovar and food category (Table 2). Among the serovars, *Salmonella* Infantis appeared to have the broadest spectrum of resistance, with frequencies of >0.1 allele per isolate for eight antimicrobial classes. Other broad spectrum resistant *Salmonella* serovars were Agona, Derby, Dublin, Heidelberg, Newport, Saintpaul, Typhimurium, and Typhimurium (O5–), which all carried resistance genes found at >0.1 allele per isolate associated with five or more antimicrobial classes (Fig. 2). A significant portion of the isolates of these serovars came from terrestrial animal sources (Fig. 1). Among the least resistant *Salmonella* serovars were Enteritidis, Johannesburg, Montevideo, and Thompson; for all of these serovars, >40% of the isolates harbored no AMR genes (Fig. 2). Alleles conferring resistance to quinolones and the MLS (macrolides-lincosamides-streptomycin) class of antibiotics (considered highest priority within the critically important class of medically important antimicrobials (40)) were rarely present in any of the top 20 serovars (Fig. 2). However, alleles conferring resistance to another critically important class, aminoglycosides, were the most abundant in all serovars. Resistance to the β-lactams group, which includes alleles coding for both narrow and extended-spectrum β-lactamases, was

present at variable levels across all serovars, with the highest levels observed in *Salmonella* Dublin (1.11 genes per isolate). The highest number of genes conferring phenicol resistance also were found in *Salmonella* Dublin (0.84 genes per isolate). Fosfomycin resistance was found at high frequencies within isolates of *Salmonella* serovars Agona, Derby, and Heidelberg (>0.95 genes per isolate; Fig. 2).

The average percentage of isolates from nonanimal foods (e.g., fruit, nuts and seeds, and vegetables) that harbored an AMR gene was 72% compared with 74% for isolates from animal foods (e.g., beef, chicken, turkey, and pork) (Fig. 3). This result was influenced in part by the presence of aminoglycoside resistance genes, which were found at roughly the same frequency (≥55% across all food types) in isolates from each food type (Fig. 3). However, when accounting for the number of AMR genes within each isolate, the average number of AMR genes per isolate was higher for isolates from animal foods than for those from nonanimal foods ($t = 2.73$, df = 12, $P = 0.018$). The chicken, beef, pork, and turkey categories had the highest percentages of isolates carrying genes conferring resistance to multiple antimicrobial classes (Fig. 3). These results were consistent even when considering all isolates, not just those from the top 20 serovars present in foods (Fig. S1). For example, regardless of serovar, within isolates from those four animal food categories, resistance genes for aminoglycosides, β-lactams, fosfomycin, sulfonamides, and tetracyclines were found in >10% of isolates. The abundance of resistance genes for fosfomycin, an antibiotic used to treat urinary tract infections in humans but not used in food animals in the United States, was highest in beef, pork, turkey, and eggs (Fig. 3). *Salmonella* serovars Agona, Derby, and Heidelberg had the highest percentage of isolates with fosfomycin resistance (Fig. 2). Although less abundant, resistance genes to the majority of antibiotic classes were also found in fish and nonanimal food products, including vegetables, fruits, and nuts and seeds (Fig. 3). Many of these isolates represented *Salmonella* serovars with a low prevalence of resistance genes (e.g., Weltevreden, Branderup, and Thompson; Fig. 2) regardless of isolate source.

The hypothesis that AMR genes could be used to predict the food source of a specific *Salmonella* serovar was generally supported; however, the strength of that predictability varied depending on the serovar being considered (Table 2). For example, isolates of *Salmonella* serovars Dublin, Reading, Typhimurium (O5–), and Kentucky could be correctly classified to their known food source with >87% accuracy. In contrast *Salmonella* Agona isolates were classified to the correct food source with only 57% accuracy. The difference in predictability may be associated with the fact that serovars with the highest predictability were more prevalent in a single food category (e.g., *Salmonella* Dublin is predominantly found in beef; Table 2 and Fig. 1). In contrast, *Salmonella* Agona is more evenly distributed across several food (host) sources. However, *Salmonella* Derby is predominantly prevalent in pork but had a predictability of only 77%, suggesting that for certain food-serovar combinations an associated distinct signature of AMR genes may not be present.

To address the question of whether the AMR gene profile in isolates of particular *Salmonella* serovars was a better predictor of isolation source than was serovar alone, we also performed a random forest analysis using only serovar as a predictor of the food of origin. Under that scenario, we were able to correctly predict isolation source with 68% accuracy, which

demonstrates that serovar is a fairly reliable predictor of food source. However, within each serovar, AMR gene profiles increase the accuracy of that prediction.

DISCUSSION

An understanding of the distribution of AMR genes across isolates from various food sources is important for developing effective methods for confronting the public health issues surrounding AMR. We determined the prevalence of resistance genes to various groups of antimicrobials across the top 20 *Salmonella* serovars isolated from foods. Because of the high prevalence of aminoglycoside resistance among *Salmonella* isolates from all food types, *Salmonella* isolates from terrestrial animals were not necessarily more likely to contain resistance genes than were *Salmonella* isolates from other food types (Fig. 3). However, when we considered the number of AMR genes per isolate we found that isolates from foods of animal origin, where various antimicrobials are more commonly used (16), had a higher prevalence of AMR genes than did isolates from nonanimal foods. This finding suggests that outbreaks of multidrug resistant (MDR) *Salmonella* would be more likely to involve animal-derived foods than nonanimal foods. This suggestion is concordant with the conclusions of Brown et al. (2), who found that strains causing MDR outbreaks were more likely to be linked to food from land animals (29%) than to plant-based foods (8%).

The use of antibiotics in animal agriculture is a longstanding practice, and many countries have implemented surveillance programs, such as NARMS, to track the development and spread of antibiotic resistance in animals, meat products, and humans. For major food animals (cattle, chickens, turkeys, and swine), our results mirror those of the NARMS data set, which comprises a significant portion of the isolates in our data set. Similar to the NARMS 2015 report (33), we found that the genes conferring resistance to critically important antimicrobials (macrolides and quinolones) were largely absent from food animal isolates. Likewise, nonanimal food isolates also bore few to none of these genes. We confirmed the NARMS finding that *Salmonella* Dublin isolates are highly resistant to multiple drug classes, including the broad β-lactams class (33). We also found high AMR among *Salmonella* Infantis isolates (Fig. 2), supporting growing evidence that a poultry-associated clonal MDR strain of *Salmonella* Infantis is currently in circulation around the globe (1, 31). We found a high prevalence of phenicol resistance genes in beef isolates, which supporting the hypothesis that antimicrobial use is the main driver of AMR. In animal agriculture, florfenicol is mainly used in beef and nonlactating dairy cattle, where it is approved for the treatment and control of bovine respiratory disease and foot rot (35). However, the finding of a high prevalence of fosfomycin resistance genes in poultry, swine, and egg isolates is ambiguous. Although fosfomycin is used mostly in South American countries (23) to treat infectious diseases of broilers and swine, it is not approved for use in the United States, where the majority of the swine, poultry, and egg isolates in our data set originated. However, the presence of *fosA* on a large MDR plasmid in *Salmonella* Infantis isolates from chicken indicates that these genes may be coselected through use of other unrelated antimicrobial agents (31, 35). The limited distribution of *fosA* may also be a consequence of biological phenomena rather than drug use. Other researchers have found that *fosA* genes are restricted to certain *Salmonella* serovars (25).

Isolates from game and other poultry and meat isolates had similar AMR gene classes as did isolates from other animal food sources, but the prevalence of those gene classes was reduced. Although these sources yielded *Salmonella* serovars with a high diversity of AMR gene classes, there was also a higher likelihood that these meats would be wild caught or raised organically. Our observation that dairy isolates contained fewer resistance genes supports those of other studies in which most *Salmonella* recovered from bulk tank milk were pansusceptible, although that finding was highly dependent on serovar distribution (29, 36). Egg isolates also had a lower prevalence of resistance genes than did chicken isolates. This difference could be due to differences in antibiotic use practices in hens laying eggs for human consumption, as has been suggested previously (1). A notable exception is the high frequency of fosfomycin resistance genes in isolates from eggs.

Because many *Salmonella* isolates from aquatic and nonanimal food sources also carried AMR genes, such foods should also be considered for possible AMR surveillance. For example, antimicrobials are used in the production of crops such as fruits, and AMR has been found in plant pathogens (38). Therefore, exposure to these antimicrobials in the environment by food species that carry *Salmonella* may also need to be considered (20, 38). Additional possible sources of AMR genes found in crops include wildlife, manure from treated animals used as fertilizer, and farm runoff into irrigation systems. In the United States, three antibiotic classes (tetracyclines, sulfonamides, and phenicols) are approved for use in fish (35), but unrestricted use of other antibiotic classes is permissible in some other countries (3, 11). In our study, fish and shellfish *Salmonella* isolates, which mostly belonged to serovar Weltevreden, were resistant to aminoglycosides, suggesting that the samples from which the isolates were recovered may not have been from the United States. Because many AMR genes (e.g., *mcr-1* [colistin] and *qnrB* [quinolones]) are thought to have aquatic origins (4, 5), imported fish and shellfish certainly should be among those foods evaluated as potential AMR reservoirs.

Our ability to accurately predict the food source based on the type of AMR genes in recovered *Salmonella* isolates was highly dependent on the distribution of food sources in which each serovar was found and further illustrates the complexities of AMR bacteria within foods. For some *Salmonella* serovars, AMR genes could be used to predict with high accuracy the food source of an isolate, which suggests that different foods harbor isolates with distinct AMR profiles (Table 2). However, for other serovars prediction of food source was less certain; for example, there is little differentiation among foods in the prevalence of AMR gene classes in isolates of *Salmonella* Typhimurium and *Salmonella* Braenderup. Although the serovars with the highest prediction accuracy were those whose isolates were primarily from a single food category (e.g., 90% of isolates of *Salmonella* Kentucky were found in chicken; Table 2 and Fig. 1), that pattern does not hold for all serovars. For example, *Salmonella* Infantis has a predictability of 84%, but only 56% of *Salmonella* Infantis isolates came from chicken, which is the most frequently observed food source for this serovar.

Some limitations are evident in our study. The majority of the data were not obtained by random sampling but by risk-based sampling, where certain food sources were likely the focus of intense inspections and thus might be overrepresented. Some food categories

and serovars also were selectively sequenced based on phenotypic results from traditional antimicrobial susceptibility tests, and our results may be biased toward higher levels of AMR. We also did not assess whether AMR changed over time or differed by geographic origin of the isolate. Industry- and regulation-driven changes in antibiotic administration impact AMR over time. For instance, NARMS data indicate a decrease in certain classes of AMR genes among *Salmonella* isolates from poultry, which could be the result of decreased use of third-generation cephalosporins in the poultry industry and greater emphasis on "no antibiotics ever" production (33). We also expect antibiotic use to vary within and among countries, so our findings may differ if we were to focus on only domestically produced foods. The degree to which AMR gene prevalence differs across food sources in relation to geography and time would be an appropriate topic for future research.

In this study, we characterized the prevalence of AMR genes among *Salmonella* isolates present in the NCBI Pathogen Detection database. We also assessed associations between specific AMR alleles and specific food categories. The results presented here may assist public health agencies develop illness prevention programs by contributing to a better understanding of the prevalence and distribution of antimicrobial resistant nontyphoidal *Salmonella* in contaminated food. Further utilization of this rich database by academia, industry food scientists, and other partners will help further define the increase in AMR among foodborne bacterial pathogens and inform efforts to address these issues and the clinical ramifications of increased AMR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Aminoglycoside resistance was found in *Salmonella* isolates from nearly all food types.
- The expected higher multiclass resistance was found among isolates from many animal foods.
- AMR genes could be used to predict the isolate food type of origin with 71% accuracy.

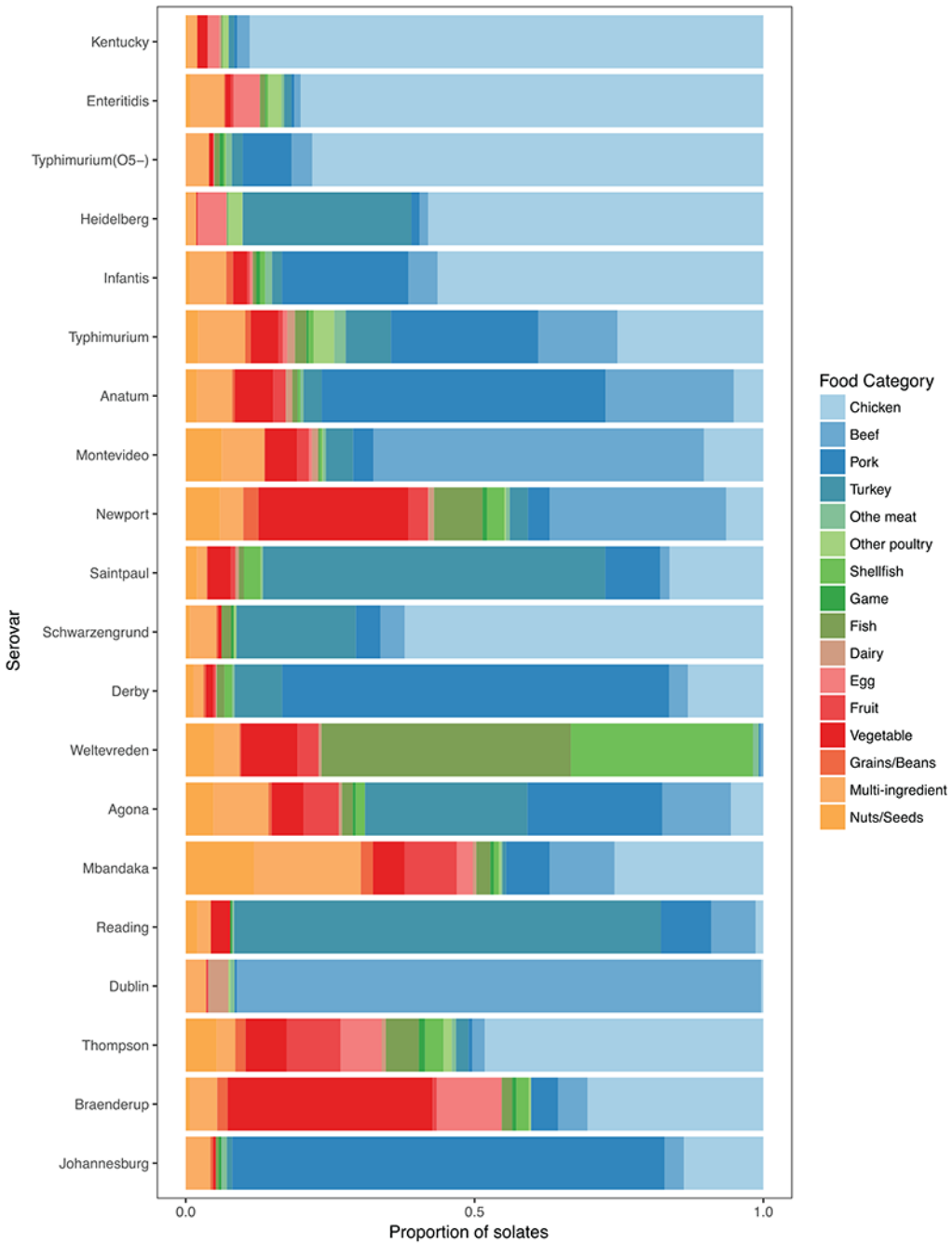

FIGURE 1.

Proportion of Salmonella *isolates of the top 20 most abundant serovars found in each of the food categories.*

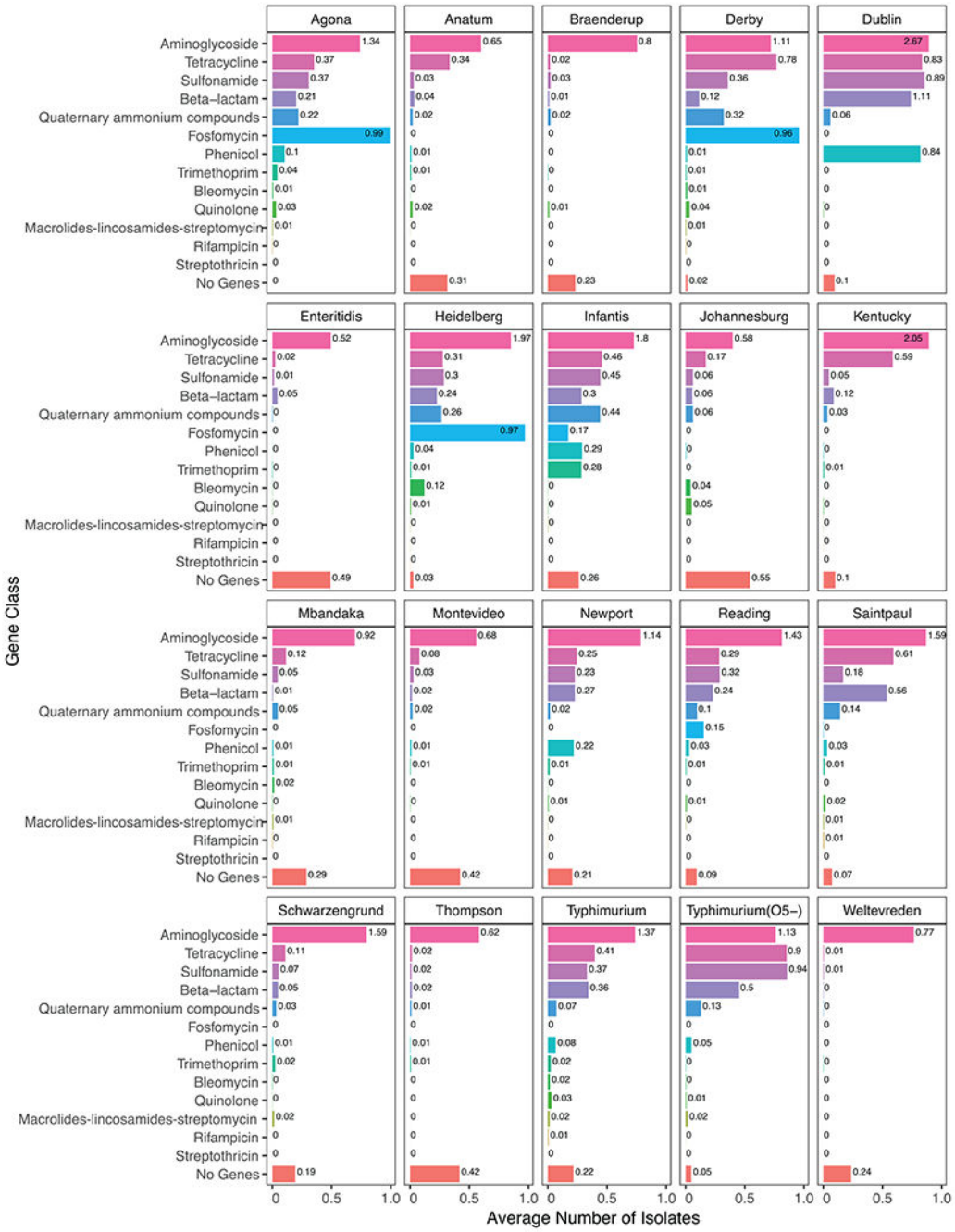

FIGURE 2.

The proportion of isolates within each serovar whose genome included a given type of AMR gene or no AMR genes. Numbers at the ends of the bars represent the average number of AMR gene classes observed within isolates of that serovar (e.g., for Salmonella *Agona, many isolates had multiple aminoglycoside AMR genes for an average of 1.34 aminoglycoside genes per isolate).*

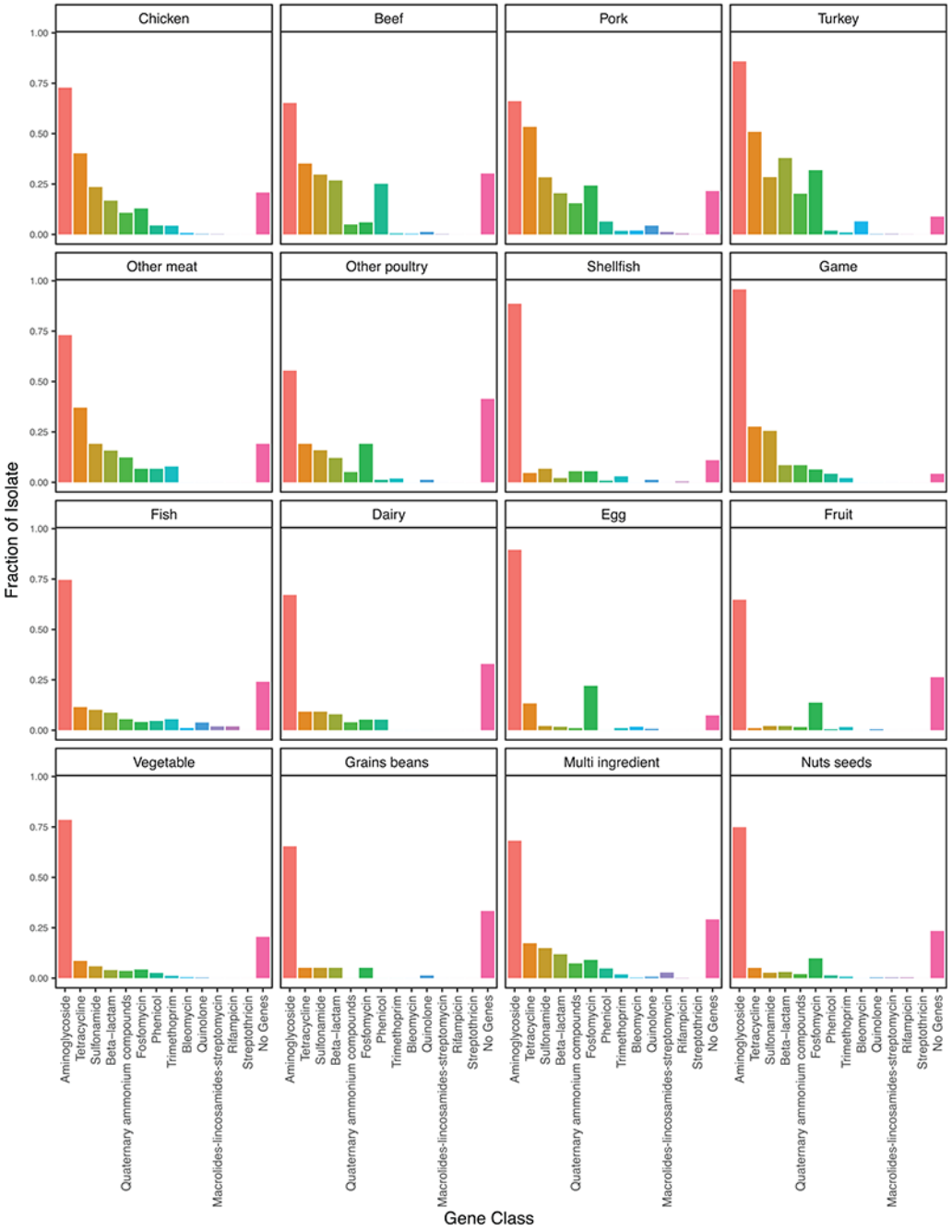

FIGURE 3.

Fraction of isolates from the top 20 Salmonella *serovars containing AMR gene classes separated by food category.*

TABLE 1.

Antimicrobials associated with AMR genes found in food isolates

Antimicrobial type	No. of unique AMR genes	Total no. of AMR genes
Aminoglycosides	44	20,325
β-Lactams	23	3,219
Bleomycin	2	201
Fosfomycin	3	2,293
Macrolides-lincosamides-streptomycin	11	74
NoGenes	1	3,219
Phenicols	8	1,035
Quaternary ammonium compounds	4	1,635
Quinolones	11	153
Rifampin	3	19
Streptothricin	2	4
Sulfonamides	4	3,726
Tetracycline	10	5,922
Trimethoprim	13	460
Total	139	42,285

TABLE 2.

Number of Salmonella *isolates across various food categories for the 20 most abundant* Salmonella *serovars*[a]

Salmonella serovar	No. of isolates																	Accuracy[b]
	Chicken	Pork	Beef	Turkey	Multi-ingredient	Vegetable	Fish	Nuts, seeds	Egg	Shellfish	Fruit	Other poultry	Other meat	Grains, beans	Dairy	Game	Total	
Kentucky	2,142	12	53	23	40	42	4	8	47	3	3	24	0	0	8	0	2,409	0.92
Enteritidis	1,720	11	23	27	126	20	19	17	97	9	10	49	10	4	2	3	2,147	0.79
Typhimurium (O5–)	1,086	118	50	26	56	11	12	0	1	4	0	2	14	0	1	10	1,391	0.88
Heidelberg	735	18	19	370	19	2	2	4	63	1	1	30	1	0	1	1	1,267	0.74
Infantis	619	240	56	19	69	27	6	8	5	10	4	0	13	13	2	7	1,098	0.84
Typhimurium	267	270	145	83	86	51	21	23	8	9	8	38	21	10	14	4	1,058	0.60
Anatum	45	434	196	28	54	59	8	17	0	3	19	0	5	4	10	1	883	0.64
Montevideo	81	28	450	37	58	44	2	49	4	3	16	1	4	1	8	1	787	0.66
Newport	49	29	233	24	31	198	64	45	1	23	26	2	5	20	7	6	763	0.71
Saintpaul	108	63	11	394	11	27	6	13	0	19	5	1	2	1	4	0	665	0.76
Schwarzengrund	411	28	28	137	30	4	11	5	0	0	0	3	0	2	0	3	662	0.80
Derby	68	349	17	43	9	7	7	7	0	7	2	0	2	2	1	0	521	0.77
Weltevreden	0	1	2	0	17	38	167	19	0	122	14	0	4	1	2	0	387	0.47
Agona	21	88	45	106	36	21	7	18	0	6	23	0	0	2	2	2	377	0.57
Mbandaka	85	25	37	2	61	18	8	39	9	3	30	1	1	7	2	2	330	0.48
Reading	4	26	23	221	7	10	0	6	0	0	0	0	1	0	0	1	299	0.89
Dublin	1	1	258	0	10	0	0	0	0	0	1	1	2	0	10	0	284	0.97
Thompson	135	2	6	6	9	20	16	15	20	9	26	4	2	5	2	3	280	0.54
Braenderup	84	13	14	0	13	98	5	2	31	6	2	1	0	5	0	2	276	0.44
Johannesburg	29	158	7	2	9	1	1	0	0	0	0	0	2	1	0	1	211	0.80
Total	7,690	1,914	1,673	1,548	751	698	366	295	286	237	190	157	89	78	76	47	16,095	0.71[c]

[a]Rows are ordered by decreasing totals.

[b]Accuracy refers to the classification success of a given isolate to food source based on its AMR gene profile within the random forest analysis.

[c]Average accuracy of classification within the random forest analyses.