Association Between Isolation Source, Clonal Composition, and Antibiotic Resistance Genes in *Escherichia coli* Samples Collected in Washington State

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Abstract

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Background:

The growing prevalence of antimicrobial resistance (AMR) represents a significant global health problem. AMR is caused by the overuse of antibiotics in humans, animals, and the environment, making it necessary to apply a One Health approach to address the problem.

Methods:

We used whole genome sequencing (WGS) data from 1,449 *Escherichia coli (E. coli)* isolates from Washington State to evaluate the relationship between isolation source (either humans, animals, food, or the environment) and the presence of antibiotic resistance genes (ARGs). We performed sequence typing using PubMLST and used ResFinder to identify ARGs. We categorized isolates as being pan-susceptible, resistant, or multidrug resistant (MDR). We used chi-square tests to assess differences between isolation sources in the frequency of antibiotic resistance genes and sequence types (STs).

Results:

The 1,449 isolates were distributed among 341 unique STs. When comparing the distribution of isolates in the six most common STs overall (ST131, ST73, Unidentified ST, ST95, ST12, and ST69), there was evidence of a difference in the frequency of STs between humans, animals, food, and environmental isolates ($p < 0.001$). In total, 60% of isolates were pan-susceptible, while 18% were resistant, and 22% were MDR. The resistance pattern varied significantly between the four isolation sources ($p < 0.001$). The most resistance was detected in isolates from humans, followed by animals, and environmental isolates showed the least antimicrobial resistance.

Discussion:

Our study demonstrates that there is a relationship between isolation source and the presence of ARGs in *E. coli* samples from Washington State and highlights the need to further characterize transmission of antibiotic resistance genes and antibiotic resistant bacteria between humans, animals, and the environment using from a One Health perspective, both in Washington State and elsewhere.

Introduction

The growing prevalence of antimicrobial resistance (AMR) represents a significant global health problem, undermining progress in healthcare and agricultural food production around the world. The CDC estimates that more than 2.8 million antibiotic-resistant infections occur each year in the United States, resulting in 35,000 deaths (1). Globally, there were around 4.9 million deaths associated with bacterial AMR in 2019, making the magnitude of AMR as a global health problem similar to that of diseases such as HIV and malaria (2). AMR infections pose a threat to people all over the world, and they make crucial healthcare procedures, like joint replacements and organ transplants, potentially life-threatening (1).

AMR is caused by the overuse of antibiotics in humans, animals, and the environment, and the spread and proliferation of resistant bacteria is driven by interactions between these sectors (3–7). Antibiotics are widely used in human medicine, and rates of antibiotic consumption are increasing around the world, largely driven by increased use in lower- and middle-income countries (LMIC) (8). In addition to their use for treating human disease, antibiotics are used in agriculture for treating animal diseases and in some cases for growth promotion and prophylaxis (4). The administration of antibiotics in agriculture results in antibiotic residues and antibiotic-resistant bacteria accumulating in soil and water, where they potentially act as environmental pollutants and promote further spread of AMR genes (4). Antibiotics are also used prophylactically in companion animals with close human contact, which could increase the risk of antibiotic resistance (9,10).

Because of the interdependent nature of AMR in humans, animals, and the environment, it is necessary to apply a One Health approach to address the problem (7). One Health is based on the interconnected nature of humans, animals, and the environment, acknowledging that we share many of the same health exposures and infectious diseases (7). A One Health approach to AMR involves the collaborative efforts of many health professionals across disciplines to prevent the spread of antimicrobial resistant pathogens (7).

One species of particular concern is *Escherichia coli* (*E. coli*), which is one of the leading pathogens causing disease and death related to AMR. *E. coli* is considered by the World Health Organization to be one of the top organisms of international concern related to AMR (2,11). *E. coli* is characterized by its genetic diversity due to its flexible gene pool of mobile genetic elements and genomic islands, which can shift and change rapidly (12). This genetic flexibility enables *E. coli* to act as a donor and reservoir of resistance genes, which is problematic because *E. coli* species are a common pathogen and commensal organism in both humans and animals (7,12,13).

There have been many studies examining resistance in *E. coli* in specific settings, such as human clinical samples (14–16), livestock and agriculture (17,18), and pre-packaged food items (5,19). However, fewer studies examine AMR from a One Health perspective, comparing resistance in multiple sources. Studies that do investigate this show that food animals and meat, particularly poultry, may be a source of *E. coli* infections in humans (20–22). In Washington State, research has separately quantified the presence of antimicrobial resistant *E. coli* in the Salish Sea ecosystem (6) and in outpatient clinical samples isolated from Washington State Public Health Laboratories (14,23). These studies demonstrate the need for continued AMR surveillance efforts, particularly from a One Health perspective.

Traditional assessment of antimicrobial susceptibility involves laboratory assays to test phenotypic resistance, although these methods involve considerable time and financial burdens, and can be difficult to standardize across laboratories and pathogens (24,25). Next generation sequencing (NGS) offers an alternative way to assess AMR by evaluating the presence of antimicrobial resistance genes (ARGs). Tools such as ResFinder can be used to screen genomes for the presence of ARGs, but cannot determine the functional status of those genes, so it is not possible to completely predict the resistance phenotypes of isolates using sequence data alone (26). Some studies have shown that predictions of AMR based on the presence of ARGs are highly correlated with phenotypic testing (24,25,27), but more recent studies have suggested that these predictions can be inconsistent, varying by antibiotic and bacterial species (6).

The goal of this study was to evaluate the relationship between isolation source and the presence of ARGs in *E. coli* samples from Washington State using whole genome sequencing data. To address this question, we first compared the clonal composition of *E. coli* isolated from animal, environmental, food, and human sources using multi-locus sequence typing (MLST). We then measured the degree of association between the isolation source and the frequency of ARGs present among all *E. coli* samples from Washington State, and among samples of the same sequence type.

Methods

Data collection:

Enterobase (28) is a large public database of submitter-supplied data, which collates all the complete genomes and Illumina paired-end reads of enteric bacteria from the NCBI Sequence Read Archive, together with available meta data. We searched Enterobase for all genomes of the species *Escherichia coli* within the state of Washington, USA, and categorized them according to their metadata

as being isolated from either animal, environmental, food, or human sources. Samples with no information about their host or isolation source were excluded.

We downloaded raw sequence files from NCBI in FASTQ format and used Trimmomatic (29) to remove Illumina adapters and clean the sequence files. We used options for a sliding window averaging across four bases with an average quality of 20, and we kept sequences with a minimum length of 25 bases.

Sequence Typing:

We used Megahit (30) for *de novo* assembly of raw reads prior to sequence typing. We performed sequence typing using PubMLST (31) with the Achtman MLST scheme (32) to identify the sequence type (ST) of each bacterial isolate. Isolates were labeled with an unidentified ST ("-") if they had a novel allele, a novel allele combination, or one or more alleles with only a partial match.

Identification of AMR Genes:

We used the ResFinder tool (26) with its associated reference databases to identify AMR genes in all isolates, using raw reads to improve sensitivity and options for both acquired resistance genes and chromosomal mutations. The threshold for gene detection was set to 90% identity and 100% coverage.

Definitions of Drug Resistance:

We categorized isolates as being pan-susceptible, resistant, multidrug resistant (MDR), or extensively drug resistant (XDR) based on guidelines adapted from the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) (33). These guidelines define epidemiologically significant categories of antimicrobial agents, listed in Table 1. Pan-susceptible isolates are defined as isolates that do not carry resistance genes for any antimicrobial agents in any of the categories, while resistant isolates are those that carry resistance genes to at least one antimicrobial agent in at least one category. Multidrug resistance is defined as carrying resistance genes for at least one agent in three or more antimicrobial categories, and extensive drug resistance is defined as carrying resistance genes for at least one agent in thirteen or more antimicrobial categories.

Resistance Profile:

We developed an antimicrobial resistance profile for a genome based on binary resistance (presence of resistance gene(s)) or susceptibility to five key antimicrobial agents representative of their class: ampicillin, ceftazidime, ciprofloxacin, gentamicin, and tetracycline. These antibiotics were selected because they are commonly used in human and animal medicine. Predicted resistance to each of these drugs (presence of resistance genes to that antibiotic) is represented by a 1, while predicted

susceptibility is represented by a 0, resulting in a five-digit resistance profile. We compared the distribution of resistance profiles in each of the four isolation sources.

Statistical Analysis:

For our first aim, we assessed differences in the number of isolates in each ST between isolation sources using Fisher's exact test. For our second aim, we used a chi-square test to compare the numbers of pan-susceptible, resistant, and multidrug resistant isolates in each isolation source.

To examine changes in levels of resistance over time, we conducted multinomial logistic regression to analyze the relationship between antimicrobial resistance category (either resistant or multidrug resistant compared to pan-susceptible) and collection year. We included isolation source in the model with isolates from humans as the reference category.

All effects were evaluated using a significance level of 0.05.

Ethical Approval

This research does not involve human subjects as defined by the Institutional Review Board (IRB) of the University of Washington, and therefore does not require IRB approval or exemption.

Results

Characteristics of *E. coli* isolates

In total, the database search yielded 1,517 isolates of *E. coli* from Washington State with genomic data in NCBI. Of these, 68 were eliminated due to poor read quality or missing information about bacterial isolation source. Of the remaining 1,449 isolates, 296 (20.4%) were isolated from animals, 327 (22.6%) were isolated from environmental sources, 54 (3.7%) were isolated from food, and 772 (53.3%) were isolated from humans. Examples of common specific isolation sources are listed in Table 2.

Temporally, isolates were collected between 1947 and 2022. There were 101 isolates (7.0%) collected before 2005, 343 isolates (23.7%) collected between 2005-2015, and 579 isolates (40.0%) collected after 2015. There were 418 isolates (28.8%) with no data for collection year (Table 2). All isolates were collected within Washington State, but most isolates (70.5%) were missing additional geographic information. Of those with additional geographic information, the only two Washington counties represented were King County (n = 264, 18.2%) and Thurston County (n = 164, 11.3%).

Clonal Composition of *E. coli* Isolates

The 1,449 isolates were distributed among 341 unique STs. The STs with the most isolates overall were ST131 (n = 131, 9.0%), ST73 (n = 80, 5.0%), and ST95 (n = 65, 4.5%). Most STs (n = 172, 50.5%) had only one isolate, while 59 STs (17.3%) had two isolates. There were 66 isolates (4.6%) whose ST was unidentified ("-"). The isolates with unidentified STs came from all four isolation sources, but most commonly (59%) from environmental sources. Human isolates had the fewest samples with an unidentified ST.

Several of the most common STs in human isolates are known to be lineages of extraintestinal pathogenic *E. coli* (ExPEC), including STs 131, 69, 95, and 73, which are associated with common urinary tract and bloodstream infections (34). ExPEC group STs were also commonly found in animal and environmental isolates, including STs 10, 117, 69, 73, and 58.

The most common STs differed between isolation sources (Figure 1). Isolates from animal and food sources had three of their six most common STs in common: ST21, ST11, and Unidentified ST. The most common STs among environmental isolates were least likely to be shared with other isolation sources; of the 12 most common STs in environmental samples, nine were unique to environmental samples. When comparing the distribution of isolates in the six most common STs overall (ST131, ST73, Unidentified ST, ST95, ST12, and ST69), there was evidence of a difference in the frequency of STs between the four isolation source categories ($p < 0.001$).

Predicted Antimicrobial Resistance

In total, 60% of isolates were pan-susceptible, while 18% were predicted resistant, and 22% were predicted multidrug resistant. No isolates were categorized as extensively drug resistant. The resistance pattern varied significantly between the four isolation sources ($p < 0.001$). The most resistance was detected in isolates from humans, with 54% of these samples categorized as either resistant or multidrug resistant, followed by animals, with 37% of samples either resistant or multidrug resistant (Figure 2). About one third (33%) of human isolates were multidrug resistant, the most of any other isolation source. Environmental isolates showed the least antimicrobial resistance, with only 12% of environmental samples categorized as either resistant or multidrug resistant.

Within each of the most common STs, the resistance pattern also varied between the four isolation sources. The variation in resistance between isolation sources was statistically significant among isolates of ST131 ($p = 0.003$) and ST73 ($p = 0.002$). Within the other most common STs (ST 95 and Unidentified ST), there was not significant evidence of an association between resistance and isolation source within each ST.

Resistance Over Time

Isolate collection can be broadly grouped by peaks in three time periods: 1947-2004, 2005-2015, and 2016-2022. Comparing isolates across these three time periods, our data shows that the greatest levels of resistance overall were seen in the period 2005-2015. However, this relationship is different within each isolation source (Figure 3). In humans, the largest isolation source, the trend mirrors the data as a whole, with the greatest proportion of resistant and multidrug resistant isolates in 2005-2015. However, in animal isolates, the next largest isolation source, the greatest proportion of resistant and multidrug resistant isolates were collected in 1947-2004. In environmental and food isolates, small sample sizes make it difficult to discern a clear pattern.

A multinomial logistic regression shows that as collection year increases, the odds of an isolate being resistant vs pan-susceptible decline about 2% while holding isolation source constant ($p < 0.001$; data not shown). However, the odds of an isolate being multidrug resistant vs pan-susceptible increase about 1% every year while holding isolation source constant ($p < 0.001$). Isolates from animals have 58% lower odds of being categorized as multidrug resistant compared to isolates from humans within the same collection year ($p < 0.001$). Similarly, compared to human isolates, environmental samples have 85% lower odds of being categorized as multidrug resistant ($p < 0.001$) and food samples have 87% lower odds of being categorized as multidrug resistant ($p < 0.001$).

Resistance Profile

There were 23 unique resistance profiles based on binary resistance indicators to five key antimicrobial agents: ampicillin, ceftazidime, ciprofloxacin, gentamicin, and tetracycline. The most common resistance profile overall was a lack of resistance genes to all five antimicrobial agents (profile 00000, 64% of isolates), followed by resistance genes for ampicillin alone (profile 10000, 6% of isolates) and tetracycline alone (profile 00001, 5% of isolates). Around 1% of isolates had resistance genes to all five profile drugs. Human isolates had the greatest number of unique resistance profiles, followed by environmental isolates (Figure 4). When comparing the distribution of isolates in the six most common profiles overall (lack of resistance genes to all five drugs, resistance genes for ampicillin alone, resistance genes for tetracycline alone, resistance genes for ampicillin and ciprofloxacin, resistance genes for ampicillin and tetracycline, and resistance genes for ciprofloxacin alone), there was evidence of a significant difference in the frequency of resistance profiles between the four isolation source categories ($p < 0.001$).

Discussion

This study of 1,449 *E. coli* isolates from Washington State found a total of 341 unique STs, and the distribution of the most common STs differed significantly between isolates from animal, environmental, food, and human sources. We also found a significant difference in the proportion of isolates that were resistant and multidrug resistant comparing samples from the four isolation sources. Samples from humans showed the most resistance, followed by animals, food, and lastly environmental samples.

The distribution of STs within human isolates is unsurprising, with many of the most common STs being known as ExPEC lineages and commonly found in human infections. Food can be a vector of transmission of ExPEC, but interestingly, in our data, none of the most common food STs were ExPEC group STs. However, several ExPEC lineages, including STs 10, 58, and 117, were commonly found in environmental and animal isolates, potentially suggesting other means of transmission to humans. One study suggests that ST372, one of the most prevalent lineages in dogs, is a potential zoonotic pathogen that causes extraintestinal infections in humans (35), and our data supports this, as ST372 is among the most common STs in both humans and animals.

Even though environmental isolates were not the largest group, they had the greatest number of unique STs and the greatest number of isolates with an unidentified ST. This suggests that environmental isolates are highly diverse and not well characterized, indicating a potentially valuable avenue of future research. The number of ExPEC lineages found in environmental isolates also indicates that they represent a potential reservoir of disease-causing bacteria, even though resistance gene frequency overall was lower in environmental isolates compared to humans and animals. Environmental *E. coli* samples are not as thoroughly studied as human clinical samples, but there have been some studies examining *E. coli* in marine environments (6,36). These studies support the notion that environmental sources are an overlooked reservoir of epidemiologically relevant bacteria.

Our overall AMR results support the idea that there is a relationship between isolation source and the presence of ARGs in *E. coli* samples from Washington State. Our finding that the proportion of resistant and multidrug resistant isolates was highest in human samples is consistent with other research that shows high levels of antimicrobial resistance in human isolates (14,37). Past studies have shown that demographic factors such as patient age and sex, as well as clinical factors like hospitalization status, are also associated with levels of drug resistance seen in bacterial isolates from humans (14,37). However, while most human isolates in our data are from clinical samples, additional metadata about the reason for sampling, patient demographic factors, and geocodes are not available, illustrating one of the limitations of this data source for epidemiologic studies.

Our study confirmed that antibiotic resistance genes to broad-spectrum antibiotics such as beta-lactams, aminoglycosides, and tetracyclines are present in all four isolation sources. However, as Ludden et al. (13) point out, while this supports the ubiquity of these genes, it does not necessarily provide evidence of recent transfer between isolation sources. Their research found distinct mobile genetic elements between humans and livestock, suggesting limited genetic transfer between isolation sources (13). Similarly, our data showed that the distributions of resistance profiles in each of the four isolation sources were distinct from one another, potentially indicating separate silos of antibiotic resistance. On the other hand, Jakobsen et al. (20) suggests that in their data, human antibiotic consumption does not account for the resistance patterns seen in human isolates, and food animals and meat may be an important source of antibiotic resistance in humans. Though our study is not able to determine how drug resistance spreads between isolation sources, it does show that antimicrobial resistance is a problem that requires a One Health approach.

Our study also showed that the proportion of isolates that are resistant or multidrug resistant compared to pan-susceptible has changed over time since 1947. Overall, the likelihood of an isolate being multidrug resistant increases every year, but the likelihood of an isolate being resistant decreases slightly every year. This trend seems to depend on the bacterial isolation source, as isolates from humans were more likely to be resistant or multidrug resistant over time compared to animal, food, and environmental isolates. Other studies also provide some evidence that resistance changes over time. For example, one meta-analysis of 15 studies shows an increase in resistance to ciprofloxacin, one of the most widely used broad-spectrum antibiotics, between the years 2000-2018 (38). While that analysis did consider isolates from humans, animals, food, and the environment, it did not differentiate between isolation source when examining rates of resistance over time. That analysis also included data from all over the world, and it is known that antibiotic consumption is significantly different in low- and middle-income countries (LMICs) compared to high income countries. Our findings suggest that there may also be a significant trend in Washington, but it is important to differentiate between bacterial isolation sources when considering trends over time.

This study has several limitations. Because of the non-systematic nature of how sequences get deposited in the NCBI GenBank database, this data cannot be interpreted as defining the underlying prevalence of resistance in type of host or media in Washington. As a whole, these data were largely from human sources. Small sample sizes in some other isolation sources, such as food, limited some comparisons. Categorizing samples by isolation source also simplified possible differences in microbial ecology between different ecological niches, potentially obscuring differences between and within

groups. For example, there were a small number of samples from wild animal species, likely from zoos, which we categorized along with livestock and domesticated animals. However, this heterogeneity masks potential relationships that may exist between specific isolation sources; for example, the microbiome of retail meat may have more in common with livestock than with wild animal species.

The available metadata also reflects the limitations of using pathogen genomic data in epidemiological studies. Although the integration of these fields for pathogen surveillance is of rising interest, some epidemiologically relevant data fields, such as the reason for collecting a sample, are scarce or missing in genomic metadata. In our study, there were also many samples that were missing relevant metadata about the timing and location of sample collection. Lastly, there are inherent limitations with the use of genotypic data without phenotypic confirmation; we do not know which resistance genes present in our sample are functional, and genotypic prediction relies on the completeness of the reference database. Although most studies indicate that the presence of ARGs closely corresponds with phenotypic resistance (24,25,27), some more recent studies have found a lack of correlation between the results of phenotypic susceptibility testing and the ARGs present according to whole genome sequencing (6). It is possible that some of the resistance genes identified in this study do not confer functional resistance, and some functional resistance genes may not have been identified. However, ResFinder is a large, frequently updated database, and several benchmarking studies show that it had high accuracy and performance (39,40), particularly on *E. coli* genomes (41).

Our results build on existing evidence that the problem of antibiotic resistance is interdisciplinary, requiring surveillance, prevention, and mitigation efforts across multiple sectors. Our study highlights the need to further characterize bacterial isolates from a One Health perspective, both in Washington State and elsewhere. As the speed of sequencing technologies and the number of available isolates continue to increase rapidly, research into genomic surveillance methods will become increasingly important.

Tables and Figures

Table 1: Antimicrobial categories and agents used to define drug resistance, multi-drug resistance (MDR), and extensive drug resistance (XDR) in *E. coli*. These categories are adapted from guidelines from the European Centre for Disease Prevention and Control (ECDC) and Centers for Disease Control and Prevention (CDC), described in Magiorakos et al (33).

Antimicrobial Category	**Antimicrobial Agents**
Aminoglycosides	Streptomycin Gentamicin Tobramycin Amikacin Netilmicin
Antipseudomonal penicillins + beta-lactamase inhibitors	Ticarcillin-clavulanic acid Piperacillin-tazobactam
Carbapenems	Ertapenem Imipenem Meropenem Doripenem
Extended-spectrum cephalosporins (3rd and 4th generation cephalosporins)	Cefotaxime Ceftriaxone Ceftazidime Cefepime
Cephamycins	Cefoxitin
Fluoroquinolones	Ciprofloxacin
Folate pathway inhibitors	Trimethoprim-sulphamethoxazole
Glycylcyclines	Tigecycline
Monobactams	Aztreonam
Penicillins	Ampicillin
Penicillins + beta-lactamase inhibitors	Amoxicillin-clavulanic acid Ampicillin-clavulanic acid
Phenicols	Chloramphenicol
Phosphonic acids	Fosfomycin
Polymyxins	Colistin
Tetracyclines	Tetracycline Doxycycline Minocycline

Table 2: Examples of common specific isolation sources and distribution of isolation year within each category of isolation source.

Isolation Source	Animal	Environmental	Food	Human
Examples of common sources	Cattle, chicken, sheep, swine, canine, feline, horse, giraffe, gorilla; abscess, urine, feces, bile	Water, soil, environmental swab	Ground beef, raw turkey, raw chicken breast, retail milk, lettuce	Clinical samples: blood, feces
Number of strains isolated between 1947-2004	31	0	8	70
Number of strains isolated between 2005-2015	74	6	11	252
Number of strains isolated between 2016-2022	188	315	34	42
Number of strains with no known isolation year	3	6	1	408
Total number of strains	**296**	**327**	**54**	**772**

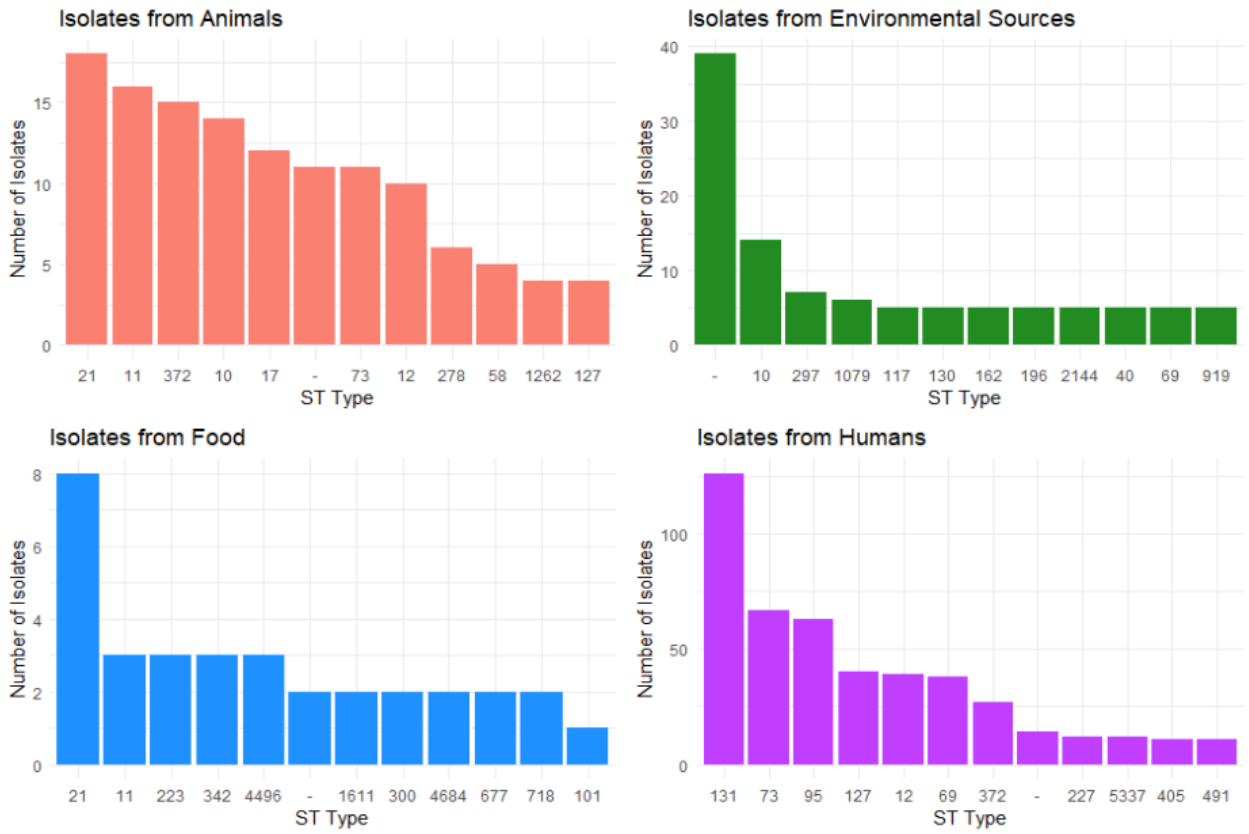

Figure 1: The number of isolates in each of the 12 most common STs within each isolation source.

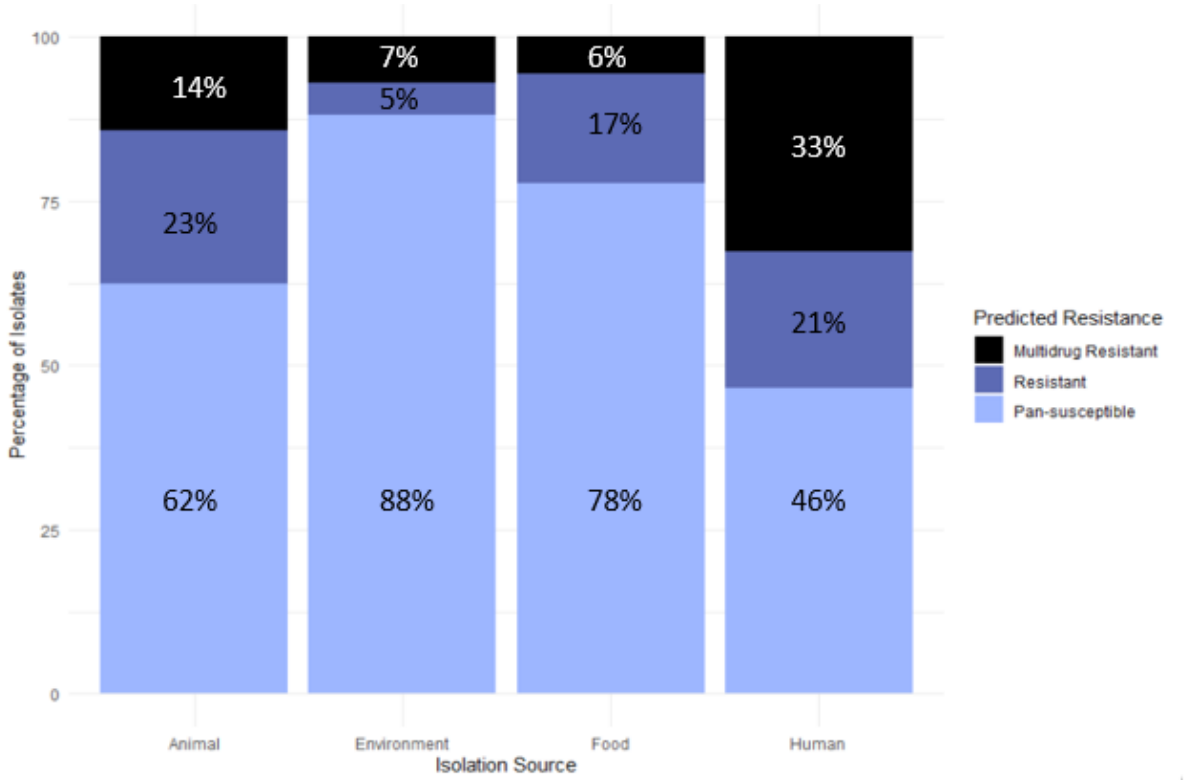

Figure 2: The proportion of isolates within each isolation source that are categorized as pan-susceptible, resistant, or multidrug resistant.

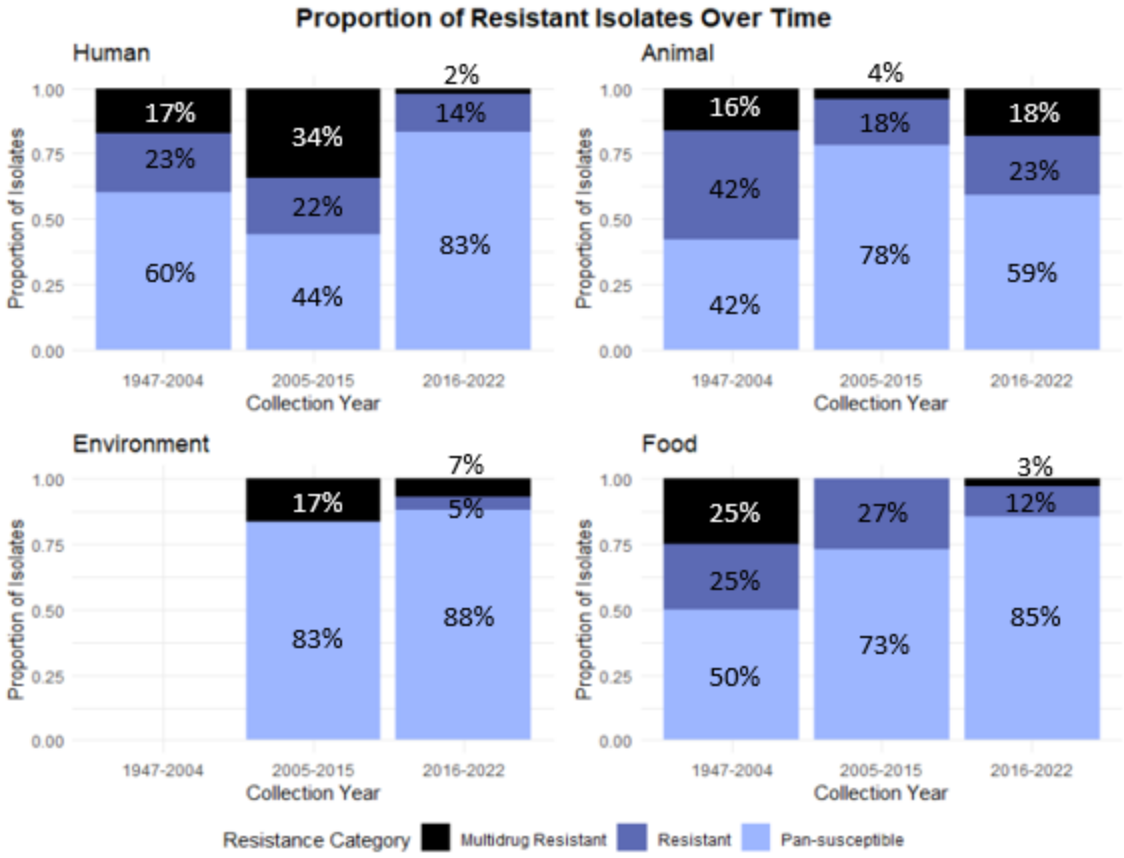

Figure 3: The proportion of isolates collected in each time period (1947-2004, 2005-2015, or 2016-2022) that are categorized as pan-susceptible, resistant, or multidrug resistant.

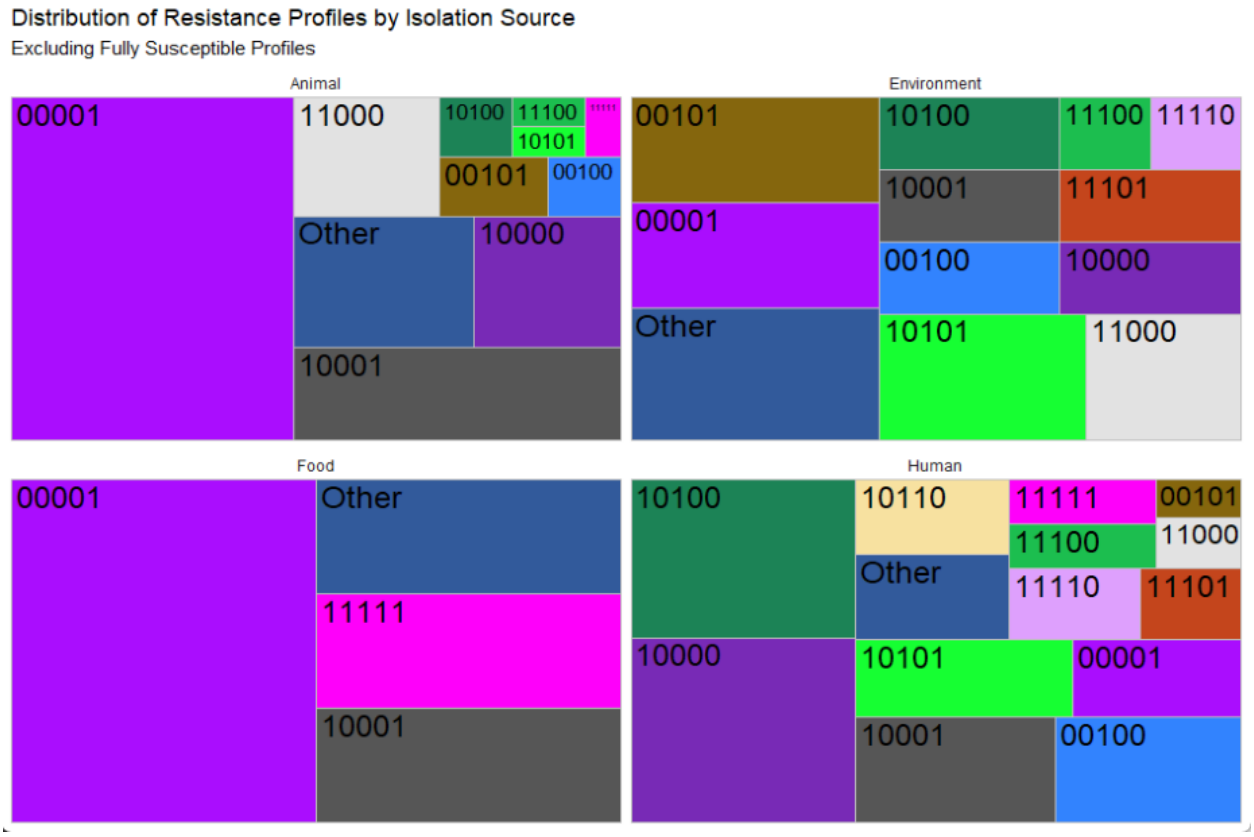

Figure 4: Distribution of resistance profiles by isolation source, excluding fully susceptible profiles (profile 00000).

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