Establishing Genotypic Cutoff Values To Measure Antimicrobial Resistance in *Salmonella*

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ABSTRACT Whole-genome sequencing (WGS) has transformed our understanding of antimicrobial resistance, helping us to better identify and track the genetic mechanisms underlying phenotypic resistance. Previous studies have demonstrated high correlations between phenotypic resistance and the presence of known resistance determinants. However, there has never been a large-scale assessment of how well resistance genotypes correspond to specific MICs. We performed antimicrobial susceptibility testing and WGS of 1,738 nontyphoidal *Salmonella* strains to correlate over 20,000 MICs with resistance determinants. Using these data, we established what we term genotypic cutoff values (GCVs) for 13 antimicrobials against *Salmonella*. For the drugs we tested, we define a GCV as the highest MIC of isolates in a population devoid of known acquired resistance mechanisms. This definition of GCV is distinct from epidemiological cutoff values (ECVs or ECOFFs), which currently differentiate wild-type from non-wild-type strains based on MIC distributions alone without regard to genetic information. Due to the large number of isolates involved, we observed distinct MIC distributions for isolates with different resistance gene alleles, including for ciprofloxacin and tetracycline, suggesting the potential to predict MICs based on WGS data alone.

KEYWORDS *Salmonella*, antibiotic resistance, breakpoints, whole-genome sequencing

Antimicrobial resistance is a major public health threat to patients suffering from bacterial illness. Resistance complicates antimicrobial therapy, increases health care costs, and ultimately costs lives (1). The U.S. National Strategy to Combat Antimicrobial Resistant Bacteria (CARB) identified enhanced surveillance and improved detection methods as priority efforts to address the global challenge of resistance (2). Developing new tools to quickly and accurately detect antimicrobial resistance is extremely important, as it can inform appropriate and timely treatment efforts and enhance antimicrobial resistance surveillance.

Traditional laboratory methods to measure antimicrobial susceptibility involve *in vitro* tests that measure the biological response of a pure culture of bacteria to a range of antibiotic concentrations. This is most commonly performed with disk diffusion or broth microdilution techniques (3). For dilution methods, the result is a MIC for each antimicrobial, which defines the lowest concentration of a drug necessary to kill or inhibit the growth of a particular bacterial isolate. This information is combined with pharmacological information and clinical outcome data to determine appropriate therapeutic options.

For antimicrobial resistance surveillance purposes and for drugs without formally established clinical breakpoints, there is value in the information from MIC distributions

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alone. Epidemiological cutoff values (ECVs) are laboratory susceptibility values that discriminate wild-type populations of bacteria from non-wild-type populations, with the latter exhibiting elevated MICs resulting from acquired traits. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) also defines ECVs (called ECOFFs by EUCAST) as the highest MIC for organisms devoid of phenotypically detectable acquired resistance mechanisms (4). Despite the reference to resistance mechanisms in these definitions, resistance genotypes typically have not been available or considered when establishing ECVs.

The advent of inexpensive whole-genome sequencing (WGS) methods has now made it feasible to generate comprehensive genetic data on clinical pathogens, making it easier to catalogue their resistance traits on a routine basis and to evaluate their contribution to resistance. It also has been used to identify bacterial species, determine serotype, and uncover virulence factors, along with other phenotypic traits (5–7). For most bacteria, the evidence for using WGS to infer antimicrobial susceptibility data is sparse, although some studies have shown a high correlation between resistance phenotypes and the presence of resistance genes or resistance-associated mutations. This agreement has been best demonstrated with enteric bacteria, including *Escherichia coli*, *Campylobacter* spp., and nontyphoidal *Salmonella* (8–12). In addition, other DNA-based methods have been used to detect resistance determinants and even guide therapy for infections caused by *Mycobacterium tuberculosis*, *Staphylococcus aureus*, HIV, and other pathogens (13–15). While more focused studies are needed to improve our knowledge, the data demonstrate the potential for WGS to supplant or enhance *in vitro* antimicrobial susceptibility testing for some bacterium-drug combinations.

Given the power of WGS to predict resistance phenotypes, we sought to use WGS to correlate resistance genotypes with MICs, a necessary step if WGS testing eventually is to guide clinical decision-making. These data can be used to establish genotypic cutoff values (GCVs) that can be used to differentiate isolates with and without resistance determinants. We define the GCV as the highest MIC at which an isolate is unlikely to possess an acquired resistance mechanism. This technique has previously been applied to *Salmonella* and *E. coli* to establish GCVs for streptomycin (16). We sought to establish GCVs for 13 additional antimicrobials for *Salmonella* by analyzing genotypic and phenotypic data from more than 1,700 isolates.

RESULTS

From retail meat (1,216), food animal (337), and human (104) sources, 1,657 *Salmonella* isolates collected during routine National Antimicrobial Resistance Monitoring System (NARMS) surveillance from 2011 to 2015 were subjected to broth microdilution MIC testing and whole-genome sequencing on the MiSeq platform. An additional 81 *Salmonella* isolates from retail meats isolated from 2002 to 2011 were included in this study based on the presentation of rare MICs, resulting in a total of 1,738 isolates (see Materials and Methods). This builds on a prior analysis we had conducted in which we compared resistance genotypes and phenotypes for 640 *Salmonella* isolates (12). In this study, we measured the correlation of specific MICs with the presence of resistance genes as defined in the ResFinder database (17). Resistance-associated mutations in *gyrA* and *parC* were also analyzed due to their importance in quinolone resistance.

β -Lactam antimicrobials. For ampicillin, a NARMS testing range of 1 to 32 mg/liter was used, and all but two isolates lacking resistance mechanisms had MICs of ≤ 8 mg/liter (99.8%) (Fig. 1A). While 99.0% of isolates with β -lactam resistance genes had MICs of >32 mg/liter, all four isolates with MICs of 16 or 32 mg/liter still carried β -lactam resistance genes, including the only three isolates with *bla*_{OXA-2} or *bla*_{LAP-1} (see Data Set S2 in the supplemental material). Thus, we propose a GCV of 8 mg/liter, with isolates with MICs of >8 mg/liter being likely to have resistance genes. In total, 12 unique resistance alleles were identified, with several of these found in combination. These included *bla*_{CMY-2}, *bla*_{TEM-1}, *bla*_{CARB-2}, *bla*_{HERA-3}, *bla*_{FOX-5}, *bla*_{LAP-1}, *bla*_{OXA-2}, *bla*_{SHV-2}, *bla*_{SHV-12}, *bla*_{CTX-M-1}, *bla*_{CTX-M-14}, and *bla*_{CTX-M-65}.

FIG 1 MIC distributions for ampicillin (AMP) (A) and amoxicillin-clavulanic acid (AMC) (B). “All mechanisms” refers to all known resistance mechanisms against a particular drug. For AMC, “R not predicted” indicates that the isolate expresses a β -lactamase but that the enzyme is not predicted to confer resistance to this antimicrobial. The MICs depicted for AMC only list the amoxicillin concentration for the combination drug. The lines depict the GCVs.

For amoxicillin-clavulanic acid, we divided isolates into three groups: those without β -lactam resistance genes, those with β -lactam resistance genes not expected to confer resistance, and those with β -lactam resistance genes expected to confer resistance. This is based on the knowledge that some β -lactam resistance genes, including most bla_{TEM} alleles and extended-spectrum β lactamases (ESBLs), such as bla_{CTX-M} , do not confer resistance to potentiated β -lactams such as amoxicillin-clavulanic acid. As shown in Fig. 1B, the isolates with distinct mechanisms differed quite markedly in their MICs. For instance, 99.6% of isolates with MICs of ≤ 2 mg/liter did not have any β -lactam resistance genes. In contrast, 97.6% (241/247) of isolates in the MIC range of 4 to 16 mg/liter contained β -lactam resistance genes such as bla_{TEM-1} , but these are not associated with high-level resistance to amoxicillin-clavulanic acid. Approximately 97% (243/251) of isolates with MICs of ≥ 32 mg/liter had β -lactam resistance genes known to confer resistance, with all but one of these isolates carrying bla_{CMY} (Data Set S2). Thus, we propose a GCV of 2 mg/liter, since isolates with resistance determinants were easily differentiated at this cutoff. However, it is worth noting that isolates with MICs of ≥ 32 mg/liter had distinct resistance genes known to confer high-level resistance. Interestingly, isolates with bla_{CARB-2} (bla_{PSE-1}) and bla_{HERA-3} had markedly higher MICs than those with bla_{TEM-1} , although none are associated with high-level resistance (Data Set S2).

For cefoxitin, ceftiofur, and ceftriaxone, not all β -lactam resistance genes confer clinical resistance, as isolates with genes such as bla_{TEM} do not have MICs that differ from isolates lacking resistance genes (Data Set S2). Of isolates with cefoxitin MICs of

≤ 8 mg/liter, all but one (1,480/1,481) lacked genes predicted to confer resistance, whereas at a MIC of 16 mg/liter, over 70% (35/48) did possess resistance genes. As a result, we propose a GCV of 8 mg/liter for cefoxitin. With ceftriaxone, 99.3% of isolates lacking mechanisms had MICs of ≤ 0.25 mg/liter. In contrast, isolates with resistance determinants had a wide range of MICs, with 99.2% encompassed between 4 and 64 mg/liter. Only one isolate had a MIC of 1 mg/liter, and this did not have a known resistance gene, while among the two isolates with MICs of 2 mg/liter, one had a known β -lactamase expected to confer resistance and one did not. As a result, we propose a GCV of 1 mg/liter for ceftriaxone. For ceftiofur, all 73 isolates with a MIC of 2 mg/liter lacked genes conferring resistance, while all 7 isolates with MICs of 4 mg/liter had such determinants (Fig. 2C). Thus, the proposed GCV is 2 mg/liter for this antimicrobial. Isolates possessing both *bla*_{CMY-2} and *bla*_{TEM-1} on average had higher MICs for the cephalosporins than those with *bla*_{CMY-2} alone, even though *bla*_{TEM-1} on its own does not confer resistance to these antimicrobials (Data Set S2).

Gentamicin. The gentamicin testing range was 0.25 to 16 mg/liter, with 98.7% of isolates lacking resistance genes having MICs of ≤ 1 mg/liter. More than two-thirds of isolates (11/16) with MICs of 2 mg/liter also lacked resistance determinants, while all seven isolates with MICs of 4 mg/liter had gentamicin resistance genes (Fig. 3A). As a result, we propose a GCV of 2 mg/liter for this antimicrobial. We identified eight unique resistance genes, including *aac(3)-Id*, *aac(3)-IId*, *aac(3)-IVa*, *aac(3)-Via*, *aacA4*, *aac(6')-IIC*, *aadB*, and *armA*, and the presence of different gentamicin resistance genes was associated with distinct MIC distributions (Data Set S2). For instance, the most common MIC for isolates with *aac(3)-VI* genes was >16 mg/liter (73/108), whereas the mode for isolates with *aac(3)-IV* was 8 mg/liter (7/14). Isolates with the gene *aacA4*, also called *aac(6')-Ib*, had a wide range of gentamicin MICs, from 2 to >16 mg/liter. This gene historically has not been designated a gentamicin resistance gene, but we agree with others who found it to be associated with elevated gentamicin MICs (18).

Tetracycline. The MIC testing range for tetracycline was 4 to 32 mg/liter (Fig. 3B). Most isolates lacking determinants had MICs of ≤ 4 mg/liter (700/710; 98.6%), while 91.9% (945/1,028) with known tetracycline resistance genes had MICs of >32 mg/liter. A total of eight different tetracycline resistance genes were identified, including *tetA*, *tetB*, *tetC*, *tetD*, *tetG*, *tetM*, *tetO*, and *tetX*. Interestingly, isolates with either *tetA* or *tetB* almost exclusively had MICs of >32 mg/liter (98.6% and 99.1% of isolates, respectively), whereas only 35.4% of isolates with *tetC* and 25.0% with *tetG* had MICs of >32 mg/liter (Table 1). Since 100% of isolates with resistance mechanisms had MICs of ≥ 8 mg/liter, we propose a GCV of 4 mg/liter for tetracycline.

Chloramphenicol. For chloramphenicol, all 1,582 isolates with MICs of ≤ 8 mg/liter lacked resistance determinants, but the majority of isolates with MICs of 16 mg/liter still did not possess resistance genes (38/40; 95.0%) (Fig. 3C). Only nine isolates had MICs of 32 mg/liter, but seven of these did have resistance genes, so we propose a GCV for chloramphenicol of 16 mg/liter. All seven of these isolates had *cmIA* genes, encompassing most of the 11 isolates with this gene (Table 2). In contrast, all but one isolate with either *floR* or *catA* genes had MICs of >32 mg/liter.

Quinolones. For ciprofloxacin, the testing range was from 0.015 to 4 mg/liter, and all but one isolate (1,694/1,695) lacking known mechanisms had MICs of ≤ 0.06 mg/liter. All 43 isolates with known resistance determinants had MICs of ≥ 0.12 mg/liter, so 0.06 mg/liter is the proposed GCV for ciprofloxacin (Fig. 4A). For nalidixic acid, 92.1% (35/38) of isolates with a MIC of 8 mg/liter lacked resistance determinants, whereas 78.9% (15/19) of isolates with MICs of 16 mg/liter had mechanisms (Fig. 4B). Accordingly, 8 mg/liter was identified as the proposed GCV. MIC distributions for both quinolone antimicrobials differed by the mechanisms involved. For instance, all 16 isolates with DNA gyrase (*gyrA*) mutations in the quinolone resistance-determining regions (QRDRs) had MICs of >32 mg/liter for nalidixic acid. In contrast, 24 of 27 isolates with plasmid-mediated quinolone resistance (PMQR) genes had nalidixic acid MICs of ≤ 32 mg/liter, which included isolates with *qnrA1*, *qnrB2*, *qnrB19*, *qnrS1*, *qnrS2*, and *oqxAB* genes. For

FIG 2 MIC distributions for ceftiofur (FOX) (A), ceftriaxone (AXO) (B), and ceftiofur (TIO) (C). “All mechanisms” refers to all known resistance mechanisms against a particular drug. The lines depict the GCVs.

ciprofloxacin, isolates with PMQRs typically had ciprofloxacin MICs between those of isolates with one and two QRDR mutations (Table 3). This is consistent with previous studies that found isolates with PMQRs have elevated ciprofloxacin MICs that are not necessarily accompanied by nalidixic acid clinical resistance (19, 20). Although the number of isolates was limited, *qnrB* genes appeared to be associated more frequently with ciprofloxacin MICs of 0.5 to 1 mg/liter than were *qnrS* genes, which were found in strains with MICs of 0.12 to 0.25 mg/liter (Data Set S2).

Azithromycin. The azithromycin testing range was from 0.12 to 16 mg/liter, with 99.0% of isolates lacking mechanisms having MICs from 2 to 8 mg/liter. Only eight isolates carried azithromycin resistance determinants, which included *mphA*, *mphB*,

FIG 3 MIC distributions for gentamicin (GEN) (A), tetracycline (TET) (B), and chloramphenicol (CHL) (C). "All mechanisms" refers to all known resistance mechanisms against a particular drug. The lines depict the GCVs.

mphE, and *msrE* (Data Set S2). Six of these isolates had MICs of >16 mg/liter, while all of the isolates with MICs of 16 mg/liter lacked resistance determinants (Fig. 4C). As a result, we propose a GCV of 16 mg/liter for this antimicrobial.

Folate synthesis inhibitors. For sulfisoxazole, all but one isolate lacking *sul* resistance genes had MICs of ≤ 256 mg/liter (1,095/1,096) (Fig. 5A). In contrast, all but one isolate with resistance genes (618/619; 99.8%), which included *sul1*, *sul2*, and *sul3*, had MICs of >256 mg/liter, so we propose a GCV of 256 mg/liter for sulfisoxazole.

For trimethoprim-sulfamethoxazole, 99.9% (1,686/1,688) of isolates with MICs of ≤ 0.5 mg/liter lacked resistance genes, whereas 93.2% (41/44) of those with MICs of >4

TABLE 1 Tetracycline MIC distribution for isolates with varied resistance genes

MIC (mg/liter)	No. of isolates positive for:								Multiple mechanisms
	No mechanisms	All mechanisms	<i>tetA</i>	<i>tetB</i>	<i>tetC</i>	<i>tetD</i>	<i>tetG</i>	<i>tetM</i>	
≤4	700								
8	5	9		1	7				1
16	3	12		2	6		3	1	
32	1	62	8		38		15	1	
>32	1	945	555	322	28	3	6		31

mg/liter had resistance genes (Fig. 5B). Although relatively few isolates had MICs from 1 to 4 mg/liter, half of the isolates in this range had dihydrofolate reductase (*dfr*) genes that confer trimethoprim resistance, resulting in our proposing 0.5 mg/liter as the GCV. In total there were 10 different *dfr* genes, including *dfrA1*, *dfrA5*, *dfrA8*, *dfrA12*, *dfrA14*, *dfrA15*, *dfrA17*, *dfrA18*, *dfrA29*, and *dfrB3*. Although trimethoprim-sulfamethoxazole is a combination drug, the three isolates with trimethoprim resistance genes alone (in the absence of sulfamethoxazole resistance genes) all had MICs of ≥ 1 mg/liter, differentiating them phenotypically from wild-type strains lacking resistance determinants.

DISCUSSION

Antimicrobial susceptibility testing interpretive criteria are critical for the accurate reporting of antimicrobial resistance in bacterial pathogens, informing clinical decision-making and resistance surveillance. To support determinations of phenotypic resistance, CLSI and EUCAST provide clinical breakpoints and ECVs/ECOFFs, which are designed to guide therapy and detect emerging resistance mechanisms, respectively (21, 22). Each group also alludes to the presence of known resistance mechanisms in their definitions of resistance and ECV/ECOFF interpretive categories. However, no comprehensive MIC-genotype comparison has been performed to evaluate CLSI or EUCAST cutoffs. In our study, we established GCVs as a new measure of resistance and found just 0.36% of phenotypic MIC values (81/22,486) failed to correlate with the assigned GCVs (Data Set S2).

The detection of resistance genes or mutations is already of some importance clinically. For instance, the presence of *rpoB* mutations in *Mycobacterium tuberculosis* is used to guide treatment, sometimes independent of any susceptibility testing (23). Thus, the use of genetic information obtained by WGS has the potential to refine clinical breakpoints as new resistance mechanisms emerge. For instance, until recently isolates with ciprofloxacin MICs of 0.12 to 0.5 mg/liter were considered clinically susceptible by CLSI. However, after poor clinical outcomes with quinolones were attributed to *Salmonella* isolates with ciprofloxacin MICs in this range, isolates with these MICs are now considered of intermediate susceptibility (24). This finding is supported by genetic data, as the vast majority of isolates with PMQRs have MICs of 0.12 to 0.5 mg/liter (Table 3).

CLSI and EUCAST breakpoints/ECOFFs were established without the benefit of knowing whether isolates possessed resistance determinants at any given MIC. Recently, EUCAST has sought comments on ways to incorporate WGS data to infer

TABLE 2 Chloramphenicol MIC distribution for isolates with varied resistance genes

MIC (mg/liter)	No. of isolates positive for:						Multiple mechanisms
	No mechanisms	All mechanisms	<i>floR</i>	<i>catA1</i>	<i>catA2</i>	<i>cmlA1</i>	
≤2	16						
4	613						
8	953						
16	38	2	1			1	
32	2	7				7	
>32		107	92	4	1	3	7

FIG 4 MIC distributions for ciprofloxacin (CIP) (A), nalidixic acid (NAL) (B), and azithromycin (AZI) (C). “All mechanisms” refers to all known resistance mechanisms against a particular drug. The lines depict the GCVs.

antimicrobial susceptibility (25). In many cases, the GCVs we propose do align with the ECOFFs and/or CLSI clinical breakpoints (Table 4); however, there was disagreement for several drugs. Although relatively few isolates are affected by the differences between these cutoffs, these data, used to produce GCVs, provide additional information about the range of MICs in strains possessing specific resistance gene sequences. In addition, there are no ECOFFs for ceftriaxone, amoxicillin-clavulanate, azithromycin, or sulfisoxazole for *Salmonella*. The application of GCVs to drugs that lack EUCAST ECOFFs or CLSI clinical breakpoints can be informative, such as with azithromycin, which has shown value in treating infections caused by nontyphoidal *Salmonella* (26).

TABLE 3 Ciprofloxacin MIC distribution for isolates with varied resistance mechanisms

MIC (mg/liter)	No. of isolates positive for:				
	No mechanisms	All mechanisms	<i>qnr</i> genes	One <i>gyrA</i> mutation	Two <i>gyrA</i> mutations
≤0.015	1,333				
0.03	344				
0.06	17				
0.12		8	1	7	
0.25	1	9	4	5	
0.5		18	17	1	
1		4	4		
2		1	1		
4					
>4		3			3

In addition to providing GCVs, the correlation between resistance genotypes and MICs provided valuable information about differences in MIC distributions between isolates with distinct resistance mechanisms. These resistance genotypes therefore can provide additional information beyond the binary resistant/susceptible determination, including prediction of the likely MICs of isolates with different mechanisms (Tables 1 to 3). This was evident with a number of resistance genes (Data Set S2), particularly for amoxicillin-clavulanate, where isolates with specific β -lactamases had clearly differentiated MICs both from each other and from isolates lacking any resistance genes (Fig. 1B).

Despite the significance of these findings, there are several limitations to the

FIG 5 MIC distributions for sulfisoxazole (FIS) (A) and trimethoprim-sulfamethoxazole (COT) (B). “All mechanisms” refers to all known resistance mechanisms against a particular drug. The MICs depicted for COT only list the trimethoprim concentration for the combination drug. The lines depict the GCVs.

TABLE 4 Comparison of susceptible CLSI clinical breakpoints, EUCAST ECOFFs, and GCVs

Drug	MIC (mg/liter) according to:		
	CLSI susceptible: treatment success likely	EUCAST ECOFF: wild type	GCV: no resistance mechanism
Ampicillin	≤8	≤4	≤8
Amoxicillin-clavulanate	≤8	None	≤2
Cefoxitin	≤8	≤8	≤8
Ceftriaxone	≤1	None	≤1
Ceftiofur	≤2	≤2	≤2
Gentamicin	≤4	≤1	≤2
Tetracycline	≤4	≤4	≤4
Chloramphenicol	≤8	≤16	≤16
Ciprofloxacin	≤0.06	≤0.06	≤0.06
Nalidixic acid	≤16	≤16	≤8
Azithromycin	None	None	≤16
Sulfisoxazole	≤256	None	≤256
Trimethoprim-sulfamethoxazole	≤1	≤1	≤0.05

analyses. For instance, although a large number of isolates from different sources were analyzed, all isolates were collected in the United States (albeit in 40 states) and tested in only three laboratories. It is possible that isolates from other locations possess unique resistance mechanisms with MIC distributions that differ from those described here, or that serotypes not well represented in our collection have different MIC distributions. Genetic changes associated with new mechanisms also may have been missed by our analysis. Nevertheless, our use of a large isolate bank from human, retail meat, and animal sources yielded a wide variety of strains with differing phenotypes and resistance mechanisms, indicating the potential for the resulting GCVs to be widely applied. One additional consideration is that we used CLSI-standardized methods to perform susceptibility testing, whereas methods that are standard in other countries may yield different MICs and therefore different conclusions. An additional limitation is that for some drugs we relied on relatively few isolates at MICs above or below the GCV. In most cases we were unable to analyze whether isolates with more than one resistance mechanism to a given antimicrobial had greater MICs than isolates with only one mechanism, and additional susceptibility testing with higher drug concentrations is necessary to perform this analysis.

Due to the benefits demonstrated here, we support the use of WGS to establish GCVs for bacteria with well-defined resistance mechanisms. This will be useful for many bacterium-MIC combinations, since in many cases no interpretive criteria exist. As additional WGS and susceptibility data are generated and made publicly available, these MIC-genotype comparisons will become more robust and include a greater number of organism-MIC combinations. This will continue to strengthen the correlations between genotype and phenotype, permitting more predictions of susceptibility based solely on the presence of specific DNA sequences. This can greatly augment, and in some cases may replace, traditional *in vitro* antimicrobial susceptibility testing.

MATERIALS AND METHODS

***In vitro* antimicrobial susceptibility testing.** A total of 1,738 *Salmonella* isolates were isolated in the United States from 40 states from retail meat (1,297), food animal (337), and human (104) sources; these included 640 isolates from a prior analysis (12). Retail meat isolates were from 2002 to 2015, food animal isolates from 2014, and human isolates from 2011 to 2012. Isolates were selected for analysis from routine surveillance of the National Antimicrobial Resistance Monitoring System (NARMS) from 2011 to 2015, except for 81 of the retail meat isolates from 2002 to 2011. These were earlier NARMS surveillance isolates selected based on MICs for which few isolates were available, including the following: ampicillin, 4 to 16 mg/liter; ceftriaxone, 0.5 to 2 mg/liter; gentamicin, 2 to 4 mg/liter; tetracycline, 8 to 16 mg/liter; chloramphenicol, 32 mg/liter; sulfisoxazole, 256 mg/liter; and trimethoprim-sulfamethoxazole, 0.5 to 4 mg/liter. Each isolate was subcultured twice on blood agar plates and subjected to broth microdilution testing, using dried panels purchased from Trek Diagnostics. Standardized testing methods and quality control testing followed established CLSI procedures, including incubation of panels for 16 to 20 h at 35°C and performing quality control testing at least weekly with appropriate QC organisms (21). Testing

ranges for the antimicrobials in 2-fold serial dilutions were the following: ampicillin, 1 to 32 mg/liter; amoxicillin-clavulanic acid, 1/0.5 to 32/16 mg/liter (dual numbers indicate ratios of the two drugs); cefoxitin, 0.5 to 32 mg/liter; ceftriaxone, 0.25 to 64 mg/liter; ceftiofur, 0.12 to 8 mg/liter; gentamicin, 0.25 to 16 mg/liter; tetracycline, 4 to 32 mg/liter; chloramphenicol, 2 to 32 mg/liter; ciprofloxacin, 0.015 to 4 mg/liter; nalidixic acid, 0.5 to 32 mg/liter; azithromycin, 0.12 to 16 mg/liter; sulfisoxazole, 16 to 256 mg/liter; and trimethoprim-sulfamethoxazole, 0.12/2.4 to 4/76 mg/liter. For combination drugs, only concentrations of the first drug are displayed in the figures and text. Isolate-level MIC values are displayed in Data Set S1 in the supplemental material. Some isolates from prior to 2011 were tested with different MIC ranges, so these MICs were excluded; therefore, there are only 1,661 MIC values for azithromycin, 1,715 for sulfisoxazole, and 1,730 for cefoxitin.

Whole-genome sequencing. DNA extractions were performed using Qiagen DNeasy blood and tissue kits, with library preparations performed using either Illumina v2 or v3 reagent kits with paired-end reads. Each isolate was subjected to whole-genome sequencing on the MiSeq platform, as described previously, with minimum N50 values of 30 kb and 20-fold coverage required for each assembled genome (9).

Whole-genome sequence analysis. WGS data were imported into CLC Genomics Workbench, and assembly was performed with automated assembly parameters. The publicly available resistance gene database ResFinder was used for identification of antimicrobial resistance genes, using a 90% sequence identity and 60% length minimum to identify resistance genes (ResFinder version 3-10-16) (17), with additional BLAST analysis of hits with less than 100% length and/or identity to identify the most accurate resistance alleles. Isolates at each MIC were analyzed for the presence of accompanying resistance genes. Mutations in the quinolone resistance-determining regions of *gyrA* and *parC* were also queried, as described previously (9). In isolates for which one or more susceptibility phenotypes did not correlate with WGS-based analysis, susceptibility testing and/or WGS was repeated as appropriate.

Accession number(s). WGS data for each isolate have been submitted to the Short Read Archive at GenBank, and accession numbers for individual isolates are listed in Data Set S1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02140-16>.

DATASET S1, XLS file, 0.64 MB.

DATASET S2, XLS file, 0.1 MB.

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