Using whole-genome sequencing to determine appropriate streptomycin epidemiological cutoffs for *Salmonella* and *Escherichia coli*

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Abstract

For Enterobacteriaceae such as *Salmonella* spp. and *Escherichia coli*, no unified interpretive resistance criteria exist for streptomycin, an epidemiologically important antibiotic. As part of the National Antimicrobial Resistance Monitoring System, we had previously used a minimum inhibitory concentration of 64 μg mL^{-1} as an epidemiological cutoff value (ECV) to define non-wild-type isolates. To identify whether this ECV correlated with genetic determinants of resistance, we performed whole-genome sequencing of 463 *Salmonella* and *E. coli* isolates to identify streptomycin resistance genotypes. From this analysis, we found that using a streptomycin resistance breakpoint of 64 μg mL^{-1} classified over 20% of strains possessing *aadA* or *strA/strB* resistance genes as wild-type. Therefore, to improve the concordance between genotypic and phenotypic data, we propose reducing the phenotypic cutoff values to 32 μg mL^{-1} for both *Salmonella* and *E. coli*, to be used widely as ECVs to categorize non-wild-type isolates.

One sentence summary:

We used whole-genome sequencing to identify streptomycin resistance genotypes of *Salmonella* and *Escherichia coli*, combining this information with susceptibility data to establish new streptomycin epidemiological cutoff values for these bacteria.

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Keywords

Salmonella ; *E. coli* ; whole-genome sequencing; streptomycin; resistance; genotyping

INTRODUCTION

Streptomycin was one of the first natural antibiotics discovered, and was introduced into clinical medicine in 1943. As an aminoglycoside, streptomycin works by binding to the bacterial 30S ribosomal subunit to disrupt protein synthesis. This effectively kills many types of bacteria, but human clinical use has been limited due to its toxicity (Bottger *et al.* 2001). However, streptomycin has been widely used to treat bacterial infections of plants and animals (Sundin and Bender 1996).

Streptomycin resistance has become prevalent among Enterobacteriaceae due to the expression of aminoglycoside modifying enzymes (Mingeot-Leclercq, Glupczynski and Tulkens 1999). In *Escherichia coli* and *Salmonella*, streptomycin resistance is often mediated by *aadA* genes, which are usually present on integrons and result in streptomycin adenylation (Hollingshead and Vapnek 1985). Other common genes include *strA* (*aph(3′)-Ib*) and *strB* (*aph(6′)-Id*), which are typically found together and produce phosphotransferases that modify streptomycin at different positions (Scholz *et al.* 1989). Streptomycin resistance genes are frequently associated with mobile genetic elements that disseminate multiple antimicrobial resistance genes in Enterobacteriaceae. As a result, streptomycin resistance has been used as an important epidemiological marker to indicate the likelihood of multidrug-resistance in pathogens (Scholz *et al.* 1989). For instance, *Salmonella* Typhimurium DT104, a significant human and animal pathogen, has several co-expressed resistance genes, yielding the resistance phenotype of ACSSuT (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline) (Hsu *et al.* 2013). Tracking and identifying the origins and sources of such important multidrug-resistant pathogens is important for foodborne outbreak investigations, and can help reduce the burden of foodborne illness.

Although there is no clinical streptomycin resistance breakpoint for Enterobacteriaceae, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) utilizes an epidemiological cutoff value (ECV) of 16 μg mL^{-1} to identify wild-type strains of both *Salmonella* and *E. coli* (www.eucast.org). These ECVs are used to define the upper limit of the wild-type populations of each bacterium for streptomycin, and are intended to refer to organisms that are likely to be missing resistance mechanisms. However, EUCAST sets ECVs based solely on the distribution of minimum inhibitory concentrations (MICs) among populations of bacteria, not based on the presence of known resistance mechanisms in these strains.

The National Antimicrobial Resistance Monitoring System (NARMS) and others use 64 μg mL^{-1} to categorize streptomycin-resistant strains of Enterobacteriaceae (Zhao *et al.* 2006; Deckert *et al.* 2010). Several groups have attempted to establish streptomycin ECVs for *E. coli* and/or *Salmonella* based on genotypic data, but have had vastly different conclusions (Sunde and Norstrom 2005; Doran *et al.* 2006; Garcia-Migura *et al.* 2012). One potential

source of these ambiguous results is that they relied on PCR tests to detect resistance genes, and thus may not have comprehensively evaluated their presence. Instead we used whole-genome sequencing (WGS) to identify streptomycin resistance genes to more rigorously evaluate the correlation between streptomycin resistance genotypes and phenotypes.

MATERIALS AND METHODS

WGS and analysis

WGS was performed on the Miseq platform for 337 *Salmonella* isolates of 36 serotypes (Table S1 Supporting Information) and 126 *E. coli* of 104 serotypes (Table S2 Supporting Information). Isolates were grown on blood agar plates at 35°C, and genomic DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. WGS was performed using the Miseq platform with v2 or v3 reagent kits (Illumina, San Diego, CA, USA). Assembly was performed *de novo* for each isolate with CLC Genomics Workbench version 7.5 using automated assembly parameters. All assemblies were of at least 30-fold coverage and contained fewer than 400 contigs. The *strA/strB* and *aadA* genes were identified using BlastX analysis with an in-house resistance gene database containing 44 *aadA*, 12 *strA* and 14 *strB* variants (Tyson *et al.* 2015). *Salmonella* serotypes were determined by traditional serology (FDA 2015), while *E. coli* serotypes were identified by sequence-based methods (Joensen *et al.* 2015).

Antimicrobial susceptibility testing

Streptomycin MICs were determined by broth microdilution using a Sensititre® system (Trek Diagnostic Systems, Cleveland, OH, USA) according to standardized protocols (CLSI 2015). Plate CMV3AGNF was used, containing streptomycin drug concentrations from 2 to 64 μg mL^{-1} in 2-fold increments. The ECOFFinder tool was used to calculate ECVs statistically based on MIC distributions (Turnidge, Kahlmeter and Kronvall 2006).

RESULTS AND DISCUSSION

A total of 337 *Salmonella* isolates of 36 serotypes from retail meats were subjected to WGS and antimicrobial susceptibility testing, and displayed a wide variety of streptomycin susceptibility phenotypes (Fig. 1A). Most isolates lacking known streptomycin resistance genes had MIC values 16 μg mL^{-1} (189/191, 99.0%), with two isolates having MICs of 32 μg mL^{-1} (Fig. 1A). As previously established, the presence of *aadA* genes was associated with decreased susceptibility to streptomycin (Hollingshead and Vapnek 1985), as a vast majority of isolates with *aadA* genes had MIC values of 32 μg mL^{-1} (89.5%) (Fig. 1A). Similar to previous results (Sunde and Norstrom 2005), isolates with *strA/strB* had elevated MICs relative to those with *aadA* genes, with most isolates (88.0%) having MICs of 64 μg mL^{-1}. The combined expression of *aadA* and *strA/strB* further increased streptomycin MIC levels, as 93.8% of isolates with both types of genes had MIC values >64 μg mL^{-1} (Fig. 1A).

Genotypic and phenotypic data generally agreed for strains carrying *strA/strB* with the original NARMS ECV of 64 μg mL^{-1}, although this was not the case for isolates with *aadA* genes. At the 64 μg mL^{-1} ECV, genotypes predicted all resistance phenotypes,

since all isolates that were phenotypically resistant at this threshold possessed resistance genes. However, many isolates with resistance genes had MICs below this level, resulting in an overall correlation between genotypes and phenotypes of 93.8% (Table 1). Reducing the threshold to 32 μg mL^{-1} resulted in increasing genotype–phenotype correlations to 98.2% (Table 1), so we suggest establishing the *Salmonella* ECV at 32 μg mL^{-1} to denote non-wild-type strains.

From animal sources, 126 *E. coli* isolates of 104 serotypes were selected for WGS and antimicrobial susceptibility testing (Table S2 Supporting Information). Among the 68 isolates without any streptomycin resistance genes, all had MIC values from 4 to 16 μg mL^{-1} (Fig. 1B), similar to what was observed with *Salmonella* (Fig. 1A). Isolates with *aadA* genes had a broad range of streptomycin MIC values, from 8 to >64 μg mL^{-1}. Although most had elevated MIC levels relative to isolates lacking resistance mechanisms, three isolates had MICs of 8 μg mL^{-1}. These three isolates appeared to be highly related, as each was of the same serotype and possessed identical class 1 integrons containing *aadA* genes. A different isolate, N34557PS, had the same gene cassette yet had a streptomycin MIC of >64 μg mL^{-1}. This suggests that additional unknown genetic factors may be present that disrupt *aadA* activity or otherwise influence streptomycin susceptibility in the three isolates with MICs of 8 μg mL^{-1}. As observed with *Salmonella*, *E. coli* isolates with *strA/strB* genes had higher streptomycin MICs than those expressing *aadA* (Fig. 1B). The additional presence of *aadA* genes further increased MICs, as all isolates with both *aadA* and *strA/strB* genes had MICs 64 μg mL^{-1}, the original resistance breakpoint. Although most isolates with resistance genes had MIC values of 64 μg mL^{-1}, a decreased ECV resulted in better agreement between resistance genotypes and phenotypes (Table 2). In fact, decreasing the resistance threshold to 32 μg mL^{-1} raised the correlation from 86.5% to 94.4%. Reducing the ECV further, to 16 μg mL^{-1}, resulted in a correlation of 92.9%, which is only slightly lower than that achieved with the 32 μg mL^{-1} cutoff (Table 2). This is because several isolates both with and without streptomycin resistance mechanisms had MICs of 16 μg mL^{-1} (Fig. 1B). We chose 16 μg mL^{-1} to designate the wild-type population since 16 μg mL^{-1} fits the definition of the upper limit of the isolates lacking resistance mechanisms. It is also only one dilution away from the most common MIC for isolates lacking mechanisms (8 μg mL^{-1}), and therefore within the error range of broth microdilution for isolates with that MIC (Steward *et al.* 1999).

The ECVs determined by genotypic methods were compared to those from using ECOFFinder, a statistics-based method that identifies ECVs based on MIC distributions (Turnidge, Kahlmeter and Kronvall 2006). Based on this analysis, a *Salmonella* ECV of 32 μg mL^{-1} and *E. coli* ECV of 16 μg mL^{-1} would refer to wild-type strains, using the typical 97.5% wild-type value cutoff (Fig. 2). The *E. coli* ECV agrees with our results, whereas the *Salmonella* ECV from ECOFFinder is higher, suggesting that statistical methods do not always concur with conclusions based on resistance genotyping, which we argue is more valuable in determining the true wild-type population of bacteria. Our ability to use WGS-based techniques to dramatically improve the genotype–phenotype correlations underscores the importance of identifying appropriate ECVs to more accurately report emerging trends in antimicrobial resistance.

CONCLUSIONS

In this work, we investigated the correlation between genotypic and phenotypic resistance to streptomycin for *E. coli* and *Salmonella*. To create a better concordance between genotypic and phenotypic data as well as unify streptomycin resistance reporting, we suggest that susceptibility testing results of 32 μg mL^{-1} for both *Salmonella* and *E. coli* be used to denote non-wild-type strains. These results contrast with some conclusions made by other groups, likely due to their reliance on PCR-based techniques to identify streptomycin resistance genes (Garcia-Migura *et al.* 2012). In contrast, our use of WGS resulted in a more unbiased ability to broadly identify resistance determinants, confirming the robustness of this technique in identifying important genes. Overall, our data demonstrate the power of WGS in predicting resistance phenotypes and should complement existing methods to expand our knowledge of antimicrobial resistance mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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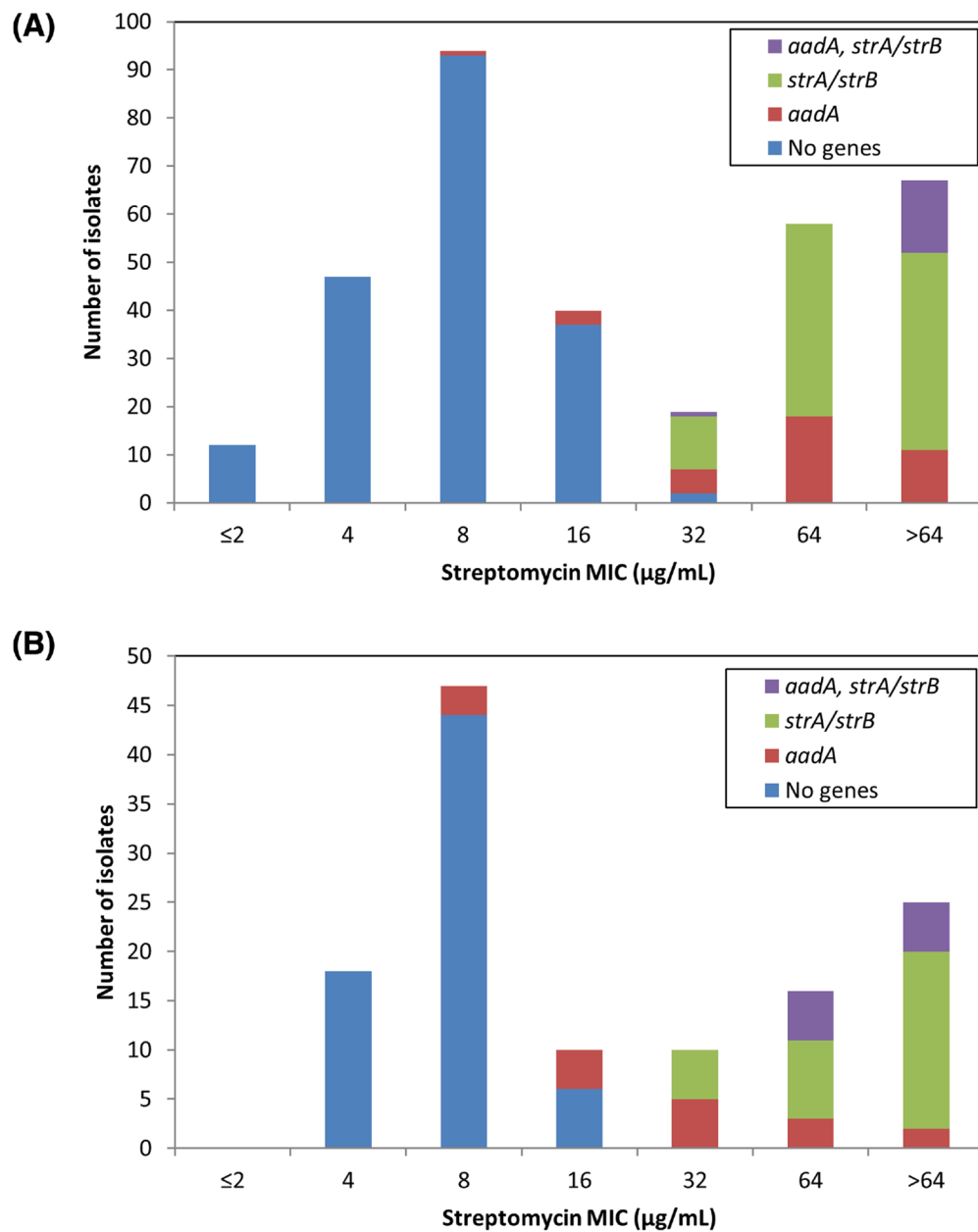
Figure 1.
Association of streptomycin resistance genes and MIC. (**A**) *Salmonella* isolates were evaluated for susceptibility to streptomycin, and categorized based on those without known streptomycin resistance genes, with *aadA* genes, with *strA/strB* genes, or with both. Numbers in parentheses indicate the number of isolates with each genotype. (**B**) The streptomycin MIC distribution for *E. coli* is similarly depicted.
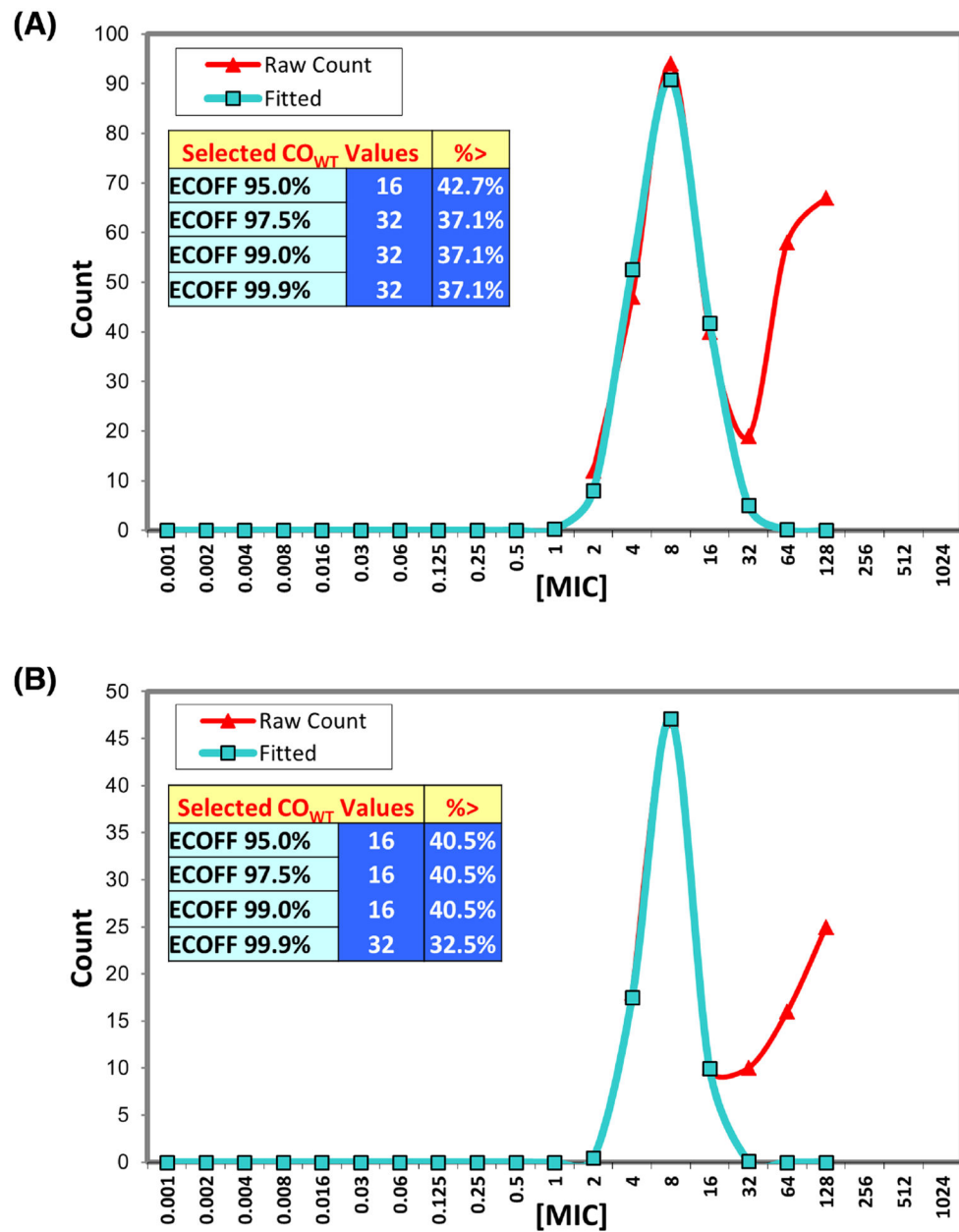
(A)

Selected CO$_{WT}$ Values		%>
ECOFF 95.0%	16	42.7%
ECOFF 97.5%	32	37.1%
ECOFF 99.0%	32	37.1%
ECOFF 99.9%	32	37.1%

(B)

Selected CO$_{WT}$ Values		%>
ECOFF 95.0%	16	40.5%
ECOFF 97.5%	16	40.5%
ECOFF 99.0%	16	40.5%
ECOFF 99.9%	32	32.5%

Figure 2.

Using ECOFFinder to determine ECVs. (**A**) *Salmonella* MIC distributions were input into the ECOFFinder tool (Turnidge, Kahlmeter and Kronvall 2006) to determine potential ECVs. (**B**) *Escherichia coli* MIC distributions were similarly input into ECOFFinder.

Table 1.

Genotype–phenotype correlation for *Salmonella* at different streptomycin cutoff values.

ECV (µg mL^{-1})	Phenotype:WT		Phenotype:NWT			
	Genotype:R	Genotype:S	Genotype:R	Genotype:S	Correlation (%)	
64	21	191	125	0	93.8 (316/337)	
32	4	189	142	2	98.2 (331/337)	
16	1	152	145	39	88.1 (297/337)	
8	0	59	146	132	60.8 (205/337)	

WT refers to wild-type, and NWT refers to non-wild-type.

Table 2.

Genotype–phenotype correlation for *E. coli* at different streptomycin cutoff values.

ECV (µg mL)	Phenotype:WT		Phenotype:NWT		Correlation (%)
	Genotype:R	Genotype:S	Genotype:R	Genotype:S	
64	17	68	41	0	86.5 (109/126)
32	7	68	51	0	94.4 (119/126)
16	3	62	55	6	92.9 (117/126)
8	0	18	58	50	60.3 (76/126)

WT refers to wild-type, and NWT refers to non-wild-type.