Evaluation of Apramycin Activity Against Carbapenem-Resistant and -Susceptible Strains of *Enterobacteriaceae*

Running Title: Activity of apramycin against *Enterobacteriaceae*

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Abstract

We evaluated activity of apramycin, a non-ototoxic/non-nephrotoxic aminocyclitol against 141 clinical *Enterobacteriaceae* isolates, 51% of which were non-susceptible to carbapenems (CRE). Among CRE, 70.8% were apramycin susceptible, which compared favorably to aminoglycosides in current clinical use. Our data suggest that apramycin deserves further investigation as a repurposed, anti-CRE therapeutic.

Keywords: apramycin; aminoglycoside; carbapenem-resistant Enterobacteriaceae; repurposing; multidrug-resistance; activity spectrum

Abbreviations: CRE - carbapenem-resistant Enterobacteriacae; CLSI - Clinical and Laboratory Standards Institute

Treatment options for carbapenem-resistant *Enterobacteriaceae* (CRE) infections are severely limited (1, 2). Aminoglycosides are among the few drugs that retain *in vitro* activity against CRE (3), and combination therapy that includes gentamicin appear particularly efficacious (4, 5). However, up to 33% of patients treated with clinically-approved aminoglycosides develop some degree of irreversible hearing loss (6) and up to 25% develop kidney damage (7).

Interestingly, structurally unique aminoglycosides, specifically apramycin and spectinomycin, appear to have significantly reduced ototoxicity and nephrotoxicity effects (8-12). Further, they are unaffected by most commonly occurring aminoglycoside modifying enzymes (13) and activity may therefore potentially be preserved in multidrug-resistant pathogens.

Previous evidence suggests resistance to apramycin, a veterinary aminocyclitol, is low among human CRE isolates in the United Kingdom (3) and animal extended-spectrum beta-lactamase producing *Escherichia coli* isolates from Germany (14). Furthermore, in a recent high throughput screening effort, we identified apramycin as a potent inhibitor of a highly resistant CRE screening strain (15). Nevertheless, the activity spectrum of apramycin among human *Enterobacteriaceae* isolates, specifically CRE, in the United States has been underexplored.

We therefore evaluated a collection of 141 strains of *Enterobacteriaceae* of United States origin for their susceptibility to apramycin and aminoglycosides in clinical use. Of these strains, 114 were collected at our institution between years 2008-2014 under IRB-approved protocols, and 27 were from the Biodefense and Emerging Infections (BEI) Research Resources, NIAID, NIH isolated between years 2004-2013 with the exception of a single strain isolated in 1981. In total, 72 were CRE (meropenem MIC $\geq$ 2 μg/ml). Carbapenem resistance mechanisms were

identified in 41 of 44 CRE strains for which genome sequences were available. Of the identified resistance mechanisms, 51% (n = 21) were KPC-3 and 39% (n = 16) were KPC-2. Other resistance elements represented included KPC-4 (n = 2) and SME-2 (n = 2). Apramycin was tested against all strains using the broth microdilution method according to CLSI guidelines (16). All experiments included *E. coli* ATCC 25922 as a quality control strain with MIC assay acceptability limits (4-8 μg ml^{-1}) as defined previously (17). Apramycin categorical breakpoints were based on the most recent National Antibiotic Resistance Monitoring Study (NARMS) report in which strains were classified as apramycin susceptible (MIC ≤ 8 μg ml^{-1}), intermediate (MIC = 16-32 μg ml^{-1}), or resistant (MIC ≥ 64 μg ml^{-1}) (18). Notably, pharmacokinetics of apramycin has been investigated in both mammals and birds where important parameters including volume of distribution, area under the curve (AUC), and half-life are similar to other aminoglycosides such as gentamicin and kanamycin (19-21). As such, breakpoints are potentially generalizable to human infections.

Amikacin, gentamicin, tobramycin, and meropenem Vitek 2 (bioMerieux, Inc., Durham, NC) susceptibility data for strains isolated at our institution were obtained from clinical laboratory records. Non-susceptible meropenem results were confirmed using Microscan broth microdilution panel testing (Beckman Coulter, Inc, Brea, CA). For the BEI strains, aminoglycoside and meropenem susceptibility data were determined in parallel with apramycin testing using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution reference method (16) with assays quality controlled against *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (22). CLSI breakpoints were used for categorical susceptibility interpretation (22): in which categorical susceptibility breakpoints for gentamicin, tobramycin, and amikacin were ≤ 4, ≤4, and ≤16 μg ml^{-1}, respectively.

Overall, 78% of bacteria tested were susceptible to apramycin (see table 1). Importantly, among 72 CRE (carbapenem MIC $\geq$ 2 μg ml^{-1}), 70.8% and 7.0% were apramycin susceptible and intermediate respectively. Among the 69 carbapenem-susceptible strains, 85.5% and 13.1% were apramycin susceptible and intermediate, respectively. Only one carbapenem susceptible strain was apramycin resistant (MIC = 64 μg ml^{-1}). The MIC distribution for all strains tested is summarized in Figure 1.

The apramycin susceptibility rate among CRE was also compared to other aminoglycosides using Fisher's exact test with significance defined as $P < 0.05$. Notably, the 70.8% apramycin susceptibility rate was significantly higher than the 47.2% gentamicin (P = 0.003) and 34.7% tobramycin ($p < 0.001$) susceptibility rates, but not significantly different from the 65.3% amikacin ($p > 0.05$) susceptibility rate. A total of 10 strains (7.1%), all of which were carbapenem-resistant *K. pneumoniae*, were non-susceptible to all aminoglycosides inclusive of apramycin. Importantly, we found 7 strains (10% of the CRE collection) which were susceptible to apramycin but otherwise resistant to all other aminoglycosides tested.

Interestingly, high-level apramycin resistance (MIC > 256 μg ml^{-1}) was restricted to carbapenem-resistant *Klebsiella pneumoniae* (n = 14) and a single strain of *Enterobacter* suggesting that these strains may have specific genetic determinants contributing to high-level apramycin resistance. We therefore searched for specific aminoglycoside resistance mechanisms in the 98 strains for which genome sequences were available either through NCBI or the Broad Institute (Carbapenem Resistance initiative, Broad Institute, broadinstitute.org). Each genome was queried against all proteins annotated as conferring resistance to aminoglycosides in the Comprehensive Antibiotic Resistance Database (CARD) (23) using a custom Python script controlling the BLAST+ analysis software (e-value cutoff = 10^{-20}) (24, 25). As expected, Aac(3)-

IVa, one of the few previously identified apramycin resistance enzymes (26), was detected in the majority (9 of 13) of highly apramycin resistant strains (MIC > 256 μg ml^{-1}) and none of the apramycin intermediate or susceptible strains. These strains were also resistant to gentamicin and tobramycin, consistent with the substrate specificity of this enzyme (27). Other apramycin resistance determinants, Aac(1) (28) or ribosomal methylases (29), were not detected in the strain set.

Two strains of *Klebsiella pneumoniae* (apramycin MIC > 256 μg ml^{-1}) were non-susceptible to all aminoglycosides tested, but contained no detectable apramycin modifying enzyme. This phenotype may potentially be explained by reduced permeability to and/or active efflux of aminoglycosides, resistance mechanisms which are more commonly associated with *Pseudomonas* spp. (30, 31). Unexpectedly, we also identified two strains with susceptibility to all aminoglycosides except for apramycin (MIC > 256 μg ml^{-1}). We hypothesize that these latter strains may carry uncharacterized resistance mechanisms highly specific to apramycin which do not appear in the CARD database.

In this work, we found that apramycin shows excellent *in vitro* activity against carbapenem-susceptible strains of *Enterobacteriaceae* and retains equal or better activity against CRE compared to gentamicin, tobramycin and amikacin. Furthermore, it is putatively less toxic than these other aminoglycosides and as a scaffold may be amenable to medicinal chemistry modification to further increase bacterial selectivity (32). As such, apramycin or derivatives appear worthy of further investigation for treatment of *Enterobacteriacae* infection inclusive of CRE.

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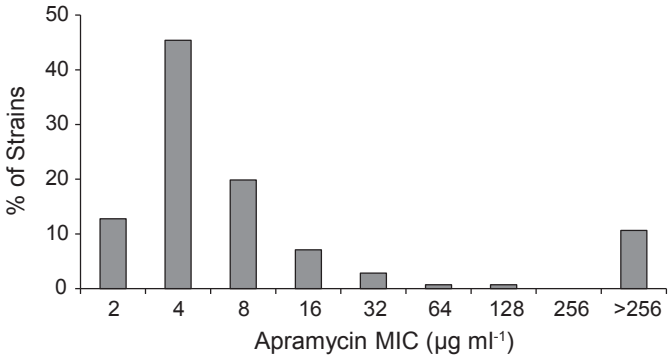
245

246 **Figure Legend.**

247

248 **Figure 1. Apramycin MIC distribution for *Enterobacteriaceae* strains (n = 141) examined in**

249 **this study.**


50
40
30
20
10
0
% of Strains
2
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8
16
32
64
128
256
>256
Apramycin MIC (µg ml-1)