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Supplementary appendix

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Supplementary appendix

In vitro Antimicrobial Susceptibility of *Treponema pallidum* subsp. *pallidum*

Contents

Supplementary methods 2
Origin of T. pallidum strains 2
Antibiotic selection 2
Cell culture and T. pallidum inoculation for susceptibility assay 2
Harvest, re-inoculation, and DNA extraction/quantification 3
Harvest 3
Re-inoculation 3
DNA extraction and quantification 3
Assessment of cytotoxicity of antibiotics on Sf1Ep cells 4
Incubation employing selective pressure with linezolid 4
Table S1. Probabilities of mutation Events in 96 wells over 9 generations based on two mutation rates in T. pallidum cultivation. 5
Whole genome sequencing of strains propagated in sub-therapeutic linezolid concentrations 5
Outcomes and statistical analyses 6
Table S2. Information sources for estimated plasma concentrations 7
Supplementary results 8
Figure S1. WST-1 assay showing lack of cytotoxicity of selected antibiotics. 8
Supplementary references 9

Supplementary methods

Origin of T. pallidum strains

Live treponemes were retrieved from frozen stocks obtained through intratesticular strain propagation in New Zealand White rabbits (*Oryctolagus cuniculus*). Although no animals were used for the study described here, care for the animals used to propagate the study strains was provided according to the Guide for the Care and Use of Laboratory Animals. Animal procedures for those experiments were approved and covered by the University of Washington IACUC protocol # 4243-01 (PI: Lorenzo Giacani).

The SS14 strain, isolated in 1977 in Atlanta from a patient's secondary skin lesion, was originally provided by Sandra A. Larsen (Center for Disease Control and Prevention, Atlanta, GA), and it was selected because it carries the A2058G mutation on the 23S rRNA gene that confers resistance to macrolides. The UW330B strain was isolated in Seattle in 2005 from a blood sample of a patient with early latent syphilis [1] and kindly provided by Christina Marra (University of Washington, Seattle, WA). This strain carries the A2059G macrolide resistance mutation on the 23S rRNA gene. The Chicago C strain was derived from the parental Chicago strain, as previously reported, to facilitate pathogenesis-related studies [2]. The parent strain was isolated in Baltimore in 1951 from a primary lesion [3] and was originally provided by Paul Hardy and Ellen Nell (Johns Hopkins University, Baltimore, MD, USA). This strain is sensitive to macrolides as it lacks mutations related to macrolide resistance.

Antibiotic selection

We aimed to test at least one FDA-approved drug from each class and subclass of antibiotics. Two prioritization criteria were applied to selecting the antibiotics for testing: first, pharmacological properties that would make an antibiotic suitable for repurposing to treat syphilis; and second, antibiotics used for other common conditions, regardless of their pharmacological properties, to gain a better understanding of their potential effects on syphilis. Pharmacological properties for prioritization included very high oral absorption (90-100%) and high volume of distribution (≥0.7 litres/Kg), capacity to reach the peripheral compartment (i.e., the skin), and high cerebrospinal fluid (CSF)-to-plasma ratio (>0.7) to enable treatment of central nervous system infections. Finally, we included zoliflodacin because of its reported efficacy against *Neisseria gonorrhoeae* that may benefit patients with syphilis co-infections[4], and spectinomycin. Table S1 lists the pharmacological properties of approved options for syphilis, as well as alternative options tested in this study. For testing, tissue culture-grade drugs were obtained from Sigma-Aldrich (St. Louis, MO). Pharmaceutical grade zoliflodacin was provided by Dr. John Mueller (Innoviva Specialty Therapeutics).

Cell culture and T. pallidum inoculation for susceptibility assay

Two sets of cultures were prepared: one for the susceptibility assay, and one for the bactericidal/recovery assay. For the susceptibility assay, we used columns of 96-well plates (Corning Inc., 8x12 format) to test different drug concentrations for each selected antimicrobial. Each drug concentration was considered as a separate experimental group and tested eight times in eight replicate wells within a column. Additionally, we included five control groups where treponemes were grown in the absence of antibiotics, each tested in eight replicate wells within a column. Four control groups were harvested at different times (Day 0, Day 1, Day 4, and Day 7) post-inoculation, and one group contained the antibiotic solvent (DMSO or water) instead of the test drug was harvested on Day 7. To avoid unnecessary exposure to oxygen, Day 1 and Day 4 replicates were plated in independent plates.

Each antibiotic solution (1.5 µl) was added from a 100X concentrated stock to achieve the final concentration to be tested without significantly altering the final volume of the culture. Azithromycin, balofloxacin, cefetamet, cefixime, ivermectin, linezolid, metronidazole, tedizolid, and zoliflodacin were solubilized in DMSO, while amoxicillin, cefuroxime, ceftriaxone, cephalexin, ertapenem, dalbavancin, isoniazid, spectinomycin, and pyrazinamide were solubilized in sterile water.

After the addition of the treponemes and agents/solvents, the culture wells were incubated at 34ºC in the tri-gas incubator until harvest. Treponemes from experimental wells with the selected antibiotic concentration range were harvested to perform DNA quantification after a week-long incubation. Controls without antibiotics were harvested after 1-, 4-, and 7-days of incubation, and control with solvent alone after a week-long incubation. DNA from inoculum treponemes (Day 0) was extracted to allow proper control of treponemal growth.

The day before treponemal inoculation, wells (96-well plates) were seeded with $3x10^3$ rabbit Sf1Ep cells in 150 µL of MEM culture media. The plates were then incubated overnight in a 5% CO_2 atmosphere within a HeraCell 150 incubator (Thermo Fisher Scientific, Waltham, MA) to allow Sf1Ep cell adhesion to the well surface. On the same day, TpCM2 treponemal media was prepared as previously reported [5] and equilibrated overnight in a HeraCell 150i tri-gas incubator (Thermo Fisher Scientific) at 34ºC in a microaerophilic environment (1.5% O_2, 3.5% CO_2 and 95% N_2). The following day, MEM was removed from the Sf1Ep-containing wells, and cells were rinsed with 150 µL equilibrated TpCM2 media. Subsequently, 150 µL of equilibrated TpCM2 media were added to each well, and the plates were placed in the tri-gas incubator for at least three hours. *T. pallidum* cells grown on Sf1Ep cultures inoculated the previous week were separated through trypsinization to allow the release and quantification of spirochetes and to prepare the inoculum for the 96-well test plates. Treponemes were counted using dark field microscopy on a Leica DM2500 LED microscope (Leica, Wetzlar, Germany) and diluted in TpCM2 to $3.3x10^5$ *T. pallidum* cells/ml. The desired inoculum (150 µL) was seeded to the corresponding wells of the 96-well test plates for drug activity assays. Eight inoculum aliquots (Day 0) were retained for subsequent DNA extraction and *T. pallidum* quantification by qPCR. Treponemes were pelleted from each aliquot at 20,000 x g for 10 minutes and, after supernatant removal, pellets were resuspended in 200 µl of genomic lysis buffer provided with the Quick-DNA 96 kit (Zymo Research, Irvine, CA) and stored at -20ºC until DNA extraction.

Harvest, re-inoculation, and DNA extraction/quantification

Harvest

The exhausted TpCM2 was removed from wells and discarded. A total of 200 µL of genomic lysis buffer (Zymo Research) was added to each well, and wells were incubated for 30 minutes at room temperature to allow for cell lysis as per the protocol provided by the kit manufacturer. Processed plates were sealed and stored at -20°C until DNA extraction. In earlier studies [6], we determined that most (~85%) of *T. pallidum* cells *in vitro* adhere to the rabbit epithelial cell monolayer, as also reported by Edmondson *et al.* for other cultivated strains [7]. This evidence allowed us to discard the culture media without concern that the experimental results would be significantly affected.

Re-inoculation

For the bactericidal/recovery assay, a second set of plates were prepared to determine whether exposure to a given drug concentration was treponemicidal, which was achieved by sub-culturing treponemes exposed to the therapeutic agent into freshly prepared antibiotic-free recovery plates. The recovery plate was returned to the tri-gas incubator for a further seven day-incubation period before being processed for DNA extraction. The day before sub-culturing for the bactericidal assay, sufficient wells of a 96-well plate were prepared with Sf1Ep cells in TpCM2 as described above. The following day, a 7-day-old plate used for the susceptibility assay was processed by removing the exhausted TpCM2 media and rinsing the cells briefly with 20 µL of warm, sterile trypsin before removal. Cell separation was completed by adding 20 more microliters of trypsin and incubating wells at 37°C for five minutes. Cells in the wells were resuspended using a multichannel pipet and 10 µL were transferred to the freshly prepared plate. The recovery plate was returned to the tri-gas incubator for a further seven day-incubation period before being processed for DNA extraction as described above.

DNA extraction and quantification

Treponemes were pelleted from each aliquot at 20,000 x g for 10 minutes and, after supernatant removal, pellets were resuspended in 200 µL of genomic lysis buffer provided with the Quick-DNA 96 kit (Zymo Research) and stored at -20ºC until DNA extraction. To extract the DNA, the 96-well plates were thawed at 37°C and spun briefly to remove condensation drops on the plate sealers. DNA was extracted using the Quick-DNA 96 kit (Zymo Research) according to the manufacturer's instructions. DNA was eluted in 100 µL of molecular water and stored at -20ºC until analysis. DNA obtained from each sample was quantified by qPCR targeting the *T. pallidum*-specific *tp0574* gene as previously described [8]. The Powerup SYBR Green Master Mix (Thermo Fisher Scientific) was used for amplification. Amplifications were run on a QuantStudio3 or QuantStudio5 thermal cycler (Thermo Fisher Scientific), and results were analyzed using the instrument software. Data were imported into Prism 8 (GraphPad Software, San Diego, CA) and further analysed to assess the statistical significance of the values from test and no-antibiotic control groups using one-way ANOVA with the Dunnett test for correction of multiple comparisons or t-test, with significance set at $p<0.05$ in both cases.

Assessment of cytotoxicity of antibiotics on Sf1Ep cells

An additional 96-well culture plate was seeded with Sf1Ep cells in TpCM2, but without *T. pallidum* cells. As a control we used two no-antibiotic well columns, and a column with only TpCM2 but no Sf1Ep cells to use as spectrophotometric blank. After seven days incubation, to one of the control columns we added 30 µl of sterile 100% ethanol to shock the Sf1Ep cells. We then added to all test and control wells 15 µl of WST-1 cell proliferation reagent (Sigma-Aldrich, Inc., St. Louis, MO) that is efficiently cleaved by metabolically active Sf1Ep cells not exposed to ethanol, increasing the solution absorbance. Finally, absorbance of each well was read on a Synergy HTX multi-mode plate reader (Agilent Technologies, Inc., Santa Clara, CA) at 450 nm.

A 96-well culture plate was seeded with Sf1Ep in TpCM2 as described above, but without *T. pallidum* cells. Antibiotics and control solutions were also added as described above. One no-antibiotic well control column was also present, along with a column with only TpCM2 but no Sf1Ep cells to use as spectrophotometric blank. After seven days in the tri-gas incubator, 30 µL of supernatant were removed from the control column (with Sf1Ep cells in TpCM2), and 30 µL of sterile 100% ethanol were added to impair cellular homeostasis. To all test and control wells, 15 µL of WST-1 cell proliferation reagent (Sigma-Aldrich, Inc., St. Louis, MO) were added. Metabolically active Sf1Ep cells efficiently cleave the tetrazolium salt WST-1 to formazan, increasing the solution absorbance. Cells shocked with ethanol have reduced ability to cleave WST-1 and provided a toxicity control for the assay. The plates were incubated at 37°C in 5% CO_2 for 2 hours, then absorbance was read on a Synergy HTX multi-mode plate reader (Agilent Technologies, Inc., Santa Clara, CA) at 450 nm. Mean blank values from TpCM2+WST-1 wells were subtracted from all readings. Data were imported into Prism 8 (GraphPad Software, San Diego, CA) and further analyzed to assess the statistical significance of the values from test and no-antibiotic control groups using one-way ANOVA with the Dunnett test for correction of multiple comparisons. The threshold for significance was set at $p<0.05$.

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Incubation employing selective pressure with linezolid

Based on our calculations, it would be highly probable (0.710 – 1.000) that the mutation conferring resistance to linezolid occurred in at least one cell within one of the wells after 2 weeks under selective pressure. Linezolid concentration was then lowered to 0.03 mg/L for an additional eight weeks of *in vitro* propagation to allow treponemal recovery. After a total of 10 weeks, *T. pallidum* cells exposed to linezolid underwent a new *in vitro* susceptibility assay in parallel to non-exposed *T. pallidum* cells. DNA was extracted to perform whole genome sequencing (WGS) as an alternative way to investigate whether mutations that could be associated with linezolid resistance were selected or had occurred during propagation.

We aimed to investigate the potential of prolonged exposure to sub-therapeutic concentrations of linezolid in selecting for less susceptible or resistant strains of *T. pallidum*, or inducing genetic changes associated with linezolid resistance. These genetic changes are expected to be primarily related to mutations in the 23S rRNA gene[9,10].

Based on preliminary data that linezolid was effective at limiting treponemal growth at a concentration of 0.5 mg/L or higher, during the initial two weeks of propagation we employed antibiotic pressure using a concentration of 0.2 mg/L of linezolid in the media. We assumed that this concentration of linezolid would inhibit the growth of *T. pallidum* cells without the mutation, thereby creating selective pressure for the proliferation of only those cells that carry the mutation conferring resistance. The duration of 2 weeks for applying antibiotic pressure to select resistance strains was chosen based on (1) previous studies demonstrating successful selection of resistance in other bacteria[11,12], (2) the anticipated exposure time in humans during a 10-day course of linezolid which would be potentially used for treating syphilis, (3) calculations on the probability of mutation occurrence as presented below.

We performed calculations to determine the probabilities of occurrence of the targeted mutation after two weeks in 6 wells with an inoculum of 5,000,000 cells per well based on the following assumptions:

1. The *in vitro* generation time of *T. pallidum* is estimated to be around 35-40 hours [13]. Therefore, after 2 weeks of cultivation, it is expected that 9 generations of *T. pallidum* would have occurred (8.4 - 9.6 generations). As a result, the number of cells derived from a single *T. pallidum* cell would have multiplied to approximately 256 cells (2^8) by this time.
2. There are no significant differences in mutation rates among different genomic sites of the bacteria (i.e., the mutation rate of the 23S rRNA is similar to any other gene).
3. The concentrations of the antibiotic do not impact the fitness of the wild-type bacteria.

For calculations, two mutation rates were employed, encompassing a range of 10^{-8} to 10^{-9} mutation rates observed in bacterial species. Subsequently, we calculated the probability of a mutation event taking place in a single well and, based on this, we extrapolated the probability of that mutation occurring in any cell within any of the 96 wells over the course of nine generations.
Based on the calculations (table), it would be very probable (1.00) that the mutation conferring resistance to linezolid occurred in at least one cell within one of the wells.

Table S1. Probabilities of mutation events in 6 wells over 9 generations based on two mutation rates in *T. pallidum* cultivation.

A. Generation	B. Factor	C. No. of cells	D. Probability of mutation in a single well (Low Mutation Rate)	E. Probability of mutation in a single well (High Mutation Rate)	F. Probability of mutation in any of the 6 wells (Low Mutation Rate)	G. Probability of mutation in any of the 6 wells (High Mutation Rate)
1	1	5000000	0.005	0.050	0.029	0.265
2	2	10000000	0.010	0.100	0.058	0.469
3	4	20000000	0.020	0.200	0.114	0.738
4	8	40000000	0.040	0.400	0.217	0.953
5	16	80000000	0.080	0.800	0.394	1.000
6	32	160000000	0.160	1.000	0.649	1.000
7	64	320000000	0.320	1.000	0.901	1.000
8	128	640000000	0.640	1.000	0.998	1.000
9	256	1280000000	1.000	1.000	1.000	1.000

Legend. We assumed exponential growth during approximately 9 generations of culture under these conditions (columns A and B, generation and factor), resulting in the total number of bacteria cells in each well (Column C, cells). To calculate the likelihood of mutation in a well, we employed two mutation rates, representing a spectrum rate documented in bacteria (low rate 10^{-9}, high rate 10^{-8}). Subsequently, we calculated the probability of a mutation occurring in a well under both low mutation rate (column D) and high mutation rate (column E). Based on this, we estimated the probability of mutation occurring in any cell in any of the 6 wells after 9 generations (columns F and G).

Whole genome sequencing of strains propagated in sub-therapeutic linezolid concentrations

Prior to sequencing, pre-capture libraries were prepared from up to 100 ng input genomic DNA using the Kapa Hyperplus kit (Roche), using a fragmentation time of 8 minutes and standard-chemistry end repair/A-tailing, then ligated to TruSeq adapters (Illumina). Adapter-ligated samples were cleaned with 0.8x Ampure beads (Beckman Coulter) and amplified with barcoded primers for 14–16 cycles, followed by another 0.8x Ampure purification. The capture of *T. pallidum* genomes was performed according to Integrated DNA Technology's (IDT's) xGen Hybridization Capture protocol. Briefly, pools of 3–4 libraries were created by grouping samples with similar treponemal load for a total of 500 ng DNA, and Human Cot 1 DNA and TruSeq blocking oligos (IDT) were added prior to vacuum drying. The hybridization master mix, containing biotinylated probes from a custom IDT oPool tiling across the NC_010741.1 reference genome, was then added overnight (>16 hr) at 65ºC. The following day, streptavidin beads were added to the capture reaction, followed by extensive washing, 14–16 cycles of post-capture amplification, and purification with 0.8x Ampure beads. Pool concentration was determined by Qubit assay (Thermo Fisher Scientific) and size was verified by Tapestation (Agilent). Libraries were sequenced on a 2x150 paired-end run on a HiseqX. Fastqs were processed, and genomes were assembled using a custom pipeline, available at https://github.com/greninger-lab/*T.pallidum*_WGS. Filtered reads were mapped to the *T. pallidum* SS14 reference genome, NC_021508.1, using Bowtie2 v2.4.1 [14] with default parameters and converted to bam with samtools v1.6 [15], followed by deduplication by MarkDuplicates in Picard v2.23.3 (http://broadinstitute.github.io/picard).

Outcomes and statistical analyses

Data on qPCR values from the drug susceptibility assay were imported into Prism 8 (GraphPad Software, San Diego, CA). The sample size for our lab study consisted of 8 replicates for each tested drug concentration and control group, which is larger than what is typically employed in this type of research.[16,17] Each sample in our study represented a technical replicate derived from the same source mixture. Due to the rigorous control of experimental conditions, there were no major sources of variation among samples that needed to be accounted for.

We used the Kruskal-Wallis mean-rank test to compare the distribution of qPCR values among independent groups with different antibiotic concentrations and the non-antibiotic control groups. We then conducted pairwise comparisons using Dunn's test to compare the distribution of qPCR values between each group with a specific antibiotic concentration and either the distribution of values for the control group at Day 0 or the control group at Day 7. We also tested the comparison between the DSMO/H2O group at Day 7 and the control group at Day 7. We used the False discovery rate (FDR) Benjamin-Hochberg correction for multiple comparisons, setting a significance level of 0.05. For the cytotoxicity experiment, we used the same methodology to compare absorbance from the antibiotic group and the non-antibiotic control group. For each experiment, the median blank value from wells containing only TpCM2 and WST-1 reagent was subtracted from all experimental readings.

Table S2. **Information sources for estimated plasma concentrations**

Natural Penicillins	**Reference**
Benzathine penicillin G	[18]
Aminopenicillins	
Amoxicillin	[19]
Cephalosporins	
Ceftriaxone	[20]
Cefetamet	[21]
Cephalexin	[22]
Cefuroxime	[23]
Cefixime	[24]
Carbapenems	
Ertapenem	[25]
Tetracyclines	
Doxycycline	[26]
Fluoroquinolones	
Moxifloxacin	[27]
Balofloxacin	[28]
Macrolides	
Azithromycin	[29]
Oxazolidinones	
Linezolid	[30]
Tedizolid	[31]
Lipoglycopeptides	
Dalbavancin	[32]
Aminoglycosides	
Spectinomycin	[33]
Antimycobactierials	
Isoniazid	[34-36]
Pyrazinamide	[37-39]
Clofazimine	[40-42]
Antiparasitics	
Ivermectin	[43-45]
Nitroimidazoles	
Metronidazole	[46]
Spyropyrimidinetriones	
Zoliflodacin	[47]

Supplementary results

Figure S1. WST-1 assay showing lack of cytotoxicity of selected antibiotics.
Amoxicillin (A), cefixime (B), ceftriaxone (C), balofloxacin (D), tedizolid (E), dalbavancin (F), isoniazid (G), pyrazinamide (H), ivermectin (I), metronidazole (J), zoliflodacin (K), azithromycin (L), spectinomycin (M), and ertapenem (N) on rabbit Sf1Ep cells that support *T. pallidum* viability and growth in culture. Ivermectin (I) was found to be progressively cytotoxic to Sf1Ep cells, although statistical significance was achieved only at concentrations ≥20 mg/L. Values represent mean OD_{450} +/- SEM of eight biological replicates. EtOH indicates cells pre-treated with 20% ethanol to provide a positive cytotoxicity control. DMSO/H_2O bars are cultures to which the compound solvent was added instead of the tested antibiotic.

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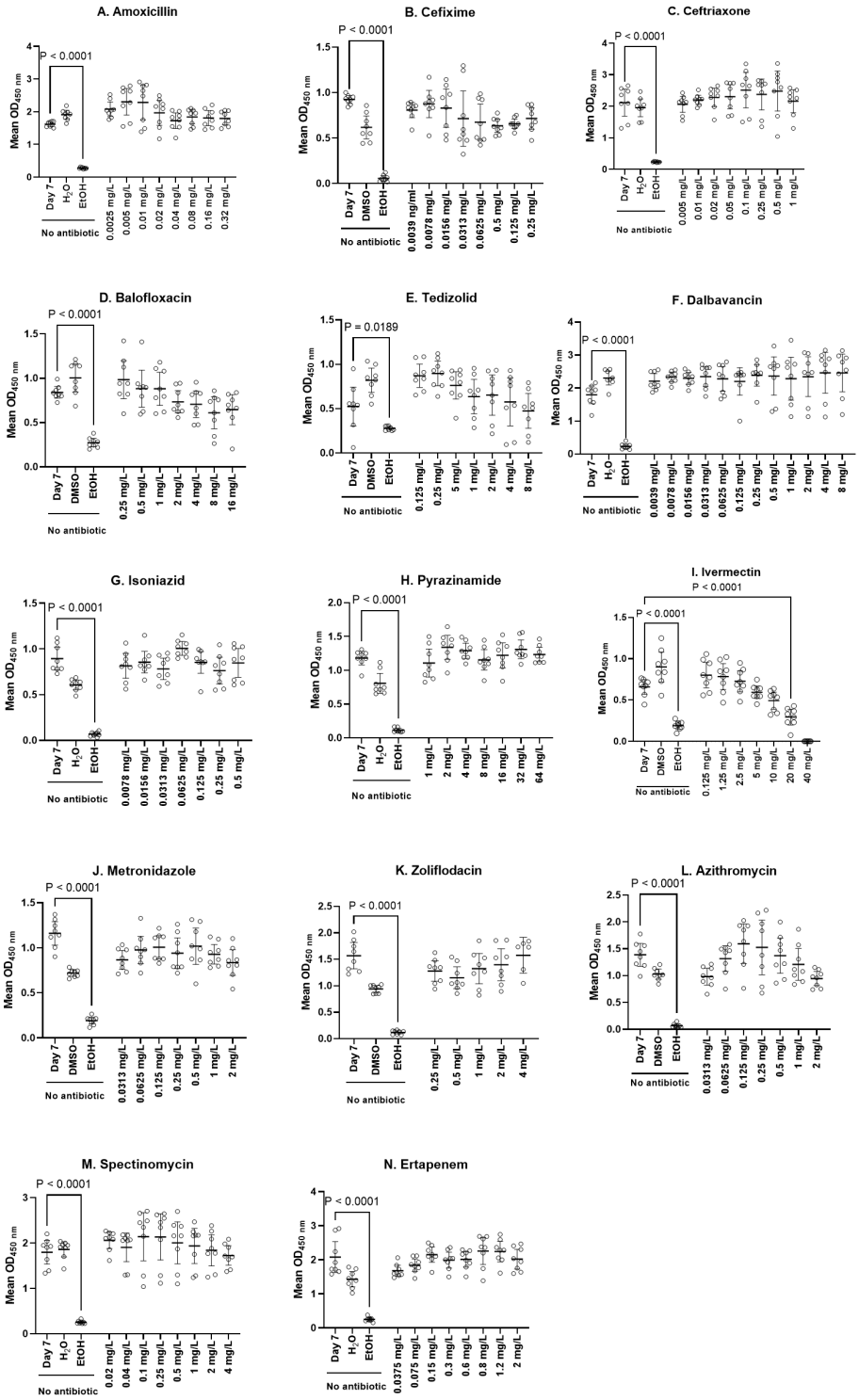

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