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Angewandte Chemie

Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

Digital Quantification of DNA Replication and Chromosome Segregation Enables Determination of Antimicrobial Susceptibility after only 15 Minutes of Antibiotic Exposure

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Experimental Section

Materials and reagents

All reagents purchased from commercial sources were used as received unless otherwise stated. BBL Trypticase Soy Agar plates with 5% Sheep Blood and Bacto Brain Heart Infusion (BHI) media were purchased from BD (Franklin Lakes, NJ, USA). BHI was dissolved in deionized water at the manufacturers recommended concentration and autoclaved prior to use. All antibiotic stock solutions and PCR reactions were prepared using sterile, nuclease-free water (NF-H_2O) purchased from Thermo Fisher (Waltham, MA, USA).

All antibiotics and clavulanic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), with the exception of amoxicillin, which was purchased from Alfa-Aesar (Ward Hill, MA, USA). Ciprofloxacin and clavulanic acid were prepared as a 1 mg/mL stock solutions in NF-H_2O. Nitrofurantoin was prepared as a 10 mg/mL stock solution in dimethylformamide (DMF). Sulfamethoxazole was prepared as a 10 mg/mL stock solution in dimethyl sulfoxide (DMSO). Trimethoprim was prepared as a 1 mg/mL stock solution in DMSO. All antibiotic stock solutions were stored at -20 °C. Amoxicillin was prepared fresh as a 1 mg/mL stock solution in NF-H_2O before each experiment.

QuickExtract DNA Extraction Solution and QuickExtract RNA Extraction Kit were purchased from Epicentre (Madison, WI, USA). SsoFast EvaGreen Supermix (2X) and QX200 ddPCR EvaGreen Supermix was purchased from Bio-Rad Laboratories (Hercules, CA, USA) and used for all qPCR and dPCR experiments respectively.

Pooled human urine (catalog no. 991-03-P) was obtained from Lee Biosolutions (Maryland Heights, MO, USA).

Isolate maintenance

Ten *E. coli* isolated from the urine of 10 unique patients were obtained from the University of California Los Angeles (UCLA) Clinical Microbiology Laboratory with approval from the UCLA and Veterans Affairs Institutional Review Boards and appropriate Health Insurance Portability and Accountability Act exemptions. All isolates were identified as *E. coli* using the Vitek2 GNID panel (bioMerieux, Durham, NC, USA), and chosen for use based on their determined MICs. Urine cultures were performed by routine semi-quantitative methods, by inoculating 1 μL of urine to a BBL Trypticase Soy Agar plate with 5% Sheep Blood (BAP, BD, Sparks MD) and a MacConkey plate followed by overnight incubation at 35 +/-2 °C in ambient air. In all cases, the *E. coli* grew in pure culture at >100,000 colony forming units. Minimum inhibitory concentrations (MIC) for each isolate was determined by UCLA for ciprofloxacin (cip), nitrofurantoin (nit), sulfamethoxazole/trimethoprim (sxt), and amoxicillin/clavulanic acid (amc) using the Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution method,[1] in panels prepared by UCLA with cation-adjusted Mueller-Hinton broth (MHB). BMD tests were incubated at 35 +/- 2 °C in ambient air conditions for 16-20 h. MICs were interpreted using CLSI M100S 26th edition breakpoints.[1] *E. coli* isolates were stored at -80 °C in Brucella broth with 20% glycerol (Becton, Dickinson, Sparks, MD, USA). Isolates were subcultured twice on BAP and well-isolated colonies were used for antibiotic exposure time course experiments.

Antibiotic exposure time course experiments

In order to generate liquid culture for use in experiments, *E. coli* isolates were cultured overnight (10-12 hours) after scraping a small portion of the plate and inoculating in 4 mL BHI. Overnight cultures were re-inoculated into 4 mL of fresh BHI and grown for an additional 4–6 h until early logarithmic phase. Cultures were then diluted 10 fold into pre-warmed BHI, and optical density (600 nm) was measured using a portable spectrophotometer (GE Healthcare Ultrospec 10). OD was converted to approximate cell count using the correlation factor OD600 1.0 = 8.0*10^8 cells/mL). The dilutions prepared for OD measurements were then immediately diluted a second time into 2 mL polypropylene tubes to a final volume of 500 µL (dilution factor dependent on desired final cell concentration). These tubes were incubated for 5 min at 37 °C with shaking at 500 rpm in a heating/shaking block (Thermo Fisher Digital Heating Shaking Drybath) to ensure thorough mixing. During this time, separate 2 mL polypropylene tubes containing 450 µL of BHI with and without antibiotics were prepared. All exposure time courses were conducted with antibiotic concentrations above the minimum inhibitory concentration (MIC) of the susceptible isolate and below the MIC of the resistant isolate

being tested. Ciprofloxacin exposure in media and urine was conducted at a final antibiotic concentration of 2.00 and 0.75 μg/mL respectively. Nitrofurantoin experiments were performed at 64.00 μg/mL. Sulfamethoxazole/trimethoprim experiments were performed at 76.00/4.00 μg/mL. For amoxicillin experiments, susceptible isolates were exposed to a final concentration of 12.00 μg/mL, and resistant isolates were exposed to a final concentration of 14.00 μg/mL. Cultures were then diluted a final 10 fold (50 µL culture into 450 µL) into single tubes containing media with or without antibiotics, and time was started. 10 µL aliquots were removed at 0, 15, and 30 min., and immediately mixed with 90 µL of a one-step extraction buffer suitable for direct use in PCR. Denaturing extraction conditions used Epicentre QuickExtract DNA Extraction Solution. Cells were mixed with Epicentre QuickExtract DNA Extraction Solution, pipette mixed, incubated at 65 °C for 6 min., 98 °C for 4 min., then chilled on ice. Non-denaturing extraction conditions used Epicentre Quick Extract RNA Extraction solution. Aliquots were mixed with RNA extraction immediately via pipette, gently vortexed to ensure thorough mixing, and chilled on ice. All samples were stored at -20 °C for several days during use before being moved to -80 °C for long-term storage.

DNA fragmentation

DNA was fragmented to a predicted 1000 bp fragment size using a Covaris 220M ultrasonicator. Samples were diluted 10 fold into a 130 µL microTUBE AFA Fiber Snap-Cap, and sheered for 90 seconds at 20 °C with a Peak Incident Power of 50 W, duty factor of 2%, and 200 cycles per burst. This size was chosen to ensure that all copies of the 23S gene will be separated from each other. Based on an analysis of 11 *E.coli* strains isolated from UTIs, the average distance between 23S genes is 1,169 kb with the closest genes being 38 kb apart. These genomes may be accessed with the following accession numbers: CP011018.1; HG941718.1; CP007265.1; CP007391.1; CP002797.2; CP002212.1; CP001671.1; CU928163.2; CP000247.1; CP000243.1; CP011134.1.

DNA quantification

All qPCR reactions were performed using a Roche LightCycler 96. All reactions contained only SsoFast EvaGreen Supermix at a final concentration of 1X, forward and reverse primers (forward primer TGCCGTAACTTCGGGAGAAGGC, reverse primer TCAAGGCTCAATGTTCAGTGTC) specific for Enterobacteriaceae[2] at a final concentration of 500 nM, template DNA at variable concentrations, and NF-H_2O. A single master mix containing supermix, primers, and NF-H_2O was prepared and aliquoted into PCR tubes. Template was then added, brining the final volume to 30 µL. Each tube was then mixed thoroughly via pipette and technical triplicates (9 µL each) were aliquoted into the 96 well plate. Cycling conditions consisted of an initial denaturation step at 95 °C for 3 min. followed by 30 cycles of 95 °C for 20 s, 62 °C for 20 s, and 72 °C for 20 s. Following amplification a continuous melt curve was obtained between 55 and 95 °C. Total cycling time (including melt analysis) was 60 min.

Digital PCR reactions were carried out in a BioRad QX200 Droplet Digital PCR system according to the manufacturer's instructions. Samples were prepared in identical fashion as those prepared for qPCR. For each sample, two wells of the droplet generation chip and well plate were used to generate and thermocycle droplets, respectively. This resulted in approximately 40,000 droplets being analyzed for each sample. Cycling conditions consisted of an initial denaturation step at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Following initial thermocycling, the sample was cooled to 4 °C for 5 min followed by a final heating step at 95 °C for 5 min. All thermocycling steps were performed with a 2 °C/s ramp rate. Total cycling time was 115 min.

Statistical analyses

Raw Ct values are not normally distributed; therefore, a typical plot showing the mean Ct +/- 2·SD does not mean that the true mean will lie in the confidence interval 95% of the time. Understanding this fact, we would still like to represent the variability in qPCR measurements for the raw Ct plot. We did this with a standard confidence interval calculation:

$$Ct_{U/L} = Ct_{avg} \pm t_{crit} \cdot \frac{s_{Cq}}{\sqrt{n}} \quad (1)$$

The critical t value (t_{crit}) for a 98% confidence interval with 2 degrees of freedom is 4.85; with $n = 3$ replicates, this results in the SD being multiplied by 2.80 for the confidence intervals. This does not mean that the true Ct is within this interval 98% of the time, but it does give a representation of the variability in Ct measurements.

In order to calculate the p-value for comparing treated and untreated samples, the raw Ct values (which are exponential) were linearized into a relative quantity (FC) with t = 0 min as the reference point using $FC = 2^{Ct(t)-Ct(0)}$. The log ratio of these linearized quantities was compared to ln(1.1) using a one-tailed t test. A one-tailed test was chosen because the untreated sample should have a higher concentration than the treated sample; if by some random event the treated sample has a statistically significant higher concentration than untreated, we don't want to draw the false conclusion that the isolate is susceptible. To account for pipetting variation (the treated sample could have randomly had 10% more bacteria pipetted into its media at time = 0 than the untreated sample), the null hypothesis is $\ln\left(\frac{FC_{ut}}{FC_t}\right) - \ln(1.1) = 0$ instead of $\ln\left(\frac{FC_{ut}}{FC_t}\right) = 0$. This makes the AST more conservative (reducing very major errors) by requiring that the untreated sample have at least 1.1 fold more copies than the treated sample. P-values for digital PCR were calculated with a one-tailed Z test comparing $\ln\left(\frac{FC_{ut}}{FC_t}\right)$ to $\ln(1.1)$, with FC_{ut} representing the fold change in concentration of the untreated sample with respect to time = 0 and FC_t representing the same quantity, but for the treated sample.

Discussion of mechanism of action of antibiotics tested

In addition to ciprofloxacin, we evaluated three other antibiotics used in the treatment of UTIs: (i) nitrofurantoin, which is reduced to a reactive radical inside the cell, reacting with multiple cellular targets including enzyme involved in DNA synthesis[3], which would directly affect replication; (ii) the combination of sulfamethoxazole and trimethoprim, which synergistically inhibit folic acid biosynthesis, subsequently impairing multiple metabolic reactions including thymidine synthesis[4]; and (iii) amoxicillin, which disrupts the synthesis of the peptidoglycan layer of bacterial cell walls leading to lysis[5], but is not known to specifically affect DNA replication.

Figures and Tables

Isolate Number	Specimen	AMC	CIP	NIT	SXT
1	urine	<=2	<=.25	<=16	<=1
2	urine	<=2	<=.25	<=16	<=1
3	urine	<=2	<=.25	<=16	<=1
5	urine	<=2	<=.25	<=16	<=1
6	urine	>16	>2	<=16	<=1
8	urine	<=2	>2	64	>8
10	urine	4	>2	<=16	>8
11	urine	ND	>2	>256	>8

Table S1. Minimum inhibitory concentrations for all isolates tested, as determined by broth dilution. AMC = amoxicillin/clavulanic acid, CIP = ciprofloxacin, NIT = nitrofurantoin, SXT = sulfamethoxazole/trimethoprim. ND = not determined.

Isolate Number	S or R	ABX	[ABX] (ug/mL)	DNA Extraction Type	Control or Treated	Fold Change (qPCR)	Upper Bound 98% CI (qPCR)	Lower Bound 98% CI (qPCR)	p-val (qPCR)	Fold Change (dPCR)	Upper Bound 98% CI (dPCR)	Lower Bound 98% CI (dPCR)	p-val (dPCR)
1	S	cip	2.00	denaturing	control	1.41	0.19	0.19	0.006	1.15	0.03	0.03	<0.001
					treated	0.99	0.02	0.02		0.78	0.02	0.02	
5	S	cip	2.00	denaturing	control	1.83	0.36	0.36	0.005	1.60	0.05	0.05	<0.001
					treated	0.97	0.18	0.18		1.11	0.03	0.03	
10	R	cip	2.00	denaturing	control	1.81	0.05	0.05	0.866	1.39	0.05	0.05	>0.999
					treated	1.72	0.24	0.24		1.38	0.04	0.04	
2	S	nit	64.00	denaturing	control	1.38	0.02	0.02	0.058	1.48	0.04	0.04	<0.001
					treated	1.05	0.34	0.34		1.01	0.03	0.03	
3	S	nit	24.00	denaturing	control	2.93	0.67	0.67	0.027	1.58	0.10	0.10	<0.001
					treated	1.87	0.09	0.09		1.00	0.04	0.04	
11	R	nit	64.00	denaturing	control	1.27	0.08	0.08	0.566	1.38	0.04	0.04	>0.999
					treated	1.16	0.05	0.05		1.36	0.03	0.03	
3	S	sxt	76.00	non-denaturing	control	1.34	0.07	0.07	0.302	3.37	0.25	0.25	0.001
					treated	1.19	0.17	0.17		2.67	0.19	0.19	
5	S	sxt	76.00	denaturing	control	1.63	0.38	0.38	0.883	1.31	0.03	0.03	<0.001
					treated	1.61	0.10	0.10		1.08	0.03	0.03	
8	R	sxt	76.00	denaturing	control	1.72	0.07	0.07	0.858	1.38	0.03	0.03	>0.999
					treated	1.64	0.24	0.24		1.35	0.03	0.03	
1	S	amc	12.00	denaturing	control	1.60	0.17	0.17	0.973	1.49	0.04	0.04	>0.999
					treated	1.60	0.02	0.02		1.62	0.05	0.05	
1	S	amc	12.00	non-denaturing	control	1.36	0.08	0.08	0.734	2.26	0.10	0.10	<0.001
					treated	1.28	0.26	0.26		1.33	0.06	0.06	
1	S	amc	12.00	non-denaturing*	control	3.50	0.72	0.72	0.463	3.60	0.19	0.19	>0.999
					treated	3.14	1.45	1.45		3.72	0.18	0.18	
3	S	amc	14.00	non-denaturing	control	1.60	0.20	0.20	0.535	6.56	0.28	0.28	<0.001
					treated	1.46	0.55	0.55		1.37	0.07	0.07	
6	R	amc	14.00	non-denaturing	control	1.11	0.13	0.13	0.920	1.15	0.04	0.04	>0.999
					treated	1.14	0.29	0.29		1.24	0.04	0.04	

Table S2. Raw data and additional experiments performed with multiple isolates. “S or R” refers to susceptible or resistant as determined by MIC. ABX = antibiotic. * indicates samples were sheared prior to quantification (see methods section of SI). Experiment exposing isolate 1 to ciprofloxacin was performed in 1:1 media:urine, all other experiments were performed in media.

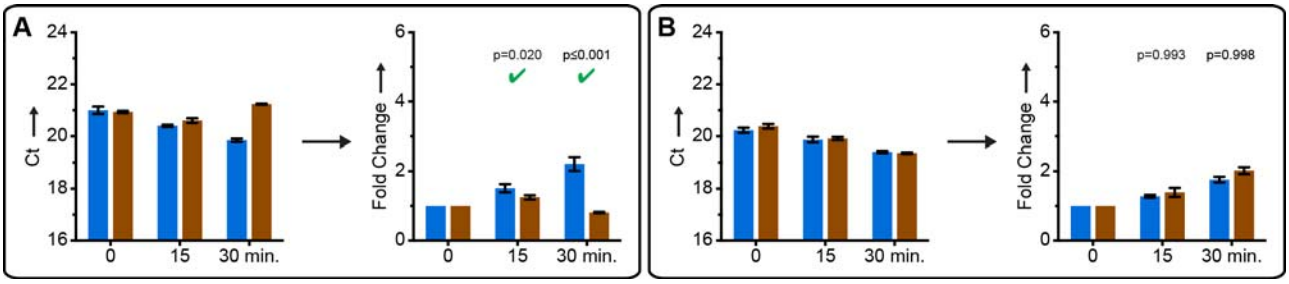

Figure S1. qPCR time course for exposure of (A) susceptible and (B) resistant UTI *E. coli* isolates to ciprofloxacin pre-cultured in urine and exposed to antibiotics in 1:1 urine:BHI. Raw cycle thresholds represent the average of technical triplicates; error bars represent 2.8 standard deviations (see SI). Fold change values represent change from t = 0 min; error bars represent the upper and lower bounds of the 98% confidence interval. Significance was defined as a p-value <= 0.02 when comparing the fold change in 23S concentration of samples incubated without antibiotics (blue) to 1.1 times the fold change in 23S concentration of samples with antibiotics (brown) at a specific time point. Significant differences detected using the susceptible isolate are marked with a green check.

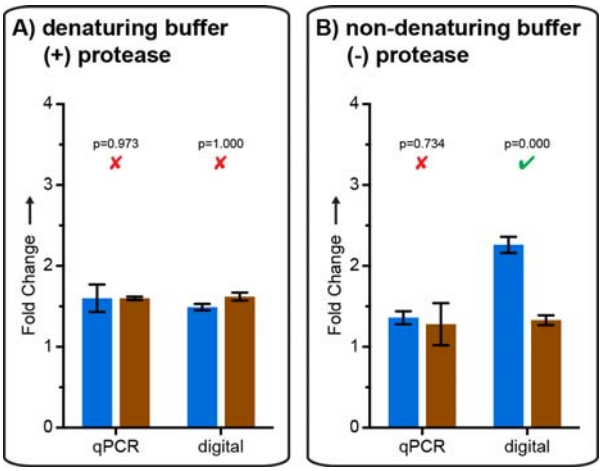

Figure S2. Comparison of susceptible isolate analyzed by qPCR and digital PCR after a 15 min exposure to amoxicillin/clavulanic acid and extracted using a denaturing buffer with protease treatment (A) and a non-denaturing buffer without protease treatment (B). Fold change values represent change from t = 0 min; error bars are 98% confidence intervals. Significance was defined as a p-value <= 0.02 when comparing the fold change in 23S concentration of samples incubated without antibiotics (blue) to 1.1 times the fold change in 23S concentration of samples with antibiotics (brown) at a specific time point. Significant and non-significant differences are marked with a green check and red x respectively.

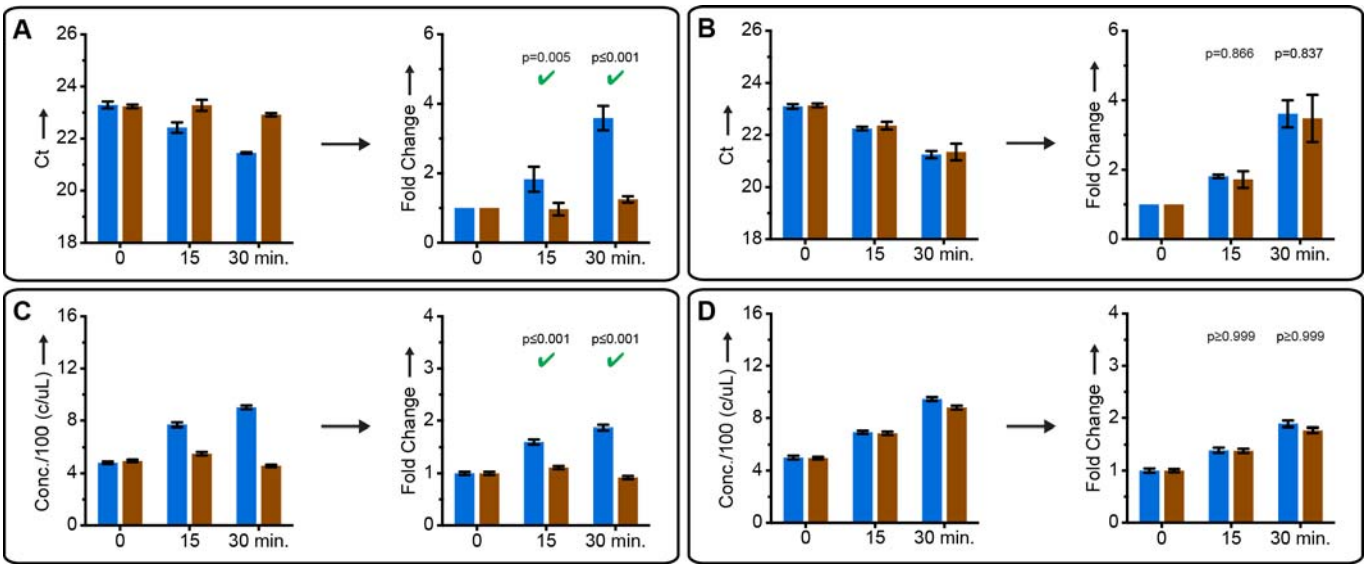

Figure S3. Fold change plots from Figures 1 and 3 with corresponding Ct and concentration plots to demonstrate conversion from either Ct or concentration to fold change. (A, B) AST results using qPCR. Time course for exposure of (A) susceptible and (B) resistant UTI *E. coli* isolates to ciprofloxacin. For cycle thresholds (Ct) error bars are 2.8 S.D. Fold change values represent change from t = 0 min; error bars represent the upper and lower bounds of the 98% C.I. Significant differences (p-value ≤ 0.02) are marked with a green check. (C, D) AST results using dPCR. Time course for exposure of susceptible (C) and resistant (D) UTI *E. coli* isolates to ciprofloxacin. Concentrations are calculated using Poisson statistics; error bars represent the upper and lower bounds of the 98% C.I. Fold change values represent change from t = 0 min; error bars represent the upper and lower bounds of the 98% C.I. Significant (≤ 0.02) p-values for susceptible isolates are denoted with a green check.

Contributions of non-corresponding authors

Nathan G. Schoepp

1. Major contributor to selecting DNA replication as AST marker, contributed knowledge on AST state of the art and effects of antibiotics on replication, contributed to digital resolution hypothesis, contributor to chromosome segregation hypothesis
2. Optimized antibiotic exposure protocols
3. Performed all antibiotic exposures
4. Maintained bacterial isolates
5. Performed all bulk and digital quantification experiments
6. Contributed all data to figures 1, 2, 3, S1, S2, S3, and table S2, contributed all non-sheared data to figure 4
7. Drew figures 1, 2, 3, 4, S1, S2, S3, and constructed table S2
8. Contributed to writing abstract, introduction, results/discussion, and conclusion sections of manuscript
9. Contributed to writing of supplemental information

Eugenia Khorosheva

1. Major contributor to selecting DNA replication as AST marker, major contributor of knowledge on AST state of the art and effects of antibiotics on replication, contributed to selecting 23S gene as a target of choice, contributed to digital resolution hypothesis, contributed to chromosome segregation hypothesis
2. Selected experimental protocols for maintenance and growth of isolates
3. Established initial AST protocols and experimental workflow from exposure to extraction
4. Selected and optimized protocols for amplification with Enterobacteriaceae specific 23S primers.
5. Contributed to optimizing DNA shearing experiments.
6. Contributed to writing introduction, results/discussion, and conclusion sections of manuscript

Travis S. Schlappi

1. Contributed knowledge on AST statistics, contributed to digital resolution hypothesis
2. Connected FDA guidelines for establishing new antimicrobial susceptibility determination methods to statistical hypothesis testing for both qPCR and dPCR.
3. Performed statistical analysis (p-values and error bars) for all data presented in the manuscript and supplemental information.
4. Performed preliminary digital PCR experiments showing that dPCR can resolve differences in concentration after 15min exposure that qPCR cannot.
5. Contributed to writing of supplemental information.

Matthew S. Curtis

1. Contributed to selecting DNA replication as AST marker, contributed knowledge on AST state of the art and effects of antibiotics on replication, contributed to digital resolution hypothesis, major contributor to chromosome segregation hypothesis
2. Selected and optimized shearing protocols on extracted DNA for the analysis of chromosome structure.
3. Performed shearing experiments to generate data for figure 4.
4. Contributed to statistical analysis
5. Contributed to writing of the introduction, results/discussion, and conclusion of the manuscript.

Romney M. Humphries and Janet A. Hindler contributed microbiological and AST expertise.

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