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

Nathan G. Schoepp⁺, Eugenia M. Khorosheva⁺, Travis S. Schlappi, Matthew S. Curtis, Romney M. Humphries, Janet A. Hindler, and Rustem F. Ismagilov*

Abstract: Rapid antimicrobial susceptibility testing (AST) would decrease misuse and overuse of antibiotics. The “holy grail” of AST is a phenotype-based test that can be performed within a doctor visit. Such a test requires the ability to determine a pathogen’s susceptibility after only a short antibiotic exposure. Herein, digital PCR (dPCR) was employed to test whether measuring DNA replication of the target pathogen through digital single-molecule counting would shorten the required time of antibiotic exposure. Partitioning bacterial chromosomal DNA into many small volumes during dPCR enabled AST results after short exposure times by 1) precise quantification and 2) a measurement of how antibiotics affect the states of macromolecular assembly of bacterial chromosomes. This digital AST (dAST) determined susceptibility of clinical isolates from urinary tract infections (UTIs) after 15 min of exposure for all four antibiotic classes relevant to UTIs. This work lays the foundation to develop a rapid, point-of-care AST and strengthen global antibiotic stewardship.

The increasingly liberal use and misuse of antibiotics (ABX) has led to widespread development of antibiotic resistance.^[1] To address this crisis, we need rapid and reliable tests of a pathogen’s susceptibility to the drugs available (antimicrobial susceptibility test (AST)) that enable healthcare providers to administer correct, life-saving treatments, in turn facilitating antibiotic stewardship^[2] and drastically decreasing hospital costs.^[1a,3] Having a rapid AST that provides results within the period of a doctor visit would improve patient outcomes and reduce the spread of antibiotic resistance.^[4] The development of such a test is currently the focus of significant research efforts^[5] that aim to supplant traditional clinical methods. To reduce the spread of resistance, one urgently

needed AST is for urinary tract infections (UTIs), which are among the most common bacterial infections, yet can progress to pyelonephritis or sepsis.^[6]

Two types of ASTs are currently used in clinical settings: traditional culture-based methods and genotypic methods. Culture-based tests remain the gold standard for determining antibiotic susceptibility because they detect phenotypic susceptibility to a drug. However, these tests require a long period of antibiotic exposure (typically 16–24 h).^[7] We^[8] and others^[5a,b,k,9] have proposed using confinement of single, or a small number of, bacterial cells in small volumes to reduce the duration of antibiotic exposure required to read out the phenotype of the target pathogen. However, these methods typically do not differentiate between the pathogen and potential contamination of the sample with commensal bacteria. Alternative genotypic methods (detecting genes responsible for known mechanisms of resistance) are more rapid than culture-based approaches.^[10] However, these resistance genes constitute only a fraction of all possible mechanisms of resistance,^[11] and new forms of resistance evolve quickly.^[12] Therefore, predicting resistance by analyzing a few known resistance genes is not a general solution.^[13]

To develop more rapid and specific phenotypic tests, hybrid approaches have been proposed that use quantification of nucleic acids to determine the susceptibility or resistance phenotype after a short antibiotic exposure. These tests do not rely on detecting specific resistance genes.^[5g,i] For example, quantification of RNA has allowed determination of susceptibility to ciprofloxacin (cip) and rifampin,^[5] which impair transcription, after exposure times as short as 15 min. However, these methods require longer incubation times when using antibiotics with different mechanisms of action. Although quantitative PCR (qPCR) has been used to quantify DNA after 2–9 h of antibiotic exposure, and thus can detect bacterial growth and determine susceptibility,^[5d,e] an ideal exposure time would be shorter than one cell division.

Herein we tested the hypothesis that digital methods of nucleic acid quantification,^[14] such as digital PCR (dPCR), would enable use of DNA markers to perform a phenotypic AST after short antibiotic exposure. Digital methods partition bacterial chromosomal DNA into thousands of compartments and then use targeted amplification to determine the number of “positive” compartments containing DNA carrying one or more copies of the target gene. This partitioning should enable more precise and robust measurements of concentrations of bacterial DNA, achieving higher statistical power

[*] N. G. Schoepp,^[†] Dr. E. M. Khorosheva,^[†] T. S. Schlappi, M. S. Curtis, Prof. R. F. Ismagilov

Division of Chemistry and Chemical Engineering
California Institute of Technology
1200 E. California Blvd., Pasadena, CA 91125 (USA)
E-mail: rustem.admin@caltech.edu

Prof. R. M. Humphries, J. A. Hindler
Pathology and Laboratory Medicine
University of California, Los Angeles
10888 Le Conte Avenue, Brentwood Annex
Los Angeles, CA 90095 (USA)

[†] These authors contributed equally to this work.

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with fewer replicates relative to qPCR.^[14c,15] Further, we hypothesized that this partitioning would provide unique capabilities for AST when analyzing target genes present in a macromolecular assembly, such as a bacterial chromosome during replication. In contrast to qPCR, dPCR results should reflect the state of the macromolecular assembly, providing a different count for a pair of segregated chromosomes (two positives) versus the chromosomal assembly just prior to segregation (one positive). We tested our hypotheses in the context of four of the main antibiotics used in UTI treatment: ciprofloxacin (cip), nitrofurantoin (nit), sulfamethoxazole/trimethoprim (sxt), and amoxicillin/clavulanic acid (amc).^[6a,7b,16]

We first determined the minimum antibiotic exposure time necessary to differentiate susceptible and resistant clinical UTI isolates using qPCR analysis of DNA after incubation in the presence (“treated”) or absence (“control”) of antibiotics (see the Supporting Information). Cycle thresholds were used to calculate the relative fold change in DNA concentration compared to $t = 0$ min (Figure 1). When treated with cip, DNA replication in susceptible isolates was significantly inhibited, resulting in an increasing difference in fold change between target concentrations in treated and control samples. If the isolate was resistant, DNA replication continued regardless of exposure. To align with US Food and Drug Administration (FDA) requirements for very major errors,^[17] we used a conservative alpha, 0.02 (see the Supporting Information). Under these parameters, susceptibility to cip could be determined after 15 min of exposure. We obtained similar results using isolates precultured in media and in urine (see Figure S1 in the Supporting Information), and chose to conduct all subsequent experiments in media to

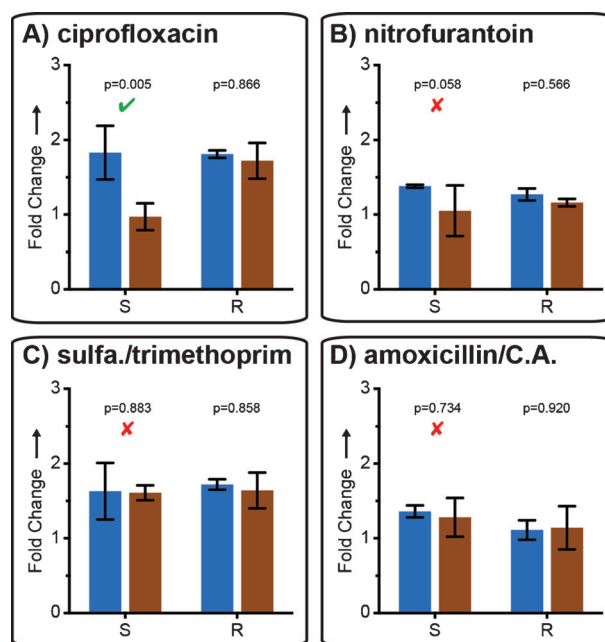


Figure 2. Comparison of susceptible and resistant isolates from UTI samples after a 15 min exposure to each of four antibiotics, analyzed by quantitative PCR. Fold change values represent changes from $t = 0$ min; error bars are 98% C.I. (see the Supporting Information), $N = 3$. Significant (p value ≤ 0.02) and nonsignificant differences detected using the susceptible isolate are marked with a green check mark and red x, respectively.

decrease the work with human samples and to ensure reproducibility.

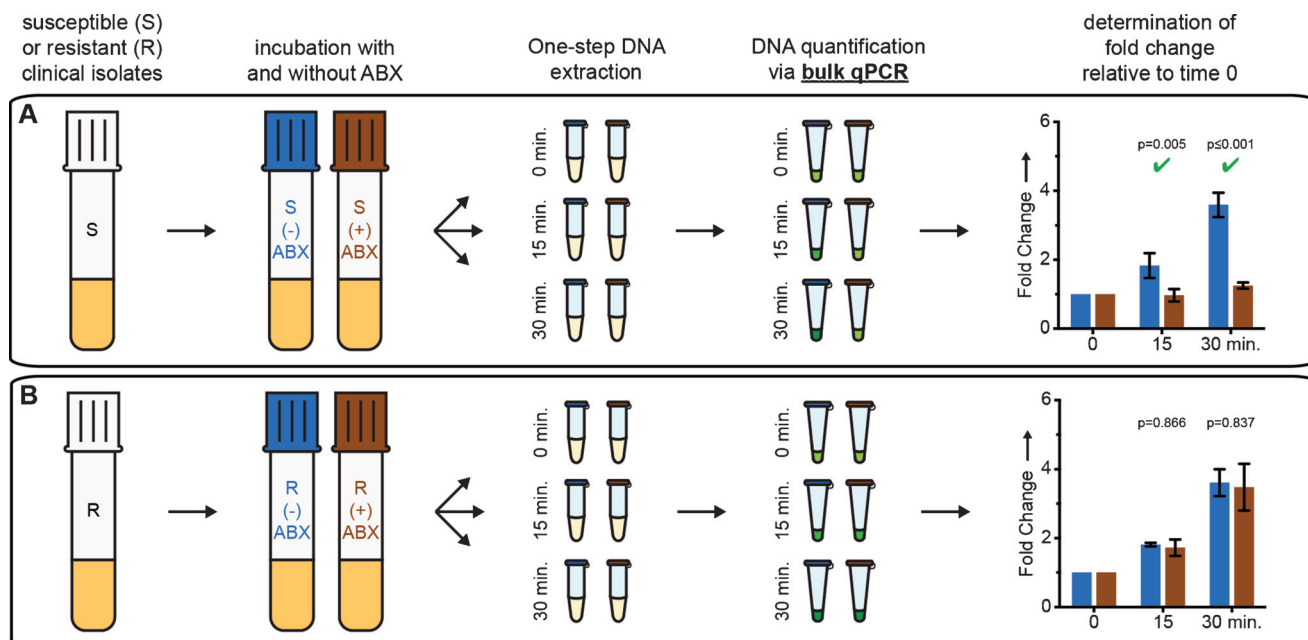


Figure 1. Quantitative PCR (qPCR) time course for exposure of A) susceptible (S) and B) resistant (R) UTI *E. coli* isolates to ciprofloxacin. Fold change values represent changes from $t = 0$ min; error bars represent the upper and lower bounds of the 98% confidence interval (C.I.; see the Supporting Information). $N = 3$ where N is the number of technical qPCR replicates. Significant differences (p value ≤ 0.02) are marked with a green check mark.

These results provide the first evidence of detection of phenotypic susceptibility based on DNA quantification after only 15 min of antibiotic exposure. The rapid effect of cip on DNA replication is logical because the drug's mechanism of action is to inhibit DNA-gyrase and topoisomerase IV, producing double stranded breaks in DNA and directly inhibiting DNA replication.^[18] To test generality, we evaluated AST with three other antibiotics: nit,^[19] sxt,^[20] and amc (amc and other β -lactam antibiotics are not known to specifically affect DNA replication; see the Supporting Information). Using qPCR, 15 min of exposure to these three antibiotics was not sufficient to detect a significant difference in DNA replication in susceptible isolates (Figure 2B–D), whereas statistically significant differences were detectable after cip treatment (Figure 2A).

A focus of this work was to evaluate the differences in minimum antibiotic exposure time necessary to determine susceptibility when quantification of changes in DNA is performed with qPCR versus dPCR. Thus, we next tested AST with digital quantification by quantifying the same DNA samples from the qPCR analysis using dPCR (Figure 3). For

cip, we detected a more statistically significant difference (smaller p value than in qPCR) between target concentrations in treated and control susceptible isolates (Figure 3A), whereas target concentrations did not differ between treated and control resistant isolates (Figure 3B). A significant difference was also detected after 15 min exposures to nit (Figure 3C) or sxt (Figure 3C).

Interestingly, neither qPCR nor dPCR detected susceptibility after exposure to amc when samples were denatured and treated with protease during extraction (Figure S2). This confirmed that genome replication proceeded (resulting in an increase in the total number of amplifiable targets) during incubation with amc regardless of phenotype. We therefore tested the hypothesis that dPCR would be sensitive not only to the total gene copy number, but also to the state of macromolecular assembly of chromosomal DNA. If exposure to amc causes changes in chromosome segregation, even without affecting replication, dPCR should still be able to differentiate susceptible and resistant phenotypes. To preserve chromosome structure and macromolecular complexes, we performed DNA extraction under nondenaturing condi-

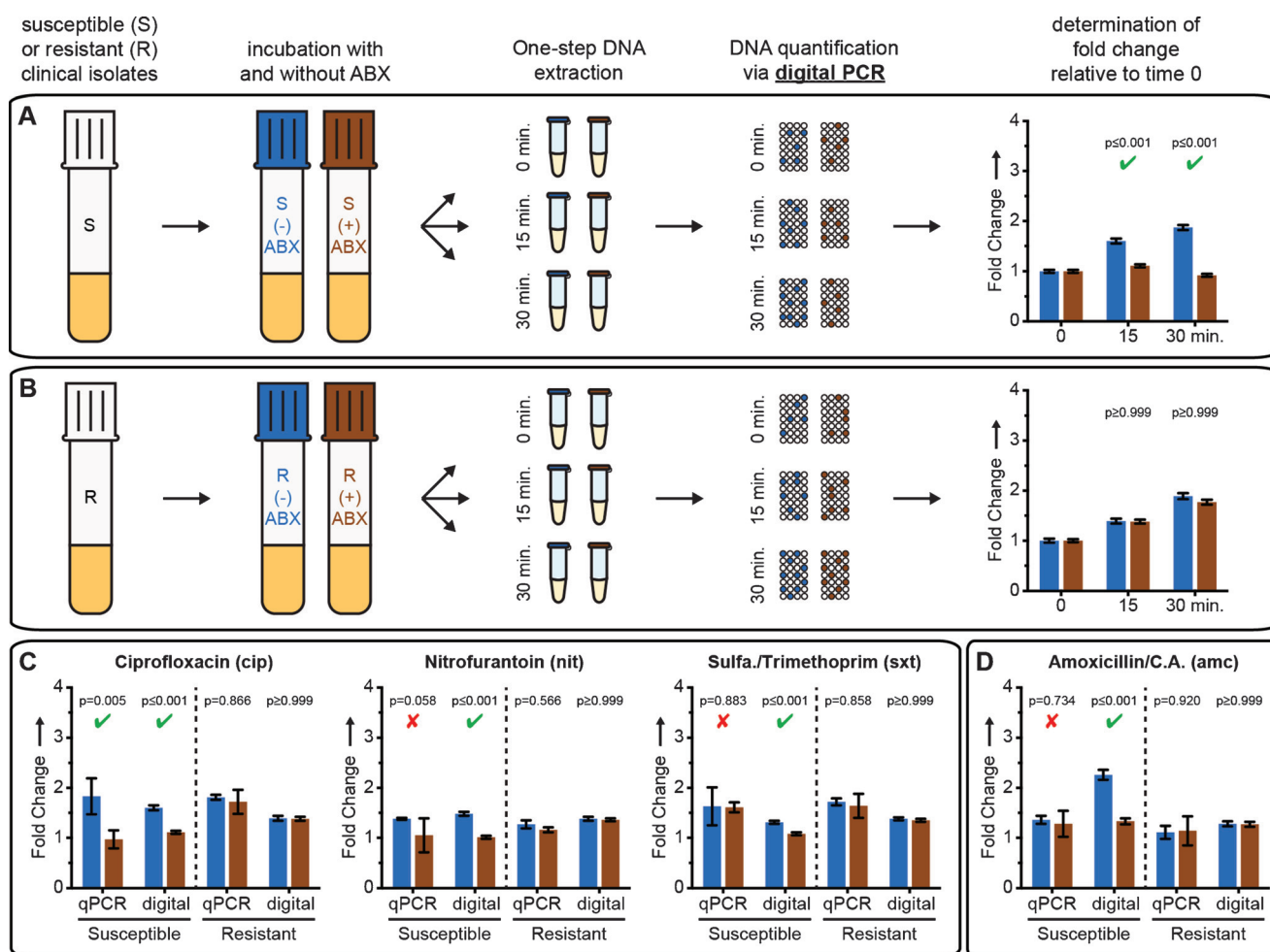


Figure 3. AST results using dPCR. A, B) Time course results for exposure of A) susceptible and B) resistant UTI *E. coli* isolates to ciprofloxacin. C, D) Fold changes after treatment with all four antibiotics tested. Significant (p value ≤ 0.02) and nonsignificant p values for susceptible isolates are denoted with a green check mark and red x, respectively. Samples treated with amoxicillin/clavulanic acid (D) were extracted using a nondenaturing procedure. Concentrations are calculated using Poisson statistics. Fold change values represent change from $t = 0$ min; all error bars are 98% C.I. (see the Supporting Information); $N = 3$ for qPCR, $N = 2$ for dPCR.

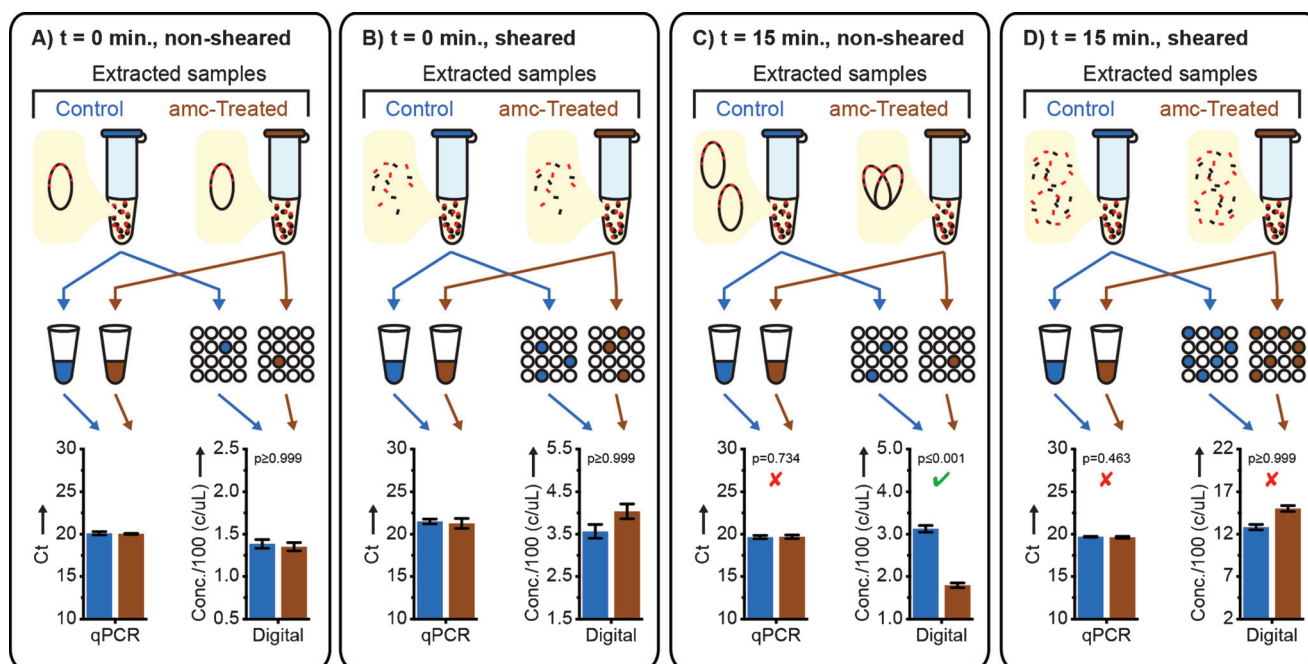


Figure 4. A mechanistic investigation of AST by dPCR after β -lactam exposure and nondenaturing DNA extraction using shearing to disrupt macromolecular assemblies; error bars for qPCR are 2.8 S.D., $N=3$; error bars are 98% C.I. for dPCR, $N=2$ (see the Supporting Information). Significant (p value ≤ 0.02) and nonsignificant p values for susceptible isolates quantified using dPCR are denoted with a green check mark and red x, respectively.

tions without protease treatment. Under these conditions, dPCR provided the susceptibility phenotype after 15 min of exposure to amc (Figure 3D).

To test whether dPCR differentiated susceptible and resistant isolates by quantifying macromolecular assemblies, we designed control experiments in which all assemblies were sheared into about 1000 bp DNA fragments, much smaller than the average distance between 23S genes within the genome (see the Supporting Information). As expected, shearing caused an increase in measured target concentration when quantified using dPCR, but not using qPCR (Figure 4A,B). In samples that were not sheared, dPCR detected the susceptible phenotype after 15 min of amc exposure (Figure 4C). Shearing these samples to disrupt macromolecular assemblies eliminated the ability to detect susceptibility (Figure 4D); qPCR measurements confirmed this was not due to loss of DNA. This suggests that in amc-susceptible isolates short exposure to amc does not result in a change of the total number of target gene copies, but does change the macromolecular assembly of these copies.

Our results suggest a previously unknown effect of brief β -lactam antibiotic exposure: a delay in chromosome segregation. Using dPCR (but not qPCR) this effect can be quantified by counting the number of macromolecular DNA assemblies containing 23S target genes, and used for AST. The high resolution of digital quantification enables measuring small (less than two-fold) changes in chromosome replication and segregation after an antibiotic exposure shorter than the average time of cell division. The dAST approach developed here adds chromosome segregation to the list of the phenotypic markers suitable for rapid antibiotic susceptibility

detection. The ability to partition macromolecular assemblies allows dAST to be used even when genome replication proceeds on the timescale of antibiotic exposure, while the high precision of digital quantification allows accurate determination of susceptibility after shorter exposure times than would be required using less-precise methods such as qPCR.

These dAST results warrant a follow-up study with a wide range of Gram-positive and Gram-negative bacterial isolates from urine, blood, and other sample types, and then a clinical study comparing dAST directly from patient samples to the gold standard culture-based methods. Elucidating the effects of using variable clinical samples with a range of background matrices is a critical next step in the development of a rapid, sample-to-answer AST. Ultimately, a sample-to-answer AST at the point of care must be robust and rapid and should require minimal sample handling and instrumentation. Ideally, such a workflow will integrate sample handling, antibiotic exposure, and quantification into a single device. We anticipate that digital isothermal amplification chemistries will replace dPCR in dAST.^[15a,21] When integrated with sample preparation^[22] and combined with simple readouts,^[23] we envision that digital quantification will establish a new paradigm in rapid point of care AST.

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