

Clinical pilot of bacterial transcriptional profiling as a combined genotypic and phenotypic antimicrobial susceptibility test

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ABSTRACT Antimicrobial resistance is a growing health threat, but standard methods for determining antibiotic susceptibility are slow and can delay optimal treatment, which is especially consequential in severe infections such as bacteremia. Novel approaches for rapid susceptibility profiling have emerged that characterize either bacterial response to antibiotics (phenotype) or detect specific resistance genes (genotype). Genotypic and Phenotypic AST through RNA detection (GoPhAST-R) is a novel assay, performed directly on positive blood cultures, that integrates rapid transcriptional response profiling with the detection of key resistance gene transcripts, thereby providing simultaneous data on both phenotype and genotype. Here, we performed the first clinical pilot of GoPhAST-R on 42 positive blood cultures: 26 growing *Escherichia coli*, 15 growing *Klebsiella pneumoniae*, and 1 with both. An aliquot of each positive blood culture was exposed to nine different antibiotics, lysed, and underwent rapid transcriptional profiling on the NanoString platform; results were analyzed using an in-house susceptibility classification algorithm. GoPhAST-R achieved 95% overall agreement with standard antimicrobial susceptibility testing methods, with the highest agreement for beta-lactams (98%) and the lowest for fluoroquinolones (88%). Epidemic resistance genes including the extended spectrum beta-lactamase *bla*_{CTX-M-15} and the carbapenemase *bla*_{KPC} were also detected within the population. This study demonstrates the clinical feasibility of using transcriptional response profiling for rapid resistance determination, although further validation with larger and more diverse bacterial populations will be essential in future work. GoPhAST-R represents a promising new approach for rapid and comprehensive antibiotic susceptibility testing in clinical settings.

IMPORTANCE Exposure to antibiotics causes differential transcriptional signatures in susceptible vs resistant bacteria. These differences can be leveraged to rapidly predict resistance profiles of *Escherichia coli* and *Klebsiella pneumoniae* in clinically positive blood cultures.

KEYWORDS antimicrobial resistance, transcriptional regulation, diagnostics, bacteremia

Infections due to antimicrobial-resistant (AMR) bacteria are a major cause of global mortality, accounting for 1.27 million deaths in 2019 alone (1), and result in increased length of hospital stays (2), higher healthcare costs (3), and higher mortality (2). Delays in appropriate antimicrobial therapy, often due to bacterial resistance to empiric antibiotic selection, directly correlate with increased in-hospital mortality (4, 5). This can push clinicians toward excessively broad-spectrum therapies that may result in worse patient outcomes (6) and the development of resistance (7), which underscores the broad potential benefits of rapid antimicrobial susceptibility testing (AST) (8–10).

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The gold standard for AST involves growing isolates in the presence of an antibiotic to determine the lowest concentration that inhibits bacterial growth (11). While reliable, growth-based AST can be time-consuming, taking up to 72 h from the time of sample collection to the final susceptibility profile. A growing number of alternative, rapid AST methods address this challenge in two broad ways: genotypic and phenotypic assays. Genotypic approaches directly identify specific genes or mutations known to confer resistance. This approach relies on detecting a limited subset of genes, however, and so is unable to detect novel or complex resistance mechanisms, especially in Gram-negative pathogens (12). Phenotypic assays, conversely, assess bacterial response to antibiotics based on different cellular properties, such as growth (13), metabolic activity (14), or bacterial motility (15, 16). Although generally applicable across resistance mechanisms, this approach does not provide information about bacterial genotypes, potentially omitting key data that could inform antibiotic selection (17) and epidemiologic inquiries (18, 19).

In recent years, our group developed a method that combines both genotypic and phenotypic information into a single, rapid AST assay termed Genotypic and Phenotypic AST through RNA detection (GoPhAST-R) (20). GoPhAST-R uses NanoString RNA hybridization chemistry (21) to quantify multiple bacterial transcripts from crude lysate samples and infer resistance patterns. To do so, it leverages the marked differences in gene expression profiles of susceptible isolates compared to species-matched resistant isolates when exposed to antibiotics: resistant isolates are relatively unperturbed, whereas susceptible isolates, physiologically distressed and dying or growth-arrested, demonstrate large transcriptional changes in response to the antibiotic. We previously showed that a small subset of genes undergo large, predictable expression changes upon exposure to a class of antibiotics (22), such that the change in expression of these marker genes reflects phenotypic antibiotic susceptibility, independent of resistance mechanism. Using this principle, we designed and validated GoPhAST-R probesets to classify the susceptibility of *Escherichia coli* and *Klebsiella pneumoniae* to aminoglycosides, fluoroquinolones, and beta-lactams (22). Additionally, transcripts from high-risk resistance genes, such as extended-spectrum beta-lactamases (ESBLs) and carbapenemases, were simultaneously interrogated to identify high-risk genotypes of epidemiologic relevance. In prior work done on blood cultures spiked with laboratory strains with pre-specified resistance patterns, GoPhAST-R achieved 94%–99% accuracy, required minimal technical expertise and hands-on time, and returned results as soon as <4 h after a positive blood culture (20).

In this effort, we sought to implement GoPhAST-R in a clinical setting for the first time. Our prior work was done on laboratory cultures or simulated blood cultures (20, 22) as well as a small cohort of archived blood cultures tested only for ciprofloxacin susceptibility, with some delay before processing (20). In this work, we processed 42 blood cultures that grew *E. coli* or *K. pneumoniae* from the clinical microbiology laboratory at Massachusetts General Hospital (MGH) in real-time. Transcriptional responses to antibiotic exposure clustered by susceptibility classification (i.e., susceptible strains and resistant strains exhibited distinct transcriptional responses), with 95% overall categorical agreement to standard clinical testing. These results are the first demonstration in a clinical setting of an assay based on the new paradigm of using bacterial transcriptional responses to predict antibiotic susceptibility.

MATERIALS AND METHODS

Sample collection and standard culture and antimicrobial susceptibility testing methods

From 1 June 2021 through 28 February 2022, sequential blood cultures from unique patients that signaled positive between 7:30 a.m. and 2:00 p.m. Mondays through Fridays when research staff were available, and showed Gram-negative rods on Gram stain, were collected for processing. In all cases, only the first positive culture from

a patient was included. A total of 66 unique positive blood cultures were collected. Samples were processed in real-time based on Gram stain before any identification or AST was available, and all processed samples that were subsequently identified as *E. coli* or *K. pneumoniae* were included in the study ($n = 42$). At the time a blood culture bottle signaled positive on the BACTEC FX system (Becton Dickinson, Sparks, MD), a 1 mL aliquot was taken for processing as below. The remainder was carried forward for routine clinical processing, including subculture to solid media followed by colony identification via matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (VITEK MS, version 3.2 *in vitro* diagnostic Knowledge Base, bioMérieux, Durham, NC) and AST using the VITEK 2 AST-GN81 Gram-Negative Susceptibility Card (bioMérieux), clinically validated for use with Clinical and Laboratory Standards Institute (CLSI) breakpoints published in M100-Ed31 (23). Aztreonam and cefazolin were tested by the CLSI disk diffusion method (24) due to limitations of the VITEK 2 AST card; aztreonam was not included on the card, and the lowest concentration of cefazolin did not permit susceptible and intermediate minimum inhibitory concentrations (MICs) to be distinguished. Cefazolin susceptibility was further tested by broth microdilution (25) in the research laboratory. Laboratory researchers remained blinded to AST profiles until after all transcriptional profiling for GoPhAST-R was complete.

Antibiotic treatment

As in prior implementations of GoPhAST-R (20), a 1 mL aliquot of each positive blood culture was first spun down at 100 g for 10 min to pellet red blood cells. The supernatant was then spun at $16,000 \times g$ for 3 min to pellet bacteria. The supernatant was removed, and the bacterial pellet was resuspended in cation-adjusted Mueller-Hinton broth (CAMHB, ThermoFisher Scientific, Lenexa, KS) to a final volume of 500 μ L. Separate 45 μ L aliquots were added to pre-diluted 5 μ L aliquots of cefazolin (CFZ), ceftriaxone (CRO), aztreonam (ATM), piperacillin-tazobactam (TZP), cefepime (FEP), ertapenem (ETP), meropenem (MEM), ciprofloxacin (CIP), levofloxacin (LVX), or gentamicin (GEN) to expose at CLSI breakpoint concentrations (23) (Table S1). Bacteria were exposed at 37°C to each non-beta-lactam antibiotic for 60 min, or 120 min for beta-lactams given the slower response for this class (22). In prior work, we found that the beta-lactam inoculum effect (26) required exposure to standard CLSI-recommended inocula in order for the transcriptional response to reflect susceptibility (22). Thus, beta-lactam treated samples and one untreated control were diluted 1:100 (targeting 5×10^5 cfu/mL) prior to incubation and plated at the time of antibiotic exposure to enumerate colony-forming units to confirm. To assess baseline transcriptional signatures, two aliquots of each sample were each added to 5 μ L of CAMHB without antibiotics, and these untreated cultures were removed at either 60 min (for comparison with aminoglycosides and fluoroquinolones) or 120 min (for comparison with beta-lactams). Lysis buffer was added, and the samples were flash-frozen and stored at -80°C for subsequent batch processing.

Lysis and hybridization

Every sample identified as *E. coli* or *K. pneumoniae* on routine clinical testing was thawed and lysed on the MP FastPrep bead beater (22). Crude lysates were carried forward on the NanoString platform per the manufacturer's protocols. RNA probe sets were designed as per prior work (20). Samples were hybridized for 1 h as per our prior protocol (20, 22), except for those treated with beta-lactams, which were hybridized for 16 h to ensure sufficient signal from the lower initial inoculum.

Quantification of transcriptional response and genotyping

Data were partitioned by each antibiotic and species combination. Raw transcript counts were first normalized to NanoString spike-in controls per the manufacturer's protocol, and then to housekeeping genes selected to have consistent expression to control for bacterial cellular density, as previously described (20). To assess transcriptional response,

we next calculated a log₂-fold change in normalized transcript counts between treated and untreated samples. To characterize resistance genotypes, probes were measured that targeted conserved regions of the carbapenemase genes *bla*_{KPC}, *bla*_{NDM}, *bla*_{VIM}, *bla*_{IMP}, and *bla*_{OXA-48} and the extended-spectrum beta-lactamase gene *bla*_{CTX-M-15} (20). If any of these probes were detected above the background, the gene was considered present.

PCR amplification of *bla*_{CTX-M-15}

To independently assess for the presence of the *bla*_{CTX-M-15} gene, we tested five patient samples identified as having the gene by GoPhAST-R (see Results), and two isolates (one each of *E. coli* and *K. pneumoniae*) that were not found to have the gene, by PCR. Individual colonies of each strain were picked from LB agar plates, diluted in 15 μ L of water, heat lysed at 95°C for 15 min, and run on a PCR reaction using *bla*_{CTX-M-15} primers (Table S2), designed to target a conserved region unique to *bla*_{CTX-M-15} (20).

Model construction and resistance prediction

To represent the entire transcriptional response profiles in a single quantitative metric, we used a method of one-dimensional projection called squared projected distance (SPD) (27) to summarize each sample's responsiveness in comparison to reference data from samples with known MICs. In brief, centroids representing the average transcriptional response of known susceptible and resistant isolates are computed for each antibiotic and species combination from highly susceptible and resistant strains from a reference set (22). Distance from the susceptible centroid is calculated for each new transcriptional profile and scaled by the distance between the sensitive and resistant centroids; thus, an SPD of 0 represents a response similar to that of the control set of susceptible strains, whereas an SPD of 1 represents a resistant-like response.

We used support vector machine (SVM) modeling for each species and drug class as a tool to report the degree to which our susceptible and resistant isolates separate from each other by their SPD values. SVM is an unbiased, algorithmic method of identifying natural separation in data that minimizes overfitting by maximizing the distance between boundaries and each class of data (28). We implemented SVM models using the *e1071* package (29) on a data set that included both the current samples as well as a larger collection with a higher proportion of resistant isolates from prior work (22), using clinical susceptibility classifications as "ground truth." We only report classifications of samples from the current study; isolates from prior work were used only to provide additional resistant isolates for SVM modeling, to avoid overtraining on the relatively few resistant isolates collected in this study. To minimize the most clinically important discrepant classifications, we set the model to penalize very major discrepancies (VMDs) (R strain misclassified as S) twice as much as major discrepancies (S strain misclassified as R); we made this decision from the outset, prior to any SVM analysis. For the genotypic portion of the assay, we incorporated whether the resistance gene of a given class was detected, and if so, we considered the isolate to be resistant to the relevant antibiotic regardless of the SVM prediction (Fig. S1). While the assay was run for cefazolin, the results were excluded from the final SVM model because (i) the CLSI breakpoint concentration falls within the MIC distribution of wild-type strains (30), which in practice leads to classification challenges, exemplified in our collection by frequent discordance between susceptible and intermediate classifications for samples tested by disk diffusion and broth microdilution methods (Fig. S2); and (ii) cefazolin is not a recommended first-line therapy for Gram-negative bacteremia, which was the focus of this study.

Retesting discrepant fluoroquinolone results *in vitro*

A subset of isolates that yielded discrepant classifications (see Results) were regrown in the research laboratory to the early log phase in MHB, exposed to ciprofloxacin or

levofloxacin at the same dose (Table S1) and for the same duration used in clinical pilot testing, and processed for NanoString exactly as in our prior *in vitro* GoPhAST-R work (22). The resulting SPDs were compared to those obtained from clinical pilot assays.

RESULTS

Sample collection and standard AST determination

A total of 66 positive blood cultures with Gram-negative rods on Gram stain were exposed in real-time in the clinical microbiology laboratory to the selected antimicrobials at CLSI susceptibility breakpoint concentrations (Table S1; see Materials and Methods). Of these 66 cultures, 26 were subsequently identified as *E. coli* and 15 as *K. pneumoniae*, for which we had previously designed probes for transcriptional susceptibility testing to beta-lactams, fluoroquinolones, and aminoglycosides (22). In one case (sample 17), a blood culture was identified as having both species and was carried forward. The other 24 samples included species for which we do not have transcriptional probes and thus were not processed further. In three cases, antibiotics were given before blood cultures were drawn, but the strains were resistant to the selected antibiotics. Routine clinical testing of these isolates found relatively low levels of resistance, with the highest rate of non-susceptibility found for ciprofloxacin at 26% (7/27) in *E. coli* and 19% (3/16) in *K. pneumoniae*, and the lowest rate seen for the carbapenems at 0% (0/27) and 6.3% (1/16), respectively (Table S3). While intermediate MICs were rare in the collection, we found four instances of intermediate MICs to levofloxacin in *E. coli* and two in *K. pneumoniae*.

Transcriptional response to drug treatment and resistance genotyping

To assess susceptibility from a transcriptional profile, we measured levels of 10–13 transcripts per drug class and calculated change in normalized expression between treated and untreated states (20) (Fig. 1A). Total hands-on time was estimated at 55 min, including 25 min for setting up antibiotic exposures in the clinical microbiology laboratory, and 30 min for lysis and processing for transcript detection in the research laboratory. While strain variation was present across drug treatments, *E. coli* and *K. pneumoniae* samples predominantly demonstrated one of two transcriptional profiles: susceptible isolates exhibited large perturbations in transcriptional responses upon antibiotic exposure, whereas resistant isolates demonstrated very little to no perturbation. When grouped by drug and species, transcripts from susceptible isolates were perturbed by an absolute log₂-fold value of 1.0 to 4.0 averaged across all responsive genes, with most drug-species pairs falling between 1.5 and 3.0; while for resistant samples these values ranged from 0.2 to 1.8, with all but one falling below 0.9 (a carbapenem-resistant *K. pneumoniae*, see below) (Fig. 1B). While uncommon, samples with intermediate MICs showed a wider average perturbation range of between 0.2 and 3.8 absolute log₂-fold change.

After condensing probe responses into SPD (27) (Fig. 2A and B; Fig. S3), a metric we previously devised to represent transcriptional response to antibiotics (see Materials and Methods), samples categorized as susceptible by clinical AST had a mean SPD value of 0.027 [median 0.0, interquartile range (IQR) ± 0.09], while resistant samples had a mean SPD of 0.975 (median 1.0, IQR ± 0.31). Samples with intermediate susceptibilities had a mean of 0.23 (median 0.1, IQR ± 0.184) (Fig. 2C).

In total, our genotypic probes detected *bla*_{CTX-M-15} in two *E. coli* isolates and three *K. pneumoniae* isolates, and a single carbapenemase gene, a *bla*_{KPC} found in sample 48, a *K. pneumoniae* that displayed a transcriptional response to both ertapenem and meropenem despite harboring this resistance gene (Fig. S4). Sample 48 underwent mCIM testing as part of the standard clinical workflow, which was positive, in keeping with our genotypic assay. In a separate study, we performed whole-genome sequencing on this isolate, which confirmed that it contained *bla*_{KPC3} (4). To independently assess GoPhAST-R's *bla*_{CTX-M-15} detection, we performed PCR on the five isolates from which

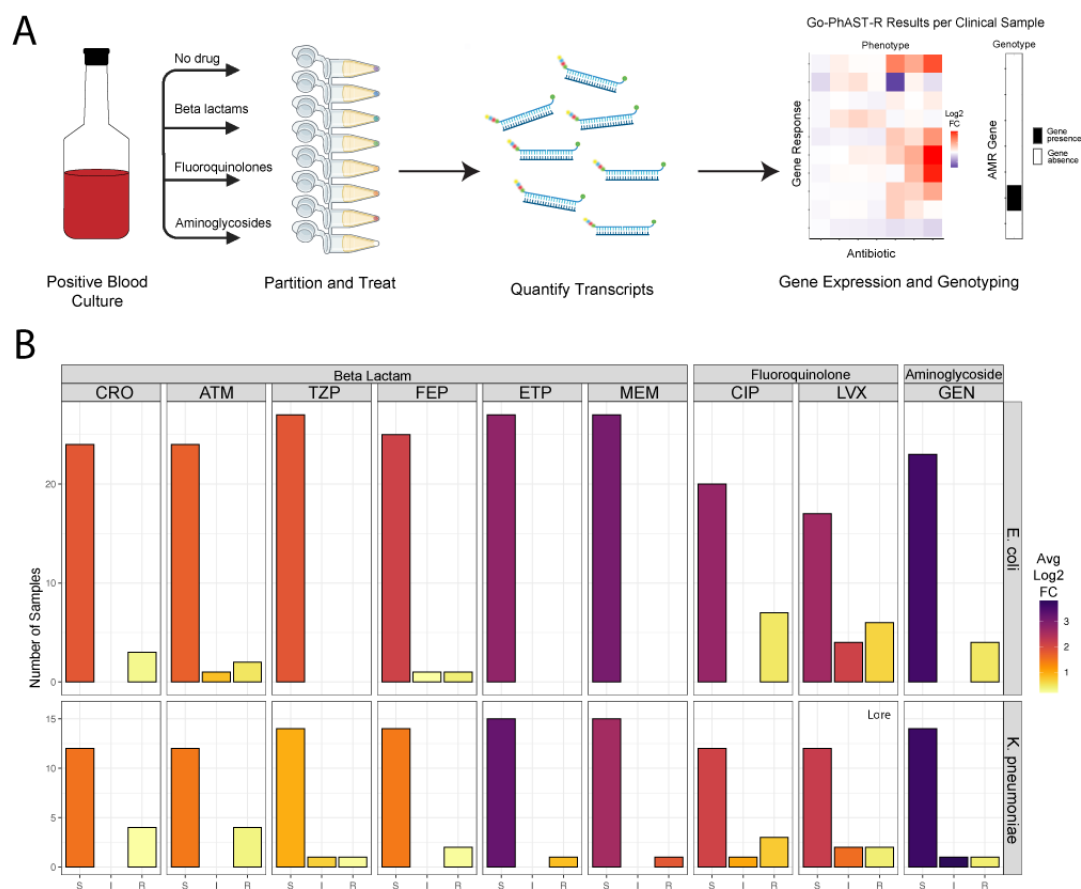


FIG 1 Assay workflow and summary of transcriptional profiles in the study population. Transcriptional response to antibiotic exposure differs by susceptibility classification. (A) Schematic workflow for an individual sample. (B) Clinical isolates show predictable transcriptional response to antibiotic treatment based on susceptibility profile. The absolute value of log₂-fold change of all transcript counts of genes targeted by the probeset upon antibiotic exposure averaged across isolates, subset by antibiotic, and grouped according to clinical resistance profile (susceptible, intermediate, and resistant) and species (*E. coli* and *K. pneumoniae*). Antibiotics are abbreviated as per Table 1 and grouped by class. The color of each bar represents the average log₂-fold change in transcript counts, while the height of each bar corresponds to the number of samples with that susceptibility profile for each antibiotic.

we detected *bla*_{CTX-M-15}, as well as two isolates from which we did not; in all cases, PCR validated the GoPhAST-R results (Fig. S5).

Susceptibility predictions

We used an SVM model to find the natural separation in the SPD values that best delineated susceptible and non-susceptible isolates (per clinical AST) and incorporated this into an algorithm for resistance classification (see Materials and Methods and Fig. S1). We had an overall categorical agreement of 95% (368/387 pairwise drug comparisons) with standard susceptibility profiling (Fig. 3). In total, there was a 5% VMD rate (2/42 resistant isolates), a 2% major discrepancy (MD) rate (7/334 susceptible), and a 3% minor discrepancy rate (Table 1). The best-performing grouping was the beta-lactam class in *E. coli*, with 99% overall categorical agreement and zero VMDs. The worst performing combination was in the fluoroquinolone class, with categorical agreement rates of 89% for *E. coli* and 88% for *K. pneumoniae* (Table 1), and VMDs of 8% and 20%, respectively, representing one VMD in each species, with 12/13 correct resistance predictions for *E. coli* and 4/5 correct predictions for *K. pneumoniae*. Both misclassifications were in ciprofloxacin for isolates with VITEK 2 MICs at the resistant clinical breakpoint (Fig. S3).

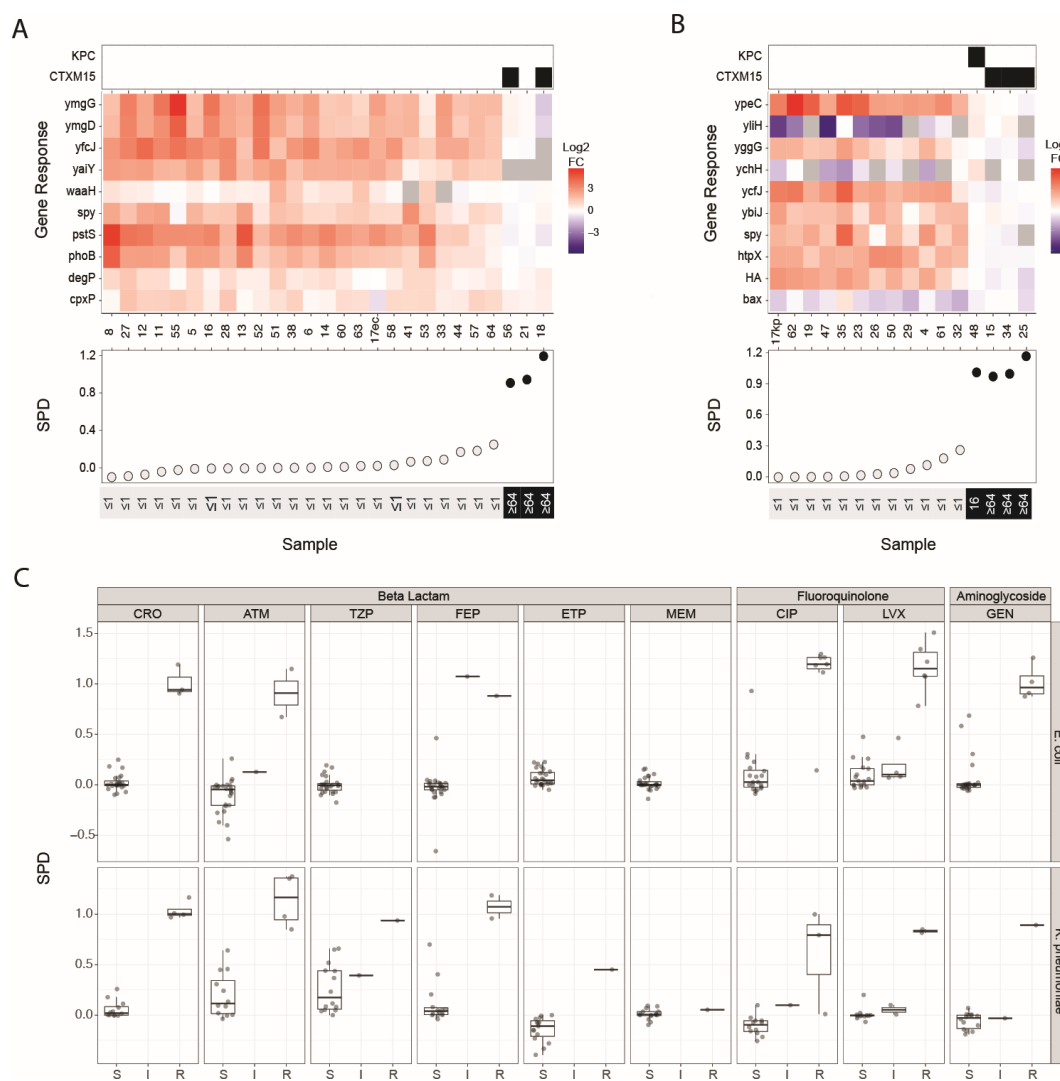


FIG 2 SPD calculation from transcriptional response and distribution. The transcriptional profile is distilled into a measure of treatment response with SPD, a single-value summary of the transcriptional response across the genes of interest (see Materials and Methods). (A and B) Heatmaps of the log₂-fold change in the transcriptional response of each (A) *E. coli* or (B) *K. pneumoniae* isolate after exposure to ceftriaxone. Each row corresponds to a different gene of interest, while each column is a different blood culture sample. KPC and CTX-M-15 genotypes are shown above the heatmap, while sample numbers, SPD values, and MICs (μg/mL) are shown below. (C) Box-and-whisker plot of SPD values for each species and antibiotic combination, grouped by clinical susceptibility profile. Antibiotic abbreviations are listed in Table 1.

Unexpectedly, we obtained one mixed culture of *E. coli* and *K. pneumoniae* during our collection (sample 17). Since each NanoString probe is species-specific in its reactivity pattern, we reasoned that we might see species-specific transcriptional responses for each species without subculture, so we carried the sample through gene expression profiling and analysis, using both *E. coli* and *K. pneumoniae* probesets for this sample (Fig. S4, samples 17ec and 17kp). Eighteen of 20 SPD-based predictions for these two strains were in categorical agreement with clinical AST testing, with discrepancies only in fluoroquinolone susceptibility of the *E. coli* strain (one major discrepancy and one minor discrepancy for ciprofloxacin and levofloxacin, respectively; Table S4). When this isolate was regrown and tested in laboratory monoculture, it demonstrated the expected susceptible transcription profile, with its SPD dropping from 0.93 to 0.15 (Fig. S6). The *K. pneumoniae* in the mixed culture, by contrast, had a perfect agreement with clinical AST results performed on the subcultured isolate (Table S4). We retested 14 other isolates in laboratory culture with GoPhAST-R against both ciprofloxacin and levofloxacin, 6 of

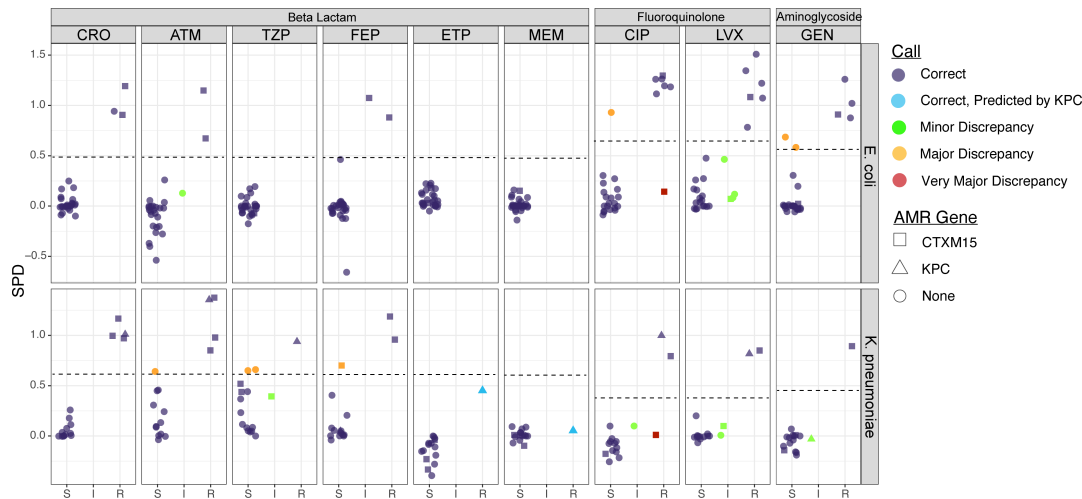


FIG 3 Predictions of drug susceptibility by SVM modeling and genotype. SVM thresholds for each drug class and bacterial species are shown by the dotted line. SVM is a model to find the optimal separation between two classes of data (see Materials and Methods). Each point corresponds to an SPD value (y-axis) and is shaped according to gene content and colored according to whether the SVM algorithm made the correct resistance assignment. Samples are grouped according to clinical susceptibility classification (x-axis).

which had resulted in minor discrepancies on initial testing, and none besides the *E. coli* in the polymicrobial sample changed classification (Fig. S6).

DISCUSSION

Here, we present the first pilot implementation of a transcription-based AST assay performed directly on positive blood cultures from clinical samples. This novel approach utilizes a single platform to concurrently assess bacterial phenotypic response to antimicrobials and identify high-risk AMR resistance genes in an integrated assay, culminating in a susceptibility prediction. The assay demonstrated strong overall concordance with routine AST methods, with some variation observed across antibiotic classes. Notably, beta-lactam antibiotics achieved 98% categorical agreement with standard clinical testing for six commonly used beta-lactams across both species and had no VMDs. Gentamicin also exhibited no VMDs in either species. However, two *E. coli* isolates classified as susceptible by standard testing were misclassified as resistant by the assay (major discrepancies), and one intermediate *K. pneumoniae* isolate was misclassified as resistant (minor discrepancy). Fluoroquinolones displayed the least favorable performance, with VMD rates of 8% (1/13) and 20% (1/5) in *E. coli* and *K. pneumoniae*, respectively, which represented the only VMDs across our entire sample set.

TABLE 1 Predictions of the SVM and KPC detection are tabulated by species and drug class, as well as cumulatively^a

	Number tested	Susc.	Int'd.	Res.	VMD %	MD %	mD %	Categorical agreement %
<i>Escherichia coli</i>								
Beta lactam	162	154	2	6	0	0	0.6	99
Fluoroquinolone	54	37	4	13	7.7	2.7	7.4	89
Aminoglycoside	27	23	0	4	0	8.7	0	93
<i>Klebsiella pneumoniae</i>								
Beta lactam	96	82	1	13	0	4.9	1	95
Fluoroquinolone	32	24	3	5	20	0	9.4	88
Aminoglycoside	16	14	1	1	0	0	6.3	94
Total	387	334	11	42	4.8	2.1	2.6	95

^aSusc., susceptible; Int'd., intermediate; Res., resistant; VMD, very major discrepancy; MD, major discrepancy; mD, minor discrepancy.

We examined each case in which the transcriptional response differed from expectation based on routine clinical susceptibility classification. In total, we observed four instances of resistant samples transcriptionally responding to treatment, which generally occurred in samples with MICs near the breakpoints (Fig. S3). However, in two of these instances, the correct susceptibility prediction was made through genotypic detection. In these strain and antibiotic pairings, the *K. pneumoniae* isolates expressing *bla*_{KPC} (#48) showed considerable perturbations in the presence of both meropenem and ertapenem. It has been noted previously that *bla*_{KPC}-producing strains may display low MICs to carbapenems (31, 32), especially at low inocula (26). Although currently available data do not allow definitive conclusions about the effectiveness of treating infections caused by carbapenemase-carrying strains with carbapenem MICs in the susceptible range (23), many experts would avoid carbapenems in this scenario, particularly in the setting of a serious infection such as bacteremia (12, 17). As such, the ability of GoPhAST-R to detect carbapenemase resistance genes in phenotypically susceptible strains is an important advantage. In a different case, one blood culture (#17) was polymicrobial, growing both *E. coli* and *K. pneumoniae*. While the *K. pneumoniae* behaved as expected, the *E. coli* was susceptible to ciprofloxacin by MIC testing but did not demonstrate a strong transcriptional response, leading to a miscall as a resistant isolate by our model. The transcriptional response of this sample, however, looks qualitatively different than the “true” resistant profiles (Fig. S3, ciprofloxacin panel, sample 17ec). On examination of the raw data from this sample, sample 17ec had low total transcript counts, likely introducing noise into the transcriptional signature, perhaps because *E. coli* was present at lower abundance than usual when the bottle signaled positive. Consistent with this hypothesis, isolate 17ec tested ciprofloxacin susceptible by GoPhAST-R when regrown in laboratory culture, whereas 14 other samples did not switch classification on retesting against either fluoroquinolone. As we have not previously included polymicrobial samples in the assay, further dedicated exploration will be needed to determine the optimal method for handling such samples. However, the ability in principle to simultaneously profile responses to multiple strains is a potentially appealing feature of this assay, as we observed 90% categorical agreement with standard AST testing across 20 phenotypic tests for these two isolates.

The majority of the remaining errors were minor discrepancies in samples with MICs near CLSI breakpoints (Fig. S3), which are inherently challenging classifications even in gold-standard AST methods (33, 34). To optimize the performance of transcriptional susceptibility testing on samples with near-breakpoint MICs, future cohorts should include a higher number of these isolates, enabling training and independent validation of a more sophisticated random forest model capable of parsing modest or partial gene responses (20).

Our assay also captured genotypic information regarding the presence of key beta-lactamases, adding several useful features beyond phenotypic testing alone. First, as outbreaks of carbapenem-resistant bacteria may be caused by the spread of epidemic beta-lactamases (18), knowledge about their presence has implications for infection control practices (35). Second, a growing number of clinical decisions are determined by bacterial genotype. Current IDSA guidelines, for example, recommend carbapenems as first-line therapy for complicated infections caused by ESBL-producing organisms (17). Finally, the emergence of novel beta-lactamase inhibitors with specificities for different carbapenemases underscores the role of gene identification to guide optimal antibiotic selection. Ceftazidime-avibactam, for instance, is effective against *bla*_{KPC} and *bla*_{OXA-48} carbapenemase-producing isolates but has no activity against metallo-beta-lactamases such as *bla*_{NDM} (36). As additional inhibitors to specific beta-lactamases are developed, the utility of defining genotype and phenotype together will likely grow in importance.

One limitation of our study is the low rates of bloodstream infections from resistant organisms within the cohort, resulting in a low number available for testing. However, we previously validated the assay on spike-in samples overrepresented for resistant isolates (20, 22). Second, the greatest discordance compared to standard testing occurred in

those samples with MIC values near the clinical breakpoints, especially in the fluoroquinolone class. The inclusion of additional samples near breakpoints will be key to improving performance for this subset of isolates. Third, as CLSI clinical breakpoints evolve, the models would need to be retrained on updated guidelines to maintain the greatest accuracy; retrospective re-adjudication of isolates would be challenging because exposure doses are chosen to match contemporaneous breakpoints. Fourth, our comparator method was not the reference standard of broth microdilution, but rather VITEK 2 (and disk diffusion in select cases), due to pragmatic considerations; however, this comparator reflects standard practices in many clinical microbiology laboratories, including our own. Finally, in this study, we used SVM modeling to separate SPD values, and in order to have a sufficient number of resistant samples for model training, we combined data from prior work (22) with the current population. This approach was necessary due to the low total number of resistant organisms, but as a result, we do not have a fully independent validation set. We previously used a more sophisticated strategy using machine learning to train random forest models on the responses of each probe to create a robust classifier for meropenem, ciprofloxacin, and gentamicin susceptibility (20). However, this approach requires larger training data sets, including a sizeable number of resistant isolates, to rigorously train and independently validate in a multi-step process. Because each antibiotic within a class elicits a distinct magnitude of transcriptional response in each species (Fig. S2), we could not use this original model without testing and retraining on hundreds of isolates, which was outside the scope of the current study. This additional training will be critical prior to clinical implementation and should improve classification accuracy.

Several technical considerations must be addressed prior to clinical implementation of this assay. First, GoPhAST-R requires species-specific probesets. In this pilot study, the samples were exposed to antibiotics and frozen in real-time, with species identified as either *E. coli* or *K. pneumoniae* prior to the selection of the corresponding probesets, which would not be possible in a prospective workflow for a clinical assay. This could be overcome by either (i) using multiple species probesets concurrently, as only those specific to the organism present would react; (ii) using a species identification probeset, which we have previously demonstrated for bacteria and yeast (37, 38), prior to GoPhAST-R; or (iii) using alternative rapid identification platforms (39, 40) prior to GoPhAST-R. Some of the initial steps in sample processing, such as bacterial isolation and even antibiotic exposure, could occur in parallel with species identification. Though species identity may affect breakpoint doses and antibiotics to test, exposing to multiple antibiotics or doses, and then selecting the relevant subset for detection once the species is known, would be a feasible way to optimize time-to-answer while requiring minimal additional resources. Second, while the current study ran NanoString detection in batches for convenience in this retrospective study, the NanoString workflow is optimally scaled to generate a conventional antimicrobial susceptibility profile for an individual isolate without batching, with 10 antibiotic-treated and two untreated samples filling a standard 12-lane cartridge. Third, while we used pre-frozen aliquots of antibiotics, a cartridge or plate pre-loaded with lyophilized antibiotics would streamline implementation. Finally, due to the limited sensitivity of the stock NanoString nCounter Sprint detector system in our laboratory, we required a 16-h hybridization time to detect signal from the lower inoculum samples used for beta-lactam testing. Pilot instruments under development offer improved RNA detection sensitivity with much shorter hybridization times (20), which we have shown can reduce total sample-to-answer times to 4 h. In the longer term, many steps performed manually for this study would benefit from laboratory automation to reduce hands-on time for the assay.

In summary, in this study, we perform the first clinical pilot using a diagnostic testing approach that simultaneously and rapidly detects bacterial phenotypic response to antibiotic exposure as well as epidemic AMR gene content. The synthesis of these complementary paradigms represents a promising step toward the next generation of clinical antimicrobial susceptibility testing. While further studies utilizing larger

cohorts are necessary to refine the predictive algorithm and enhance its robustness, and automation of the process would be required before clinical utilization, this work establishes a framework for the continued development and ultimate clinical implementation of this novel transcriptional assay.

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E. L. Young, Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | D. J. Roach, Data curation, Formal analysis, Validation, Writing – original draft, Writing – review and editing | M. A. Martinsen, Conceptualization, Data curation, Methodology,

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DATA AVAILABILITY

All data generated and the code used for analysis are available on GitHub at https://github.com/broadinstitute/Go-PhAST-R_Clinical_Pilot/.

ETHICS APPROVAL

Clinical blood culture aliquots were collected under Mass General Brigham Institutional Review Board protocol 2015P002215.

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental material (JCM00997-24-s0001.docx). Figures S1 to S6; Tables S1 to S4.

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