Molecular Detection of Antimicrobial Susceptibility: Changing Paradigm of Laboratory Testing for Multidrug Resistant Organisms

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Introduction

The increasing threat of antibiotic resistance is of worldwide public health concern. Empirical therapy for treatment of infectious diseases has helped create bacterial strains with multiple antibiotic resistance mechanisms. The assessment of antimicrobial susceptibility patterns of these organisms is one of the primary responsibilities of the clinical microbiology laboratory. Implementation of timely infection control measures can prevent possible outbreaks, decrease patients' lengths of stay, and reduce healthcare costs. Molecular testing can offer a rapid and sensitive approach compared to phenotypic testing and can have a significant impact on patient care. In this study, a molecular diagnostic Antibiotic Resistance (ABRx™) Panel was designed to screen for seventeen multidrug resistance genes encoding the most clinically prevalent mechanisms of resistance to three major classes of antibiotics. The objectives of this study were to 1) develop a panel of assays for direct detection of genes associated with resistance to β-lactams/carbapenems, macrolides, and fluoroquinolones; 2) assess the performance of the system using phenotypically and molecularly characterized clinical isolates; and 3) compare the system to phenotypic reference methods.

Materials & Methods

Samples

- Assay inclusivity was verified using 167 clinical isolates with established antibiotic resistance genotypes and phenotypic antibiotic susceptibility profiles from various sources (ATCC and BEI Resources, Manassas, VA; CDC, Atlanta, GA; IHMA, Schaumburg, IL; and Microbiologics, St. Cloud, MN).
- DNA from samples was extracted on the KingFisher™ Flex System (Thermo Fisher Scientific, Waltham, MA).

Test System

- TaqMan® assays were designed based on sequences obtained from the basic local alignment search tool (BLAST) using an algorithm evaluating melting temperature, nucleotide composition of primer-pair combinations, and specificity of genomic sequences with closely related gene subtypes.
- Assays were designed to detect the most clinically relevant gene variants within individual enzyme classes (Table 1).
- Assays were printed on OpenArray® plates for high-throughput testing on the QuantStudio™ 12K Flex instrument (Thermo Fisher Scientific).
- To increase sensitivity for low concentration samples, a target-specific preamplification step was performed prior to real-time PCR amplification on OpenArray® plates.

Table 1. ABRx™ Panel Content

Enzyme Class	Panel Target Abbreviation
Class A β-lactamase	CTX-M Group 1
	CTX-M Group 2
	CTX-M Group 8/25
	CTX-M Group 9
	KPC
Class B metallo-β-lactamase	IMP-1
	VIM
	NDM
AmpC β-lactamase	FOX
Class D oxacillinase	OXA-1
	OXA-48
Minor ESBL	PER
	VEB
	GES
Macrolide	ermB
Fluoroquinolone	qnrA
	qnrS

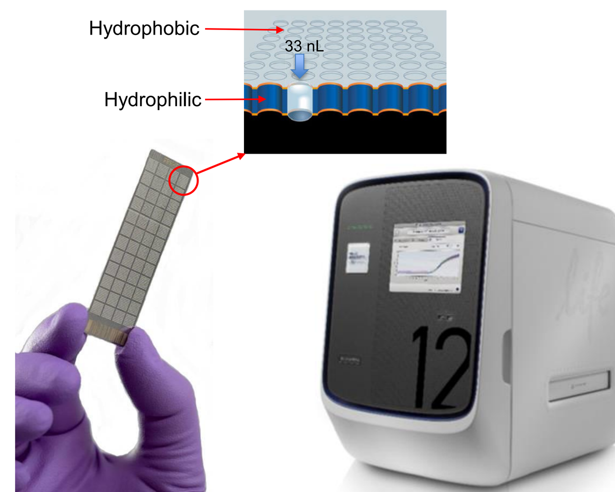

Figure 1. The QuantStudio™ 12K Flex can accommodate up to four OpenArray® Plates for simultaneous real-time PCR detection. The ABRx™ Panel contains seventeen unique TaqMan® assays printed in triplicate. Forty-eight samples can be tested on an individual OpenArray® plate.

Validation

- Plasmid DNA for each target was tested in duplicate at high concentration (1e6 copies/mL) for specificity of the ABRx Panel.
- Linearity and sensitivity for each assay was determined using nine mixes of plasmid DNA (pDNA) over a nine-log serial dilution ranging from 100 million copies to one copy (Latin Square).
- Each pDNA mix was tested in five replicates; the experiment was repeated over three days.
- Data from the three experiments were compiled to determine the linear range of each assay and were also used to assess inter- and intra-run precision.

Materials & Methods *(continued)*

- For panel sensitivity, eighteen organisms of known titer (0.5 McFarland Standard) were serially diluted from 1e6 to 1e2 CFU/mL, spiked into negative matrices, and DNA was extracted from samples using the KingFisher™ Flex System.
- The ABRx™ Panel was validated on clinical isolates, urine, stool/rectal swabs, and nasal swabs.
- Twenty-six blinded, characterized isolates (IHMA, Inc.) were used to assess the accuracy of the panel.
- Resistance profiles from the ABRx™ Panel were compared to the molecular profile provided by the vendor, as well as two phenotypic methods (Kirby-Bauer disc diffusion and Thermo Fisher Scientific Sensititre™ microbroth dilution).
- Results for phenotypic methods were interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI).

Figure 2. Workflow of the ABRx™ Panel.

Results

Inclusivity

Table 2. ABRx™ Panel Inclusivity of Gene Resistance Targets. Assays were designed *in silico* to include clinically relevant enzyme subtypes within a group. Characterized clinical isolates (n=167) were tested for inclusivity. Genotypic profiles for all tested ABRx™ assays were 100% concordant with reported phenotypic antibiotic resistance profiles, independent PCR, and sequencing tests.

Assay	Number of RefSeq Used To Design Assay	Included Strains (*in silico*/*in vitro*)	Number of Strains Tested	Resistance Genes Verified
CTX-M Group 1	495	*Acinetobacter spp., Aeromonas spp., Citrobacter freundii, Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa*	31	CTX-M-1, -3, -12, -15, -32, -55, -116
CTX-M Group 2	110	*E. coli, K. pneumoniae, P. aeruginosa, P. mirabilis, Salmonella enterica*	8	CTX-M-2, -39, -74
CTX-M Group 8/25	12	*Citrobacter amalonaticus, E. coli, K. pneumoniae, Kluyvera georgiana, S. enterica*	5	CTX-M-8, -25, -26, -40, -94 to -100
CTX-M Group 9	222	*C. freundii, E. cloacae, E. coli, K. pneumoniae, P. mirabilis*	19	CTX-M-9, -14, -24, -27, -38, -65, -67, -134
KPC	73	*C. freundii, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, P. aeruginosa, Serratia marcescens*	19	KPC-2, -3, -4, -12
IMP-1	99	*Achromobacter xylosoxidans, Acinetobacter baumannii, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, P. aeruginosa, S. marcescens*	8	IMP-1, -4, -6, -26, -28, -29, 34
VIM	327	*A. baumannii, C. freundii, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, P. aeruginosa, P. mirabilis, S. marcescens*	14	VIM-1, -2, -4, -5, -6, -10, -11, -23, 26, -27, -31, -32
NDM	174	*A. baumannii, Acinetobacter lwoffii, C. freundii, Enterobacter asburiae, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, P. aeruginosa, P. mirabilis, Providencia rettgeri, Providencia stuartii*	14	NDM-1, -2, -3, -5, -6, -7, -8
FOX	10	*E. cloacae, E. coli, K. oxytoca, K. pneumoniae*	5	FOX-3, -4, -5, -6, -7, -10
OXA-1	164	*C. freundii, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, Morganella morganii, P. aeruginosa, P. mirabilis, S. enterica, Shigella flexneri*	28	OXA-1, -30, -320
OXA-48	14	*E. cloacae, E. coli, K. pneumoniae, M. morganii, S. enterica, S. flexneri*	11	OXA-48, -162, -244
PER	13	*A. baumannii, Aeromonas spp., Alcaligenes faecalis, E. coli, K. pneumoniae, P. mirabilis, P. rettgeri, P. aeruginosa*	5	PER-1, -3, -5, -7
VEB	31	*Aeromonas spp., E. asburiae, E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa*	6	VEB-1, -2, -3, -4, -5, -6
GES	47	*A. baumannii, K. oxytoca, K. pneumoniae, P. aeruginosa*	7	GES-1, -3, -4, -5, -6, -11, -12, -13, -19, -20
ermB	347	*Clostridium difficile, Enterococcus faecalis, Enterococcus faecium, E. coli, K. pneumoniae, Pseudoclostridium difficile, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes**	10	*ermB*
qnrA	72	*A. baumannii, C. freundii, E. coli, E. cloacae, E. faecalis, K. oxytoca, K. pneumoniae, P. mirabilis*	6	*qnrA*
qnrS	59	*E. faecalis, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, S. enterica, S. flexneri*	12	*qnrS*, qnrS1, qnrS4

*Not all strains shown

Results *(continued)*

Cross-Reactivity

Table 3. Specificity of the ABRx™ Panel was determined by testing plasmid DNA at 1e6 copies/μL in duplicate. The table shows the average (n=4) cycle threshold (Ct) values for each target. Standard deviation was less than 0.36 for all targets. No cross-reactivity was observed.

	CTX-M Group 1	CTX-M Group 2	CTX-M Group 8/25	CTX-M Group 9	ermB	FOX	GES	IMP	KPC	NDM	OXA-1	OXA-48	PER-1	qnrA	qnrS	VEB	VIM	APX1
pDNA	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct
CTX-M Grp 1	20.92	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
CTX-M Grp 2	---	20.94	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
CTX-M Grp 8/25	---	---	21.95	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
CTX-M Grp 9	---	---	---	22.18	---	---	---	---	---	---	---	---	---	---	---	---	---	---
ermB	---	---	---	---	20.06	---	---	---	---	---	---	---	---	---	---	---	---	---
FOX	---	---	---	---	---	21.02	---	---	---	---	---	---	---	---	---	---	---	---
GES	---	---	---	---	---	---	21.37	---	---	---	---	---	---	---	---	---	---	---
IMP-1	---	---	---	---	---	---	---	20.88	---	---	---	---		---	---	---	---	---
KPC	---	---	---	---	---	---	---	---	21.33	---	---	---	---	---	---	---	---	---
NDM	---	---	---	---	---	---	---	---	---	21.05	---	---	---	---	---	---	---	---
OXA-1	---	---	---	---	---	---	---	---	---	---	20.39	---	---	---	---	---	---	---
OXA-48	---	---	---	---	---	---	---	---	---	---	---	21.74	---	---	---	---	---	---
PER-1	---	---	---	---	---	---	---	---	---	---	---	---	21.31	---	---	---		---
qnrA	---	---	---	---	---	---	---		---	---	---	---	---	21.40	---	---	---	---
qnrS	---	---	---	---	---	---	---	---	---	---	---	---	---	---	21.33	---	---	---
VEB	---	---	---	---	---	---	---	---	---	---	---		---	---	---	21.74	---	---
VIM	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	20.94	---
APX1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	26.43

Analytical Sensitivity

Table 4. Linear Range of ABRx™ Panel Targets (n=30 per assay/concentration). Linear range was assessed using mixes of pDNA from 1e8 to 1e0 copies/μL across three days. The Ct values from linear range experiments were used to calculate slope and correlation coefficient. Both parameters are indicative of assay performance.

Assay	Linear Range (copies/μL)	Slope	Assay Efficiency (%)	Correlation Coefficient (R^2)
CTX-M Group 1	10^8-10^1	-3.2	105.4	1.000
CTX-M Group 2	10^8-10^1	-3.4	96.8	1.000
CTX-M Group 8/25	10^8-10^1	-3.4	96.8	0.994
CTX-M Group 9	10^8-10^2	-3.2	105.4	0.998
ermB	10^8-10^2	-3.3	100.9	0.999
FOX	10^8-10^1	-3.2	105.4	0.999
GES	10^8-10^1	-3.2	105.4	0.998
KPC	10^8-10^2	-3.4	96.8	1.000
NDM	10^8-10^2	-3.4	96.8	0.999
OXA-1	10^8-10^1	-3.4	96.8	0.999
OXA-48	10^8-10^1	-3.3	100.9	1.000
VEB	10^8-10^2	-3.5	110.2	1.000
VIM	10^8-10^2	-3.3	100.9	0.999
PER-1	10^8-10^1	-3.1	110.2	0.994
IMP-1	10^8-10^1	-3.1	110.2	0.996
qnrA	10^8-10^1	-3.2	105.4	0.998
qnrS	10^8-10^1	-3.2	105.4	0.999
APX1	10^8-10^1	-3.1	110.2	0.997

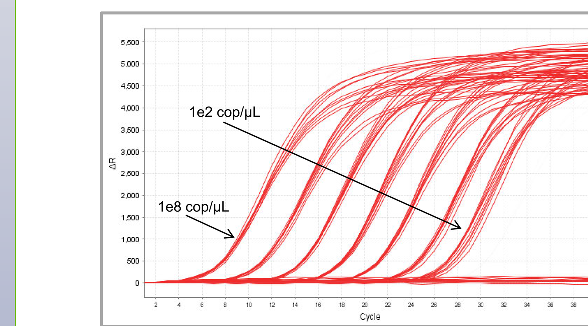

Figure 3. Representative Example of Real-Time Amplification Curves on the ABRx™ Panel: KPC Assay.

Figure 4. Standard Curve of the ABRx™ Panel KPC assay. This assay has a 96.8% primer efficiency (calculated from slope -3.4) and a 0.9999 correlation coefficient.

- All ABRx™ Panel assays have primer efficiencies between 96.8–110.2% and correlation coefficients >0.994.
- Lower limits of detection for each target were determined to be 1e5 to 1e3 CFU/mL when tested directly from negative clinical specimens spiked with pathogens.

Precision

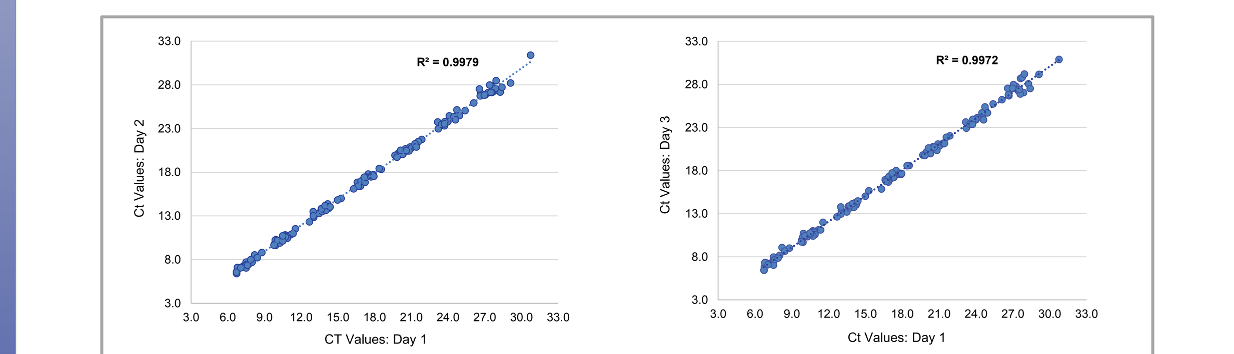

Figure 5. Precision of the ABRx™ Panel. Inter- and intra-assay variation was assessed over three technical runs (n=3570) using plasmid DNA mixes.

Accuracy

- Targeted resistance genotypes from the ABRx™ Panel demonstrated 100% correlation with the reported molecular profiles provided by IHMA, Inc.
- Additionally, discrepancies in gene detection on the OpenArray® were identified in six isolates, and gene presence was confirmed by Sanger sequencing.

Results *(continued)*

- Antibiotic resistance genotypes were consistent with observed antibiotic susceptibility profiles from both the traditional Kirby-Bauer disc diffusion method and the Thermo Fisher Scientific Sensititre™ microbroth dilution method.

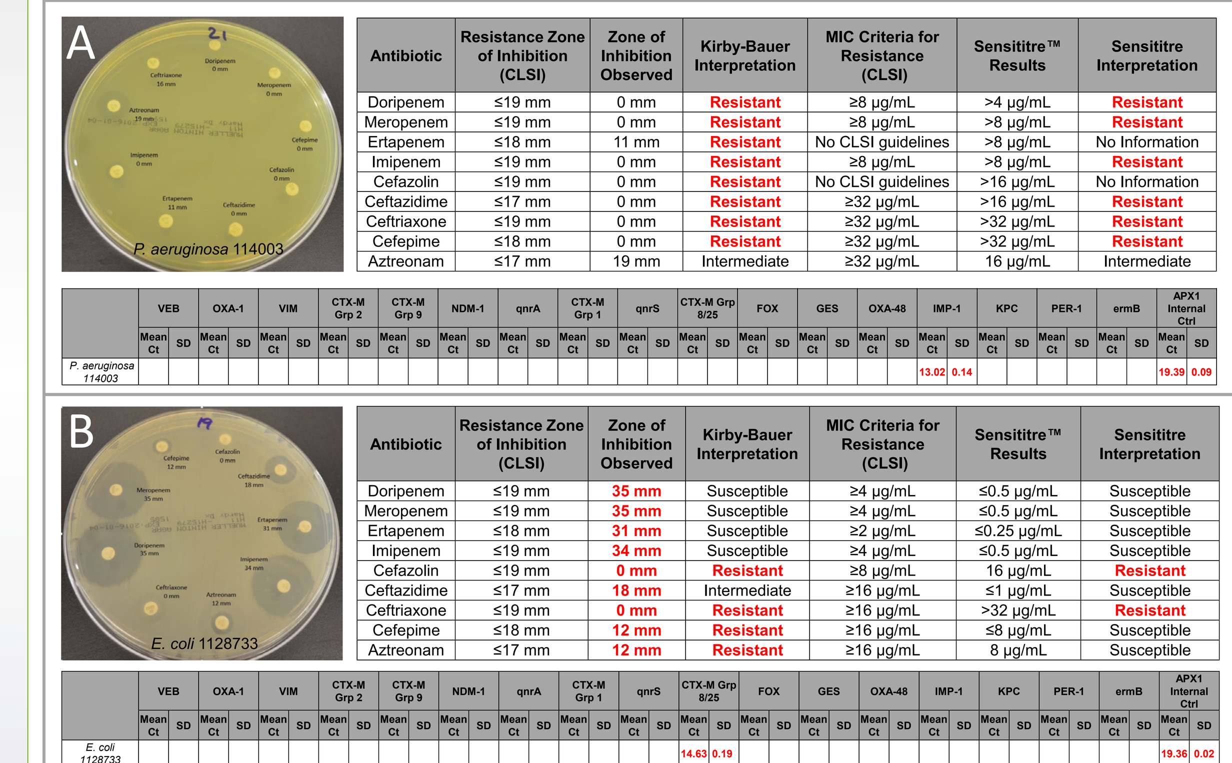

Figure 6. Linking Phenotypic and Genotypic Multidrug Resistance Profiles: IMP-1 and CTX-M Group 8/25. Blinded samples were tested and results were compared after phenotypic and genotypic analyses were concluded. **(A)** The ABRx™ Panel detected the IMP-1 target in *P. aeruginosa* 114003 which is concordant with the molecular characterization provided by the vendor. IMP-1 confers resistance to penicillins, cephalosporins, and carbapenems. Phenotypic testing confirms these results. **(B)** The ABRx™ Panel detected CTX-M Group 8/25 in *E. coli* 1128733 which is concordant with the characterization provided by the vendor. This gene confers resistance to penicillins, cephalosporins, and monobactams. Kirby-Bauer disc diffusion confirmed these results; however, the Sensititre™ method did not identify resistance to Cefepime and Aztreonam. Phenotypic methods can show differing results for the same strain resulting in improper therapy.

Conclusions

- TaqMan® assays were designed to ensure the detection of multiple clinically relevant subtypes within three major antibiotic resistance gene classes and across representative bacterial species.
- The ABRx™ Panel, consisting of seventeen TaqMan® assays printed on the OpenArray® platform, was developed and validated using 167 genotypically and phenotypically characterized clinical isolates.
- Clinical specimens can be used directly for detection of multidrug resistance gene profiles as no additional bacterial isolation or culture is required; established analytical sensitivity was between 1e5 and 1e3 CFU/mL.
- The ABRx™ Panel demonstrated excellent intra- and inter-assay precision with an R^2=0.99.
- Accuracy of the ABRx™ Panel was assessed against conventional phenotypic methods. Profiles were concordant with resistance gene identification on the ABRx™ Panel.
- Molecular testing for antibiotic resistance genes removes the variability and subjectivity found in current phenotypic test methods, allowing for appropriate therapeutic decisions.
- The ABRx™ Panel is a rapid, accurate, and sensitive tool to shift the paradigm of diagnostics for multidrug resistant organisms, advancing antibiotic stewardship programs in healthcare facilities.

References

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