Challenges Associated with Developing Rapid Molecular Diagnostics for Detection of Antibiotic Resistance

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Abstract

Introduction: Widespread use of antibiotics has led to an increased number of multidrug resistant bacteria all over the world. New resistance mechanisms emerge and spread globally, hindering the ability of healthcare professionals to treat common infectious diseases. Because accurate and timely detection of antibiotic resistance (AR) patterns are crucial for treatment of disease, as well as antimicrobial stewardship, the field is moving toward molecular-based testing. However, the development of novel diagnostic tests based on gene detection faces several challenges including a multiplicity of mechanisms and gene targets, lack of common nomenclature for gene classes, and a scarcity of organisms with well-defined phenotypic and genotypic profiles. This study highlights these challenges with emphasis on a subset of problematic gene targets.

Methods: Extensive NCBI and literature searches were performed to determine relevant gene targets and corresponding gene sequences responsible for resistance to common classes of antibiotics. For discrimination of extended-spectrum beta-lactamases (ESBL) and non-ESBL TEM variants, 4590 sequences were used to design TaqMan assays targeting four specific point mutations (E104K, R164S, G238S, E240K). For coverage of relevant CTX-M enzymes, four assays were designed using 839 sequences. Two nomenclature systems to identify aminoglycoside modifying enzymes were used in primer design. Characterized bacterial isolates were collected from commercial (n=266), public (n=336), and academic (n=113) sources.

Results: Over eighty assays that detect genes responsible for resistance to eight commonly used classes of antibiotics were developed. Out of 47 strains characterized for TEM ESBL mutations, seven publicly-sourced isolates produced discrepant results, which were confirmed by phenotypic testing. The CTX-M assays were inclusive of 69 variants that can be found across 33 bacterial species, and assay specificity and inclusivity were confirmed with 63 characterized isolates. For less common AR genes, isolates were not available; therefore, assays for select aminoglycoside, macrolide, and quinolone resistance genes were designed but could not be validated.

Conclusions: Several factors that can influence test development were identified in this study, illustrating the challenges associated with the molecular detection of AR. The complexity of mechanisms involved in AR is indisputable, and prevalence rates are needed to discern the most important genes of interest. As the field moves toward molecular-based testing, it would be beneficial for commercial and public sources to have more comprehensive, accurate genetic characterization of isolates, and a consensus on gene nomenclature is required.

Note: Abstract modified to reflect updated number of isolates.

Introduction

The increasing threat of antibiotic resistance is a worldwide public health concern. Empirical treatment has led to the evolution of multidrug resistant organisms and dissemination of plasmid-borne antibiotic resistance genes across different bacterial species. Identifying these genes in a timely manner can advance antibiotic stewardship programs, 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. This study outlines several challenges encountered while developing a molecular test to detect genes associated with resistance to eight commonly prescribed classes of antibiotics.

Materials & Methods

- Extensive NCBI and literature searches were performed to determine clinically relevant gene targets and corresponding gene sequences.
- Several databases and publications were used to reconcile gene nomenclature during assay design.
- 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.
- Over eighty assays that detect genes responsible for resistance to eight commonly used classes of antibiotics were developed.
- Characterized bacterial isolates were collected from commercial (n=266), public (n=336), and academic (n=113) sources to verify assay inclusivity.
- DNA from samples was extracted using the QIAamp UCP Pathogen Mini Kit on the QIAvac 24 Plus (QIAGEN, Hilden, Germany).
- Real-time PCR was performed using 96-well or OpenArray® plates on the QuantStudio™ 12K Flex instrument (Thermo Fisher Scientific, Waltham, MA).

Results

Challenge #1: Assay Design

- Assay design was hindered by complex resistance mechanisms, the presence of single nucleotide polymorphisms (SNPs), the presence of multiple variants within gene families, a lack of prevalence data, and a lack of a common nomenclature for gene names.

Table 1. Panel inclusivity of a subset of gene resistance targets used in the ABRx™ Panel. Assays were designed *in silico* to include clinically relevant enzyme subtypes within each assay type and across different bacterial strains. Characterized clinical isolates (n=371) were tested for inclusivity.

Assay	Number of RefSeq Used	Included Strains (*in silico/in vitro*)	Number of Strains Tested	Resistance Genes Verified
ant3	2259	*Acinetobacter baumannii, Aeromonas spp., Citrobacter freundii, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Laribacter hongkongensis, Morganella morganii, Pseudomonas aeruginosa, Salmonella enterica, Serratia marcescens, Shigella sonnei, Staphylococcus aureus, Vibrio cholerae, Yersinia pestis*	28	*aadA1, aadA2, aadA5, aadA16*
CMY	424	*A. baumannii , C. freundii, Citrobacter koseri, E. coli, Klebsiella oxytoca, K. pneumoniae, Proteus mirabilis, Providencia rettgeri, S. enterica, S. marcescens, V. cholerae*	21	CMY-2, -4, -16, -34, -42, -48, -81, -112
CTX-M Group	839	*Acinetobacter spp., Aeromonas spp., Citrobacter amalonaticus, C. freundii, Enterobacter aerogenes, E.cloacae, E. coli, K. oxytoca, K. pneumoniae, Kluyvera georgiana, P. mirabilis, P. aeruginosa, S. enterica*	63	CTX-M-1, -2, -3, -8, -9, -12, -14, -15, -24 to -27, -32, 38, -39, -40, -55, -65, -67, -74, -94 to -100, 116, -134
TEM	4597	*Achromobacter denitrificans, A. baumannii, Aeromonas hydrophila, C. freundii, E. aerogenes, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, Neisseria gonorrhoeae, P. mirabilis, P. aeruginosa, S. enterica, S. marcescens, Shigella flexneri, S. sonnei, V. cholerae, Vibrio parahaemolyticus*	47	TEM-1, -2, -3, -4, -6, -10, -11, -12, -20, -21, -24, -26, -28, -33, -43, -52, -63, -71, -89, -129, -211

Table 2. Discrimination of TEM variants: extended spectrum β-lactamases (ESBL) vs non-ESBL.[3] Point mutations at selected loci within the TEM gene give rise to the extended-spectrum phenotype. ESBL-producing organisms have been associated with poor clinical outcomes, so assay discrimination at the SNP level for TEM variants is required.

β-lactamase	Alternate Name	Phenotype	Amino acid at position 4	11	13	24	26	28	30	34	35	38	39	40	41	42	43	44	49	51	64	68	69	80	84	92	99	100	102	104	105	114	115	120	124	127	130	136	137	138	145	146	153	155	156	157	158	163	164	165
TEM-1	PTEM-1	2b	Q	A	I	F	L	E	L	K	D	D	Q	L	G	A	R	V	L	L	E	M	M	V	V	G	Q	N	L	E	Y	T	D	R	S	I	S	N	L	L	P	K	H	M	G	D	H	D	R	W
TEM-2		2b											K																																						
TEM-3	CTX-1, TEM-14	2be											K																	K																					
TEM-4		2be					F																							K																					
TEM-5	CAZ-1	2be																																															S		
TEM-6		2be																												K																			H		
TEM-7		2be											K																																				S		
TEM-8	CAZ-2	2be											K																	K																			S		
TEM-9	RHH-1	2be					F																							K																			S		
TEM-10	MGH-1, TEM-E3, TEM-23	2be																																															S		
TEM-11	CAZ-to	2be											K																																				H		
TEM-12	YOU-2, CAZ-3, TEM-E2	2be																																															S		
TEM-13		2b											K																																						
TEM-15		2be																												K																					
TEM-16	CAZ-7	2be											K																	K																				H	
TEM-17		2be																												K																					
TEM-18		2be											K																	K																					
TEM-19		2be																																																	
TEM-20		2be																																																	
TEM-21		2be											K																														R								
TEM-22		2be											K																	K																					
TEM-24	CAZ-6	2be											K																	K																			S		
TEM-25	CTX-2	2be					F																																												
TEM-26	YOU-1	2be																												K																			S		
TEM-27		2be																																															H		
TEM-28		2be																																															H		
TEM-29		2be																																															H		
TEM-30	IRT-2, TRI-2, E-GUER	2br																																																	
TEM-31	IRT-1; TRI-1; E-SAL	2br																																																	
TEM-32	IRT-3	2br																					I																												
TEM-33	IRT-5	2br																					L																												
TEM-34	IRT-6	2br																					V																												
TEM-95	IRT-4	3br																					L																												

Table 3. Lack of consensus of gene nomenclature for a subset of aminoglycoside O-nucleotidyltransferases (ANTs).[4]

ANTs	Gene Names	Genetic Location	Host
ANT(6)-Ia	*ant(6)-Ia, ant6, aadE*	Plasmid, chromosome	*E. faecium, S. aureus, Staphylococcus epidermidis, Streptococcus suis*
	ant6	Plasmid	*E. faecalis*
	aadE	Chromosome	*Streptococcus mitis*
	aadE	Plasmid	*Campylobacter jejuni*
	aad(6)	Plasmid	*E. faecalis, Streptococcus oralis*
ANT(2')-Ia	*ant(2')-Ia, aadB*	Plasmid, integron	*A. baumannii, C. freundii, E. coli, K. pneumoniae, M. morganii, P. aeruginosa, Salmonella typhimurium*
ANT(3")-Ia	*ant(3")-Ia, aadA, aadA1, aad(3")(9)*	Plasmid, integron, transposon	*A. baumannii, Enterobacteriaceae, P. aeruginosa, V. cholerae*
	aadA2	Plasmid, integron	*Aeromonas spp., C. freundii, Corynebacterium glutamicum, K. pneumoniae, Salmonella spp.*
	aadA3	Plasmid, integron, transposon	*E. coli*
	aadA4	Plasmid, chromosome	*Bordetella parapertussis, E. coli*
	aadA5	Plasmid, integron, transposon	*E. cloacae, E. coli, K. pneumoniae, K. georgiana, P. aeruginosa*
	aadA6	Integron	*P. aeruginosa*

Results *(continued)*

Challenge #2: Availability of Characterized Bacterial Isolates

- Genotypically and phenotypically characterized isolates are required for development and validation of molecular assays.
- Commercial sources with complete genotypic profiles are limited and costly.
- Isolates from public sources often lack genotypic profiles, may have inaccurate information, and require material transfer agreements.
- Custom permit approvals and legal agreements for procurement of isolates from overseas academic researchers were necessary to obtain isolates for less prevalent gene types.
- Validation of several assays could not be completed due to the unavailability of profiled strains.
- Serial passage of bacterial strains can result in plasmid loss; discrepancies between vendor-provided profiles and TaqMan assay results have to be confirmed by an orthogonal method.

Table 3. Sources of characterized isolates used in panel development (n=715). Limited genotypic information was available for fluoroquinolone and macrolide isolates from IHMA. Fifty-six percent of the isolates from BEI and 43% of the isolates from the CDC lacked genotypic information.

Vendor	# Isolates (Discrepancies)
International Health Management Association (IHMA)	216 (4)
American Type Culture Collection (ATCC)	18
ZeptoMetrix	2
Microbiologics	7
BEI Resources	23
Centers for Disease Control and Prevention (CDC)	287
Antibiotic Resistance Leadership Group (ARLG)	49 (7)
Academia (7 sources)	113

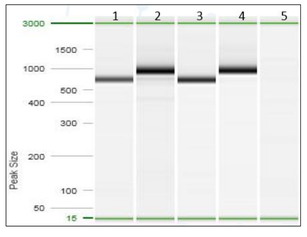
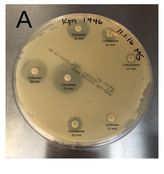


Figure 1. Confirmation of NDM plasmid loss in *K. pneumoniae.* NDM-1 was not confirmed with the ABRx™ TaqMan assay. The vendor performed confirmatory testing using capillary gel electrophoresis (QIAxcel Advanced System, QIAGEN) with original DNA and re-extracted DNA from the isolate. The bla_{NDM} gene was detected in the original DNA (Lane 1) but not in the re-extracted DNA (Lane 5). Copy number reduction and loss of bla_{NDM} has been shown to occur in the absence of strong carbapenem selection[2] and may explain the lack of bla_{NDM} detection with the TaqMan assay.



Antibiotic	Generation of Cephalosporin	MIC (µg/mL)	Kirby Bauer Interpretation (mm)
Cefazolin	1st	NA	11 (R)
Cefotetan	2nd	NA	25 (S)
Cefoxitin	2nd	NA	20 (S)
Cefuroxime	2nd	NA	20 (S)
Cefepime	3rd	4 (SDD)	31 (S)
Cefotaxime	3rd	64 (R)	31 (S)
Ceftazidime	3rd	>256 (R)	21 (S)

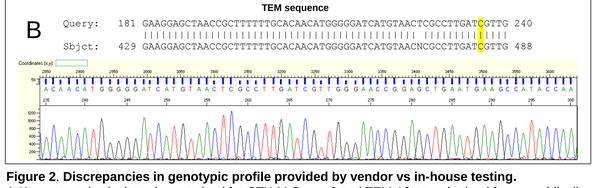
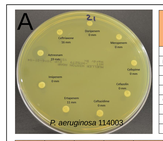


Figure 2. Discrepancies in genotypic profile provided by vendor vs in-house testing. A *K. pneumoniae* isolate characterized for CTX-M Group 2 and TEM-12 was obtained from a publically funded source. CTX-M Group 2 and TEM-12 were not amplified by ABRx™ TaqMan assays, and the published ESBL phenotype could not be confirmed by **(A)** disc diffusion. **(B)** Sanger sequencing analysis on a 3500XL Genetic Analyzer (Thermo Fisher Scientific) confirmed the non-ESBL (wild type) TEM genotype (yellow) indicating that this isolate may have undergone plasmid loss. The identified discrepancies were communicated, and no additional follow-up/feedback was provided by the vendor. R, resistant; S, susceptible

Results *(continued)*

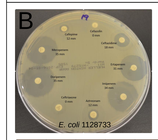
Challenge #3: Linking Phenotypic and Genotypic Profiles

- Currently, there are no accepted tools to link genotypic and phenotypic antibiotic resistance profiles.
- Statistical analyses with an expanded data set are required to predict phenotypic profiles based on genotypic data.



Antibiotic	Resistance Zone of Inhibition (CLSI)[1]	Zone of Inhibition Observed	Kirby-Bauer Interpretation	MIC Criteria for Resistance (CLSI)	Sensititre™ Results	Sensititre Interpretation
Doripenem	≤19 mm	0 mm	Resistant	≥8 µg/mL	>4 µg/mL	Resistant
Meropenem	≤19 mm	0 mm	Resistant	≥8 µg/mL	>8 µg/mL	Resistant
Ertapenem	≤18 mm	11 mm	Resistant	No Guidelines	>8 µg/mL	No Information
Imipenem	≤19 mm	0 mm	Resistant	≥8 µg/mL	>8 µg/mL	Resistant
Cefazolin	≤19 mm	0 mm	Resistant	No Guidelines	>16 µg/mL	No Information
Ceftazidime	≤17 mm	0 mm	Resistant	≥32 µg/mL	>16 µg/mL	Resistant
Ceftriaxone	≤19 mm	0 mm	Resistant	≥32 µg/mL	>32 µg/mL	Resistant
Cefepime	≤18 mm	0 mm	Resistant	≥32 µg/mL	>32 µg/mL	Resistant
Aztreonam	≤17 mm	19 mm	Intermediate	≥32 µg/mL	16 µg/mL	Intermediate

ABRx™ Targets	CTX-M Grp 1	CTX-M Grp 2	CTX-M Grp 8/25	CTX-M Grp 9	ermB	FOX	GES	IMP-1	KPC	NDM	OXA-1	OXA-48	PER	qnrA	qnrS	VEB	VIM	Internal Control
Mean Ct								13.02										19.39
Std Dev								0.14										0.09



Antibiotic	Resistance Zone of Inhibition (CLSI)	Zone of Inhibition Observed	Kirby-Bauer Interpretation	MIC Criteria for Resistance (CLSI)	Sensititre™ Results	Sensititre Interpretation
Doripenem	≤19 mm	35 mm	Susceptible	≥4 µg/mL	≤0.5 µg/mL	Susceptible
Meropenem	≤19 mm	35 mm	Susceptible	≥4 µg/mL	≤0.5 µg/mL	Susceptible
Ertapenem	≤18 mm	31 mm	Susceptible	≥2 µg/mL	≤0.25 µg/mL	Susceptible
Imipenem	≤19 mm	34 mm	Susceptible	≥4 µg/mL	≤0.5 µg/mL	Susceptible
Cefazolin	≤19 mm	0 mm	Resistant	≥8 µg/mL	16 µg/mL	Resistant
Ceftazidime	≤17 mm	18 mm	Intermediate	≥16 µg/mL	≤1 µg/mL	Susceptible
Ceftriaxone	≤19 mm	0 mm	Resistant	≥16 µg/mL	>32 µg/mL	Resistant
Cefepime	≤18 mm	12 mm	Resistant	≥16 µg/mL	≤8 µg/mL	Susceptible
Aztreonam	≤17 mm	12 mm	Resistant	≥16 µg/mL	8 µg/mL	Susceptible

ABRx™ Targets	CTX-M Grp 1	CTX-M Grp 2	CTX-M Grp 8/25	CTX-M Grp 9	ermB	FOX	GES	IMP-1	KPC	NDM	OXA-1	OXA-48	PER	qnrA	qnrS	VEB	VIM	Internal Control
Mean Ct			14.63															19.36
Std Dev			0.19															0.02

Figure 3. 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 detection of IMP-1 in *P. aeruginosa* 114003 was concordant with the genotypic profile provided by the vendor. IMP-1 confers resistance to penicillins, cephalosporins, and carbapenems which was confirmed by phenotypic testing. **(B)** The detection of CTX-M Group 8/25 in *E. coli* 1128733 was concordant with the genotypic profile 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 indicate resistance to cefepime or aztreonam.

Conclusions

- Molecular testing can offer a rapid and sensitive approach while removing the variability and subjectivity found in current phenotypic test methods, thus having a significant impact on patient care.
- Several factors that can influence test development were identified in this study, illustrating the challenges associated with molecular detection of antibiotic resistance.
- Complex resistance mechanisms, SNPs, and presence of multiple variants within gene families must be considered for assay design.
- Simultaneous use of multiple gene nomenclature systems causes confusion and may require genes to be renamed.
- There is an urgent need for a central and easily accessible database with an established nomenclature system, and it must be widely adopted.
- As the field moves toward molecular-based testing, commercial and public sources need more comprehensive and accurate genetic characterization of isolates.
- Prevalence rates are needed to discern the most important genes of interest.
- Education is required to prove that molecular detection is meaningful and can be translated into appropriate therapeutic regimens.

References

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