

Development of a TaqMan Assay Panel for Rapid Detection of Multidrug Resistance Genes

Megan Stonebraker
megan.stonebraker@diatherix.com
Leslie L. Malone, MS, MB(ASCP)^{CM}
Stefan Brzezinski, MBA
Don Stalons, PhD, D(ABMM), MPH
Elena Grigorenko, PhD
Diatherix Laboratories, LLC, Huntsville, AL

Abstract

Introduction: The progression of antimicrobial resistance is a threat to public health worldwide. The assessment of antimicrobial susceptibility patterns of bacterial isolates is one of the primary responsibilities of the clinical microbiology laboratory. Molecular testing can offer a rapid and sensitive approach compared to bacteriological testing, and such tests can have a significant impact on patient care. In this study, we developed a TaqMan® Assay panel to screen for twenty-six multidrug resistance genes encoding the most clinically prevalent mechanisms of resistance to three major classes of antibiotics: β -lactams, fluoroquinolones, and macrolides.

Methods: TaqMan® Assays were designed based on sequences obtained from the basic local alignment search tool (BLAST). Assays were printed for use on a high-throughput real-time platform (QuantStudio® 12K Flex). DNA was isolated from samples using a KingFisher™ Flex instrument and subsequently amplified using a panel-specific PreAmp primer mix. Pre-amplification products were then processed on custom OpenArray® Real-Time PCR Plates. Ligation-mediated real-time PCR (Check-Points, Netherlands) and Target-Enriched Multiplex PCR (TEM-PCR™) were used for confirmation of gene resistance profiles for selected samples.

Results: Target specificity of TaqMan Assays was validated using clinical isolates (n=145) with known, sequence-validated gene resistance profiles and patient samples (n=457) for which phenotypic data were available. No cross-reactivity was detected for following gene targets: TEM, SHV, CTX-M Groups 1, 2, 8/25, and 9, ACT/MIR, KPC, NDM, VIM, VEB, IMP, OXA-1, OXA-48, ACC, GES, FOX, *ermA-C*, *qnrA*, *qnrS*, DHA, CMY, and PER. Clinical isolates yielded 100% correlation when compared to resistance profiles. One isolate, characterized as blaNDM-1 positive, was not detected by this panel, but repeated sequencing indicated loss of the NDM-1 plasmid. Screening of patient samples revealed a complex pattern of up to fourteen genes encoding resistance for four major classes of antibiotics, with TEM and *ermA-C* being the most prevalent (48% and 91% in respiratory and gastrointestinal samples, respectively). CTX-M Groups 1, 2, and 9; SHV; ACT/MIR; *qnrA* and *qnrS* were also detected. Results generated with TaqMan Assays were confirmed with alternative PCR-based methods.

Conclusions: TaqMan chemistry in combination with high-throughput real-time PCR provides a powerful technological platform for molecular testing for a broad spectrum of multidrug resistance genes. The implementation of rapid tests for identification of antimicrobial resistance profiles can lead to targeted antibiotic therapy, reduction of unnecessary antibiotic use, and overall improvement of patient care.

Introduction

Multidrug resistant organisms present a major healthcare threat worldwide. Effective antibiotic stewardship programs within healthcare facilities should include rapid and sensitive diagnostic methods. Quick implementation of infection control measures can prevent possible outbreaks, decrease patients' lengths of stay, and reduce healthcare costs. The objectives of this study were to 1) develop a molecular screening system for detection of genes associated with resistance to three major classes of antibiotics: β -lactams, macrolides and fluoroquinolones and 2) assess the performance of the system using phenotypically characterized clinical isolates.

Materials & Methods

Samples

- Clinical isolates (n=145) from various sources (ATCC, BEI, CDC, IHMA, and Microbiologics) with characterized antibiotic resistance genotypes and phenotypic antibiotic susceptibility profiles were used for assay development.
- Nasopharyngeal (n=302) and rectal swabs (n=155) were tested from patients admitted to Alabama Infectious Disease Center and Huntsville Hospital.
- Stool and rectal swabs (n=150) were collected from healthy donors to establish the baseline prevalence of resistance genes in healthy populations.
- DNA from samples was extracted on the KingFisher™ Flex System.

Test System

- TaqMan® assay designs were based on a Thermo Fisher Scientific 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 gene classes.
- Assay specificity was verified using plasmid constructs and clinical isolates with established antibiotic resistance profiles.
- TaqMan® assays were printed on OpenArray® plates for testing.
- Samples were evaluated with and without a preamplification step prior to real-time PCR amplification on OpenArray® plates.

Materials & Methods (continued)

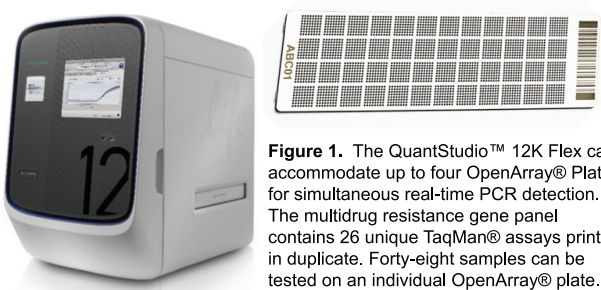


Figure 1. The QuantStudio™ 12K Flex can accommodate up to four OpenArray® Plates for simultaneous real-time PCR detection. The multidrug resistance gene panel contains 26 unique TaqMan® assays printed in duplicate. Forty-eight samples can be tested on an individual OpenArray® plate.

Results

Genotypic, multidrug resistant gene (MDRG) profiles for all tested assays were 100% concordant with reported phenotypic antibiotic resistance profiles, independent PCR, and sequencing tests.

Table 1. TaqMan® Assay Specificity for Detection of Gene Resistance Targets. Characterized bacterial strains were used to determine assay specificity.

MDRO Class	Assay	Number of RefSeq Used	Species Tested	Number of Strains Tested	Resistance Genes Verified
Class A β -lactamases	TEM	791	<i>Citrobacter freundii</i> , <i>Enterobacter asburiae</i> , <i>Enterobacter cloacae</i> , <i>Escherichia coli</i> , <i>Klebsiella oxytoca</i> , <i>Klebsiella pneumoniae</i> , <i>Proteus mirabilis</i>	32	TEM-1, TEM-2, TEM-3, TEM-10, TEM-12, TEM-99
	SHV	468	<i>C. freundii</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>K. pneumoniae</i>	24	SHV-1, SHV-11, SHV-12, SHV-18, SHV-28
	CTX-M Group 1	495	<i>C. freundii</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>K. pneumoniae</i> , <i>K. oxytoca</i> , <i>P. mirabilis</i>	26	CTX-M-1, CTX-M-3, CTX-M-12, CTX-M-15, CTX-M-32, CTX-M-55, CTX-M-116
	CTX-M Group 2	110	<i>E. coli</i> , <i>K. pneumoniae</i>	4	CTX-M-2, CTX-M-74
	CTX-M Group 8/25	12	<i>E. coli</i> , <i>K. pneumoniae</i>	3	CTX-M-8, CTX-M-40
	CTX-M Group 9	222	<i>C. freundii</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>K. pneumoniae</i>	17	CTX-M-9, CTX-M-14, CTX-M-24, CTX-M-27, CTX-M-65, CTX-M-67, CTX-M-134
Class B metallo- β -lactamases	KPC	73	<i>C. freundii</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>K. pneumoniae</i> , <i>Pseudomonas aeruginosa</i>	15	KPC-2, KPC-3, KPC-12
	IMP-1	99	<i>E. cloacae</i> , <i>E. coli</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i>	5	IMP-1, IMP-4, IMP-26
	VIM	327	<i>C. freundii</i> , <i>E. cloacae</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>P. mirabilis</i>	12	VIM-1, VIM-2, VIM-4, VIM-5, VIM-23, VIM-26, VIM-27, VIM-31, VIM-32
AmpC β -lactamases	NDM	174	<i>C. freundii</i> , <i>E. asburiae</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i> , <i>P. mirabilis</i>	14	NDM-1, NDM-5, NDM-7
	ACT/MIR	14	<i>E. cloacae</i> , <i>K. pneumoniae</i>	8	ACT-type, ACT-16, MIR-type, MIR-3, MIR-12
Class D oxacillinases	FOX	10	<i>K. pneumoniae</i> , <i>E. cloacae</i>	3	FOX-3, FOX-10
	OXA-1*	164	<i>C. freundii</i> , <i>E. cloacae</i> , <i>E. coli</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i> , <i>Morganella morganii</i>	13	OXA-1
Minor ESBLs	OXA-48	14	<i>E. cloacae</i> , <i>K. pneumoniae</i> , <i>M. morganii</i>	7	OXA-48
	PER	13	<i>E. coli</i> , <i>K. pneumoniae</i> , <i>P. mirabilis</i> , <i>P. aeruginosa</i>	4	PER-1, PER-3
	VEB	31	<i>K. pneumoniae</i> , <i>E. asburiae</i> , <i>P. mirabilis</i> , <i>P. aeruginosa</i>	4	VEB-1, VEB-2, VEB-4
Macrolides	GES	47	<i>Acinetobacter baumannii</i> , <i>K. oxytoca</i> , <i>P. aeruginosa</i>	7	GES-1, GES-5, GES-6, GES-11, GES-13, GES-18, GES-20
	<i>ermA</i>	185	<i>Staphylococcus aureus</i>	3	<i>ermA</i>
	<i>ermB</i>	347	<i>E. coli</i> , <i>E. faecalis</i> , <i>E. faecium</i>	5	<i>ermB</i>
Fluoroquinolones	<i>ermC</i>	25	<i>E. faecalis</i> , <i>E. faecium</i>	5	<i>ermC</i>
	<i>qnrA</i>	72	<i>C. freundii</i> , <i>E. cloacae</i> , <i>E. faecalis</i> , <i>K. pneumoniae</i>	6	<i>qnrA</i>
	<i>qnrS</i>	59	<i>E. cloacae</i> , <i>E. coli</i> , <i>E. faecalis</i> , <i>K. oxytoca</i> , <i>K. pneumoniae</i>	9	<i>qnrS</i>

*OXA-1 sequence TBD

Results (continued)

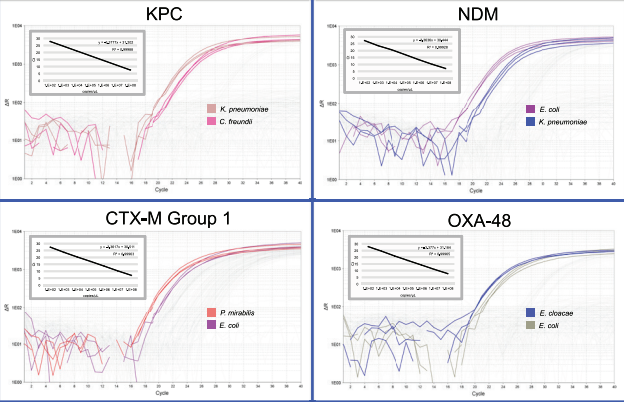


Figure 2. Representative amplification curves of target-specific TaqMan® assays using the OpenArray® platform. Samples were tested in triplicate. Plasmid constructs ranging from 1e8 to 1e0 copies/μL were used to determine reportable ranges for each target (inset). Reportable ranges are based on the linearity and slopes of standard curves.

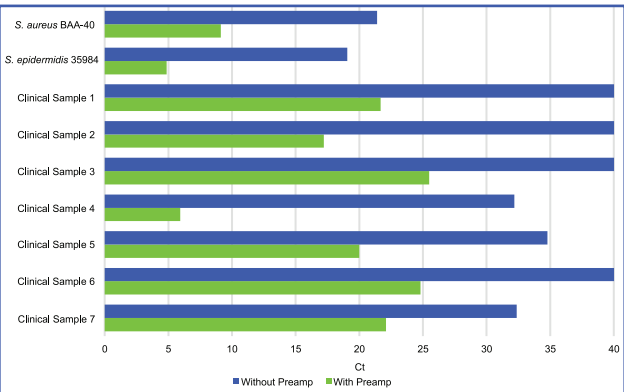


Figure 3. Preamplification increases the detection of *ermA* in clinical samples and characterized isolates. DNA was processed by qPCR with and without a target-specific preamplification step. Clinical samples 1, 2, 3, and 6 were assigned a Ct of 40 because they were not detected without preamplification. For other targets, preamplification versus non-preamplification results were similar to *ermA* results.

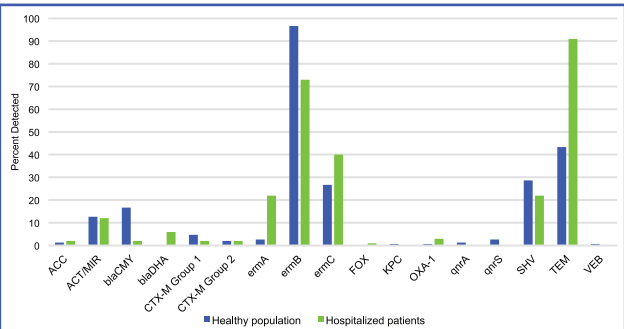


Figure 4. A comparison of multidrug resistant genes (MDRGs) detected in gastrointestinal tract samples collected from asymptomatic, healthy donors (n=150) and hospitalized patients with *Clostridium difficile* infections (n=99).

Results (continued)

The pattern and frequency of specific MDRG detection in sputum samples is related to microbiota present in respiratory tracts of tested samples.

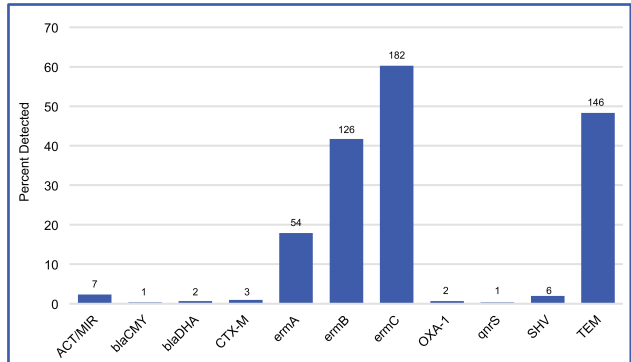
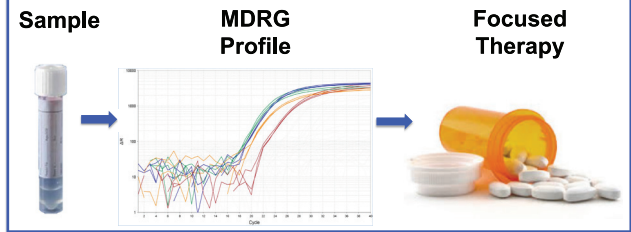


Figure 5. Multidrug resistance genes were detected in sputum samples collected from hospitalized patients (n=302; mean age=55 years). The number above the column represents the number of positive samples for each detected drug resistance gene.

Utility of a Multidrug Resistance Diagnostic Panel: Sample-to-Answer in 5 hours



Conclusions

- An initial TaqMan® assay panel for detection of up to twenty-six MDRGs was developed using the spacial multiplexing OpenArray® platform.
- TaqMan® assays were designed to capture multiple clinically relevant subtypes within each major antibiotic resistance gene class found across representative bacterial species.
- Assay specificity was validated using 145 clinical isolates with characterized phenotypic profiles and was 100% concordant with reported phenotypic antibiotic resistance profiles.
- The MDRG Panel provides direct sample testing with no additional enrichment and can be used to evaluate antibiotic resistance patterns in variable patient populations.
- Accurate and rapid detection of antibiotic resistance genes provides a powerful screening tool in clinical settings to improve antibiotic stewardship by lowering healthcare costs, reducing unnecessary antibiotic use, and focusing patient therapy.

Acknowledgements

We would like to thank Dr. Ali Hassoun from the Alabama Infectious Disease Center for generously providing samples for this study.