Development of a DNA Microarray to Detect Antimicrobial Resistance Genes Identified in the National Center for Biotechnology Information Database

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To understand the mechanisms and epidemiology of antimicrobial resistance (AR), the genetic elements responsible must be identified. Due to the myriad of possible genes, a high-density genotyping technique is needed for initial screening. To achieve this, AR genes in the National Center for Biotechnology Information GenBank database were identified by their annotations and compiled into a nonredundant list of 775 genes. A DNA microarray was constructed of 70mer oligonucelotide probes designed to detect these genes encoding resistances to aminoglycosides, β-lactams, chloramphenicols, glycopeptides, heavy metals, lincosamides, macrolides, metronidazoles, polyketides, quaternary ammonium compounds, streptogramins, sulfonamides, tetracyclines, and trimethoprims as well as resistance transfer genes. The microarray was validated with two fully sequenced control strains of *Salmonella enterica*: Typhimurium LT2 (sensitive) and Typhi CT18 (multidrug resistance [MDR]). All resistance genes encoded on the MDR plasmid, pHCM1, harbored by CT18 were detected in that strain, whereas no resistance genes were detected in LT2. The microarray was also tested with a variety of bacteria, including MDR *Salmonella enterica* serovars, *Escherichia coli, Campylobacter* spp., *Enterococcus* spp., methicillin-resistant *Staphylococcus aureus, Listeria* spp., and *Clostridium difficile*. The results presented here demonstrate that a microarray can be designed to detect virtually all AR genes found in the National Center for Biotechnology Information database, thus reducing the subsequent assays necessary to identify specific resistance gene alleles.

Introduction

ANTIMICROBIAL RESISTANCE (AR) in bacteria is an ongoing problem in human and animal health. Virtually from the inception of antimicrobial chemotherapies to treat bacterial infections, resistance was found and began to expand.[1] Despite regulations and controls of antimicrobial use designed to reduce its development and spread, bacterial resistance to antimicrobials continues to increase.[1] To help understand the development of AR and its spread, the genetic mechanisms must be identified. These studies often require assaying for dozens or hundreds of possible resistance-encoding genes to investigate the underlying genetics behind the phenotypic resistance observed in bacteria.[12] This problem has been compounded by the growth of multidrug resistance (MDR) in important pathogenic bacteria, oppor-

tunistic pathogens, commensal bacteria, and environmental bacteria. To address this issue, many researches have turned to high-density gene detection techniques, primarily DNA microarrays.[2,5,7,9,10,13,16,24,26,37,46]

Several studies have demonstrated that DNA microarrays can be used to detect resistance genes as effectively as standard techniques such as polymerase chain reaction, sequencing, conjugation, and Southern hybridization.[2,5,7,9,10,13,16,24,26,37,46] Most of these microarrays rely on short to medium-length (20mer–80mer) oligonucleotides as detection probes because they are easily synthesized without the requirement of template DNA and can be cheaply made and arrayed onto a variety of substrates such as glass slides.[13,34] Modified microscope glass slides have become the most widespread format for custom microarrays, with most universities and research institutes having access to printing robots and

TABLE 1. SUMMARY OF GENE PROBES WITH POSITIVE HYBRIDIZATIONS TO THE CONTROL STRAINS AND TEST ISOLATES

	Aminoglycoside	Beta-lactam	Chloramphenicol	Efflux	Glycopeptide	Heavy metal	Macrolide	Metronidazole	Poly-ketide	Quaternary ammonium	Sulfonamides	Tetracycline	Transfer associated	Trimethoprim
LT2	aadA1			acrR, emrR, marA, marB				nitroimidazole resistance						
CT18	aac(3), aadA, strA,strB	bla, blaPER2, blaTEM-1, tem	cat, cat4, catIII	acrR, corA, emrR, marA, marB, msrA	ble	merACDRT					sulII	tet(A), tet(A)(B), tet(R)	htdAFT, InsA, IntI, parB, putative transposase, repC, repE, sitbA, tnpA, tnpAIS26, tnpR, traI, trhBCFNUV	dfrA, dfrV, dfrV, dfrB
SE Tc JF201	aac(3), aadA, aadA, aadAb, aadA2*, aadA2, aadB, aadE, acc(6)-Ib*, aph(3)-I, aph2, aphA7, aphAI, ksgA, ntplI, strA*, strB, str3K	ampC, ampR, blaL2, bla, PER2, cmy-2*, sme-1*, tem	cat, cmlA5, flo*	acrR, corA, EmrA, emrR, marA, marB, msrA, ABC transporter, ybit	ble	merACFT			nonR	qac, qacEdelta	sul, sulII	tet(A), tet(A)(B), tet(C), tet(D), tet(R)	InsA, IntI, putative transposase, tnpA, tnpAIS26, tnpM, tnpR	dfr, dfr6, dfrA
SE Hd JF210	aac(3), aadA, aadA, aadAb, aadA2*, aadA2, aadA7, aadB, acc(6)-Ib*, aph(3)-I, aphA7, aphAI, ksgA, ntplI, orfE, SAT-2, strA*, strB, str3K	ampC, bla, blaL2, blaTEM-1, cmy-2*, sme-1*, tem	cat, cmlA5, flo*	acrR, corA, emrR, marA, marB, msrA, ABC transporter, ybit	ble	merACDFPRRT, orf5			nonR	qac, qacEdelta	sul, sulII	tet(A), tet(A)(B), tet(C), tet(D), tet(R)	InsA, IntI, mobA, putative transposase, tnpA, tnpAIS26, tnpM, tnpR,trhCF	dfr, dfr6, dfrA
EC JF220	aac(3), aac(6), aadA, aadAb, aadA2*, aadE, aph(3)-I, aphA7, aphAI, ksgA, strA*, strB, str3K	ampC, ampR, bla, blaL2, blaTEM-1, cfxA, cmy-2*, penA, sme-1*, tem	flo*	acrR, corA, EmrA, emrR,marA, marB, msrA, potassium-transporting ATPase, ABC transporter, ybit	ble, vanH	merACDFPRRT, orf5			nonR	qac, qacEdelta	sul, sulII	tet(A), tet(C), tet(D), tet(R)	InsA, IntI, putative transposase, tnpA, tnpAIS26, tnpM, tnpR	dfrA, dhf
EC JF227	aacC3, aadA, aadAb, aadA2*, aadE, aph(3)-I, aphA7, aphAI, ksgA, strA*, strB, str3K	ampC, ampR, bla, blaL2, blaTEM-1, cfxA, cmy-2*, sme-1*, tem	cat, flo*	acrR, corA, EmrA, emrR, marA, marB, msrA, potassium-transporting ATPase, ABC transporter, ybit	ble, vanH	merADFRRT, orf5			nonR	qac, qacEdelta	sul, sulII	tet(A), tet(A)(B), tet(C), tet(D), tet(R)	InsA, IntI, putative transposase, tnpA, tnpAIS26, tnpM	dfrA, dhf
CC 14-22	aad9, aphA-3	cam, cfiA		cmeBC, lsidR, marR								tet(O)	btgA, trfA2, trhU	
CC 141-27	aphA-3	blaL2, cam		cmeBC, lsdR, potassium-transporting ATPase								tet(O)	htdT	
ECF ATCC25788					vanC*	merT							tnpA, trans	

	Aminoglycoside	Beta-lactam	Chloramphenicol	Efflux	Glycopeptide	Heavy metal	Macrolide	Metronidazole	Quaternary ammonium	Poly-ketide	Sulfonamides	Tetracycline	Transfer associated	Trimethoprim
EGM ATCC49573		blaL2, MecR		acrR, EmrA, potassium-transporting ATPase, ABC transporter, ybit	ble, vanC*								putative transposase, tnpA, tnpR, trans	
LI03				msrC, potassium-transporting ATPase, ybit	ble							tet(M), tet(O), tet(S)	putative transposase, recP	
LI04		blaTEM-1		msrC, potassium-transporting ATPase, ybit	ble			nitroimidazole resistance				tet(M), tet(O)	putative transposase	
MRSA G10-C87-2		blaI, mecA, mecI, mecR, MecR, pre, penicillinase repressor, spc		marR, potassium-transporting ATPase	ble		ermA						putative transposase, tnpA, tnpAIS26, tnpB, tnpC, transposase B	
MRSA DN	aadE, aphA-3, sat4	blaI, mecA, mecR, MecR, pre, penicillinase repressor, spc	cat	marR, potassium-transporting ATPase	ble		ermA						putative transposase, tnpA, tnpB, tnpC, transposase B	
MRSA 36-1	aadE, aphA-3, sat4	blaI, mecA, MecR, pre					ermC						putative transposase, repC	
MRSA 59	aadE, aphA-3, sat4	mecA, MecR											putative transposase, repC	
CD 70														
CD 98		orfE												
CD 112		blaL2										tet(C)	parB, tnpB, tnpR, trhC	
CD 113														

Complete data in Supplemental Table S1 (available online at www.liebertonline.com/mdr).
*Indicates multiple identical probes for this gene. Genes with more than one positive probe are listed for each positive hybridization detected.
Isolates: SE, Salmonella enterica; Hd, Heidelberg; Tc, serovar Typhimurium variant Copenhagen; EC, Escherichia coli; ECF, Enterococcus casseliflavus; EGM, Enterococcus gallinarum; LI, Listeria Innocua; MRSA, Methicillin Resistant Staphylococcus aureus; CD, Clostridium difficile.

scanners designed to manufacture and analyze these arrays. With the advent of this technology, it should be theoretically possible to design microarrays for the detection of all known sequenced AR genes available in the public domain and cheaply construct them in many research facilities world wide.[13,23] It is important to note that this microarray is not intended to replace phenotypic testing in diagnostic and clinical settings, although there has been considerable progress in the development of diagnostic microarrays.[2,4,15,31] Microarray data are difficult to interpret in a clinical setting because the detection of a gene is a potentially nebulous result. Indeed, genes detected may not be functional or expressed, and negative hybridization results are even more difficult to interpret, as previously uncharacterized resistance mechanisms could lead to failure of a selected treatment. In the clinical setting, the goal is to select a successful treatment regimen, thus making phenotypic testing more informative than gene detection. However, when the goal is to study the molecular epidemiology of AR, DNA microarrays are an exceptional tool for detecting multiple AR genes in a single assay.

In the present study, a DNA microarray was designed to detect as many resistance genes as possible for use as a screening technique in studies of prevalence, epidemiology, and spread of resistance genes. The National Center for Biotechnology Information (NCBI) databases and simple bioinformatics processes were used to build a local database of target gene sequences. Unique oligonucleotide probes were designed for the detection of each AR-associated gene in this database.[13,43] Probes 70 bases in length were chosen because of their ability to tolerate possible mismatches within the probe regions and to detect as many alleles of resistance genes as possible. These sequences were used to construct the microarray utilizing the most widely available microarray format, glass slides, which was then tested on control strains and a variety of MDR bacteria. The results demonstrate that these simple and relatively inexpensive techniques yielded a highly useful research tool to study the epidemiology of AR genes in a wide range of important bacteria. This report supplies researchers in the field with information they can use to build their own arrays and improve upon this technique.

Materials and Methods

Identification and selection of target genes

Previous work demonstrated that oligonucleotide microarrays can be used to detect resistance genes in a wide variety of bacteria.[13,25,26,37,41] The major short coming of these microarrays as well as our previous array (described in Frye et al.[13]) was the limited number of genes they were designed to detect and thus the limited number of bacteria they could be used to investigate. To design a microarray representing the most comprehensive set of genes associated with AR, sequences available in the NCBI GenBank database were identified by employing several database search strategies. A query designed to yield the maximum number of nonredundant genes annotated as "bacteria & antibiotic & resist*" yielded 3,391 genes from the nonredundant DNA sequence database as well as 1,115 genes from the translated protein database. All of these sequences were sorted by nucleotide coding sequence enabling the identification and elimination

of identical duplicate genes, leaving 3,751 genes. These sequences were downloaded into a local database, and 70mer oligonucleotides were designed as described below.[35,43] All probe sequences likely to detect a homologous gene in the probe set (>90% identity over the length of the 70mer) were eliminated from the list for a final total of 1,224 gene probes. Probe sequences that would detect any of the 94 genes from our previous work (Frye et al.[13]) were excluded, as those previously designed probes were included on the new microarray and used as working control probes.[13] Next, the annotation of each gene was examined, and genes not likely to be related to AR or AR gene transfer were deleted leaving 681 genes. The sequences of these probes as well as the probes from the previous array (total $n = 775$) are included in Supplemental Table S1 (available online at www.liebertonline.com/mdr). Finally, the probe sequences were BLAST-Like Alignment Tool (BLAT) searched against the entire NCBI database to identify all close homologs to which they would likely hybridize.[18] This yielded 24,489 genes in the complete NCBI database that were likely to be detected by the microarray at the time of the query (data not shown).

Oligonucleotide probe design and microarray construction

Sequences for the 681 genes were used to design an optimized unique 70mer oligonucleotide probe for each gene using the program OligoWiz 2.0 following methods and settings recommended by the authors (probe sequences are presented in Supplemental Table S1).[35,43] Oligonucleotide probes of this length were selected, as previous work had demonstrated that 70mers have good specificity, can tolerate several nucleotide mismatches in the target sequence, require no chemical modification to adhere to the slide surface, and give good results without the need for problematic labeling or amplification of DNA samples.[11,13,19,23] These probes were synthesized (Operon, Huntsville, AL), diluted in printing buffer (35 µM in 50% dimethyl sulfoxide [DMSO]), and arrayed in triplicate onto UltraGaps amino silane–coated slides (Corning, Life Sciences, Acton, MA) with an Omnigrid robot (Genemachines, San Carlos, CA), and postprocessed as previously described.[13,38] As stated above, the 94 probes from our test microarray were added for a total of 775 unique AR-associated genes.[13] Additional controls included 3 positive control probes designed to detect bacterial 16s rDNA sequences,[22] 2 Cy3-labeled and 1 Cy5-labeled Lambda DNA controls, 12 buffer (50% DMSO) only spots, and 130 empty (background) spots. Twelve probes were also synthesized in duplicate or triplicate as controls (indicated in Table 1 and Supplemental Table S1 with an "*").

Strains, growth conditions, and antimicrobial susceptibility

The fully sequenced control strains were *Salmonella enterica* serovar Typhimurium LT2[28] and *Salmonella enterica* serovar Typhi CT18 (*S.* Typhi CT18).[36] *Enterococcus* control strains obtained from the American Type Culture Collection (ATCC, Manassas, VA) are indicated by their ATCC numbers in Tables 1 and 2. Test isolates of *Salmonella* serovars, *Escherichia coli, Enterococcus* spp., *Campylobacter coli, Listeria innocua*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Clostridium difficile* were obtained from the National Anti-

TABLE 2. ANTIMICROBIAL SUSCEPTIBILITY PHENOTYPES OF TEST ISOLATES USED IN THIS STUDY

Antimicrobial class	Antimicrobial	SE Tc JF201[b]	SE Hd JF210	EC JF220	EC JF227	CC 14-22	CC 141-27	ECF ATCC25788	EGM ATCC49573	LI03	LI04	MRSA G10-C87-2	MRSA DN	MRSA 36-1	MRSA 59	CD 70	CD 98	CD 112	CD 113
Aminoglycosides	AMK	S	S	S	S			S	ID						S				
	APR	R	S	S	S			S	ID						S				
	GEN	R	R	R	R	S	S	S	ID	S	S	S	S	S	S				
	KAN	R	R	R	R		S	S	ID	S	S	S	S	S	S				
	STR	R	R	R	R	S	S	S	ID			S	S	S	S				
Bacitracin	BAC							R	R										
Beta-lactams	AMC	R	R	R	R	S	S				S	R	R	R		S	S	S	S
	AMP	R	R	R	R					S	S	R	R	R	R	S	R	S	S
	CEF	R	R	R	R														
	CRO	S	I	S	I		S			R	S	R	R	S	R				
	FEP					S													
	FOX	R	R	R	R	S	S			R	R	R	R	R	R				
	IPM					S	S												
	OXA			S	R					R	R	R	R	R	R				
	PEN							S	R	R	R	R	R	R	R				
	TIO	R	R	R	R														
Chloramphenicol	CHL	R	R		R	S	S	S	S										
Phosphoglycolipid	FLA							R	ID										
Glycopeptides	VAN							S	I	S	S	S	S	S	S	S	S	S	S
Lincosamides	CLI					S	R			R	S	R	R	R	R	R	R	S	R
	LIN							R	R							S	S	S	S
Lipopeptide	DAP									S	S	S	S	S	S	S	S	S	S
Macrolides	AZM					S	R	I	S										
	ERY					S	R	S	S	S	S	R	R	R	R	S	R	S	S
	TYL																		
Metronidazole	MET															R	R	R	R
Nitrofurantoin	NIT							S	S										
Oxazolidinone	LZD							S	ID	S	S	S	S	S	S	S	S	S	S
Quinolones	CIP	S	S	R	R	S	S	I	S	S	S	R	S	R	S				
	GAT									S	S	R	S	S	S				
	LEV									S	S	R	S	S	S	S	S	S	S
	NAL	S	S	R	R	S	S												
Rifamycin	RIF							I	ID	S	S	S	S	S	S	R	S	R	S
Streptogramin	Q-D									S	S	S	S	S	S				
Sulfanilamide	NIL	R	R	R	R														
Tetracycline	TET	R	R	R	R	S	R	S	R	R	S	S	S	R	S	S	S	R	
Trimeth/sulfa	SXT	R	R	S	S	S	S	R	S	S	S	S	S	S	S				

Clinical and Laboratory Standards Institute (formerly NCCLS) breakpoints were used to determine resistance phenotype.

Antimicrobials: AMK, amikacin; APR, apramycin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; BAC, bacitracin; AMC, amoxicillin–clavulanic acid; AMP, ampicillin; CEF, cephalothin; CRO, ceftriaxone; FEP, cefepime; FOX, cefoxitin; IPM, imipenem; OXA, oxacillin; PEN, penicillin; TIO, ceftiofur; CHL, chloramphenicol; FLA, flavomycin; VAN, vancomycin; CLI, clindamycin; LIN, lincomycin; DAP, daptomycin; AZM, azithromycin; ERY, erythromycin; TYL, tylosin; MET, metronidazole; NIT, nitrofurantoin; LZD, linezolid; CIP, ciprofloxacin; GAT, gatifloxacin; LEV, levofloxacin; NAL, nalidixic acid; RIF, rifampin; Q-D, quinupristin–dalfopristin (Synercid); NIL, sulfanilamide; TET, tetracycline; SXT, trimethoprim–sulfamethoxazole.

Isolates: SE, Salmonella enterica; Hd, Heidelberg; Tc, serovar Typhimurium variant Copenhagen; EC, Escherichia coli; CC, Campylobacter coli; ECF, Enterococcus casseliflavus; EGM, Enterococcus gallinarum; LI, Listeria innocua; MRSA, Methicillin Resistant Staphylococcus aureus; CD, Clostridium difficile.

R, resistant; S, susceptible; I, intermediate; ID, indeterminate (due to lack of CLSI standard); empty block, not assayed.

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microbial Health Monitoring System (NARMS) bacteria collection. Phenotypic analyses were conducted as previously described (www.cdc.gov/narms/). Bacteria were grown from frozen stock cultures stored at −70°C by standard methods with appropriate media. *Salmonella* and *E. coli* were grown in the Luria Bertani (LB) medium, on LB agar, or blood agar plates (BAP) at 37°C as indicated. *C. coli* were grown on Campy-cefex plates and incubated at 42°C for 48 hours under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) in zip-top storage bags. *Enterococcus* spp., MRSA, and *L. innocua* were grown in LB, in brain heart infusion, or on BAP at 37°C with standard methods. *C. difficile* was grown on BAP at 37°C in an anaerobic atmosphere generated by AnaeroGen gas pack system (Oxoid Unipath, Basingstoke, United Kingdom).

Susceptibility testing for *Salmonella, E. coli, Enterococcus* spp., *L. innocua*, and MRSA were performed using custom-made broth microdilution plates for the Sensititre™ System (TREK Diagnostic Systems, Westlake, OH). Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards) guidelines for interpretation and recommended quality control organisms were used when available.[32,33] *C. coli* isolates were tested following CLSI guidelines using the *E*-test (AB-Biodisk, Piscataway, NJ) or Sensititer.[32,33] Susceptibility of *C. difficile* was performed by *E*-test (AB-Biodisk) following CLSI guidelines.[32,33]

DNA extraction and labeling

Genomic DNA from *Salmonella, E. coli, Enterococcus* spp., *L. innocua*, and MRSA isolates was extracted from 5 ml of overnight cultures grown in LB (Gram-negative bacteria) or brain heart infusion (Gram-positive bacteria) broth using the GenElute Bacterial Genomic DNA kit (Sigma, St. Louis, MO) as described previously.[13] *C. coli* genomic DNA was isolated from colonies collected from Campy-cefex plates using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to manufacturer's directions. *C. difficile* DNA was extracted from colonies collected from BAP using the UltraClean Microbial DNA Isolation Kit (MoBio Lab, Carlsbad, CA), following manufacturer's instructions for Gram-positive DNA isolation. DNA (1.5 μg) was labeled with either Cy-5 dye– or Cy-3 dye–labeled dCTP (Amersham, Piscataway, NJ) via random priming and extension with Klenow fragment (New England Biolabs, Beverly, MA), followed by purification with a Qiagen PCR cleanup kit (Qiagen, Valencia, CA) as previously described.[39]

Hybridization and scanning

Dye-labeled DNA (1.5 μg) was dried and resuspended in 80 μl of hybridization buffer (25% formamide, 5× sodium chloride sodium citrate [SSC], 0.1% sodium dodecyl sulfate [SDS], and 1% bovine serum albumin), boiled 5 minutes, and applied to the microarray under a LifterSlip (Erie Scientific, Portsmouth, NH). Hybridizations were performed overnight in a hybridization chamber (Corning) submerged in a 42°C water bath in the dark. Protocols suggested by the manufacturer for hybridizations in formamide buffer were used for prehybridization, hybridization, and posthybridization wash processing (step 1: 2×SSC, 0.1% SDS, 5 minutes at 42°C; step 2: 0.1×SSC, 0.1% SDS, 10 minutes at room temperature; step 3:

4×0.1×SSC, 1 minute at room temperature). Microarrays were scanned with a ScanArray Lite Laser scanner (PerkinElmer Life and Analytical Sciences, Waltham, MA) using ScanArray Express 3.0 software or with a GenePix 4100A (Molecular Devices, Sunnyvale, CA) and GenePix Pro software.

Data analysis

Images were analyzed and quantified using ScanArray Express 3.0 software (PerkinElmer). Hybridization signal intensities were measured in arbitrary intensity units (IU) by adaptive quantification mode followed by local background subtraction, and the median of the triplicate gene probes was recorded. The control strain hybridizations were used to evaluate methods for interpreting the quantitative data. Optimum calls were achieved by setting a cutoff at two times the median of intensities for all probes for each hybridization. Since the great majority of probes should not hybridize to a sample, this was an accurate measure of background (no hybridization) or cross hybridization (hybridization to nonhomologous DNA sequences) by a sample lacking a particular gene for all probes on the array. Therefore, all genes with a median intensity of its triplicate probes greater than two times the median intensity of all probes on the chip were scored as present. Additionally, hybridizations with no positive controls detected (less than one out of three 16s rDNA gene probes), too many negative controls detected (greater than two out of twelve 50% DMSO spots), or with obvious hybridization anomalies (streaks, spots, blotches, etc.) were considered as failed and were repeated.

Results

Validation of the AR gene microarray construction

The new AR gene probes were arrayed along with the 94 probes from our previous study that demonstrated the detection of resistance genes with 70mer oligonucleotides.[13] This approach allowed the 671 new probes to be tested and compared directly to the previously verified oligonucleotide probes. After printing and postprocessing, the arrays were evaluated by DNA staining.[3] Scanning and image analysis demonstrated proper quantities of oligonucleotides in spots, good spot morphology, detection of positive controls and fluorescence dye controls, and negative controls below detection limits (data not shown).

Hybridization of control strains to the AR gene microarray

Initial test hybridizations were performed using two *S. enterica* strains with published complete genome sequences: serovar Typhimurium strain LT2 (antimicrobial sensitive) and serovar Typhi strain CT18 (harboring the pHCM1 MDR plasmid).[28,36] Labeled LT2 DNA hybridized strongly to the three 16s rDNA positive control spots but not to any of the buffer-only or negative control spots on the microarray (background hybridization controls). Ten gene probes also had positive hybridizations (Supplemental Table S1 and summarized in Table 1), and all but one of these probes were found to be 100% (70/70 bp) identical to genes in the LT2 genome using a low stringency basic local alignment search tool (BLAST) of all probe sequences against the LT2 genome sequence (settings: discontinuous megablast, short queries,

word size 11 bp, match/mismatch scores 2, gap costs −3, and template length 16 bp). Probe AR2-1-0032 also showed a positive hybridization, but a BLAST search failed to find any homologous sequence in LT2. This probe was designed to detect the *marC* gene (a homolog of the family of multiple antibiotic resistance proteins) from *E. coli* (gi | 1170657:c1234-569). Conversely, nine other gene probes were found to have similarity to sequences within the LT2 complete genome, but did not hybridize above the cutoff. These probes had identity to LT2 sequences ranging from 73% to 91%. Alignments of the probe sequences to the LT2 genes in most cases revealed nucleotide mismatches along the length of the alignments that could lead to a lack of hybridization (data not shown). Five of these genes were also homologous to members of the *marBARC* multiple antibiotic resistance operon.[20]

Hybridizations of labeled *S.* Typhi CT18 DNA to the microarray resulted in positive hybridizations to all three 16s rDNA probes and to 95 gene probes; 92 of those were found to be homologous to genes in the CT18 genome ($n = 14$) or encoded by the pHCM1 MDR plasmid carried by CT18 ($n = 78$) (Table 1 and Supplemental Table S1). One hundred percent identity to CT18 or pHCM1 genes was found for 78 out of the 92, while 14 had identity ranging from 77% to 99%. Three gene probes with positive hybridizations were not found to be homologous to the CT18 or pHCM1 genome by BLAST, and five gene probes were found to have homologous sequences in CT18 by BLAST, but no hybridization was detected. Included in the positive hybridizations were the same 12 probes with positive hybridizations to CT18 from previous microarray experiments.[13] These were probes for the genes *aph6 (strB), dfrA1, aph3″ (strA)*, tnpA, bla*TEM*, intI1*, tnpM, aph3″ (strA)*, aadA1, sulII, intI1*,* and *cat4* (* indicates probes synthesized in duplicate). New microarray probes with positive hybridizations included *tet*(A) and homologs of *dfr1B, tnpR, strA, strB, dfrV, intI1, sulII, catIII, bla*TEM-1*, acc(3), bla, bla*PER3*, bla*TEM-13*, tnpA, tet*(B), *cat,* and several pHCM1 genes, including conjugation genes ($n = 15$), heavy metal resistance genes ($n = 6$), and plasmid replication genes ($n = 3$) (Supplemental Table S1 and summarized in Table 1). Overall, the new array detected all resistance genes in *S.* Typhi CT18 and also detected genes indicating the presence of an MDR plasmid (pHCM1).[36]

AR gene microarray analysis of test strains

To assess the ability of the microarray to detect AR genes in unsequenced bacteria, several of the test strains used in the previous work were re-evaluated with the new microarray as well as additional new isolates (Table 1). Hybridization results for all strains are summarized in Table 1, and complete hybridization results are available in Supplemental Table S1. Two *Salmonella* MDR strains (*Salmonella* Typhimurium JF201 and *Salmonella* Heidelberg JF210) were analyzed. In addition to strong hybridization signals to the 16s rDNA probes, many gene probes had positive hybridizations to these MDR strains with 150 IU above the cutoff for JF201 and 170 IU above the cutoff for JF210. Positive hybridizations for JF201 included 16 out of the 17 genes detected by the previous array; the *aacC1* gene probe (AR1-0025) was not above the cutoff. An additional 137 probes had positive hybridizations. These included probes designed to detect resistance genes for aminoglycosides ($n = 31$), β-lactams ($n = 12$), chloramphenicol ($n = 6$), quaternary ammonium ($n = 3$), sulfanilamide ($n = 5$), tetra-

cycline ($n = 10$), trimethoprim ($n = 4$), and heavy metals ($n = 6$). Twenty genes potentially involved in horizontal gene transfer such as integrons, transposons, and plasmid functions also had positive hybridization signals as well as 40 genes with no known function but associated with AR by their NCBI annotation. Hybridization of JF210 to the array yielded similar results. All but 1 probe of the 19 probes from the previous array (the *vanR* gene, AR1-0028) had positive hybridizations along with an additional 152 probes of the new array. Those include probes for genes encoding resistance to aminoglycosides ($n = 38$), β-lactams ($n = 12$), chloramphenicol ($n = 8$), efflux pumps ($n = 11$), quaternary ammonium ($n = 5$), sulfanilamide ($n = 5$), tetracycline ($n = 11$), trimethoprim ($n = 4$), and heavy metals ($n = 14$), as well as genes related to integrons ($n = 4$), transposons ($n = 19$), plasmid transfer ($n = 3$), and 35 other genes annotated as associated with AR.

E. coli isolates were analyzed with the array, and hybridizations were very strong to the 16s rDNA–positive control probes, indicating excellent labeling and hybridization efficiency of *E. coli* DNA samples. Isolate JF220 had positive hybridizations to genes for resistance to aminoglycosides ($n = 28$), β-lactams ($n = 18$), chloramphenicol ($n = 15$), efflux pumps ($n = 15$), quaternary ammonium ($n = 3$), sulfanilamide ($n = 5$), tetracycline ($n = 8$), trimethoprim ($n = 2$), and heavy metals ($n = 13$), and genes associated with integrons ($n = 7$), transposons ($n = 15$), as well as 45 other genes annotated as AR related. JF227 presented similar results with probes detecting resistance genes for aminoglycosides ($n = 24$), β-lactams ($n = 16$), chloramphenicol ($n = 6$), efflux pumps ($n = 16$), quaternary ammonium ($n = 3$), sulfanilamide ($n = 5$), tetracycline ($n = 6$), trimethoprim ($n = 2$) and heavy metals ($n = 8$), integrons ($n = 8$), transposons ($n = 8$), as well as 38 other genes annotated as associated with AR.

C. coli isolates assayed with the microarray hybridized above the cutoff to only two of the positive control 16s rDNA probes (16S-1333_1402 and 16S-507_576). BLAST analysis of the third 16s probe (16S-93_162) revealed only very short regions of identity to the published *C. coli* 16s rDNA gene, explaining hybridization below the cutoff (data not shown). Eighteen gene probes with hybridizations above the cutoff for *C. coli* isolate 14–22 included aminoglycoside resistance (*aphA-3* and *aad9*), β-lactam resistance (*cam1* and *cifA*), tetracycline resistance (*tet0*), efflux pumps (*cmeB, cmeC, hdsR,* and *marR*), and plasmid transfer (*btgA* and *thrU*). *C. coli* isolate 141–27 showed positive hybridizations for *aphA-3, aph, cam1, blaL2 cmeB, cmeC, tetO, htdT* (plasmid transfer), and *hsdR.* These positive hybridizations are also very similar to the results of *C. jejuni* hybridizations obtained with the previous AR microarray where *aph, cam1, cmeB, cmeC,* and *tetO* gene probes were positive.[13]

E. casseliflavus ATCC25788 and *E. gallinarum* ATCC49573 were analyzed. Both strains had hybridization signals well above the cutoffs for the positive control 16s rDNA probes. Both strains, used as positive controls for vancomycin resistance, hybridized to probes for the *vanC* gene. Each strain hybridized to several probes for transposons (*trans* and *tnpA* in ATCC25788; *trans, tnpA,* and *tnpR* in ATCC49753) and AR-associated gene sequences (*hur* and *murT* in ATCC25788; *tetO, tetM, tetS,* and *blaL2* in ATCC49753).

L. innocua isolates were also tested. These strains hybridized to all three 16s rDNA gene probes as well as to several

other probes. Strain LI03 and LI04 hybridized to tetracycline resistance probes *tetO* and *tetM*, efflux gene probes (*marC* and *ybiT*), several resistance transfer genes (IS1542, *recP*, p9123, and Tn5), six *Listeria*-specific genes, as well as several genes annotated as being resistance associated. LI03 also hybridized to a β-lactamase gene (*bla*_{TEM-1}).

MRSA isolates were tested with the microarray. Only two 16s rDNA gene probes were detected with a very strong hybridization. All four MRSA isolates had hybridizations to as many as 51 Staphylococcal methicillin resistance chromosomal cassettes (*mec*) gene probes. MRSA isolate G10-C87-2 had 51 probes with positive hybridizations, 42 of which were *mec* gene probes. These included probes for genes involved in resistance to β-lactams (*mecA, mecI*, and *mecR1*) and erythromycin (*ermA*). MRSA isolate DN had 51 positive hybridizations, 42 of those were *mec* gene probes. These included β-lactam resistance (*mecR1, mecA, spe, pre*, and *blaI*), erythromycin resistance (*ermA*), and aminoglycoside resistance (*aphA-3* and homologs). MRSA isolate 36-1 had 33 positive hybridizations, including 5 *mecA* β-lactamase genes as well as several MRSA-associated genes (*mecR1, pre, ccrA*, and *ccrB*), 7 aminoglycoside resistance genes related to *ahpA-3*, macrolide resistance gene *ermC*, 13 resistance-associated genes, and 3 transposase-associated genes. MRSA isolate 59 presented similar results with 30 positive hybridizations, 23 to *mec* genes including β-lactam resistance and aminoglycoside resistance; however, there were no *erm* genes detected in MRSA 59.

C. difficile isolates 70, 98, 112, and 113 were tested with the microarray. All three 16s rDNA probes were detected in each isolate; however, only two resistance genes were detected. In *C. difficile* isolate 98 *orfE*, a gene annotated as plasmid encoded and in isolate 112, *blaL2* was detected, both of which showed homology to β-lactamase genes. Probes for several other genes annotated as related to resistance also had positive hybridizations for these isolates, including *tet(C)* in isolate 112.

Antimicrobial susceptibility of isolates tested

All of the test strains used to evaluate the microarray were analyzed for susceptibility to various antimicrobial compounds (Table 2). Although phenotypic testing is not necessarily expected to correlate well with gene detection, agreement between the two techniques was surprisingly high. Out of 266 phenotypes tested, 203 (76.3%) were in agreement with genes detected. This included 71 (26.7%) where resistant phenotypes agree with one or more gene probe hybridizations, and 132 (49.6%) where sensitive phenotypes had no genes detected. Nonconcordant results were found for 63 (23.7%) detected phenotypes as compared to the microarray data. These fell into two categories, with 26(9.8%) having positive hybridizations to a resistance gene but no corresponding phenotype was detected, and 37(13.9%) where an isolate was resistant but no genes explaining the phenotype were detected.

Discussion

In this study, standard techniques were used to build a glass slide microarray for the detection of a large representation of all sequenced AR genes. Previous work by our group and many others has established oligonucleotide microarrays as an easy approach for high-density screening for AR genes.[2,13,24,26,30,37,42,44] Therefore, the primary challenge of the current study was to identify the resistance genes to be detected by this assay. This project used simple key word searches to compile a list of genes associated with AR based upon their NCBI annotations. This list was pared down by deletion of identical genes from different organisms, deletion of genes unlikely to be associated with resistance based upon their annotation, and deletion of probes for these genes with greater than 90% homology. This approach led us to the construction of a microarray with probes for the detection of 775 AR or resistance-associated genes.

Control and experimental hybridizations demonstrated that the array could detect a wide variety of resistance genes in diverse Gram-positive and Gram-negative bacteria. Phenotypic data correlated with the array data over 76% of the time, which was an interesting result considering the differences between the two assays, the diversity of the bacteria tested, and the variety of antimicrobial compounds tested. It is not surprising that these assays sometimes yield different results. Phenotypic testing does not include an induction step and could suffer from false-negatives when a bacterium's resistance genes are tightly regulated. The microarray assay developed in this study also lacks probes for some resistance genes and can also yield false-negative results. This occurred if a gene was absent from the NCBI database, if gene was incorrectly annotated, if the gene was erroneously deleted during review of the annotations, or if the resistance was due to a point mutation of an endogenous gene (e.g., most quinolone resistances). For example, we failed to identify resistance genes in several of the Gram-positive bacterial species (e.g., *Listeria, Clostridium*, and *Enterococcus*).[8,37,41] This probably reflects that resistance mechanisms in some of these bacteria have not been well studied, so few resistance genes attributed to them have been added to the database or properly annotated. We have also discovered that our array design missed several resistance genes identified by other researchers for macrolide, lincosamide, and streptogramin resistance.[8] A comparison of their list of genes and our list showed that our array lacked 14 out of the 23 genes identified in their study.[8] By reviewing the files generated during our design process, we found that probes for those genes had been discarded during inspection of the annotations because the sequences were annotated as RNA-methylases and not as macrolide resistance RNA-methylases. These problems will be remedied by periodic searches of the NCBI database to identify reannotated and newly discovered genes as they are submitted to NCBI. This is easily facilitated by setting up an automatic e-mail notification from NCBI. Probes for these and other omitted genes will be added to future versions of the microarray as well as probes published by other research groups.

This microarray is designed to detect as many AR-associated genes as possible. This is illustrated by some of the results of the test strains where multiple gene probes for a single class of resistances have positive hybridizations. For example, strain JF201 had positive hybridizations to 31 aminoglycoside resistance gene probes. However, these genes are fairly homologous, and it is expected that only one or a few of these genes are present in this strain.[17] In cases where the research project must identify specific alleles, data from the microarray can be used to determine a set of polymerase chain reaction primers necessary to identify an allele by amplification and sequencing.

The current array was not designed to detect single-nucleotide polymorphisms (SNPs) that are responsible for

specific alleles or for specific phenotypic resistances. For example, resistances due to mutations of endogenous genes such as the quinolone resistance determining region of *gyrA* leading to nalidixic acid or fluoroquinolone resistance would require detection of SNPs.[44,45] Similarly, SNP detection would be necessary to detect changes resulting in macrolide resistance in *Campylobacter* spp. due to mutations in ribosomal protein genes or 23S rDNA.[6,21,27] Another example would be β-lactam resistance, which is complex because alleles differing by only one of a few SNPs can result in different phenotypes. Thus, a β-lactamase gene (e.g., *bla*$_{TEM-1}$) encoding resistance to ampicillin and an extended spectrum β-lactamase gene encoding resistance to ampicillin and also to third- and fourth-generation cephalosporins (e.g., *bla*$_{TEM-37}$) differ only by an SNP. Several groups have presented microarrays capable of better discriminating specific alleles and even SNPs.[14,26,31,40] However, these arrays exhibit some limitations. Short oligonucleotide arrays printed on glass slides are more specific, but require the additional expense of modified oligonucleotides, and samples may require amplification with specific primers during labeling that would be problematic for an array designed to detect nearly 1,000 genes. Tiling arrays (e.g., Affymetrix, Inc., San Francisco, CA, and Roche Nimblegen, Inc., Madison, WI) with perfect match/mismatch probes have shown the most promise, but are cost prohibitive for most laboratories as design costs and specialized hybridization and scanning equipment are not as widely available as microscope slide arrays and scanners. The ArrayTube (Clondiag GmbH, Jena, Germany) format is also promising and could potentially be developed for diagnostic assays within the limitations of the utility of gene detection versus phenotypic testing data in the clinical setting.[2,31] However, it also requires specialized equipment and exclusive contracts with the company, and is limited to 196 probes per assay. Our design and construction process uses the most freely available databases, bioinformatics, techniques, and equipment to produce an array with the widest application at the lowest possible cost to researchers.

High-throughput sequencing may soon offer an alternative to microarray techniques, but that technology will continue to be out of reach for most researchers in the near term. Currently, microarray detection of AR genes presents the most comprehensive tool for studying the genetic causes of resistance. Even with the limitations of allele discrimination and SNP detection, it is a very powerful tool and has begun to demonstrate its utility in a variety of research studies.[2,5,7,9,10,13,24,29,37,46] In addition, the high density of data provided by microarrays also allows analysis of gene prevalence by new bioinformatic tools such as cluster analysis and linkage disequilibrium, which may be helpful in identifying factors leading to development and spread of AR.[29,46] This type of data will be necessary in the development of possible strategies to prevent increasing AR and its spread to human infections. It is our hope that other researchers will find these methods useful in their studies, and that they will continue to improve upon the technique and expand the catalog of resistance genes detectable by microarray analysis.

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Disclosure Statement

All authors state that no competing financial interests exist.

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