The background of the entire page is a close-up photograph of several petri dishes containing bacterial cultures. The cultures show various patterns of growth, including streaks and colonies, in different colors like pink, purple, and blue. The lighting is dramatic, with strong highlights and deep shadows, creating a sense of depth and focus on the microbial world.

Assessing the Microbial Enemy's Antibiotic Defenses on the Genetic Level...with Next-day Service

Antibiotic resistance is a serious public health problem; annually, 63,000 people die in the U.S. alone from antibiotic-resistant infections. Multidrug resistant (MDR) infections can overwhelm a wounded patient before an effective antibiotic is determined and administered, and using the wrong antibiotic can increase the opportunity for the infection to do so. The economic toll is immense as well: extended hospital stays, lost productivity, and the need for additional treatment.

And the toll on military readiness is untold. Every military commander asks “what are we up against here?” but when the enemy is a pathogen, NRL's Antimicrobial Resistance Determinant Microarray (ARDM) can provide the answer, and with a one-day turnaround. ARDM's singular ability is determining the genetic-based antibiotic resistance of a sample without knowing in advance the bacterial species being assayed. Using a small reader and minimal specialized equipment, ARDM is able to simultaneously detect hundreds of antibiotic genes and produce results of the assays with relative speed.

Working with Walter Reed Army Institute of Research, Mercy Hospital (Sierra Leone), and Naval Medical Research Unit-3 (Cairo), NRL researchers have used the ARDM to study antibiotic resistance on a geographical scale, and have made some startling discoveries: there are geographical differences in the resistance genes, and even worse, some of those genes confer resistance to entire classes of antibiotics. ARDM is proving invaluable as a surveillance tool in forward laboratories; knowing in advance of treatment the antibiotic resistance of the pathogens likely to be encountered can and will help save lives.



Molecular Epidemiology of Global Antimicrobial Resistance

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The emergence and spread of antibiotic resistance present a serious challenge to modern day public health. To identify resistance potential in less than a day, we have developed the Antimicrobial Resistance Determinant Microarray (ARDM) to provide DNA-based analysis for over 250 resistance genes, including those for last-resort antibiotics used to treat war trauma-associated infections. The breadth of the ARDM's content has allowed us to detect rare, emerging, or unexpected resistance genes without prior knowledge of bacterial species. A collaborative study with Walter Reed Army Institute of Research, Mercy Hospital (Sierra Leone), and Naval Medical Research Unit-3 (Cairo) showed geographic differences in resistance genes that can be used to track the prevalence and spread of antimicrobial resistance. More concerning was the presence of several genes conferring resistance to entire classes of antibiotics. This information is critical in steering medical personnel towards strategies with the highest chances of success when treating individuals deployed to those regions.

INTRODUCTION

The emergence and spread of antibiotic resistance presents a serious challenge to modern day public health. Resistant organisms are implicated in more than 63,000 deaths in the United States per year, with European Union estimates of economic losses ranging 1.5 billion € per year in lost productivity, additional treatments, and extended hospital stays.¹ Military readiness is also affected by the prevalence of resistant bacterial pathogens. Infectious diarrhea affects between 25% and 80% of deployed personnel at least once during deployment, with drug resistant strains on the rise.² Furthermore, an alarming increase in the number of multidrug resistant (MDR) infections has been noted in military personnel wounded in Operations Iraqi Freedom (OIF), New Dawn (OND), and Enduring Freedom (OEF). Estimates of MDR in non-diarrheal pathogens have exceeded 80% within some military treatment facilities.³

Currently, the standard approach for treating bacterial infections with antibiotic compounds is empirical. If the pathogens causing infection are not sensitive to the applied therapy, the delay in application of an effective treatment may cause the infection to become worse and possibly spread. Moreover, the growth of resistant strains may be encouraged due to decreased competition from sensitive strains, or from the stress-induced exchange/transfer of resistance genes. Thus, it is very important to have a full picture of the antimicrobial resistance status of both clinical isolates and local baseline patterns of resistance to aid medical

personnel in making timely, more effective therapeutic decisions.

Despite these long-standing needs, there does not exist a single comprehensive surveillance tool that can rapidly generate antimicrobial resistance profiles. Standard microbiological methods involving growth in the presence of antibiotics may take several days until results are available. More rapid molecular methods, such as polymerase chain reaction (PCR), are limited by the number of individual or multiplexed reactions that can be performed simultaneously and require a priori knowledge. On the other hand, DNA microarrays, which consist of an array of spatially localized oligonucleotide probes and use nucleic acid hybridization to enable the simultaneous interrogation of hundreds to thousands of genetic elements, provide unprecedented high-throughput analysis capabilities for the detection of antibiotic resistance genes in a single test.

THE ANTIMICROBIAL RESISTANCE DETERMINANT MICROARRAY (ARDM)

Scientists at the Naval Research Laboratory's Center for Bio/Molecular Science and Engineering have developed the ARDM to provide the military's forward laboratories with the capability to detect hundreds of antibiotic resistance genes simultaneously using a small reader and minimal specialized equipment. The ARDM chip has been designed to test four samples for 278 different resistance genes at the same time. Each of the four subarrays on the chip harbors 2,240 different DNA oligonucleotide "capture" probes immobilized on an

array of microelectrodes. Furthermore, each of the 278 resistance genes targeted by the ARDM is represented by 10 unique probes that were designed to hybridize to conserved and nonconserved portions of each targeted gene. In this manner, identical, closely related, and less conserved versions of the resistance determinants can be detected. Genes represented on the ARDM include those conferring resistance to 12 different families of antibiotics and are derived from species most commonly associated with hospital-acquired infections and war wound infections from Iraq and Afghanistan, as well as the most often encountered diarrheal pathogens (Fig. 1). A key advantage of the ARDM's broad coverage is elimination of any need for a priori knowledge of what might be present (which is required for PCR).

breadth of the ARDM's content, allows us to detect rare, unexpected, or emerging resistance genes without knowing or assuming what might be present.

GLOBAL SURVEILLANCE OF ANTIBIOTIC RESISTANCE

We have conducted studies to determine the prevalence and spread of resistance determinants in multiple geographic regions to monitor the global evolution of MDR in militarily relevant pathogens. For this purpose, we have initiated collaborative studies with the Walter Reed Army Institute of Research/Multidrug Resistant Organism Surveillance Network (WRAIR/MRSN), Mercy Hospital (Bo, Sierra Leone), and Naval Medi-

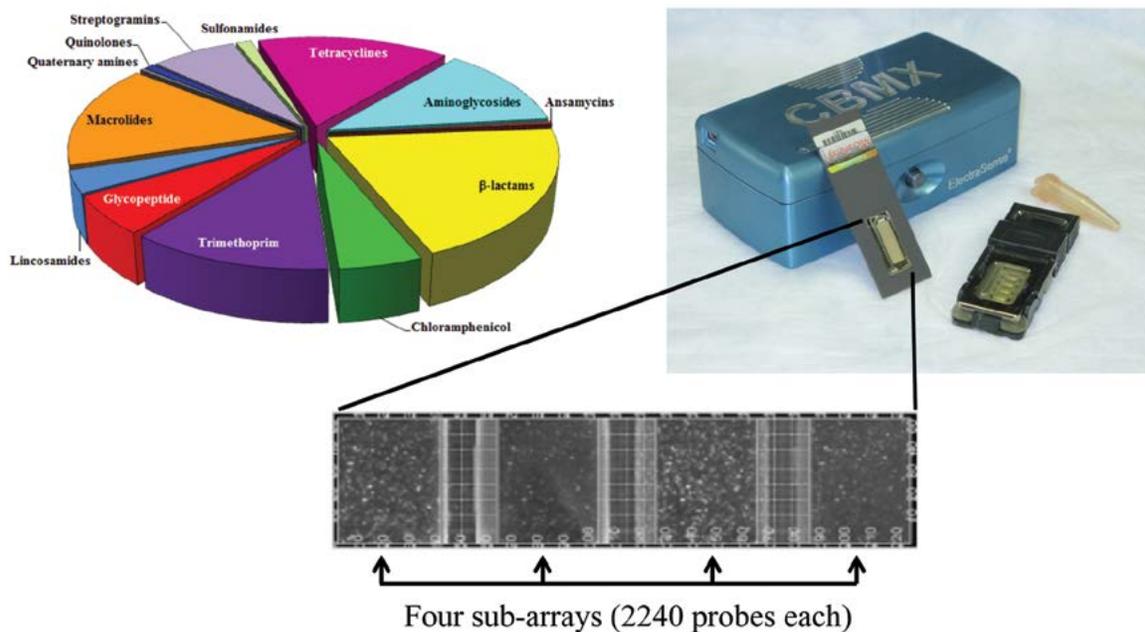


FIGURE 1 Clockwise, from right: ARDM chip with reader in the background; false-color image of the signals from four samples analyzed on the same chip (four subarrays/chip); pie chart of resistance gene content on each subarray [2,240 oligonucleotide probes; shown are different classes of antimicrobial compounds and mechanisms of resistance]. (Photo courtesy of Dr. Joel Golden, NRL)

Extracted DNA samples to be analyzed on the ARDM are processed using an unbiased amplification method, fragmented for efficient hybridization to the microarray probes, and then labeled with a tag that allows the bound DNA to be detected. After hybridization to the microarray, the DNA-DNA hybridization duplexes are detected electrochemically using a small, brick-sized reader that is commercially available. The time-to-result ranges from 11 to 24 hours depending on the length of hybridization, and beyond the small reader, only standard microbiological lab equipment is needed for all processing steps. Furthermore, the unbiased amplification method, combined with the

cal Research Unit-3 (NAMRU-3; Cairo, Egypt). Of the samples obtained from WRAIR/MRSN, approximately 20% were *Acinetobacter* isolates from war wounds. All of the samples collected to date have been selected for their resistance to common antibiotics, and DNA from each sample was extracted, processed, and analyzed on the ARDM to determine its unique genetic profile for antibiotic resistance.

We observed clear differences in resistance determinant profiles when assessing samples taken from different geographic regions (Fig. 2), with each region presenting with a different set of antibiotic classes for which resistance genes were encountered. The most

obvious difference was the broader spectrum of resistance genes from samples collected in Sierra Leone; resistance genes for 11 different classes of antibiotics were observed within this population. Multidrug efflux pumps and determinants directed against glycopeptides such as vancomycin — not detected in the other two populations — were observed in the Sierra Leonean samples. On the other hand, a gene conferring resistance to quinolone antibiotics such as ciprofloxacin was detected in samples from Egypt but not in the other populations. Conversely, a much lower percentage of OIF/OEF samples possessed chloramphenicol resistance determinants, but genes conferring resistance to macrolides, lincosamides, and streptogramins were detected in samples from this region only.

six or more genes conferring aminoglycoside resistance. Importantly, we unexpectedly observed the *armA* gene in one of the WRAIR/MRSN samples; this gene confers resistance to the entire class of aminoglycoside antibiotics and has clear implications when considering chemotherapeutic countermeasures.

The majority of samples from all three populations also carried at least one gene conferring resistance to β -lactam antibiotics but the overall distribution of specific β -lactamase genes varied (Table 1). Due to their cost, broad clinical spectrum, and low toxicity, β -lactams are generally considered as the starting point for treatment of Gram-negative bacterial infections. As expected, the *Acinetobacter* samples from WRAIR/MRSN harbored *Acinetobacter*-specific β -lactamases

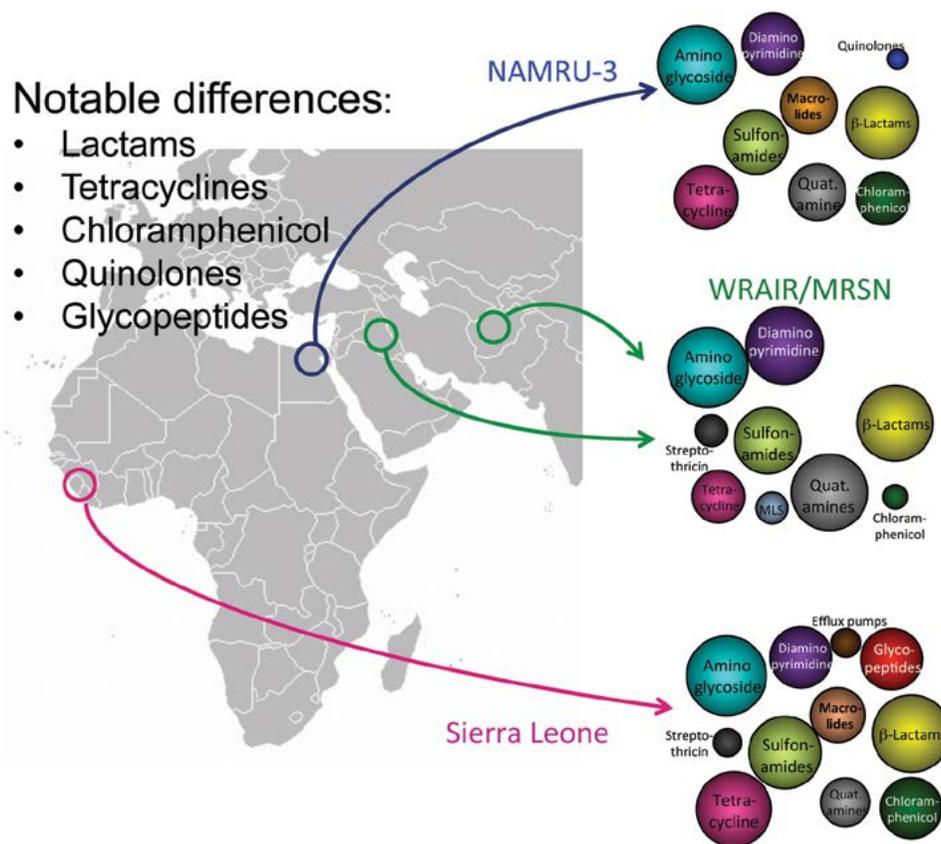


FIGURE 2
Geographic profiles of antibiotic resistance gene content. Bubble size indicates the percentage of samples from each site harboring at least one gene that confers resistance for each class of antimicrobial compound.

Genes directed against aminoglycosides such as gentamicin and amikacin were common throughout the three sample sets tested. At least 80% of all tested samples contained at least one aminoglycoside resistance gene, and over half possessed three or more; moreover, a small number of samples from Sierra Leone and WRAIR/MRSN (approximately 5%) harbored

such as *bla*_{OXA-51} and *bla*_{OXA-23}, which confer resistance to the most potent lactam antibiotics such as imipenem and meropenem. On the other hand, the majority of Egyptian strains possessed genes for extended-spectrum β -lactamases (ESBLs), which hydrolyze all third-generation cephalosporins.⁴ The possession of multiple ESBLs with overlapping specificities made

TABLE 1 — Resistance Determinants Detected in >10% Isolates Tested

<u>WRAIR</u>	<u>NAMRU-3</u>	<u>Sierra Leone</u>
β-lactam resistance determinants		
<i>Acinetobacter</i> -specific carbapenemases: <i>bla</i> _{OXA-51} , <i>bla</i> _{OXA-23}	ESBL families: <i>bla</i> _{TEM} , <i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-9} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M-1} , <i>bla</i> _{CTX-M-9}	ESBL family: <i>bla</i> _{TEM}
Aminoglycoside resistance determinants		
<i>aac(C1)</i> , <i>aadA1</i> family <i>aadB</i> , <i>aphA1</i> <i>strA</i> , <i>strB</i>	<i>aac(3)-III</i> , <i>aac(6)-ib</i> family <i>aadA1</i> family <i>aadB</i> , <i>aphA1</i> <i>strA</i> , <i>strB</i>	<i>aac(6)-ib</i> family, <i>aadA1</i> family <i>aadB</i> , <i>aphA1</i> <i>strA</i> , <i>strB</i>
Macrolide resistance determinants		
	<i>macA</i> , <i>macB</i> <i>mphA/mphK</i>	<i>macA</i> , <i>macB</i> <i>mphA/mphK</i>
Tetracycline resistance determinants		
<i>tet39</i> , <i>tetA</i> , <i>tetB</i>	<i>tet39</i> , <i>tetA</i> , <i>tetB</i>	<i>tet39</i> , <i>tetA</i> , <i>tetB</i> <i>tetS</i> , <i>tetX</i>
Multidrug resistance efflux pumps		
		<i>vcaM</i>
Chloramphenicol resistance determinants		
	<i>catA1/cat4</i> family	<i>catA1/cat4</i> family
Quarternary ammonium compound resistance determinants		
<i>qacEΔ1</i>	<i>qacEΔ1</i>	<i>qacEΔ1</i>
Sulfonamide resistance determinants		
<i>sulI</i> , <i>sulII</i>	<i>sulI</i> , <i>sulII</i>	<i>sulI</i> , <i>sulII</i>
Trimethoprim resistance determinants		
<i>dfrA1</i> , <i>folA</i>	<i>dfrA1</i> , <i>dfrA14</i> , <i>dfrA17</i>	<i>dfrA1</i> , <i>dfrA14</i> , <i>dfr20</i>

many of these Egyptian isolates virtually untreatable with standard β -lactam antibiotics. A significant number of the isolates from Sierra Leone harbored genes for only one of the ESBLs, *bla*_{TEM}, but were negative for the other ESBLs detected in the Egyptian samples.

The carriage of tetracycline resistance determinants also varied between the three regions. Whereas the Egyptian samples harbored four different genes, those from WRAIR/MRSN were limited to only three of those. Almost 80% of the Sierra Leonean samples harbored tetracycline resistance genes, with over half of them positive for the presence of multiple genes, including two additional determinants. The high prevalence of multiple tetracycline determinants clearly distinguishes this region from the others tested here, as well as other Gram-negative bacterial populations isolated throughout the world.⁵ Perhaps more importantly, the ARDM detected the presence of the *tetX* gene in the Sierra Leonean isolates. The *tetX* gene encodes an enzyme that hydrolyzes all known tetracycline antibiotics, including the most recently approved third-generation glycylycylcline antibiotic, tigecycline. Although the *tetX* gene had previously never been found in any Gram-negative human pathogens, our results confirmed its presence in 21% of the hospital isolates collected from Sierra Leone.⁶ Therefore, its presence in a large number of samples from clinical infections was both surprising and alarming, as it may portend the eventual failure of all tetracycline antibiotics in this region. The ability of the ARDM to detect an unexpected resistance determinant in samples where it otherwise might be missed speaks to the impact of the broad-based surveillance capability enabled by this technology.

DETECTION OF GENETIC ASSEMBLAGES

Bacteria exchange genetic information — including antibiotic resistance genes — through lateral transmission of clusters of genes, or genetic assemblages. The ARDM's broad coverage allowed us to deduce the likely presence of different genetic assemblages by identifying clusters of characteristic resistance genes. Integrons are genetic elements that capture antibiotic resistance gene “cassettes” through site-specific recombination; in this study, we observed assemblages indicative of two types of integrons. Class 1 integrons (markers - *sull*, *qacEΔ1*) were found in approximately half of the samples from Egypt and WRAIR/MRSN, but at a much lower rate amongst the Sierra Leone sample collection. Class 2 integrons (markers - *dfrA1*, *sat2*, *aadA1*), on the other hand, were observed in only approximately 7% of the WRAIR/MRSN samples. Furthermore, genes corresponding to several *Acinetobacter*-specific resistance islands, potential hotspots for the integration of resistance genes, were observed in two of the Egyptian samples (AbaR3 [seven markers] and AbaR6/7 [four

markers]). The presence and types of such genetic assemblages can not only help epidemiologists track the spread of related species, but can indicate the potential efficacy of different antibiotic combination therapies, which are now common practice for the treatment of MDR infections.

SUMMARY

In collaboration with researchers from NAMRU-3, WRAIR/MRSN, and Mercy Hospital, we have tested over 120 MDR bacteria from different geographic regions using NRL's Antimicrobial Resistance Determinant Microarray. In doing so, we identified significant differences in overall patterns of resistance, as well as in the individual genes present within each population. Of particular concern were both the presence of genes responsible for resistance to last-resort antibiotics and co-localization of genes directing resistance to the antibiotics most commonly used in combination therapy. While the ARDM is not intended as a diagnostic test, our studies have demonstrated its utility as a surveillance tool, enabling detection of unexpected resistance determinants (e.g., *tetX*, *armA*) with obvious and high potential impact. The single-test screening and surveillance capabilities that are afforded by the ARDM can lead to more effective therapeutic decisions, decreased use of inappropriate antimicrobials, and early detection of emerging drug-resistant pathogens, thus decreasing the threat of MDR infections to military personnel deployed to these regions. On the merits of these findings, we have expanded our surveillance efforts in the form of ongoing collaborations with Naval Medical Research Unit-2 (NAMRU-2; Phnom Penh, Cambodia), Naval Medical Research Unit-6 (NAMRU-6; Lima, Peru), the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia), the University of Chicago hospital pavilion (Chicago, Illinois) and the Landstuhl Regional Medical Center (Germany).

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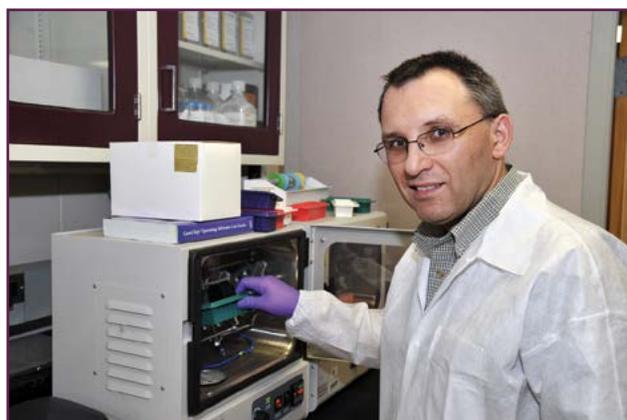
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