

Analysis of Antibiotic Resistance Genes in Multidrug-Resistant *Acinetobacter* sp. Isolates from Military and Civilian Patients Treated at the Walter Reed Army Medical Center[∇]

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Military medical facilities treating patients injured in Iraq and Afghanistan have identified a large number of multidrug-resistant (MDR) *Acinetobacter baumannii* isolates. In order to anticipate the impact of these pathogens on patient care, we analyzed the antibiotic resistance genes responsible for the MDR phenotype in *Acinetobacter* sp. isolates collected from patients at the Walter Reed Army Medical Center (WRAMC). Susceptibility testing, PCR amplification of the genetic determinants of resistance, and clonality were determined. Seventy-five unique patient isolates were included in this study: 53% were from bloodstream infections, 89% were resistant to at least three classes of antibiotics, and 15% were resistant to all nine antibiotics tested. Thirty-seven percent of the isolates were recovered from patients nosocomially infected or colonized at the WRAMC. Sixteen unique resistance genes or gene families and four mobile genetic elements were detected. In addition, this is the first report of *bla*_{OXA-58}-like and *bla*_{PER}-like genes in the U.S. MDR *A. baumannii* isolates with at least eight identified resistance determinants were recovered from 49 of the 75 patients. Molecular typing revealed multiple clones, with eight major clonal types being nosocomially acquired and with more than 60% of the isolates being related to three pan-European types. This report gives a “snapshot” of the complex genetic background responsible for antimicrobial resistance in *Acinetobacter* spp. from the WRAMC. Identifying genes associated with the MDR phenotype and defining patterns of transmission serve as a starting point for devising strategies to limit the clinical impact of these serious infections.

The emergence and rapid spread of multidrug-resistant (MDR) isolates of *Acinetobacter* spp. causing nosocomial infections are of great concern worldwide (1, 23). The Centers for Disease Control and Prevention recently highlighted the enormity and gravity of MDR *Acinetobacter baumannii* infections in military medical facilities treating civilians and service personnel injured in Iraq/Kuwait and Afghanistan (7). Antimicrobial susceptibility testing of these isolates originally reported that more than half were resistant to three or more classes of antibiotics. This resistance profile leaves clinicians with limited therapeutic options. Another significant problem is the large number of military personnel that may be colonized by MDR *Acinetobacter* spp. (16). This reservoir would serve to widely disseminate MDR *Acinetobacter* spp. As has been seen with methicillin-resistant *Staphylococcus aureus* isolates and vancomycin-resistant enterococci (VRE), patients who are colonized can spread MDR pathogens (5, 6, 30).

Here, we report an analysis of the antibiotic susceptibility profile and genetic determinants of antibiotic resistance in 75 isolates of *Acinetobacter* spp. obtained from military and civilian patients at the Walter Reed Army Medical Center (WRAMC). This hospital was the major U.S. site receiving casualties from the conflict in Iraq/Kuwait and Afghanistan. Understanding the genetic background of antibiotic resistance is an important step in defining the impact of MDR *Acinetobacter* spp. in military and civilian patients.

MATERIALS AND METHODS

Study population, bacterial isolate identification, and susceptibility testing. The *Acinetobacter* sp. isolates were collected from 75 unique civilian and military patients treated at the WRAMC in Washington, D.C. A medical record review was performed for each case. For the purpose of this particular study, (i) clinical infections were defined using standard criteria set by the National Nosocomial Infections Surveillance System (40) and (ii) hospital-acquired infection was defined as infection that occurred at least 72 h after arrival at the WRAMC. The injury severity score (values from 0 to 75) was calculated based upon the method of Baker et al. (3). Institutional Review Board approval for this retrospective medical chart review was obtained at the WRAMC.

All isolates were collected from March 2003 through February 2005. Fifty-two of the 75 isolates examined were collected from February 2004 to February 2005 and represent 67% of all isolates of *Acinetobacter* spp. collected at the WRAMC during that time period. An additional 9% of *Acinetobacter* sp. isolates from that

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TABLE 1. Primers for amplification of genes from *Acinetobacter* sp. isolates

Primer name	Primer sequence (5' to 3') ^a	Annealing temp (°C)	Target gene(s)	Source or reference
SHV PROX	ATGCGTTATATTCGCCTGTG	60	All <i>bla</i> _{SHV} genes	33
SHV DIS	TGCTTTGTTATTCGGGCCAA			
TEM 285	AAACGCTGGTGAAAGTA	45	All <i>bla</i> _{TEM} genes	33
TEM 2023	AGCGATCTGTCTAT			
CTX-M2-1	ATGATGACTCAGAGCATTTCGCCGCT	70	<i>bla</i> _{CTX-M-2} , <i>bla</i> _{CTX-M-4} , <i>bla</i> _{CTX-M-5} , <i>bla</i> _{CTX-M-20} , <i>bla</i> _{CTX-M-31} , <i>bla</i> _{CTX-M-35} , <i>bla</i> _{TOHO-1}	33
CTX-M2-2	TCAGAAAACCGTGGGTTACGATTTTCG			
VEB-1 FOR	ATGAAAAATCGTAAAAAGGATATT	47	<i>bla</i> _{VEB-1} , <i>bla</i> _{VEB-1A} , <i>bla</i> _{VEB-1B} , <i>bla</i> _{VEB-2} , <i>bla</i> _{VEB-3}	This study
VEB-1 REV	TTATTTATTCAAATAGTAATTCC			
PER F-1	ATGAAATGTCATTATAAAAAG	44	<i>bla</i> _{PER-1}	This study
PER R-2	TTGGGCTTAGGGCAG			
IMP FOR	GTTTATGTTTCATACWTCG	45	<i>bla</i> _{IMP-1} , <i>bla</i> _{IMP-2} , <i>bla</i> _{IMP-4} , <i>bla</i> _{IMP-5} , <i>bla</i> _{IMP-6} , <i>bla</i> _{IMP-7} , <i>bla</i> _{IMP-8} , <i>bla</i> _{IMP-9} , <i>bla</i> _{IMP-10} , <i>bla</i> _{IMP-11} , <i>bla</i> _{IMP-15} , <i>bla</i> _{IMP-19} , <i>bla</i> _{IMP-20} , <i>bla</i> _{IMP-21}	This study
IMP REV	GGTTTAAAYAAAACAACCAC			
VIM FOR	TTTGGTTCGCATATCGCAACG	66	<i>bla</i> _{VIM-1} , <i>bla</i> _{VIM-2} , <i>bla</i> _{VIM-4} , <i>bla</i> _{VIM-5} , <i>bla</i> _{VIM-6} , <i>bla</i> _{VIM-8} , <i>bla</i> _{VIM-9} , <i>bla</i> _{VIM-10} , <i>bla</i> _{VIM-11} , <i>bla</i> _{VIM-12}	This study
VIM REV	CCATTCAGCCAGATCGGCAT			
GIM FOR	ATATTACTTGTAGCGTTGCCAGC	61	<i>bla</i> _{GIM}	This study
GIM REV	TTAATCAGCCGACGCTTCAG			
ADC-7 FOR	ATCGGATTTAAAAAATTTCTTGT	50	<i>bla</i> _{ADC-1} , <i>bla</i> _{ADC-2} , <i>bla</i> _{ADC-3} , <i>bla</i> _{ADC-4} , <i>bla</i> _{ADC-5} , <i>bla</i> _{ADC-6} , <i>bla</i> _{ADC-7}	21
ADC-7 REV	TTATTTCTTTATTGCATTCAG			
OXA SET A FOR	ATGAAAAAATTTATACTTCC	47	<i>bla</i> _{OXA-24} , <i>bla</i> _{OXA-25} , <i>bla</i> _{OXA-26} , <i>bla</i> _{OXA-33} , <i>bla</i> _{OXA-40} , <i>bla</i> _{OXA-72}	This study
OXA SET A REV	TTAAATGATTCGAAGATTTTC			
OXA SET B FOR	TCTGGTTGTACGGTTTCAGC	51	<i>bla</i> _{OXA-23} , <i>bla</i> _{OXA-27} , <i>bla</i> _{OXA-49}	This study
OXA SET B REV	AGTCTTTCCAAAAATTTTG			
OXA SET C FOR	ACAGAARTATTTAAGTGGG	47	<i>bla</i> _{OXA-51} , <i>bla</i> _{OXA-58} , <i>bla</i> _{OXA-64} , <i>bla</i> _{OXA-69} , <i>bla</i> _{OXA-70} , <i>bla</i> _{OXA-71} , <i>bla</i> _{OXA-75} , <i>bla</i> _{OXA-78}	This study
OXA SET C REV	GGTCTACAKCCMWTCGCCA			
OXA SET D FOR	TAGCACTGCTTTTCTCAGCTG	51	<i>bla</i> _{OXA-20} , <i>bla</i> _{OXA-37}	This study
OXA SET D REV	TTGACGGATTGAAGAATAGCACG			
OXA1F	ACACAATACATATCAACTTCGC	50	<i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-4} , <i>bla</i> _{OXA-30} , <i>bla</i> _{OXA-31} , <i>bla</i> _{OXA-47}	This study
OXA1R	GTGTGTTTAGAATGGTGATC			
OXA58-5'	ATGAAATTTAAAAAATTTGAGTTTAG	53	<i>bla</i> _{OXA-58} , <i>bla</i> _{OXA-96}	This study
OXA58-3'	TTATAAATAATGAAAAACACCCAAC			
Hep35	TGCGGGTYAARGATBTKGATTT	49	Conserved region <i>intI1</i> , <i>intI2</i> , <i>intI3</i>	53
Hep36	CARCACATGCGTRTARAT			
Hep58	TCATGGCTTGTTATGACTGT	55	Class 1 integron cassette array	53
Hep59	GTAGGGCTTATTATGCACGC			
IS _{ABA1a}	ATGCAGCGTCTTTTGCAGG	55	IS _{ABA1}	19
IS _{ABA1b}	AATGATTGGTGACAATGAAG			
IS1133 for	AGTACAAAAGCTGTGAGATTTTCAG	58	IS1133	This study
IS1133 rev	GATATTCATGAGCGCAATATTGGCT			
adeR-5'	ATGTTTGATCATTCTTTTCTTTTG	46	<i>adeR</i> (regulatory gene of AdeABC efflux gene cluster)	This study
adeR-3'	TTAATTAACATTTGAAATATG			
adeE-5'	ATGTTGTCGAGTTTTTTTTATTGCACG	60	<i>adeE</i> (efflux gene of AdeDE)	This study
adeE-3'	TCATTTTGCCTTTTGGTATTTAAC			
aacA4 FOR	ATGACTGAGCATGACCTTGCG	65	<i>aacA4</i>	This study
aacA4 REV	TTAGGCATCACTGCGTGTTCG			
aacC1-5'	ATGGGCATCATTTCGCACATGTAGG	64	<i>aacC1</i>	This study
aacC1-3'	TTAGGTGGCGGTACTTGGGTC			
aadA1-5'	ATGAGGGAAGCGGTGATCG	62	<i>aadA1</i>	This study
aadA1-3'	TTATTTGCCGACTACCTTGGTG			
aadB-5'	ATGGACACAACGCAGGTCCG	68	<i>aadB</i>	This study
aadB-3'	TTAGGCCGCATATCGCGACC			
aphA6 FOR	ATGGAATTGCCCAATATTATTC	55	<i>aphA6</i>	This study
aphA6 REV	TCAATTCATTCATCAAGTTTTA			
aacC2 FOR	ATGCATACGCGGAAGGCAATAAC	65	<i>aacC2</i>	This study
aacC2 REV	CTAACCGGAAGGCTCGCAAG			
gyrA-1	AAATCTGCCCGTGTCTGGT	63	<i>gyrA</i> (QRDR ^b)	52
gyrA-2	GCCATACCTACGGCGATACC			
parC-1	AAACCTGTTTCAGCGCCGATT	58	<i>parC</i> (QRDR)	51
parC-2	AAAGTTGCTTGGCATTCACT			
QnrA	GGGTATGGATATTATTGATAAAG	52	<i>qnrA</i>	27
QnrB	CTAATCCGGCAGCACTATTA			
CarO 5'	ATGAAAGTATTACGTGTTTTAGTG	50	<i>carO</i>	This study
CarO 3'	TTACCAGTAGAAGTTTACACC			

^a Y = C or T; M = A or T; R = A or G; B = C, G, or T; W = A or T; K = G or T.^b QRDR, quinolone resistance-determining region.

time period were received but eliminated from this study due to insufficient patient information or being duplicate patient samples. In the case of duplicate patient samples, the first collected *Acinetobacter* sp. isolate was chosen to eliminate bias.

Antimicrobial susceptibility testing was performed on the 75 isolates according to the method established by the CLSI (formerly NCCLS) and interpreted with criteria published in 2005 (9, 31). Isolates were tested using cation-adjusted Mueller-Hinton agar (BBL MH II; Becton Dickinson Microbiology Systems, Sparks, MD). Meropenem, imipenem, ampicillin, ampicillin-sulbactam, ciprofloxacin, ceftazidime, cefepime, amikacin, and tobramycin disks (Becton Dickinson Microbiology Systems) were used.

Multidrug resistance was defined in this analysis as resistance to three or more representatives of the following classes of antibiotics: quinolones (ciprofloxacin), extended-spectrum cephalosporins (ceftazidime and cefepime), β -lactam/ β -lactamase inhibitor combination (ampicillin-sulbactam), aminoglycosides (amikacin and tobramycin), and carbapenems (imipenem and meropenem).

PCR amplification, cloning, DNA sequencing, and restriction endonuclease digestion. All target genes and corresponding primers for PCR amplification are listed in Table 1. Positive controls were used for β -lactamase gene amplification as follows: *bla*_{SHV-1} cloned into pBC SK(-) (Stratagene, La Jolla, CA), *bla*_{TEM-1} cloned into the commercially available pBR322 (New England BioLabs, Beverly, MA), *bla*_{ADC-7} cloned into pBC SK(+), *bla*_{CTX-M-2} cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA), RGN238 *bla*_{OXA-1} construct, the isolate containing *bla*_{IMP-2} (a kind gift from G. Rossolini), and *Pseudomonas aeruginosa* PU21 possessing *bla*_{PER-1} (a kind gift from F. Danel) (11, 21, 38, 39, 45). Positive control reactions for *bla*_{VIM}, *bla*_{GIM}, and *bla*_{VEB-1} were not performed. For OXA set A to D, positive controls were also not used. Presumptive positive PCR products for β -lactamase genes were identified according to size and either cloning into the pCR-XL-TOPO vector followed by sequencing or direct sequencing of the PCR product. PCR product identification via pCR-XL-TOPO vector cloning and sequencing was carried out for representative *bla*_{ADC}, *bla*_{OXA-23}-like, *bla*_{OXA-69}-like, *bla*_{OXA-58}-like, and *bla*_{PER} positive amplicons. Direct sequencing of select positive amplicons was carried out for *bla*_{SHV} and *bla*_{TEM}. For all other PCR amplifications, the products obtained were considered presumptive positives based on amplicon size. These include PCR amplification reactions for all aminoglycoside-modifying enzyme (AME) genes, all quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* genes, *adeE*, *adeR*, *carO*, and *int* genes, variable regions of class 1 integrons, and insertion (IS) elements. In the case of a negative PCR, in which a positive control was not used, PCR amplifications were repeated at least twice for these genes. A negative control was run with every PCR.

For PCR, a 1:10 dilution of an overnight culture was boiled for 10 min. Amplification was then performed with 10 μ l of this dilution as the DNA template. PCR conditions included 30 cycles of amplification under the following conditions: denaturation at 95°C for 30 s, annealing for 1 min at primer set-specific temperatures (Table 1), and extension at 72°C for 1 min/kb product. Cycling was followed by a final extension at 72°C for 10 min. PCR products were resolved on 1.0% agarose gels, stained with ethidium bromide, and photographed with UV illumination. Either the 1-kb DNA ladder or the 100-bp DNA ladder (Promega, Madison, WI) was used to assess PCR product size.

Amplification and Hinfl (Promega) digestion of the QRDRs of topoisomerase IV *parC* and *gyrA* genes in *Acinetobacter* spp. were performed as follows. For the restriction endonuclease digestion, 40 μ l of PCR amplification product was purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The eluant was incubated at 37°C for 2.5 h with 10 U of Hinfl. The digests were then separated by electrophoresis in 1.5% NuSieve (FMC BioProducts, Rockland, ME) plus 1.0% agarose gel stained with ethidium bromide and photographed with UV illumination. To serve as a control, the remaining 10 μ l of undigested PCR product from each sample was resolved on the same agarose gel.

PCR products generated and cloned into pCR-XL-TOPO were sequenced using an ALF Express II automated DNA sequencer (Amersham Biosciences, Piscataway, NJ) with a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham Biosciences) and Cy5-labeled M13 reverse and M13 universal primers (21). pCR-XL-TOPO clones containing the *bla* genes were cycle sequenced under the following conditions: DNA was heated to 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 60°C for 2 min. Direct sequencing was performed for *bla*_{TEM} and *bla*_{SHV} genes by using previously described Cy5-labeled TEM104 reverse, TEM164 forward, and A forward primers under the same cycling conditions as those described above (20, 21).

Clonal determination by PCR ESI-MS. To determine clonal relatedness by PCR electrospray ionization mass spectroscopy (PCR ESI-MS), the conserved regions of six bacterial housekeeping genes (*efp*, *trpE*, *adk*, *mutY*, *fumC*, and *ppa*)

TABLE 2. Characteristics of the study population^a

Parameter (units unless otherwise specified)	No. (%) of patients	Mean \pm SD (range [minimum, maximum])
Duration of hospitalization (days)	75	53 \pm 48 (3, 205)
Duration of ICU stay (days)	75	19 \pm 35 (0, 205)
Time febrile (days)	75	11 \pm 11 (0, 51)
Total surgeries (no.)	75	6 \pm 6 (0, 23)
Different antibiotics used (no.)	75	6 \pm 4 (1, 17)
Duration of antibiotic use (days)	75	38 \pm 37 (1, 205)
Changes to antibiotic regimen (no.)	75	5 \pm 5 (0, 21)
Debridement procedures (no.)	75	4 \pm 5 (0, 22)
Injury severity score	58	21 \pm 12 (4, 59)
APACHE II score ^b	17	15 \pm 10 (0, 30)
Hospital locations		
Surgical ICU	24 (32)	
Medical ICU	26 (35)	
ICU step-down	3 (4)	
ONP ^c	11 (15)	
Surgery	4 (5)	
Internal medicine	4 (5)	
Emergency room	3 (4)	

^a Patients in this study were civilians eligible for care (contractors injured in Iraq, family members of active duty soldiers, or retirees) and active duty military personnel hospitalized at the WRAMC.

^b The APACHE (Acute Physiology and Chronic Health Evaluation) II Score was calculated at the time of admission to ICU (medical and surgical).

^c ONP, orthopedics/neurology/physical medicine and rehabilitation.

were amplified from each isolate by using eight sets of previously validated primers (14). The amplification products were then desalted and purified, and the mass spectra were determined using previously established protocols (13). These base compositions were compared to those of 23 isolates representing three major *A. baumannii* clone types (I, II, and III) that have spread throughout Europe (50). In addition, 26 American Type Culture Collection (ATCC) *Acinetobacter* sp. reference strains were used for *Acinetobacter* species verification. The European isolates and ATCC control strains used for this analysis were recently described by Ecker et al. (14).

RESULTS

Characteristics of the study population. *Acinetobacter* sp. isolates were recovered from 75 patients: 67 men (89%) and 8 (11%) women. Patients in this study included active duty soldiers or civilians (e.g., contractors injured in Iraq) sent to Iraq/Kuwait or Afghanistan and civilians (family members of active duty soldiers and retirees eligible for care) hospitalized at the WRAMC. One patient was deployed to Afghanistan and 57 (76%) to Iraq/Kuwait, while 17 (23%) were nondeployed. More than half (63%) of the patients had positive *Acinetobacter* sp. cultures less than 3 days from the time of admittance.

The relevant descriptive statistics of the study population are summarized in Table 2. The mean age (\pm SD) of the population was 35 (\pm 18) years, with a range of 5 to 86 years. When *Acinetobacter* spp. were isolated, 53 patients (71%) were in an intensive care unit (ICU) or step-down unit, 51 (68%) were mechanically ventilated, and 66 patients (88%) had an indwelling central venous catheter. Sixty-seven patients (89%) had clinical infections and eight (11%) were colonized. Overall, five deaths (7% mortality rate) were observed in patients with *A. baumannii* infection (Table 3). Taken as a unique group, these five patients were older and were immunosuppressed. The death rate of the nondeployed patients was 29%. It is also

TABLE 3. Mortality associated with MDR *A. baumannii* infections in nonmilitary personnel^a

Isolate	Age (yr)	Underlying condition(s)	Hospital day of death	Attributable mortality
AB001	71	Chronic obstructive pulmonary disease	27	Ventilator-associated pneumonia
AB002	35	Renal transplant	44	Pneumonia and lung abscess
AB029	85	Abdominal aortic aneurysm	88	Ventilator-associated pneumonia
AB052	72	Congestive heart failure, chronic renal failure	45	Pneumonia and sepsis
AB056	76	Dementia	93	Anoxic brain injury and bacteremia

^a The sixth death was a 21-year-old active duty soldier admitted due to a severe trauma blast. This patient died on hospital day 17 due to a pulmonary embolus. He previously had *A. baumannii* bacteremia (AB017) during his hospital course. All isolates except for AB029 were bloodstream infections and were acquired in the hospital.

notable that these isolates were from patients hospitalized for a prolonged period of time (Table 3).

Antimicrobial susceptibility. Table 4 summarizes the sites of isolation and antibiotic resistance patterns found in these isolates compared to Meropenem Yearly Susceptibility Test Information Collection (MYSTIC) data for *Acinetobacter* spp. in the United States during 2004. Isolates obtained at the WRAMC demonstrated significantly more resistance to ciprofloxacin, ceftazidime, cefepime, amikacin, tobramycin, imipenem, and meropenem (ampicillin-sulbactam was not available for comparison) than the U.S. *Acinetobacter* spp. collected in 2004.

Overall, 89% of the isolates were resistant to at least three classes of antibiotics, hence meeting the criteria for multidrug resistance. More than 90% of the isolates were resistant to ciprofloxacin, and at least 80% were resistant to extended-spectrum cephalosporins. Nearly half (49%) of the isolates were also resistant to ampicillin-sulbactam. Notably, imipenem resistance was observed in 20% of isolates and meropenem resistance was detected in 25%. Eighty-one percent of isolates were resistant to at least one aminoglycoside (amikacin or tobramycin). Quite alarmingly, 11 isolates (15%) were resistant to the five classes of antibiotics tested.

Genotypic analysis: β-lactamase genes. Table 5 lists the detected β-lactamase genes and the genes encoding the AMEs. Detailed genetic and phenotypic analyses of specific isolates

can be found in Table 6. The *ampC* *Acinetobacter*-derived cephalosporinase, *bla*_{ADC}, was detected in 99% of the collection by PCR amplification (21). This cephalosporinase is responsible for resistance to ceftazidime and other extended-spectrum cephalosporins. Three distinct *bla*_{OXA}-like genes were also identified. Using PCR, a *bla*_{OXA-69}-like gene was found in 97% of the isolates (17). In two carbapenem-susceptible isolates (AB002 and AB006), *IS*_{ABAI} was found to be proximal to the *bla*_{OXA-69}-like gene. The *bla*_{OXA-23}-like carbapenemase gene was detected in 11% of strains (12, 18), and the *bla*_{OXA-58}-like carbapenemase gene was found in an additional 12% of the isolates (10, 35). Seventeen of 19 isolates (90%) that were imipenem and/or meropenem resistant by disk diffusion possessed evidence for the presence of a *bla*_{OXA-23}-like or a *bla*_{OXA-58}-like gene (Table 6). All eight isolates that contained a *bla*_{OXA-23}-like gene were shown to have insertion sequence *IS*_{ABAI} proximal to the gene, as previously reported by others (18, 49).

PCR amplification of class A β-lactamase genes revealed that 40% (30/75) of the isolates contained *bla*_{TEM}, only one isolate contained *bla*_{SHV}, and two isolates contained *bla*_{PER} (24, 34). This is also the first description of *bla*_{PER} in the United States. Evidence for other common *Acinetobacter*

TABLE 5. Percentage of each antibiotic resistance gene detected in the collection of *Acinetobacter* sp. isolates from the WRAMC

Gene	% Detection in isolates
Genes encoding β-lactamases	
<i>bla</i> _{ADC}	99
<i>bla</i> _{OXA-69} -like ^a	97
<i>bla</i> _{OXA-23} -like ^b	11
<i>bla</i> _{OXA-58} -like ^c	12
<i>bla</i> _{TEM}	40
<i>bla</i> _{SHV}	1
<i>bla</i> _{PER}	3
Genes encoding AMEs^d	
<i>aacC1</i>	56
<i>aacC2</i>	5
<i>aadA1</i>	39
<i>aadB</i>	48
<i>aphA6</i>	71

^a Includes *bla*_{OXA-51}, *bla*_{OXA-58}, *bla*_{OXA-64}, *bla*_{OXA-69}, *bla*_{OXA-70}, *bla*_{OXA-71}, *bla*_{OXA-75}, and *bla*_{OXA-78}-like genes.

^b Includes *bla*_{OXA-23}, *bla*_{OXA-27}, and *bla*_{OXA-49}-like genes.

^c Includes *bla*_{OXA-58} and *bla*_{OXA-96}-like genes.

^d *aacC1* confers gentamicin resistance. *aacC2* confers tobramycin and gentamicin resistance. *aadA1* confers streptomycin and spectinomycin resistance. *aadB* confers tobramycin, gentamicin, and kanamycin resistance. *aphA6* confers amikacin, gentamicin, kanamycin, and neomycin resistance.

TABLE 4. Anatomical sites of isolation and antibiotic resistance patterns of isolates in this study

Anatomical site	No. of isolates	% Resistance to ^a :							
		CIP	CAZ	FEP	SAM	AMK	TOB	IPM	MEM
All	75	91	91	84	49	53	67	20	25
Bloodstream	40	90	90	88	58	50	70	23	25
Skin	18	89	94	78	56	61	72	17	28
Wound	8	100	100	75	25	50	75	13	13
Respiratory ^b	5	80	60	80	40	60	60	20	20
Urine	2	100	100	100	100	100	50	50	50
Intravenous catheter	1	100	100	100	100	0	0	0	0
Cerebrospinal fluid	1	100	100	100	100	0	0	0	0
MYSTIC ^c	44	42	32	NR ^d	14	13	8	8	8

^a CIP, ciprofloxacin (quinolone); CAZ, ceftazidime (cephalosporin); FEP, cefepime (cephalosporin); SAM, ampicillin-sulbactam (β-lactam/β-lactamase inhibitor); AMK, amikacin (aminoglycoside); TOB, tobramycin (aminoglycoside); IPM, imipenem (carbapenem); MEM, meropenem (carbapenem).

^b Includes bronchoalveolar lavage fluid, trachea, and sputum.

^c Includes *Acinetobacter* spp. in the United States during 2004 in all specialties (http://www.mystic-data.org).

^d NR, not reported.

TABLE 6. Summary of resistance: phenotypic and genetic characteristics of the isolates^a

Isolate ^b	Source ^c	PCR ESI-MS type	Resistance phenotype ^e													Genes encoding AMEs						QRDR of ^f :	Class 1 integron	Efflux gene			
			AMP	CIP	CAZ	FEP	AMK	TOB	SAM	MEM	IPM	TEM	SHV	PER	ADC	OXA-23	OXA-58	OXA-69	aphA6	aadA1	aadB				aacC1	aacC2	gyrA
AB001	B	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB002	B	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB003	B	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB004	B	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB005	B	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB006	B	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB007	B	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB008 ^g	SS	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB009 ^g	SS	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB010	SS	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB011	SS	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB012	SS	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB013	W	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB014	W	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB015	BAL	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB016	S	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB017	B	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB018	B	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB019	B	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB020	B	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB021	B	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB022	SS	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB023 ^g	SS	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB024 ^g	SS	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB025	SS	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB026	SS	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB027	W	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB028 ^g	W	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB029	S	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB030	C	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB031	CSF	11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB032	B	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB033	B	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB034	B	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB035	B	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB036	B	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB037	B	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB038	B	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB039	B	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB040	U	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	+	+	adeR
AB041	B	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	S	+	adeR
AB042	B	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	S	+	adeR
AB043	B	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	S	+	adeR
AB044	B	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	S	+	adeR
AB045	B	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	S	+	adeR
AB046	B	24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	R	R	S	+	adeR

TABLE 7. Distribution of *Acinetobacter* sp. PCR ESI-MS clone types isolated at the WRAMC^a

European type	PCR ESI-MS type	No. of isolates	Hospital location(s)	Time course
I	15 ^b	4	SICU, MICU, ICU step-down	May 2003–August 2004
I	16	1	ONP	July 2004
I	46	2	MICU, ONP	April 2003–May 2003
II	1	1	MICU	October 2003
II	10	9	SICU, ONP, surgery, ICU step-down, internal medicine, ER	August 2003–February 2005
II	11	15	SICU, MICU, ONP	May 2003–November 2004
II	12 ^b	4	SICU, MICU, internal medicine	August 2004–December 2004
III	14 ^b	16	SICU, MICU, ONP, surgery, ICU step-down	May 2003–December 2004
§	3	6	SICU, MICU, ONP	July 2003–February 2005
§	9	1	MICU	October 2004
§	24	11	SICU, MICU, ONP	June 2004–February 2005
§	39	1	MICU	September 2003
§	47	1	SICU	January 2004
§	48	1	SICU	April 2004
§	<i>Acinetobacter</i> genome sp. 3	1	ER	August 2003
§	<i>A. johnsonii</i>	1	ER	January 2004

^a All isolates are *A. baumannii* except *Acinetobacter* genome sp. 3 and *A. johnsonii*. §, Isolates not related to European strains. SICU, surgical intensive care unit; MICU, medical intensive care unit; ONP, orthopedics/neurology/physical medicine and rehabilitation; ER, emergency room.

^b Exact matches to European reference strains.

β -lactamase genes was absent (*bla*_{CTX-M} and other *bla*_{OXA} genes), including the carbapenem-hydrolyzing metallo- β -lactamase genes *bla*_{IMP}, *bla*_{VIM}, and *bla*_{GIM}.

DNA topoisomerase mutations and quinolone resistance. More than 90% of our isolates were resistant to ciprofloxacin. *Hinf*I digestion of the QRDR amplification products of *gyrA* and *parC* in these isolates indicated that 88% contain a point mutation at Ser83 in *gyrA* or Ser80 in *parC* leading to quinolone resistance. Except for two isolates, this analysis correlated with susceptibility studies (Table 6). Our primers did not detect the presence of the plasmid-mediated quinolone resistance gene *qnrA* in these 75 isolates (27, 42).

Genes encoding AMEs. Screening for genes encoding AMEs demonstrated that 97% of the isolates that are amikacin resistant contained the phosphotransferase gene *aphA6* (Tables 5 and 6). Other genes encoding AMEs included the adenylyltransferase genes *aadA1* (39%) and *aadB* (48%) and the acetyltransferase genes *aacC1* (56%) and *aacC2* (5%). Seventy-eight percent (40/51) of the tobramycin-resistant isolates contained either the *aacC2* or *aadB* gene.

Genes encoding efflux pumps. An important resistance determinant in *A. baumannii* is the AdeABC efflux pump (26, 28). *A. baumannii* isolates demonstrating enhanced expression of this pump are resistant to aminoglycosides, quinolones, tetracyclines, and trimethoprim. The AdeABC operon possesses two genes that encode proteins that act as a sensor (*adeS*) and a regulator (*adeR*) of the pump. This regulatory gene was identified in 95% of the isolates. The expression of the AdeABC efflux pump is being further evaluated.

We also detected the efflux pump gene *adeE* (part of the AdeDE pump in *Acinetobacter* genome species 3) (8). This amplicon was found in two isolates that did not contain the AdeABC pump (Table 6). By PCR ESI-MS, these two isolates were determined not to be *A. baumannii* (see below).

Mobile genetic elements. Integrons are genetic elements harboring multiple antibiotic resistance genes. Central to the genetic organization of the integron is the integrase gene (*int*).

The isolates were screened for *intI1*, *intI2*, and *intI3* genes, class 1 integron specific gene cassettes, and the insertion sequence elements (IS_{I133} and IS_{ABA1}). Sixty-one percent of the isolates demonstrated evidence of *int* genes. Nearly 70% of these contained class 1 integron gene cassettes of various sizes (data not shown).

Ninety-five percent of the isolates contained IS_{ABA1} and 5% of the isolates contained IS_{I133}, another IS element associated with increased expression of the ADC β -lactamase in *A. baumannii* (19, 44). These two IS elements were found together in four isolates. We did not uncover evidence that the IS elements were located 5' to the *bla*_{ADC} genes, but we are continuing to map the location of the IS elements. We also showed that in certain isolates, IS_{ABA1} is proximal to the *bla*_{OXA-69}-like gene and to the *bla*_{OXA-23}-like resistance determinant as described above.

Species identification and clonality by PCR ESI-MS. PCR ESI-MS is a form of high-throughput multilocus sequence typing that is able to distinguish *Acinetobacter* isolates at the species level as well as to determine clonality (13). Using this technique, we identified at the genetic level 73 *A. baumannii* isolates, 1 *Acinetobacter* genome species 3 isolate, and 1 *Acinetobacter johnsonii* isolate. There was a distribution of 16 different clone types identified within the collection. Further analysis revealed eight main clone types. All eight main types were involved in nosocomial acquisition (28 patients, 37% of isolates) (Table 6). It is notable that carbapenem-resistant isolates were composed of four distinct clones while three of these types possessed *bla*_{OXA-23}-like genes; the fourth, ESI-MS type 24, contained the *bla*_{OXA-58}-like gene.

Interestingly, the base composition analysis of 21 of 23 reference strains representing European clones I, II, and III matched 32% of the isolates exactly for all six housekeeping loci tested (*efp*, *trpE*, *adk*, *mutY*, *fumC*, and *ppa*). Related clones of European type I (ESI-MS types 16 and 46) and II (ESI-MS types 1, 10, and 11) are found in an additional 37% of the isolates (Table 7). These isolates are identical in five of the

six genetic loci examined. Sequence variations occurred only within the *mutY* locus, suggesting that these isolates are variants of the well-characterized European strains. Consistent with previous studies comparing the relatedness and aminoglycoside resistance of these pan-European strains, clone type III (ESI-MS type 14) remains the most uniform in its antibiogram and genetic profiles, while clones I and II have slightly diverse susceptibility and genetic patterns within each group (32, 50).

DISCUSSION

The goal of our study was to link the resistance phenotypes and the genetic determinants of resistance in *A. baumannii*, giving a "snapshot" of the complex nature of this endemic situation. From these data, it is clear that the potential impact of *A. baumannii* infections on military and civilian personnel in receiving hospitals is significant.

A unique characteristic of our study is that bloodstream infections were the most common clinical source of *Acinetobacter* spp. This may reflect the nature and severity of injury among these patients: more than two-thirds of the patients were critically ill, had central venous catheters, and were on mechanical ventilation when their isolates were obtained. Hence, in light of our susceptibility testing, empirical monotherapy for bloodstream infections would be inappropriate. Only two isolates in this collection are resistant to both amikacin and imipenem-cilistatin, and therefore, combination therapy with these antibiotics would ensure that at least one agent is effective for the majority of isolates. It is notable these isolates (from the WRAMC) have a broader range of resistance than those (from the MYSTIC) typically found in U.S. hospitals (Table 4).

A second striking feature of this study is the large number of antibiotic resistance genes found in these isolates. Forty-nine *A. baumannii* isolates had eight or more resistance determinants. Our genetic analysis revealed that the *bla*_{ADC} and *bla*_{OXA-69}-like genes were present in nearly all of the strains (17). More significantly, we have also detected for the first time in the United States the presence of the *bla*_{OXA-58}-like gene (4, 10, 12, 18, 29, 35, 37, 47, 49). OXA-23- and OXA-58-like β -lactamases contribute to meropenem and imipenem resistance in 90% of the carbapenem-resistant isolates in this collection. We also detected the presence of an IS element, IS_{ABAI}, located proximal to the *bla*_{OXA-23}-like genes in our isolates. This IS element may act as a strong promoter and be linked to higher levels of carbapenem resistance by increasing β -lactamase expression. This analysis is similar to contemporary studies by Turton et al. (49), wherein IS elements (IS_{ABAI} upstream of *bla*_{OXA-23}-like genes) have been found in carbapenem-resistant *A. baumannii* isolates in the United Kingdom. Insertion elements have also been shown to flank *bla*_{OXA-58} and enhance its expression (36). Interestingly, this is the first description of *bla*_{PER} in the United States.

It is possible that the carbapenem-resistant isolates also have reduced permeability of the outer membrane, altered penicillin binding proteins, or upregulated efflux pumps. We did not find evidence for common metallo- β -lactamases (IMP, VIM, and GIM type) that confer high levels of carbapenem resistance. Investigations analyzing the outer membrane proteins in carbapenem-resistant isolates are in progress.

Many of our isolates were resistant to quinolones due to mutations in the QRDR. Although two isolates do not have these mutations, an upregulated efflux pump might account for their quinolone resistance.

Aminoglycoside resistance (secondary to the presence of genes encoding AMEs) was also prevalent. Many of these genes are widespread in *Pseudomonas aeruginosa* and *A. baumannii* and mirror those described in a collection of MDR *A. baumannii* isolates from Europe (clone types I, II, and III) (22, 32). Unlike the European strains, we did not always find the aminoglycoside gene *aadA1* in conjunction with *aacC1*. As previously shown, a significant proportion of the genes encoding AMEs are contained in class 1 integrons. There was a 93% correlation with the presence of *aadA1* and *int* genes as previously described (2, 43, 48, 54).

Our analysis of the genetic relatedness of these 75 isolates by PCR ESI-MS against 26 ATCC reference strains and 23 European *A. baumannii* isolates firmly establishes the complexity of the transmission dynamics of this endemic situation. It is remarkable that more than 60% of the isolates were related to the pan-European strains that have been collected from more than 25 countries, including Spain, Greece, Portugal, Poland, France, South Africa, The Netherlands, and Italy, over the last 20 years (32, 50). We further demonstrate that isolates from each of the eight main clones (including all three pan-European types) were also responsible for the hospital-acquired infections at the WRAMC. During the 13-month period, January 2004 to February 2005, seven of the eight main clone types of *A. baumannii* simultaneously circulated at the WRAMC (Table 7). Although we identified a total of 16 unique clone types, one limitation of our study is that the isolates we analyzed may not have included all *Acinetobacter* sp. clone types present at the WRAMC during the entire collection period (March 2003 to February 2005).

Why are these MDR isolates spreading so readily? It has been suggested that the epidemic potential of *A. baumannii* may be linked to the presence of class 1 integrons that contain antibiotic resistance genes (25). In an environment in which antibiotics are frequently used, possessing integrons with multiple resistance determinants confers a strong selective advantage (46). We found genetic evidence for the integron-encoded integrase genes *int11*, *int12*, and *int13* in 60% of our collection (Table 6). Class 1 integron cassettes were found in 43% of our MDR isolates. As warned by Richet and Fournier, the major concern regarding *A. baumannii* is the large repertoire of resistance genes carried by a series of mobile genetic elements in the genome (15, 41).

The majority of our population were previously healthy military personnel that were wounded and became critically ill (58 patients sustained trauma and 53 patients were in an ICU). Despite the large number of bloodstream isolates, mortality rates were not high. Nevertheless, it is important to keep in mind that mortality rates were higher in immunosuppressed patients possessing MDR *A. baumannii* isolates (Table 3). Also alarming is that MDR *A. baumannii* isolates with at least eight resistance genes were frequently recovered from patients who were intubated.

This study highlights the need for careful epidemiological and molecular surveillance among receiving hospitals and renewed infection control policies with regard to *Acinetobacter*

spp. It is significant that 37% of the isolates were nosocomial in origin (obtained from patients colonized or infected at the WRAMC). In light of these observations and the nature of the multiclonal spread, we suspect that incomplete disinfection procedures and environmental transmission are playing important roles in the dissemination of *Acinetobacter* spp. in military medical facilities. As with recounts of the genetic basis of multidrug resistance in hospital outbreaks reported by Turton et al., our paper describes isolates from patients that have been transported from the combat theater hospitals in Iraq and Afghanistan to the U.S. military medical facility in Landstuhl, Germany (47). In Germany, they are stabilized prior to further transport to the WRAMC for additional treatment (8,000 miles, three hospital settings). The various environments afford multiple opportunities for acquisition and spread of *Acinetobacter* spp. The next challenge is to discern the impact of resistance determinants on clinical outcomes.

Our comprehensive analysis is the first in depth study to link the MDR phenotypes and clonality to the genetic determinants of resistance to β -lactams, aminoglycosides, and quinolones in *A. baumannii* in the U.S. As we have learned with methicillin-resistant *S. aureus* isolates and vancomycin-resistant enterococci, many of these *Acinetobacter* sp. isolates may also serve as reservoirs for antibiotic resistance genes that can be transmitted to other pathogens. Hence, the potential introduction of MDR *Acinetobacter* sp. isolates into European and American civilian, military, and Veterans Affairs medical centers has far reaching consequences. The implementation of specific educational programs, strategies for prevention of primary and secondary acquisition, and interventions designed to control this emerging nosocomial pathogen needs to be an urgent imperative in the care of civilian and military personnel in both European and U.S. hospitals. The diversity and complexity of the resistance determinants found and the potential for widespread dissemination of these strains through Europe and North America could lead to important changes in the global epidemiology of *A. baumannii*-associated diseases.

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