Fatal Outbreak of an Emerging Clone of Extensively Drug-Resistant *Acinetobacter baumannii* With Enhanced Virulence

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(See the Editorial Commentary by Paterson and Harris on pages 155–6.)

Background. Severe *Acinetobacter baumannii* infections in immunocompetent patients are uncommon, and the virulence mechanisms of this organism are not fully understood.

Methods. Following an outbreak of fatal *A. baumannii* infections in a cohort of relatively immunocompetent patients (low comorbidity and illness severity scores), isolates were investigated with comparative genomics and in animal models.

Results. Two unrelated *A. baumannii* clades were associated with the outbreak. The clone associated with the majority of patient deaths, clade B, is evolutionarily distinct from the 3 international clonal complexes, belongs to multilocus sequence type (MLST) 10, and is most closely related to strains isolated from the Czech Republic, California, and Germany in 1994, 1997, and 2003, respectively. In 2 different murine models, clade B isolates were more virulent than comparator strains, including the highly virulent reference strain AB5075. The most virulent clade B derivative, MRSN 16897, was isolated from the patient with the lowest combined comorbidity/illness severity score. Clade B isolates possess a unique combination of putative virulence genes involved in iron metabolism, protein secretion, and glycosylation, which was leveraged to develop a rapid and specific clinical assay to detect this clade that cannot be distinguished by MLST.

Conclusions. Clade B warrants continued surveillance and investigation.

Keywords. *Acinetobacter baumannii*; virulence; outbreak; extensively drug resistant.

Acinetobacter baumannii (AB) is refractory to outbreak control, persistent in the hospital built environment, and readily develops high-level antimicrobial resistance [1]. However, when compared to other "ESKAPE" species, such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, or *Pseudomonas aeruginosa*, AB is considered a low-virulence pathogen [2–4]. In fact, there were no AB-related fatalities in patients with devastating blast-related injuries and inflammatory dysregulation, despite recurrent or recalcitrant infections with extensively drug-resistant (XDR) isolates [5–7].

The intrinsic virulence and attributable mortality of AB remain controversial [3, 8–10], and few virulence factors have been identified [11]. Most studies using animal models have employed uncharacterized clinical isolates, with few or no patient data, making it difficult to correlate those results with patient outcomes. Although the mouse pulmonary model is widely used to study AB virulence [12–16], most strains of AB do not infect immunocompetent mice and induce only a self-limiting pneumonia with no or very limited local bacterial replication and systemic dissemination. Previously, all AB strains required additional manipulation of the host immune system for murine pathogenicity, except

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LAC-4 (isolated in Los Angeles, California, 1997). Unfortunately, there are no clinical or epidemiologic details available for this isolate [17, 18].

We describe a clade of AB associated with a fatal outbreak in relatively immunocompetent patients who received appropriate antimicrobial therapy and an early infectious disease consultation, which have both been associated with improved outcomes [19]. The virulence observed in patients was reproduced in animal models. Clade B isolates were more virulent than the highly virulent reference strain AB5075 [14]. Furthermore, MRSN 16897, isolated from the patient with the lowest combined illness severity and comorbidity scores, showed hypervirulence and also killed conventional, immunocompetent BALB/c mice. The genome of this evolutionarily distinct clade contains unique features that we exploited to develop (and herein make available) a rapid, polymerase chain reaction (PCR)–based diagnostic assay to facilitate earlier detection of this clade. Putative targets for countermeasure development are also suggested.

METHODS

This study was undertaken as a quality-improvement, infection-control initiative authorized by policy memoranda 09-050, 11-035, 13-016, and Walter Reed Army Institute of Research Institutional Review Board and International Animal Care and Use Committee protocols 1812, IB02-10 and 14-BRD-01S. Antibiotic susceptibility testing was performed according to Clinical and Laboratory Standards Institute guidelines in a College of American Pathologists–accredited laboratory as previously described [20]. Pulsed-field gel electrophoresis (PFGE), whole-genome mapping, sequencing, and comparative genomics were performed as previously described [20–22] and as detailed in the Supplementary Data. Illness severity and comorbidity scores were calculated after death. To reflect status of the patient before terminal infection occurred, physiologic values, ventilator status, and other components of the scoring system present 5 days before the first positive culture were used.

Mouse Infections

Eight-week-old female BALB/c mice, housed in presterilized filter-top cages and given sterile food and water ad libitum, were used. For the immunocompromised mouse model, mice were injected intraperitoneally with cyclophosphamide prior to infection, as previously described [14]. Mice were then anesthetized and intranasally inoculated with 5.0×10^6 colony-forming units (CFU) for neutropenic mice or 1.0×10^8 CFU for nonneutropenic mice of an inoculum prepared in sterile phosphate-buffered saline. Infected animals were observed twice daily for signs of morbidity. To assess bacterial dissemination, organs from infected mice were plated after 24 hours, and the bacterial burden was determined by CFU enumeration. Paraffin-embedded organs were cut into 5-μm sections and stained with hematoxylin and eosin for visualization by light microscopy.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 5.01 (La Jolla, California).

PCR Assay for Clade B Strains

Primer sets were developed for 3 genes unique to clade B, and tested in silico by using BLASTN to search the National Center for Biotechnology Information (NCBI) nonredundant and whole-genome shotgun nucleotide databases for AB genome sequences that encode 1 or more of the PCR products. This analysis verified that only clade B strains possess all 4 target sequences. Multiplex assays were carried out in 30-μL reactions containing 24 μL of Thermo DreamTaq master mix; 3.0 μL of DNA (20 ng/μL); and 0.5 μL of 1347 primers, 1.0 μL of 2622 primers, 0.5 μL of 3134 primers, and 1.0 μL of secE primers (each at 10 μM). Amplification conditions were as follows: denaturation at 95°C for 4 minutes; 95°C for 15 seconds, 56°C for 15 seconds, 72°C for 5 minutes (35 cycles); and final extension at 72°C for 5 minutes. The following primers were used for each PCR reaction: *MRSN16897_P2622*, 5′-CCCCGTTCTACACTAC GAATTAAG-3′ and 5′-CCACGGCATTCCCATAAA-3′; *secE*, 5′-GATAAATCGCGTGACGCATT-3′ and 5′-CCTTGATGGG TGGCGTATA-3′; *MRSN16897_P1347*, 5′-CGAGGTCATCG CAGTAAGAAAT-3′ and 5′-CGAGCTTCATGCCAAGTTAG A-3′; *MRSN16897_P3145*, 5′-TACCGCTTTAGCATCGGATT-3′ and 5′-ACGCCAAGACAACCATTCT-3′. We further validated the specificity of the assay with a diversity panel of AB strains representing 100 different PFGE clades (defined using a 90% similarity cutoff) from our global collection of >2500 multidrug-resistant AB strains [20]. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition. The sequence of MRSN 16897 was submitted to NCBI under BioProject PRJNA238593.

RESULTS

Patient Characteristics

The outbreak occurred at a tertiary care facility in the northwest United States. All 6 patients with AB infection–related death, as defined by Chuang et al, were included [23]. All patients were white, born in the United States, and were living in the northwest United States at the time of their terminal hospitalization. All had at least 1 previous hospital admission in the previous 90 days. One patient was from the south-central United States,

Table 1. Patient and Isolate Characteristics

Case	Age/ Sex	Primary Diagnosis	CCI[a] Survival Rates 1 y/ 10 y[a]	SAPS II[b] Predicted Mortality[b]	Pitt Bacteremia Score[c]	Isolate /PFGE/ST[a] Phenotype	Culture Type	Date of Isolation (Hospital Admission Day)	Patient Outcome	Fatal in Neutropenic (Nonneutropenic) Murine Model
1	28/M	Wound infection with sepsis	2 99/90	6 0.5	2	16874/A/ ST2 XDR+	Blood	05/06/11 (1)	Death	Yes (No)
2	61/F	Cholecystitis with peritonitis and severe sepsis	3 81/77	59 66	3	16875/B/ ST10 XDR	Resp.	08/30/11 (50)	Death	Yes (No)
2						16881/B/ ST10 XDR	Tissue	10/04/11 (85)		Yes (No)
2						16882/B/ ST10 XDR	Tissue	10/04/11 (85)		Yes (No)
3	61/M	Chronic obstructive pulmonary disease, pneumonia, and severe sepsis	3 81/77	29 10	5	16896/B/ ST10 XDR	Resp.	11/26/11 (10)	Death	Yes (No)
3						16897/B/ ST10 XDR	Blood	11/29/11 (13)		Yes (Yes)
4	64/M	Congestive heart failure and chronic obstructive pulmonary disease	4 81/53	39 23	2	16898/B/ ST10 XDR	Resp.	12/16/11 (6)	Death	Yes (20%)
5	63/M	Myelodysplastic syndrome and pancytopenia	4 81/53	40 25	4	16901/B/ ST10 XDR	Blood	12/17/11 (9)	Death	Yes (No)
6	81/F	Pneumonia with bacteremia	8 63/<20	33 14	2	17592/B/ ST10 XDR	Blood	2/27/13 (10)	Death	Yes (No)
6						17593/B/ ST10 XDR	Blood	2/27/13 (10)		Yes (No)

Abbreviations: CCI, Charlson comorbidity index; PFGE, pulsed-field gel electrophoresis; Resp., respiratory; SAPS II, Simplified Acute Physiology Score; ST, sequence type; XDR, extensively drug resistant.

[a] Charlson comorbidity index with 1- and 10-year survival rates (%). The CCI can range from 0 to 37. (A patient <40 who has no other medical or surgical conditions would have a score of 0; an 81-year-old with AIDS and cancer would have a score of 37).

[b] Simplified Acute Physiology Score II with predicted in-hospital mortality (%). SAPS II comprises 12 physiological variables and 3 disease-related variables. The worst physiological variables were collected 5 days before the first positive culture. The "worst" measurement was defined as the measure that correlated to the highest number of points. The SAPS II score ranges from 0 to 163 points.

[c] A Pitt bacteremia score >4 indicates severe illness. The Pitt bacteremia score is based on temperature, hypotension, mental status, and presence or absence of mechanical ventilation.

and 5 were from the northwest United States. Patient 1 had extended stays at long-term acute care and skilled nursing facilities and the broadest array of antibiotic exposure (5 classes). Patient 5 had previous healthcare contact in Seattle, Washington. No patient had recently traveled outside the United States prior to terminal admission. Their average age was 60 years (range, 28–81 years). None had scores indicating excessive comorbidities or critical physiologic dysfunction (Table 1). Their Charlson comorbidity index scores [24] ranged from 2 to 8, with a mean of 4 (standard deviation [SD], 2.1; 95% confidence interval [CI], 1.80–6.20). Their Simplified Acute Physiology Score (SAPS II) [25] ranged from 6 to 59, with a mean of 34.33 (SD, 17.29; 95% CI, 16.18–52.48). Two patients had a Pitt bacteremia score [26] >4, indicating severe infection (Table 1). Pitt bacteremia score values ranged from 2 to 5, with a mean of 3 (SD, 1.3; 95% CI, 1.67–4.33).

Isolate Characteristics

All isolates met definitions of hospital acquired or healthcare associated and XDR. (Supplementary Table 1) [27]. Both PFGE and multilocus sequence typing (MLST) showed that the outbreak included isolates from 2 separate clades (Table 1; Supplementary Figure 1); whole-genome sequencing analysis confirmed the PFGE and MLST findings (Supplementary Figure 2). The single clade A strain, MRSN 16874, is sequence type (ST) 2 (Table 1) and belonged to ICC2, one of the major international clonal complexes (ICCs) [28]. This strain was most similar to strains MDR-JZ06, BJAB07104, and MDR-TJ (Figure 1*A*) [29].

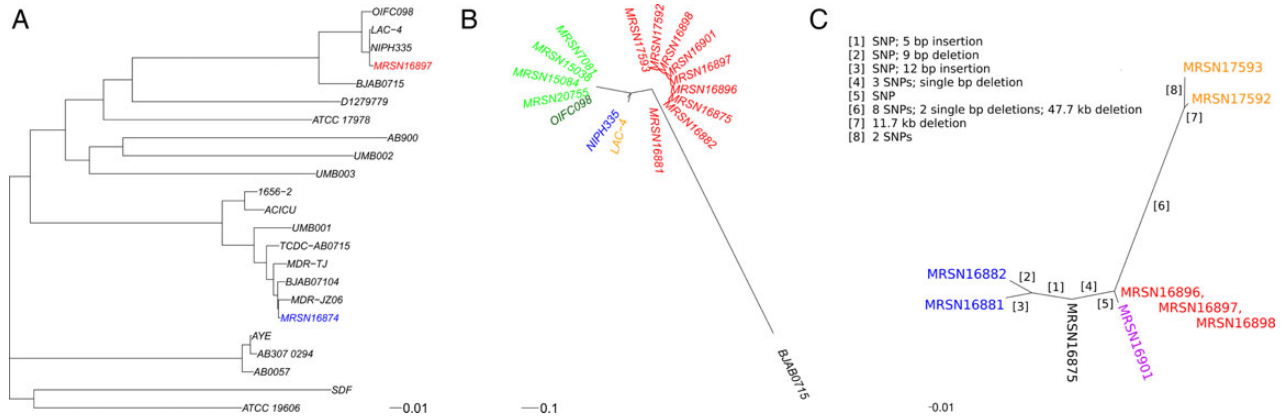

Figure 1. Phylogenetic analysis of the outbreak strains. *A*, Relationships based on whole-genome analysis between the clade A strain (blue), clade B strains (red), and a set of 34 reference isolates representing the genomic diversity of *Acinetobacter baumannii* (described in Supplementary Table 4). *B*, Phylogenetic relationships between clade B strains (red), NIPH 335 (blue), LAC-4 (orange), and the OIFC strains (green). Strain BJAB0715 represents the root of the tree. *C*, Evolutionary relationships among the clade B isolates. Strain names are color-coded by patient (patient 2: blue; patient 3: red; patient 4: black; patient 5: purple; patient 6: orange). The time of isolation after the start of the outbreak, in days, is indicated in parentheses after the strain name. MRSN 16875 is the progenitor strain of the outbreak. Abbreviation: SNP, single-nucleotide polymorphism.

Clade B, the predominant outbreak clade, showed a PFGE pattern unique among 1800 AB isolates in our collection and belonged to MLST ST10, which is distinct from the 3 ICCs. ST10 is an uncommon ST type that may be emerging or reemerging. Genome analysis revealed that clade B isolates are clonal and are most closely related to the sequenced strains NIPH 335 (Czech Republic; 1994), LAC-4 (California; 1997), OIFC098 (US military hospital in Germany; 2003), and BJAB0715 (China; 2007) (Figure 1*B*). NIPH 335, LAC-4, and OIFC098 were ST10, and the more distantly related BJAB0715 was ST23. Ecker et al identified 7 *Acinetobacter* isolates (hereafter called "OIFC isolates") with the same genetic profile as OIFC098 [30]. All 6 OIFC isolates in our repository were closely related to clade B at the genome level (Figure 1*B*). None, however, had a PFGE pattern similar to those of clade B strains (data not shown). The PFGE profile of OIFC193 was closest to those of clade B strains, yet had only 61% similarity. Genome sequencing of clade B isolates revealed the presence of 21 antibiotic resistance genes that correlated with the XDR phenotype (Supplementary Table 2). The 2 clade B isolates that were also resistant to imipenem, MRSN 16881 and MRSN 16882, had acquired a knockout mutation in the AB *oprD* homologue.

Phylogenetic and Clinical-Temporal Analysis of Isolates

Phylogenetic analysis and date of isolation suggested that MRSN 16875 was the progenitor of clade B (Figure 1*C*). Among strains derived from this isolate, MRSN 16897 was of particular interest because it exhibited hypervirulence in mice (see below). MRSN 16897 was isolated from the blood of patient 3, 15 days after hospital admission and 207 days after the progenitor clade B strain was isolated from patient 2. Patient 3 had the lowest combined comorbidity and illness severity scores. MRSN 16897 differed from the progenitor by 4 mutations: (1) a predicted knockout mutation in a transcriptional repressor, (2) a knockout mutation in a protein of unknown function, (3) a mutation causing a conservative amino acid change in a putative transport system protein, and (4) a mutation producing a conservative amino acid substitution in a homologue of the GTP binding protein Hflx (green box in Supplementary Table 3).

Potential Virulence Markers and Countermeasure Targets

Clade B strains encoded 208 genes homologous to known virulence factors: 186 loci from the Virulence Factor Database [31] and 22 genes identified by Marti and coworkers [32] (Figure 2*A*). The most common functional categories of virulence factors were iron acquisition (n = 68), adhesion and invasion (n = 67), and secretion systems and effectors (n = 45). Twenty-six virulence genes were present in less than half of a set of 34 evolutionarily diverse AB reference strains (Table 2). Five genes are present only in clade B isolates and/or the most closely related strains; 3 of these are unique to clade B.

Protein glycosylation is important for biofilm formation, capsule production, and other aspects of virulence. AB strains are quite diverse with respect to glycosylation; >20 versions of the glycosylation gene complex have been identified [33]. Clade B strains possessed a novel gene cluster encoding enzymes for O-linked protein glycosylation (Figure 2*B*) that was most similar to the PSgc24 version of the glycosylation complex.

Clade B isolates also possessed a unique variant of the type VI secretion system (T6SS) gene cluster (Figure 2*C*, top). Different,

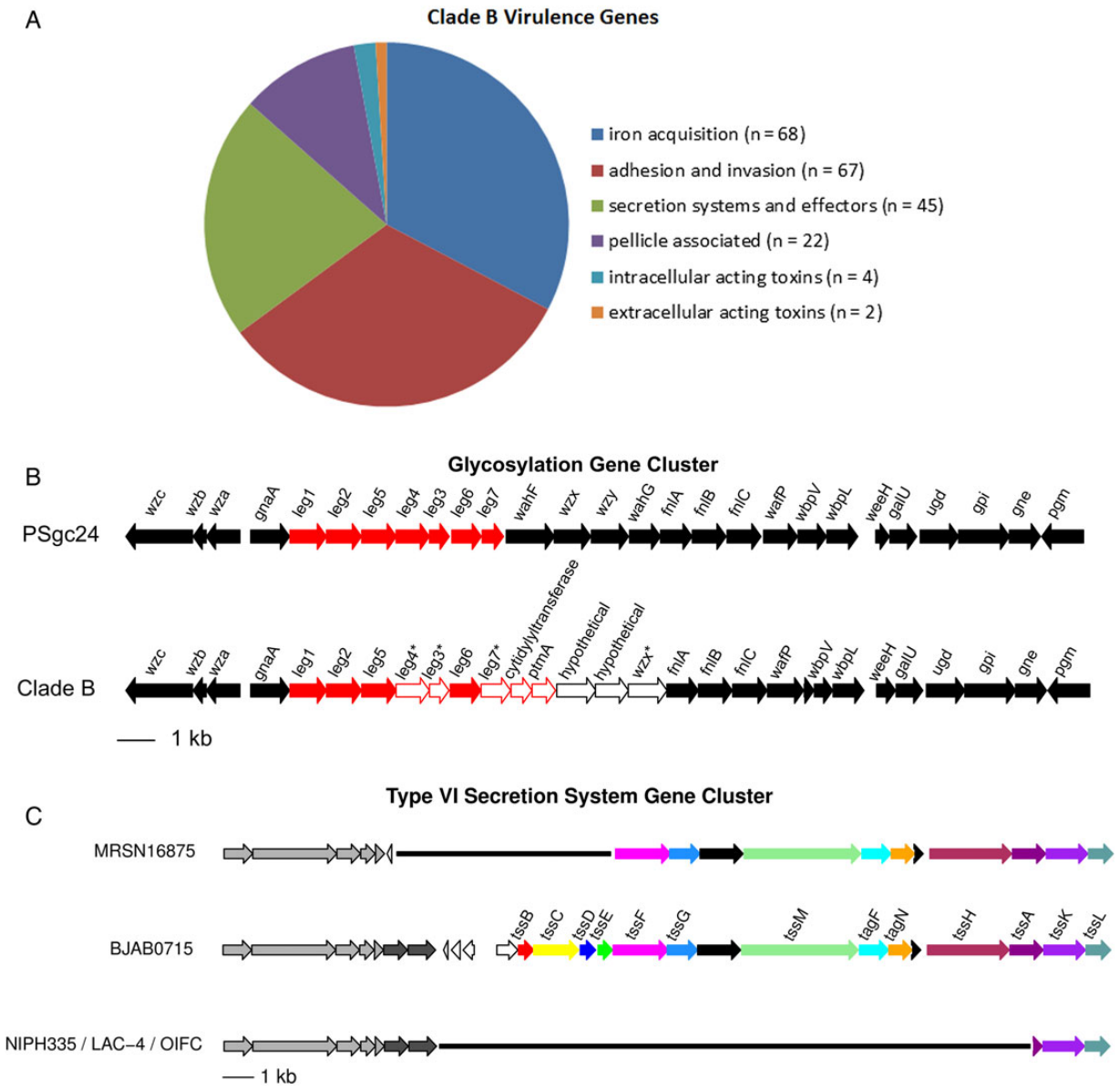

Figure 2. Clade B virulence loci. *A*, Distribution of virulence loci in clade B strains by Virulence Gene Database functional category. *B*, Glycosylation gene cluster of clade B strains. Shown are the PSgc24 version of the glycosylation gene cluster (top) and the glycosylation gene cluster present in clade B strains (bottom). Predicted clade B genes, drawn as unfilled arrows, encode proteins showing <90% identity to the corresponding protein products of the PSgc24 gene cluster. Genes colored red are involved in the production of carbohydrates containing legionaminic acid. *C*, Variant type VI secretion system (T6SS) gene clusters of clade B and related strains. Shown are the T6SS clusters of clade B strain MRSN 16875 (top), BJAB0715 (middle), and NIPH 335, LAC-4, and OIFC strains (bottom) . The T6SS gene cluster in BJAB0715 consists of the core *Acinetobacter baumannii* T6SS gene cluster (*tssB–tssL*) with an additional approximately 10 kb of sequence, encoding 11 predicted genes of novel sequence (gray and unfilled arrows), immediately upstream of the *tssB* locus. The clade B and NIPH 335/LAC-4/OIFC gene clusters appears to be derived from this T6SS gene complex via independent deletion events (solid bars). The T6SS gene cluster in clade B strains was produced by a 7.25-kb deletion that removes 6 novel genes and the first 4 genes of the core T6SS cluster. The gene cluster in NIPH 335, LAC-4, and the OIFC strains resulted from a 19.15-kb deletion that removes 4 novel genes and nearly the entire set of core T6SS loci. One novel gene retained in LAC-4 encodes a protein with 6 Sel1 protein–protein interaction domains.

yet evolutionarily related, variant T6SS gene complexes were found in the Chinese strain (Figure 2*C*, middle), and NIPH 355, LAC-4, and the OIFC isolates (Figure 2*C*, bottom). MRSN 17592 and MRSN 17593 differed from other clade B isolates by a 47.7-kb deletion that completely removed the T6SS gene cluster.

Table 2. Virulence Proteins That Are Unique to or Poorly Conserved in Clade B Strains

Protein	Description	Virulence Function	No. of Strains
P1165	Type IV pilin PilA	Adhesion and invasion	0[a]
P3145	Legionaminic acid cytidylyltransferase (EC 2.7.7.43)	Adhesion and invasion	0[a]
P3488	Cell filamentation protein Fic	Secretion systems and effectors	0[a]
P955	Outer membrane protein E	Pellicle	1[b]
P2770	Two-component response regulator	Secretion systems and effectors	1[b]
P2757	PhaK-like protein	Adhesion and invasion	3
P3193	VgrG protein	Secretion systems and effectors	3
P3333	FilF	Adhesion and invasion	3
P3144	Legionaminic acid biosynthesis protein PtmA	Adhesion and invasion	4
P3381	Chaperone protein DnaK	Adhesion and invasion	4
P1479	Outer membrane protein E	Pellicle	5
P1957	Outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid	Iron acquisition	5
P1306	Ferrichrome-iron receptor	Iron acquisition	6
P3149	*N*-acetylneuraminate synthase (EC 2.5.1.56)	Adhesion and invasion	6
P105	Ferrichrome-iron receptor	Iron acquisition	7
P3324	VgrG protein	Secretion systems and effectors	7
P3409	Outer membrane protein G1b	Pellicle	7
P3705	Conserved domain protein	Secretion systems and effectors	7
P3140	UDP-*N*-acetylglucosamine 4,6-dehydratase (EC 4.2.1)	Adhesion and invasion	9
P279	DNA-binding heavy metal response regulator	Secretion systems and effectors	12
P289	Response regulator receiver:transcriptional regulatory protein, C-terminal	Secretion systems and effectors	12
P602	Ferrichrome-iron receptor	Iron acquisition	12
P1540	Fimbrial adhesin precursor	Adhesion and invasion	15
P1885	FIG006045: Sigma factor, ECF subfamily	Secretion systems and effectors	15
P399	Sigma-fimbriae tip adhesin	Adhesion and invasion	16
P507	Copper-sensing 2-component system response regulator CusR	Secretion systems and effectors	16

Protein identifiers are based on the annotation of the MRSN 16897 genome. Predicted functions are from the Virulence Factor Database [31] (adhesion and invasion; iron acquisition; secretion systems and effectors) and Marti et al [32] (pellicle). The last column shows how many of 34 *Acinetobacter* reference strains possess a close homologue (ie, 90% similarity over 90% of the length of the shorter protein) of the virulence protein.

Abbreviation: ECF, extracytoplasmic function.

[a] The protein is specific to clade B isolates.

[b] The protein is present in clade B strains and the evolutionarily related BJAB0715, NIPH 335, LAC-4, and OIFC isolates.

Virulence in Animal Models

Two murine pulmonary infection models were used to assess the virulence of clade B isolates. In the first model, mice were rendered neutropenic by cyclophosphamide treatment prior to inoculation with 5.0×10^6 CFU. Mice infected with MRSN 16897, MRSN 16898, and MRSN 17593 all succumbed to infection by day 3, but only 50% of mice infected with MRSN 16881 died at that time (Figure 3*A*). To determine whether differences in murine susceptibility to AB infection were associated with bacterial burden, the lungs, spleen, and liver of infected mice were collected at 24 hours postinfection, and the bacterial burden was assessed by quantifying CFU. Mice infected with MRSN 16897, MRSN 16898, and MRSN 17593 had approximately 1–2 logs higher CFU in the lungs compared with MRSN 16881 (Figure 3*B*). Extrapulmonary dissemination from the lungs to the spleen and liver was observed in all mice infected with clade B (Figure 3*C*–*E*). However, the bacterial burden in the blood, spleen, and liver was significantly lower in MRSN 16881 compared with other clade B isolates, suggesting that the level of bacterial colonization and dissemination directly correlated with murine susceptibility to infection.

Next, we used an immunocompetent model to further compare virulence of clade B and other AB clinical isolates. We found that 80% of mice intranasally inoculated with MRSN 16897 and 20% of mice infected with MRSN 16898 died by day 2, but mice infected with all other tested strains, including the reference strains ACICU and highly virulent AB5075, survived infection (Figure 3*F*). Histological analysis (Figure 3*G*–*J*) indicated that rapid death of mice infected with MRSN16897 was due to AB bacteremia, with end-organ involvement.

Novel Diagnostic Assay for Clinical Use

Clade B cannot be distinguished from other, less virulent ST10 strains by conventional MLST analysis. Therefore, we developed

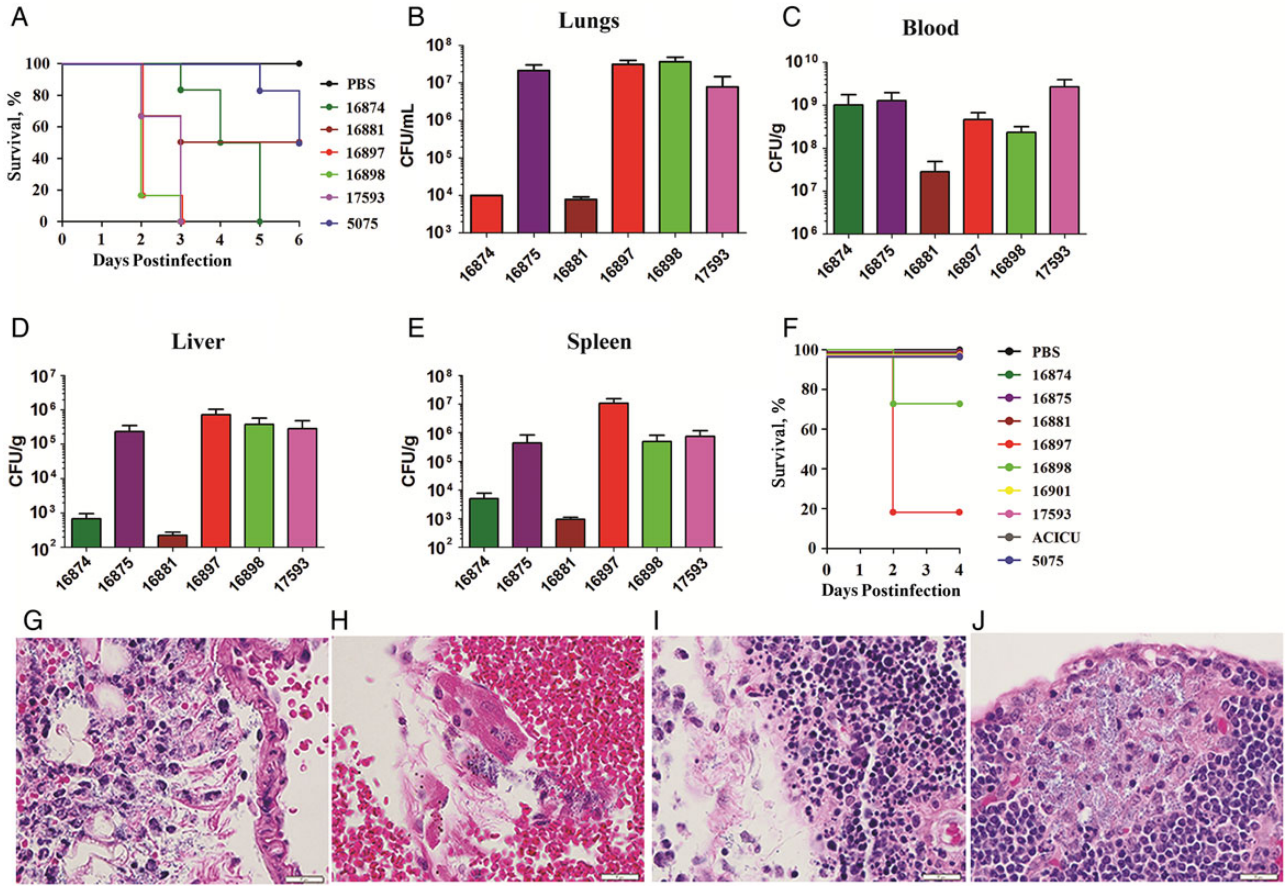

Figure 3. Extrapulmonary dissemination and mortality of mice infected with the *Acinetobacter baumannii* fatal cluster. *A*, Survival curve of mice intranasally infected with 5×10^6 colony-forming units (CFU) of *A. baumannii* and monitored twice daily for signs of morbidity ($P < .0001$, log-rank test). At 24 hours post infection (hpi), the lungs (*B*), blood (*C*), liver (*D*), and spleen (*E*) of infected mice were harvested, and the bacterial burden was determined by CFU enumeration. Data are representative of 3 independent experiments. *F*, Mice were intranasally infected with 1×10^8 CFU of bacteria and monitored twice daily (n = 11; $P = .0031$, log-rank test). At 24 hpi, the lungs (*G*), heart (*H*), thymus (*I*), and pancreaticoduodenal lymph nodes (*J*) were collected and stained with hematoxylin and eosin. All images are ×100 magnification. Abbreviation: PBS, physiologic buffered saline.

and validated an inexpensive PCR assay to rapidly and specifically identify these strains in clinical laboratories. We used template from 5 of the OIFC clinical isolates and 6 reference AB strains to validate the specificity and reproducibility of this assay (Figure 4). Using 100 genetically diverse AB strains, only clade B strains were positive for the 4 genes. In fact, not a single isolate amplified any product other the *sec*E positive control. This was further confirmed using assays with real-time PCR primers, which were developed alongside conventional PCR primers.

DISCUSSION

A key finding of our work was that AB strains which are genetically closely related may show significant differences in virulence, suggesting that small genomic changes can profoundly affect virulence. To our knowledge, this is the first report leveraging comparative genomics, animal models, and a surveillance network with isolate repository to characterize an emerging clade of XDR AB associated with a fatal outbreak in patients not severely immunocompromised. Clade B strains were highly virulent in the neutropenic murine pneumonia model. MRSN 16897 and MRSN 16898 also killed immunocompetent BALB/c mice and were more virulent than a recently isolated highly virulent reference strain, AB5075 [14]. Finally, the isolate that demonstrated the highest virulence in animal models, MRSN 16897, was isolated from the patient with the lowest combined severity indices (patient 3) (Table 1). Clade B isolates possessed unique and biologically plausible genomic correlates of pathogenicity, which were used to develop a surveillance-based countermeasure.

In addition to the intrinsic properties of the pathogen, the host response to the pathogen is also a major driver of virulence and outcomes. We attempted to account for the host response by using 2 independent murine models and 3 clinical scoring

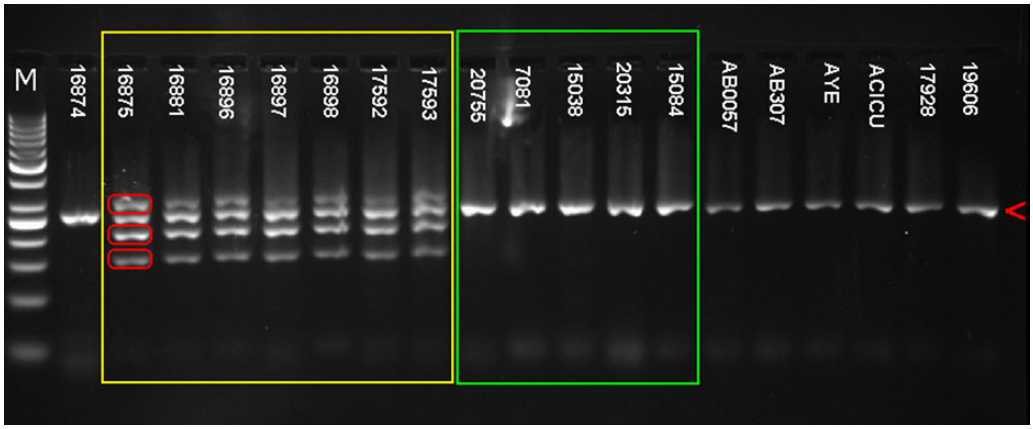

Figure 4. Multiplex polymerase chain reaction (PCR) assay for the identification of clade B strains. The 4 PCR products visible in the agarose gel, from highest to lowest molecular weight, are amplified from *MRSN16897_P2622*, *secE*, *MRSN16897_P1347*, and *MRSN16897_P3145*. The 3 PCR products specific to clade B strains are circled in red. The product from the *secE* gene (red arrowhead) is amplified from DNA extracted from all *Acinetobacter baumannii* strains and serves as a positive control. PCR products from clade B strains are enclosed by the yellow box. PCR products of the OIFC isolates are enclosed by a green box. Amplification products of laboratory reference strains lie in the rightmost 6 lanes of the gel.

systems. Together, the Charlson comorbidity index, SAPS II, and Pitt bacteremia score entailed 15 physiologic and disease-related variables and provided a comprehensive, objective assessment of the status of the patients [24–26].

Our current understanding of AB pathogenicity is primarily based on mutagenesis studies using reference strains ATCC 19606 and ATCC 17978, which were isolated >50 years ago. Considering the ease with which AB can alter its genetic structure and acquire resistance or virulence determinants [34], these models of AB pathogenesis may have limited clinical relevance [14]. The AB strain LAC-4, isolated in 1997, showed hypervirulence in immunocompetent mice. The lack of clinical history available for that isolate [17, 18], however, complicates the application of these research findings to patient care.

The structures of potential virulence gene clusters can provide insights into the evolutionary relationships and pathoadaptation of ST10 and clade B strains. This was most evident with the gene cluster encoding the T6SS. T6SS are specialized bacterial secretion systems that mediate the transport of effector proteins into bacterial and eukaryotic cells via a needle-like apparatus [35]. T6SS are thought to influence bacteria–bacteria and bacterial–host interactions. Bacteria use T6SS to kill or degrade other species, thereby enhancing their ability to colonize or invade by suppressing competing or predatory bacteria [36, 37]. The T6SS gene cluster in clade B and the variant T6SS cluster in NIPH 335, LAC-4, and the OIFC strains appear to have arisen from an ancestral T6SS cluster through independent deletion events (Figure 3C). Interestingly, the gene cluster in clade B included 5 novel genes that were not homologous to any bacterial genes known to be involved in T6SS. Notably, their presence was accompanied by the loss of the first 4 genes of the canonical T6SS. The gene cluster in the other ST10 isolates had an even more extensive deletion that removed all but 2 canonical T6SS loci. Perhaps the presence of 1 virulence factor such as a modified capsule (see below) may demand the alteration or loss of another (T6SS) for fitness or membrane integrity. Alternatively, deletion of genes encoding T6SS components may enhance immune evasion. AB may rearrange its genome in response to ambient selection pressures. Complete loss of T6SS genes occurred in 2 clade B strains isolated more than a year after the initial fatal infection (Supplementary Table 3). In this case, the organism may have adapted to a specialized niche (the bloodstream or nosocomial milieu) by jettisoning metabolically costly genes needed to ward off competitors normally present in more "hostile" environments such as the soil resistome. Clade B, as well as NIPH 335 and LAC-4, possessed a unique set of genes for protein modification by the addition of carbohydrates containing legionaminic acid, a sugar not typically produced by *Acinetobacter* [33]. Recent work has confirmed that LAC-4 polysaccharides contain this sugar [38]. We speculate that polysaccharide modifications may contribute to the enhanced virulence of clade B strains and LAC-4, but further investigation is necessary.

Our study is limited by the lack of mutagenesis studies, which are beyond the scope of our surveillance mission. Attributable mortality cannot be determined; however, the crude mortality 18% (6/34) is consistent with other reports, and higher than similar cohorts of non–intensive care unit patients. The distinctive genetic features of clade B could inform the development of specific countermeasures targeting virulence factors such as compounds or antibodies against capsular carbohydrates containing legionaminic acid, protein P1165 (type IV pilin), and

protein P955 (outer membrane protein E). A vaccine based on attenuated clade B strains such as MRSN 17592 and MRSN 17593, which were less virulent in mice but contained genetic determinants universal to clade B strains, might merit investigation. Perhaps clade B isolates resist early innate effectors, leading to sustained bacteremia, Toll like receptor 4–mediated hyperinflammation, and lethality [39]. Together, these findings support the contention that the first dose of antibiotic is the most crucial and so should be rationally dosed for greatest impact [40]. Finally, the PCR-based assay described will facilitate surveillance because it is faster and more specific than MLST, and less technically demanding and expensive than PFGE or whole-genome sequencing. Clinicians and infection preventionists should remain vigilant for XDR and highly virulent clade B.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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