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Comparison of molecular typing methods for the analyses of *Acinetobacter baumannii* from ICU patients

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Abstract

Acinetobacter baumannii has emerged as an important cause of healthcare-associated infections causing great morbidity and mortality. Despite its clinical importance, it is still unknown which molecular typing method is the best to determine or confirm institutional outbreaks as well as to identify epidemiologically related isolates from different geographical areas. To determine the most discriminatory molecular typing method, we isolated *A. baumannii* from perianal swabs collected from intensive care unit (ICU) patients in a cohort study during 2002 and 2008. Strains from each year were analyzed by pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and multi-locus variable-number tandem repeat analysis (MLVA). Genetic relatedness of the isolates was consistent between PFGE and MLST as well as between analyses of loci containing MLVA and MLST. Our data show that PFGE and MLVA are similar when discriminating between isolates and are both good methods to use when questioning whether two isolates are indistinguishable.

1. Introduction

Acinetobacter baumannii has emerged as a significant multidrug-resistant pathogen in healthcare institutions globally as well as the cause of many community acquired infections. It is also known for its resistance to nearly all existing antibiotics as well as its ability to cause great morbidity and mortality.^{1–4} *A. baumannii* is among the ten most common Gram-negative bacteria causing nosocomial infections worldwide and accounts for 2–10% of all nosocomial infections in the United States and Europe.^{5–7} The proportion of nosocomial infections caused by *A. baumannii* has increased over the past decade while no other Gram-negative bacteria demonstrated a similar increase.⁸ Because of its clinical significance and

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its propensity to cause outbreaks, *A. baumannii* has been the topic of molecular epidemiology studies which use techniques like pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and multi-locus variable-number tandem repeat analysis (MLVA).^{9–13}

Understanding of how transmission occurs, a necessary step in the effort to prevent the spread of a pathogen, requires the ability to distinguish genetic differences within a species. Currently, PFGE is the gold standard for identifying the genetic relatedness of many strains of pathogenic bacteria. However, with the emergence of other molecular methods, such as MLVA and MLST, it is necessary to test the discriminatory powers of each method. For *Escherichia coli* O157¹⁴ and *Vibrio cholerae*,^{15,16} the analyses of loci containing variable number tandem repeats (VNTR), the building blocks of MLVA, is a better discriminatory method than either PFGE or MLST for determining genetic relatedness and tracking isolates in epidemiological context. Therefore, when a new MLVA method for rigorously differentiating *A. baumannii* was demonstrated,^{17,18} there was a clear need to determine whether it may be useful during epidemiological studies.

In the current study, we applied MLVA, MLST, and PFGE typing methodologies to a large group of *A. baumannii* isolated from patients in the intensive care unit (ICU). We sought to assess the discriminatory power of these methods in order to establish a standard for *A. baumannii* and to evaluate the potential utility of these techniques in defining relationships among isolates for epidemiological studies of patient-to-patient transmission.

2. Materials and methods

2.1 Bacterial Isolates.

A subset of surveillance perianal swab specimens taken from a cohort of patients admitted to the medical and surgical ICUs at the University of Maryland Medical Center (UMMC) in 2002 and 2008 were used. Patients from this cohort received admission, weekly, and discharge perianal swabs as part of an infection control plan for active surveillance of vancomycin-resistant enterococci. Specimens were frozen for future research use.¹⁹ This study was approved by the University of Maryland Institutional Review Board. From this cohort, we examined three different sub-groups of *A. baumannii* isolates that were epidemiologically linked in different ways in order to test the discriminatory powers of the selected molecular tests. The first sub-group was from patients that were colonized with *A. baumannii* during a non-outbreak setting from April 1, 2002 to January 31, 2003. The second sub-group was from patients that were colonized with *A. baumannii* during an outbreak setting defined by UMMC infection control by increase in clinical infections and linked by epidemiological factors including unit, room, and medical staff from February 1, 2008 to February 28, 2008. The third sub-group was from patients that were colonized with *A. baumannii*, but also had blood-stream infections (BSI) with *A. baumannii* from January 1, 2008 to June 30, 2008. For the patients with BSI, isolates collected from blood samples were also included in this cohort.²⁰ Stored frozen perianal cultures were thawed, plated onto MacConkey agar (Remel, Lenexa, KS), and MacConkey agar supplemented with 6 µg/ml imipenem and incubated at 37°C for 24 to 48 hours. Non-lactose fermenting organisms were

identified as *A. baumannii* using API 20NE Identification Strips (BioMérieux Inc.; Hazelwood, MO, USA).

2.2. Pulsed Field Gel Electrophoresis (PFGE).

PFGE was performed as previously described by the Center for Disease Control and Prevention's PulseNet.²¹ DNA from each isolate was digested with the restriction enzyme *ApaI*, and the resulting fragments and control lambda ladder were separated by electrophoresis in 1% agarose gels with a contour-clamped homogeneous-field machine (CHEF-DR II, Bio-Rad, Hercules, CA). Electrophoresis was performed at 200 V for 18.5 hours, with pulse times ranging from 7–20 seconds. Photographic images of the gels were saved digitally with Geldoc EQ software (Bio-Rad, Hercules, CA) and saved as a TIFF files for gel analysis with GelCompar II (Applied Maths, Austin, TX). The band patterns were compared by the use of the Dice coefficient by using the unweighted pair group method to determine band similarity. The criteria outlined by Tenover et al. to define the pulsed-field type (PFT) clusters.²² Isolates with band patterns that were 100% identical were considered to be of identical PFGE types and delineated by the same PFGE number (example: 1, 2, 3), and isolates that had band patterns with a 85% similarity were clustered into PFGE groups and delineated by a PFGE number and letter to show the subtype (example 1A and 1B).^{17,23}

2.3 Multilocus sequence typing (MLST).

MLST was performed by using primers from Bartual et al. described previously except for *cpn60-R*, *gdhB-R*, *rpoD-F* and *rpoD-R* primers, which were designed in house (Table 1), and *gpi*, which was described by Karah et al.^{24,25} Briefly, 3–5 colonies are placed into a 96 well plate containing 100 µl of deionized water. The bacteria were then boiled at 100°C for 15 minutes to release the genomic DNA. Standard DNA amplification and sequencing using an ABI Prism 3730xl DNA sequencer (Applied Biosystems, Foster City, CA) with BigDye fluorescent terminators of the seven housekeeping genes: *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi* and *rpoD*, was performed on all 68 isolates. The nucleotide sequences were compared to existing sequences in the MLST database (<http://pubmlst.org/abaumannii/>) and assigned a sequence type (ST) number according to their allelic profiles. For cluster analysis and assignment into clonal complexes, which differed by one allele, eBurst was used: http://eburst.mlst.net/v3/mlst_datasets/.

2.4 Multi-locus variable-number tandem repeat analysis (MLVA).

The MLVA method was performed using primers and PCR conditions described by Pourcel et al. 2011.¹⁷ Instead of using gel electrophoresis, the described primers were tagged with a dye-label and multi-colored capillary electrophoresis was used¹⁸ in the ABI 3730 XL DNA analyzer (Applied Biosystems, Foster City, CA) to size the fragments using the software GeneMapper V4.0 (Applied Biosystems, Foster City, CA). Eight loci named 3530, 3002, 2240, 1988, 0826, 0845, 2396, and 3468 were shown to have a variable number of tandem repeats and were divided into two running panels. Panel one included 3530, 3002, 2240, and 1988. Panel two included 0826, 0845, 2396, and 3468. The 5' end of the forward primer of 3530 and 2396 was labeled with PET, 3002 and 3468 with FAM, 2240 and 0826 with VIC, and 1988 and 0845 with NED, and PCR was performed. The number of repeats in each allele was obtained by subtracting the flanking regions and then dividing the value by the

length of each repeat. Strains were compared using MLVAnet: <http://mlva.u-psud.fr/MLVAnet/> and were assigned into MLVA-8 complexes based on the criteria suggested by Pourcel et al. Strains were assigned to the same MLVA-8 complex if they have identical L-repeat VNTR profiles (3530,3002,2240 and 1988) and the S-repeat VNTRs (0826,0845,2396, and 3468) have no more than two repeats with a maximum difference of three.¹⁷

3. Data analysis

To compare the discriminatory powers of PFGE, MLST, and MLVAs, Simpson's index of diversity (D) was utilized by use of the following formula^{26,27}:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^S n_j(n_j - 1)$$

Where N is the total number of strains in the sample population, S is the total number of types described, and n_j is the number of strains belonging to the j th type. To measure the overall equivalence between the two tests, the Adjusted Rand was used. The Wallace coefficient was also performed to calculate the probability that two different of strains which are assigned to the same type by one method are categorized as the same type by another method.²⁸ Strains were analyzed at the clonal complex level, meaning that PFGE groups, MLST clonal complexes, and MLVA-8 complexes were used. All three tests can be calculated by inputting the data into the online tool located at: <http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool#>.

4. Results

Sixty-eight isolates of *A. baumannii* were compared using three different sub-groups, (1) 13 from 2002–2003 representing ICU colonized patients, (2) 21 from the 2008 outbreak and (3) 34 from patients with an *A. baumannii* BSI from January to June 2008 including both perianal and BSI isolates. These 68 isolates were cultured from a total of 25 unique patients. The median time between a patients first swab positive for *Acinetobacter baumannii* and their last for sub-groups 1, 2, and 3 were 12, 13, and 30 days respectively. All 68 isolates were analyzed by the PFGE, MLST, and MLVA methods (Table 2). See Figure 1 for a comparison of PFGE, MLST, and MLVA methods by the three separate sub-groups.

Overall, the PFGE method revealed the most unique types, followed by MLVA and MLST, which had the least unique types. There were a total of 34 different MLVA types which were grouped into 20 MLVA-8 complexes. Complexes 15, 10, and 9 were comprised of the most isolates. PFGE analysis using ApaI digestion of the 68 *A. baumannii* isolates identified 33 unique PFGE types. These 33 types were grouped into 22 PFGE groups. The largest group was 12, consisting of 10 isolates, 2 unique types, and 4 unique patients. MLST revealed a total of 11 different STs. Among these 11 STs, 9 were not previously submitted to the *A. baumannii* database (<http://pubmlst.org/abaumannii/>). Of the 11 different STs, 6 could be clustered by analysis with the eBURST program into three different clonal complexes while the remaining five STs were classified as singletons, i.e. their alleles differed from all other

STs at 3 or more loci. Results also showed that patients can harbor more than one genotype of *A. baumannii*.

The discriminatory abilities of PFGE, MLST and MLVAs were compared by the number of MLST clonal complexes, MLVA-8 complexes or PFGE groups determined by each method. When comparing the three molecular typing methods using Simpson's *D* value, PFGE had a higher discriminatory power than the MLVA method (*D* values, 0.929 and 0.881, respectively). PFGE and MLVA had more discriminatory power than MLST which had a *D* value of 0.519.

To measure the agreement between two tests, the Adjusted Rand coefficient was calculated. The adjusted Rand for the comparison of the clustering by MLST and MLVA-8 is 0.255 and MLST and PFGE is 0.152. The adjusted Rand for the comparison of the clustering by MLVA-8 and PFGE is 0.344. The Wallace coefficient calculates the probability that two different strains are the same using one test, are the same using another test. These results are shown in table 3. The probability that two isolates that share the same MLVA-8 complex or the same PFGE groups will share the same MLST clonal complex is 100% based on our results. However, the reverse is not the same. The probability that two isolates that share the same MLST clonal complex will share the same PFGE group or MLVA group is <30%.

There were a total of 25 patients. Of these, there were 16 patients that had more than one swab positive for *A. baumannii* by culture. Interestingly, of these 16 patients, 14 had two or more genetically indistinguishable *A. baumannii* isolates by all three methods. Of the eleven patients from the outbreak period (B in Table 2), there were 21 isolates with 13 distinct combined genotypes on the clonal complex level. Among the 4 MLST types, ST349 comprised the largest group with 14 and ST355 as the smallest group with only 1 isolate. Among ST349, there were 7 MLVA-8 complexes and 2 PFGE groups. Of patients that came from time period C (Table 2), the non-outbreak period where patients had colonization (positive perianal swab) and a blood stream infection, four of the six had a genetically related isolate from both sources on the clonal level. Of the two patients with genetically distinct isolates, the MLST type was the same. However, one patient's blood isolate (patient Y) differed by PFGE and the other patient's blood isolate (patient X) did not match any of the perianal isolates by either PFGE group or MLVA-8 complex.

5. Discussion

We compared three different typing methods, PFGE, MLVA, and MLST on 68 *A. baumannii* isolates that were obtained from surveillance peri-rectal swabs and isolates from blood stream infections. Using the Simpson's *D* value, we determined that PFGE and the MLVA methods had similar discriminatory abilities and were more discriminatory when compared to MLST with *D* values of 0.929, 0.881, and 0.519 respectively.

Our study has shown that both PFGE and MLVA methods are highly discriminatory when used for typing *A. baumannii*. The *A. baumannii* isolates collected from patients in the ICU exhibited substantial variability in a short time frame. This differed from the study performed by Hu et al. who showed MLVAs had greater discriminatory power than PFGE by

resolving their isolates into 64 MLVA types and 43 PFGE types.²⁹ In their study, the isolates were non-outbreak related, limited to one isolate per patient, and were from three different Chinese cities. An explanation in the differences in our studies could be that our isolates are more epidemiologically linked by hospital, patient, and time, showing less variability.

Although the current study showed that MLST had lower discriminatory power than both PFGE and MLVA, MLST and MLVA supplied information about the relationship of isolates that is unavailable from PFGE. If the goal is to assess the relationship between many isolates from different time periods, MLST and MLVA may be the more useful typing methods.

PFGE and MLVA genotypes of isolates from the same patient confirm the presence of multiple genetic lineages in a single patient and a single outbreak. This simple observation has wide ranging implications. When using clinical surveillance of *A. baumannii* during an outbreak, testing of multiple colonies to accurately assess transmission may be needed.

Based on the adjusted Rand coefficient, there is not much agreement when comparing one test to the other, with MLVA-8 complex and PFGE groups having the highest agreement at 30%. Also, PFGE groups and MLVA-8 complexes are not good predictors of the opposite test, whereas they are excellent predictors of the MLST clonal complex. This leads to the assumption that when molecular typing isolates, performing MLVA and PFGE can further distinguish isolates, but adding MLST will add no further value.

When both MLVA and PFGE were combined, the power to discriminate between the isolates increased. However, based on our data, using more than one method to determine an outbreak may not be needed. MLVA alone or PFGE alone showed an outbreak was occurring in time period A and B. When the two methods were used together, the outbreak was less apparent in time period B and all three methods agreed in time period A.

In this study, we compared PFGE, MLST, and MLVA using epidemiologically linked isolates to maximize the discriminatory powers of these molecular methods. These are not the only molecular tools to type isolates, repPCR^{30,31} and whole genome sequencing¹⁰ are also gaining ground as potential methods for typing. However, we did not choose these methods for several reasons. RepPCR performed using agarose gels may not be sufficiently reproducible unless performed using the specialized DiversiLab equipment, used for PFGE, which provides a semiautomated method. Whole genome sequencing requires specialized analysis to interpret the large datasets created that is time-intensive.³²

In summary, we determined that PFGE is more discriminatory than MLST or MLVAs overall. However, both PFGE and MLVA are suitable methods for discriminating isolates during an outbreak. Both require specialty equipment, with PFGE being a more labor intensive method, is relatively subjective, and it is difficult to compare strains across different hospitals unless the same protocol and standard was used. We also found that patients often had more than one genetic lineage when colonized leading us to conclude that typing more than one isolate of *A. baumannii* may be needed during an outbreak investigation.

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Figure 1.

Comparison of PFGE, MLST, and MLVA for three unique time periods.

Letters represent unique patients. Circles represent MLST types. Lines represent MLVA allelic change.

Table 1:

Primers made in house for performing MLST on cultured *A. baumannii* strains.

Name	Target gene	Primer
CPN60-R	cpn60	CGCTTCACCTTCAACAT
GDHB-R	<i>gdhB</i>	TGGAATACTTCCATCAAGATTTA
rpoD-F	<i>rpoD</i>	AATGGGTACAGTAGAACTG
rpoD-R	<i>rpoD</i>	ACGCACTTTTCCAAGTG

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Table 2:

Assignment of different MLST, PFGE groups, and MLVA-8 types for each isolate.

Patient Identifier	MLVA-8 allelic profile								MLST ST	MLST complex	PFGE groupsubtype	MLVA-8 complex	Number of Isolates	Time Period of Culture*	Source	Year
	Abaum_3530	Abaum_3002	Abaum_2240	Abaum_1988	Abaum_0826	Abaum_0845	Abaum_2396	Abaum_3468								
A	7	7	4	8.5	12.5	13	16	13	350	3	8B	20	1	A	Perianal	2002
A	7	7	4	8.5	13.5	13	16	13	350	3	8A	20	3	A	Perianal	2002
B	6	8	2	5.5	9.5	20	25	13	352	6	4	4	1	A	Perianal	2002
B	7	7	4	8.5	13.5	13	16	13	350	3	8A	20	2	A	Perianal	2002
C	7	7	4	8.5	13.5	12	17	13	350	3	8A	20	2	A	Perianal	2002
D	7	7	4	8.5	13.5	12	17	13	350	3	8A	20	1	A	Perianal	2002
E	6	8	2	8	0	0	25	11	351	5	2	3	1	A	Perianal	2002
F	4	8	3	3	0	20	17	17	229	4	13	2	1	A	Perianal	2003
G	7	6	3	5	0	15	18	14	355	8	3	1	1	A	Perianal	2002
H	7	7	4	8.5	15.5	10	29	10	281	2	7	11	1	B	Perianal	2008
H	7	7	4	8.5	15.5	9	28	10	281	2	7	12	1	B	Perianal	2008
I	7	7	4	8.5	7.5	15	28	10	281	2	9	14	1	B	Perianal	2008
J	7	7	4	8.5	18.5	14	29	10	349	2	5B	10	1	B	Perianal	2008
J	7	7	4	8.5	17.5	14	29	10	349	2	5B	15	1	B	Perianal	2008
J	7	7	4	8.5	19.5	15	30	10	349	2	6D	9	1	B	Perianal	2008
K	7	7	4	8.5	18.5	14	29	10	349	2	5C	10	1	B	Perianal	2008
K	7	7	4	8.5	18.5	14	29	10	349	2	6B	10	1	B	Perianal	2008
K	7	7	4	8.5	18.5	14	29	10	349	2	6C	10	1	B	Perianal	2008
L	7	7	4	8.5	16.5	13	28	10	349	2	5B	13	1	B	Perianal	2008
L	7	7	4	8.5	16.5	13	28	10	349	2	6C	13	2	B	Perianal	2008
M	7	7	4	8.5	19.5	14	29	10	349	2	5A	10	1	B	Perianal	2008
M	7	7	4	8.5	19.5	15	29	10	349	2	6A	10	1	B	Perianal	2008
O	7	7	4	8.5	17.5	14	29	10	349	2	5B	12	1	B	Perianal	2008
P	7	7	4	8.5	19.5	14	29	10	349	2	5A	10	1	B	Perianal	2008
P	7	7	4	8.5	19.5	14	28	11	349	2	5A	16	1	B	Perianal	2008
Q	7	8	2	5	26.5	18	21	8	354	1	1	5	1	B	Perianal	2008

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Patient Identifier	MLVA-8 allelic profile								MLST ST	MLST complex	PFGE groupsubtype	MLVA-8 complex	Number of Isolates	Time Period of Culture*	Source	Year
	Abaum_3530	Abaum_3002	Abaum_2240	Abaum_1988	Abaum_0826	Abaum_0845	Abaum_2396	Abaum_3468								
R	7	8	2	5	26.5	18	21	8	354	1	1	5	2	B	Perianal	2008
S	7	7	4	8.5	17.5	13	27	10	349	2	12A	15	3	C	Perianal	2008
S	7	7	4	8.5	17.5	13	27	10	349	2	12A	15	1	C	Blood	2008
S	7	7	4	8.5	17.5	13	27	10	349	2	18A	15	1	C	Perianal	2008
S	7	7	4	8.5	17.5	13	27	10	349	2	19	15	1	C	Perianal	2008
T	7	7	4	8.5	13.5	13	27	10	349	2	12A	15	1	C	Blood	2008
T	7	7	4	8.5	17.5	13	27	10	349	2	12A	15	1	C	Perianal	2008
T	7	7	4	8.5	16.5	13	19	12	353	7	15	17	1	C	Perianal	2007
T	7	7	4	8.5	17.5	13	27	10	349	2	18A	15	1	C	Perianal	2008
T	7	8	2	5	22.5	14	20	8	356	1	21	7	1	C	Perianal	2008
U	7	7	4	8.5	18.5	15	29	10	349	1	10A	10	3	C	Perianal	2008
U	7	7	4	8.5	18.5	15	29	10	349	2	10C	10	1	C	Perianal	2008
U	7	7	4	8.5	18.5	15	29	10	349	2	16A	10	2	C	Perianal	2008
U	7	7	4	8.5	18.5	15	29	10	349	2	16A	10	1	C	Blood	2008
V	7	6	3	5	0	18	18	14	355	8	3	1	1	B	Perianal	2008
W	7	7	4	8.5	15.5	14	23	12	348	3	14	18	1	C	Blood	2008
X	7	7	4	8.5	17.5	13	29	10	349	2	10A	15	1	C	Perianal	2008
X	7	7	4	5	17.5	12	24	10	349	2	12A	19	1	C	Blood	2008
X	7	7	4	8.5	17.5	13	29	10	349	2	16B	15	1	C	Perianal	2007
X	7	7	4	8.5	15.5	12	29	10	349	2	17	11	1	C	Perianal	2008
X	7	7	4	8.5	17.5	12	29	10	349	2	17	15	1	C	Perianal	2007
X	7	7	4	8.5	17.5	12	28	10	349	2	18A	15	1	C	Perianal	2007
X	7	7	4	8.5	17.5	13	29	10	349	2	18A	15	1	C	Perianal	2007
X	7	7	4	8.5	17.5	14	29	10	349	2	18B	15	1	C	Perianal	2008
X	7	8	2	5	25.5	16	21	8	354	1	20	5	1	C	Perianal	2008
X	7	8	2	5	25.5	22	22	8	354	1	22	6	1	C	Perianal	2007
Y	7	7	4	8.5	8.5	14	30	10	349	2	10B	8	1	C	Perianal	2008
Y	7	7	4	8.5	8.5	14	30	10	349	2	11	8	1	C	Blood	2008
Z	7	7	4	8.5	16.5	13	28	10	349	2	12B	13	2	C	Perianal	2008

Patient Identifier	MLVA-8 allelic profile								MLST ST	MLST complex	PFGE groupsubtype	MLVA-8 complex	Number of Isolates	Time Period of Culture*	Source	Year
	Abaum_3530	Abaum_3002	Abaum_2240	Abaum_1988	Abaum_0826	Abaum_0845	Abaum_2396	Abaum_3468								
Z	7	7	4	8,5	16,5	13	28	10	349	2	12B	13	1	C	Blood	2008
ATCC 17978	6	8	2	5	19	2	19	13								

* A: non-outbreak period, patients are colonized with *A. baumannii*. B:outbreak period, patients are colonized with *A. baumannii*. C: non-outbreak period, patients are colonized and have an BSI with *A. baumannii*.

Table 3:

Wallace coefficients for the methods used to characterize the 68 *Acinetobacter baumannii*

Typing Method	Typing Method		
	MLVA-8 complex	PFGE group	MLST clonal complex
MLVA-8 complex	1.000	0.320	1.000
PFGE group	0.540	1.000	1.000
MLST clonal complex	0.248	0.147	1.000