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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 (Eveillard et al., 2013; Giamarellou et al., 2008; Lin and Lan, 2014; Neonakis et al., 2011). *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 (Gaynes and Jonathan, 2005; Magill et al., 2014; Manikal et al., 2000). 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 (Fournier and Richet, 2006). Because of its clinical significance and 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) (Azimi et al., 2016; Fitzpatrick et al., 2016; Hammerum et al., 2015; Kanamori et al., 2015; Villalon et al., 2015).

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* O157 (Noller et al., 2003) and *Vibrio cholerae* (Ghosh et al., 2008; Stine et al., 2008), 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 (Hauck et al., 2012; Pourcel et al., 2011), 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

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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 (Green et al., 2007). 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 (Thom et al., 2010). 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 24–48 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 (Ecker et al., 2006). 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 to 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 (Tenover et al., 1997). 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) (Minandri et al., 2012; Pourcel et al., 2011).

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. (Bartual et al., 2005; Karah et al., 2011). 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/.

Table 1
Primers made in house for performing MLST on cultured *A. baumannii* strains.

Name	Target gene	Primer
CPN60-R	cpn60	CGCTTCACCTTCAACAT
GDHB-R	*gdhB*	TGGAATACTTCCATCAAGATTTA
rpoD-F	*rpoD*	AATGGGTACAGTAGAACTG
rpoD-R	*rpoD*	ACGCACTTTTTCCAAGTG

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). Instead of using gel electrophoresis, the described primers were tagged with a dye-label and multi-colored capillary electrophoresis was used (Hauck et al., 2012) 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 2 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 (Pourcel et al., 2011).

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 (Hunter and Gaston, 1988; Johnson et al., 2007):

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

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 (Carrico et al., 2006) 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, (Lin and Lan, 2014) 13 from 2002 to 2003 representing ICU colonized patients, (Neonakis et al., 2011) 21 from the 2008 outbreak and (Giamarellou et al., 2008) 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