

Review

Genotyping methods for monitoring the epidemic evolution of *A. baumannii* strains

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Abstract

Acinetobacter baumannii is clustered with other phenotypically similar species into what has commonly become known as the ACB complex: *A. calcoaceticus*, *A. pittii* and *A. nosocomialis*. The ecology and pathology of most of these species are not well understood, mainly because current specific phenotypic techniques have, to date, been insufficient. This has inhibited both the precise identification of, as well as the ability to discriminate between, these clinically important and closely related *Acinetobacter* strains. However, new genotypic methods have greatly enhanced our capacity to identify the ACB complex. This has resulted in the implementation of more rational infection control programs. Several genotypic identification methods are explored in this study, including non-polymerase chain reaction (PCR)-based and PCR-based methods. These methods include ribotyping, pulsed-field gel electrophoresis, 16S rRNA identification, multilocus sequence typing, single locus sequence typing, restriction fragment length polymorphism analysis, restriction analysis of 16S-23S rRNA intergenic spacer sequences, rapid amplification of polymorphic DNA, and repetitive extragenic palindromic PCR; however, there is no current single ideal genotyping method. Each one has its own advantages and disadvantages. With this in mind we reviewed current and new genotyping methods used to characterize the *Acinetobacter* species.

Key words: nosocomial infection; genotyping method; *Acinetobacter baumannii*.

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Introduction

The clinically important and closely related genus of the *Acinetobacter* strain, *Acinetobacter baumannii*, is a most troublesome pathogen, causing hospital-acquired infections worldwide [1]. It can survive for longer periods in hospital settings and is potentially capable of causing serious hospital outbreaks. Over the last 10 years, the clinical significance of *A. baumannii* has increased through its ability to acquire multi-drug resistance and so reduce therapeutic options [2]. In most hospital settings, it is increasingly associated with nosocomial pneumonia in intensive care units, particularly in patients with ventilator-associated pneumonia [3,4].

Currently, *Acinetobacter* is split into large groups of species. Taxonomy of the genus *Acinetobacter* has revealed 23 valid names and 11 genomic species that have been delineated by DNA-DNA hybridization [5]. However, identification of *Acinetobacter* at species level is still erratic and for clinical reasons because it obscures possible differences in the biology and pathology of the individual species. In clinical settings, precise identification of species may be

subject to ramifications in diagnosis, antimicrobial therapy, and infection control policies [6]. Associated with the *A. baumannii* strain is a group of phenotypically and genetically closely related Gram-negative bacteria. These are non-motile, aerobic, rod-shaped bacteria with polar fimbriae, which can have oxidase-negative and catalase-positive biochemical reactions [7,8]. These particular species have been named *Acinetobacter calcoaceticus*, *Acinetobacter pittii* (formerly referred to as the *Acinetobacter* genomic species 3), and *Acinetobacter nosocomialis* (formerly referred to as the *Acinetobacter* genomic species 13TU). Collectively, they are now referred to as the ACB complex, having a 16S rRNA sequence identity value of between 97% and 99.9% and inter-species values of between 65% and 75% [7]. Although the ACB complex is considered to be an important nosocomial infectious agent, the clustering of *A. nosocomialis* and *A. pittii* together in an ACB complex is unsatisfactory because of the ambiguity in biological and pathological differences within the species. An ACB complex is often associated with nosocomial

infections [9]; however, *A. calcoaceticus* is predominantly an environmental isolate [10]. Thus, precise identification of species in an ACB complex is essential to determine the pathology, epidemiology, and ecology within these species.

Only few phenotypic techniques have been validated to identify clinically important *Acinetobacter* species, with large numbers of strains already having been identified by DNA-DNA hybridization [7]. A different and largely adopted 19 biochemical test pattern developed by Bouvet and Grimont [11] identified 12 different *Acinetobacter* species. Further studies revealed its inability to discriminate all genomic species identified by DNA-DNA hybridization [11,12]. Unfortunately, the differentiation of genetically closely related species in an ACB complex is not possible using this method. In addition, commercially available identification platforms were also found to be insufficient and inaccurate; they were found to incorrectly identify 25% of the species belonging to the ACB complex [13].

Genotypic analysis

Several genotypic methods have been developed for identification of *Acinetobacter* species. Nevertheless, despite significant advances in genotypic methodology, genotypic identification has not, to date, been used very extensively. This is quite surprising, as genotypic methods have several significant advantages compared to phenotypic methods: they have a faster turnaround time and are highly sensitive. For example, pulsed-field gel electrophoresis (PFGE) is a genome-based non-polymerase chain reaction (PCR) method and is considered to be the gold standard for genotyping [14]. Also, the use of phylogenetic analysis using particular DNA sequences for identification and genetic relatedness has improved over the last ten years. Several target DNA sequences are also used for species identification studies. In this review, current and new genotypic methods used to identify and characterize the *Acinetobacter* species are discussed, and a comprehensive assessment of the methods is presented.

Ribotyping

Ribotyping is based on an application of southern blotting. Its efficacy in identifying and genotyping several bacterial strains has already been proven [15]. In this technique, genomic DNA is digested using restriction enzymes. The DNA fragments are then

separated by electrophoresis and transferred to a membrane. Next, hybridization using a labelled probe specific for ribosomal DNA is done. Non-radioactive labels are used, which makes the method easier to perform in a well-equipped microbiology laboratory. The resulting ribotype profiles could be species or strain specific. Gerner-Smidt initiated the identification of *Acinetobacter* species using *EcoRI*, *ClaI* and *SalI* as restriction enzymes followed by a digoxenin-11-UTP-labeled cDNA probe for the ribosomal DNA [16]. Ribotype profiles using *HindIII* were in accordance with amplification and restriction fragment length polymorphism analysis (AFLP) results for European clones [17]. Furthermore, an automated ribotyping system, Riboprinter (Dupont, USA), is also marketed, and has been used in several studies using *EcoRI* restriction enzymes [18]. Another study, in which 83 isolates were identified using the PCR method, showed 100% specificity compared to ribotyping, which had 85.5% sensitivity and 93.5% specificity. This showed that ribotyping was less efficient and less rapid than the PCR method. Although ribotyping is laborious, results can be compared between laboratories. Species from *Acinetobacter* can be easily differentiated. However, this method is less discriminatory than the PFGE method [19]. Therefore at present, ribotyping has been replaced by the PFGE method.

Pulsed-field gel electrophoresis (PFGE)

In this method, genomic DNA digested by restriction enzymes and large DNA fragments are separated by a pulsed electric field. Currently, the most commonly used technique is counter-clamp homogeneous electric field (CHEF) electrophoresis. In this system, the DNA fragments are migrated in a zigzag formation through an agarose matrix in a pulsed alternating electric field that is kept at a consistent temperature of 120°C. Although PCR-based methods are currently used because they are expeditious, PFGE still remains the preferred method of choice and is considered to be the gold standard for genotyping. Usually, *ApaI* and *SmaI* restriction enzymes are used for macrorestriction of the genomic DNA for *Acinetobacter* species typing. When used on a set of ACB complexes with the *ApaI* restriction enzyme, PFGE was found to be more discriminatory than was the ribotyping method [20]. A comparative study, in which a set of ACB complex strains were studied and analyzed for inter-laboratory studies, showed that PFGE profiles can be compared if scrupulously standardized [21]. Thus, this procedure

could be a future tool in establishing an international database for regional and international monitoring strains. However, PFGE equipment and its reagents are expensive, and inter-laboratory reproducibility possibly still remains a process for implementation at some future date. Furthermore, PFGE takes more than five days to generate results and needs expensive software for such analysis. PFGE is therefore laborious and expensive. Other PCR-based genotyping methods are usually used, but sometimes both methods are used together [20,21].

16S rRNA sequencing

16S rRNA gene amplification and sequencing is the most commonly used method for bacterial identification. The gene is 1,550 base pair (bp) long and is composed of both variable and conserved regions. It is long enough to provide unique and statically compelling measurements. It is a universal gene in bacteria, and universal primers are used to complement the conserved regions for the identification of bacterial species that are difficult to identify by ordinary phenotypic methods [22]. The efficiency of 16S rRNA gene sequencing depends on the application of the correct label to each sequence, the deposition of a complete explicit nucleotide sequence, and the accurate collation of this information into a private or public database. The sequencing of 16S rRNA gene method has been used for determining a large number of strains. As a result, GenBank, the open access databank, has over 90,000 16S rRNA genes. Due to the successful use of 16S rRNA, gene differentiation of many major phyla in bacteria at the genus level can now also be done. However, there have been some drawbacks with this differentiation method, particularly in the case of species that have similar sequences. 16S sRNA sequencing is not satisfactorily polymorphic to all *Acinetobacter* species because of its extremely slow rate of base substitution [12]. On the other hand, the RNA polymerase β -subunit (*rpoB*) gene sequences seem to possess better discriminatory powers and reliability than does 16S rRNA gene sequencing in these specific cases [23]. In another study [24], 99 well-identified strains of ABC complex were studied to assess inter- and intra-species variability using the *rpoB* gene for resultant comparison and 16S rRNA differential sequencing. It was found that *rpoB* sequencing identified ABC complex strains more accurately than did the 16S rRNA gene sequencing methodology.

Multilocus sequence typing (MLST)

This is a high-resolution genotypic method for sequencing microorganisms; it has been applied successfully for clinical characterization of many important nosocomial pathogens including the *Acinetobacter* species. MLST is a relatively new technique that is used as an alternative to PFGE. It was designed initially for global epidemiology and surveillance. MLST is a process based on multilocus enzyme electrophoresis, during which internal fragments of housekeeping genes are compared. These genes are present within all isolates, and mutations within them are considered to be neutral. For every gene fragment, different sequences are assigned as alleles. Fragments are approximately 500 bp in length, and five to eight loci are sequenced. Each isolate is characterized by the alleles of each housekeeping loci or sequence type [25,26]. The main advantage of MLST when compared to other genotyping methods is that the data can be shared between laboratories and on the internet (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/>). The internal fragments of the seven loci that can be selected for an MLST platform are *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* [26,27]. However, according to Hamouda *et al.* [28], the exclusion of the *gyrB* and *gpi* genes from the MLST platform could improve the accuracy of detection.

Furthermore, an *adeB* gene from an efflux pump protein could also be used as a marker for MLST. The *adeB* gene makes up a part of the ABC efflux pump cluster, which has a built-up resistance to tetracycline and aminoglycosides. Huys *et al.* [29] used *adeB* gene MLST to study 50 pan-European (pE) multidrug-resistant *Acinetobacter baumannii* (MDRAB) strains, mostly from European hospitals. An internal fragment of the *adeB* gene was investigated to establish whether it contained potential polymorphic sites for sequence-based identification of intraspecific groups in MDRAB. Of 50 MDRAB isolates, 11 different *adeB* sequence types were defined, establishing 5 genotypically unrelated groups and 6 previously delineated intraspecific groups. However, little is known about the distribution of the *adeB* gene in geographically unrelated isolates. Furthermore, this gene is regulated in multidrug-resistant isolates due to stringent controls of gene regulation, and it is difficult to predict the presence of this gene in an aminoglycoside resistance spectrum [30]. MLST is a powerful tool to discriminate closely related genomic species, and it is comparable to PFGE and amplification and restriction fragment length

polymorphism analysis (AFLP) [26]. For rapid identification (around four hours), multilocus PCR can be followed by electrospray ionization mass spectrometry (PCR-EIMS). Notwithstanding this, PCR-EIMS generates slightly less resolution power and less information than does MLST [31].

Single locus sequence typing (SLST)

Unlike MLST, which requires amplification and sequencing of seven housekeeping genes for population structures, SLST requires just one gene. In SLST, amplification and sequencing of the *bla*_{OXA-51-like} gene is performed; this gene is unique to *A. baumannii*. Although *bla*_{OXA-51-like} has been occasionally identified in *A. nosocomialis* and *A. pittii*, it is powerful marker for identification of *A. baumannii* if SLST is considered. Previously, 585 isolates of *A. baumannii* obtained from Greece, Lebanon, Italy, and Turkey were studied and evaluated using SLST [32]. The performance of SLST was compared with Pasteur's MLST, and PubMLST scheme resulted in detection of the same alleles. The reliability of SLST was further validated by the analysis of *A. baumannii* whole genome sequence available in GenBank. This method can be used in developing countries because it is less laborious, faster, and more reliable.

Restriction analysis of PCR-amplified sequences

Restriction of PCR-amplified DNA sequencing is an important genotyping marker used for the characterization of microorganisms. In this method, various conserved sequences are amplified and restricted by one or several restriction enzymes. Restricted DNA fragments are obtained by electrophoresis, and these fragments are used to build libraries. Several sequences within the genome are targeted, including 16S rDNA, 16S-23S intergenic spacer sequences, 16-23S rDNA, and the *recA* gene [17].

Amplification and restriction fragment length polymorphism analysis

In this method, amplification of internal sequences of the *recA* gene are used for the identification of *Acinetobacter* species, followed by a restriction fragment length polymorphism (RFLP) analysis with two restriction enzymes. In this method, chromosomal DNA is extracted and amplified, DNA sequences are restricted with *MboI* and *HinfI* restriction enzymes, and electrophoresis is performed on 8% agarose gel. This technique was documented using 40 clinical

important *Acinetobacter* strains [33], where all strains were correctly identified to a genospecies level. This proved to be a simple tool for the identification of *Acinetobacter* species. However, in another study [34], 32 strains were typed using *recA* amplification and RFLP analysis, and this method could not identify the genospecies of most isolates belonging to the *Acinetobacter* species. Recently, 77 strains, including 43 *Acinetobacter* reference strains, were examined. In this study, three restriction enzymes were used: *MboI*, *HinfI*, and *Tsp5091*. The discriminatory powers of *Tsp5091* were found to be very satisfactory, generating an individual profile for 23 genomic species. Furthermore, PFGE is also used as an auxiliary method, which adds to the cost of this method [12,35]. However, there are unresolved issues in terms of reproducibility and inter-laboratory sharing of results. In addition, there is no national or international database for comparing isolate profiles.

16S-23S rRNA intergenic spacer sequences

Restriction analysis of 16S-23S rRNA intergenic spacer sequences is also a promising approach for the identification of *Acinetobacter* species. The 16S rRNA gene sequences of the members of the *Acinetobacter* species have 94% similarity identity. In this technique, genomic DNA is extracted and 16S-23S rRNA intergenic spacer genes are amplified. Restriction analysis of the amplified sequences is done using two or more restriction enzymes. In a previous study [35], *Acinetobacter* strains from ACB complex were restricted with *AluI* and *NdeII* restriction enzymes, which yielded satisfactory restriction patterns. Furthermore, southern blotting was performed for five to six targets in the DNA of the identified strains, and only one fragment per strain was detected. It was found that 16S-23S rRNA intergenic spacer sequences have a low degree of intra-species variation and high degrees of inter-species divergence. In another study [7], 28 types of strains, including 11 reference strains, were used; amplification and restriction of sequences was followed by DNA sequencing, revealing low-level similarity within the ACB complex. This study also demonstrated that internal transcribed spacer (ITS) sequences have better discriminatory power than a 16S rRNA gene. However, this method takes more than two days to complete and it is not suitable for a routine diagnostic laboratory [35].

Rapid amplification of polymorphic DNA (RAPD)

This is one of the easiest genotyping methods to evaluate strain-level fingerprinting, using PCR and consequently performing gel electrophoresis. No knowledge of the DNA target sequence is required, as a primer anchors to a non-specific site in the DNA sequence. The primer target sites may vary in number and location over the genome; accordingly, the size and number of the amplified fragments differ. Usually, decamer (10 nucleotides) lengths of non-specific short sequence primers are used under low annealing temperature, and electrophoresis RAPD profiles can be used for strain genotyping. Various primers and protocols have been used to type the *Acinetobacter* species [27,36]. Recently, a melting curve analysis was performed for the amplified DNA fragments that were generated during RAPD. This approach does not require electrophoresis and ethidium bromide by real-time PCR, thus greatly reducing workload and overall time [37]. RAPD has many advantages: it requires low-cost primers, only a small quantity of DNA for analysis, and it requires no blotting or hybridization. However, the introduction of commercial and standardized reagents made RAPD fingerprinting more time consuming to standardize. Thus, exchange and comparison of RAPD profiles between laboratories has become more difficult, and it has become impossible to produce accurate results. A multicenter study was conducted by Grundmann *et al.* [38] in seven laboratories across six European countries to evaluate the reproducibility of RAPD profiles of 40 isolates within the ACB complex using standardized protocols and reagents. The outcome of these efforts by three of the centers to improve inter-laboratory reproducibility was unsatisfactory. Nonetheless, PCR with standardized protocol, reagents, and primers (DAF4 and M13) produced unhindered banding patterns [39]. These patterns are strain specific; RAPD could therefore be a useful approach for epidemiological genotyping. However, inter-laboratory data exchange remains an unresolved issue.

Repetitive extragenic palindromic (REP)-PCR

There are many repetitive (strain coding and non-repetitive) DNA sequences spread throughout the genome that can be used as markers for identification. PCR primers can be constructed for REP sequences to amplify the DNA between these sequences when two REP sequences are in close proximity [40]. The regions occupied between the REP sequences vary in

size due to heterogeneity among the separate strains; thus, different sizes of the fragments are amplified. Fragment amplification generates unique profiles following gel electrophoresis. These profiles (band patterns) can be compared to other genotypes. The REP-PCR results are comparable to other genotypic characterization techniques, including PFGE [41,42]. Intra-laboratory reproducibility in commercially available REP-PCR-based methods occurred more than 98% of the time when the process was tested in triplicate. However, it was not made clear how the triplicates performed [42]. One of the main drawbacks of REP-PCR is the use of traditional agarose gel electrophoresis, which lacks reproducibility; this may result in the variation and use of different electrophoresis systems.

The DiversiLab (Biomérieux, Marcy L'Etoile, France) system using the REP-PCR approach is a semi-automated technique used in many hospitals worldwide. A number of commercially available detection kits have been developed for various bacterial species including *Acinetobacter*. In this method, PCR is followed by a separation of the amplified genomic DNA regions between the repetitive elements using chip-based micro-fluidic capillary electrophoresis. This process increases reproducibility and the resolution of the REP-PCR approach in comparison with the traditional gel electrophoresis method. The resulting data is automatically collected and analyzed by DiversiLab software. Various studies have shown many advantages of using DiversiLab: it is easy, simple, rapid, and yields reproducible results. In addition, when DiversiLab was compared with PFGE and arbitrary PCR methods for the characterization of an outbreak (caused by resistant and susceptible *A. baumannii*) involving 29 patients, isolates showed similar antibiotic resistance patterns. In another study [43], in which 21 isolates of *A. baumannii* were studied and results were compared with ribotyping, DiversiLab appeared to be more discriminatory than ribotyping was, distinguishing eight different clusters compared to only six different clusters distinguished by ribotyping. However, the installation of the DiversiLab system is expensive, especially in resource-limited laboratories. The overall cost of reagents and consumables for this system is also much higher than for PFGE. Since inter-laboratory reproducibility of REP-PCR approaches are limited, large-scale inter- and intra-laboratory studies are required to assess the efficiency of the DiversiLab system.

Conclusions

In recent years, substantial improvements in genotyping methods have changed the current approaches to identifying *Acinetobacter* species. This has involved major improvements in bioinformatics, automation, and technical advances in genotyping methods. The steadily increasing genotypic databases allow for rapid and easy assessments between laboratories to enable comparisons and epidemiological surveillances of *Acinetobacter* species to be made. However, it remains regrettable that there is no absolute genotypic method. Every genotypic approach has its own benefits and drawbacks. Accordingly, one or more genotypic approaches can be used, but this is dependent upon the laboratory setting and the available financial support. Ribotyping also has good discriminatory powers in assessing the ACB complex; however, a banding patterns complex and libraries of profiles of well-defined strains are required for species identification. If a quick analysis is important (*e.g.*, in the event of an all-encompassing local outbreak), then PCR-based methods such as DiversiLab can be used. However, if the outbreak is disseminating to various geographical regions, more robust techniques such as PFGE should be used, because this method makes it easier to compare results obtained in different laboratories. New methods such as MLST are as effective as PFGE because immediate results can be obtained. Although MLST is quick and reliable, it still requires an expensive system to be implemented, well-trained staff, quality controlled laboratories, and a large budget for consumables. It is easier to implement the MLST and PFGE methods locally, but national or international surveillance needs to standardize its techniques. In addition, availability of resources is a question in developing countries; SLST could be another option for provisional genotyping, since it is cheaper and faster. Currently, PFGE and MLST remain the most widely used genotyping methods for the *Acinetobacter* species.

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