The genomic identification of Colombian *Acinetobacter baumannii clinical* isolates by RFLP-PCR analysis of the 16S-23S rRNA gene spacer region

Identificación genómica de aislamientos colombianos de *Acinetobacter baumannii* mediante RFLP-PCR de la región intergénica espaciadora de los genes 16S y 23S rRNA

María Andrea Hernández[*,**], *Emilia María Valenzuela*[**], *Ingrid Yamile Pulido*[**], *María Teresa Reguero*[**], *Silvia Restrepo*[*], *Sandra Gualtero Trujillo*[***], *Dagoberto Santofimio Sierra, Martha Ramirez Plazas*[****], *Luz Eneida Quintero*[*****], *José Ramón Mantilla*[**]

Abstract

The 16S-23S rRNA gene intergenic spacer (ITS) was analysed by RFLP in this study to identify *A. baumannii* from 139 isolates from four hospitals (identified as A, B, C and D). One hundred and twenty of these isolates (86.3%) belonged to the *A. baumannii* species; those identified as being *A. baumannii* were found to be polyclonal (19 clone groups) when determining the genetic relationships, 16 of them being found in hospital C. Hospitals A, B and D shared two clone groups isolated during different years. This study describes a rapid and easy method for genospecies identification of *Acinetobacter baumannii*.

Keywords: *Acinetobacter baumannii-Acinetobacter calcoaceticus* complex*;* 16S-23S rRNA gene intergenic spacer (ITS); RFLP-PCR.

Resumen

Con el objeto de identificar la genomoespecie *Acinetobacter baumannii*, se estudiaron 189 aislamientos pertenecientes al Complejo Acinetobacter baumannii-Acinetobacter calcoaceticus provenientes de cuatro hospitales colombianos (denominados A, B ,C, D) mediante el análisis por RFLP-PCR de la región intergénica espaciadora (ITS) de los genes 16S y 23S rRNA. Se encontraron 120 aislamientos (86.3 %) pertenecientes a la especie *A. baumannii*. La estructura de la población fue policlonal, con 19 grupos clonales, 16 de los cuales se hallaron en el hospital C . En los hospitales A, B, y D se encontra-

* Mycology and Phytopathology Laboratory, Universidad de los Andes, Bogotá-Colombia

** Instituto de Biotecnología, Universidad Nacional, Bogotá, Colombia

*** Fundación Clínica Abbod Shaio, Bogotá, Colombia

**** Facultad de Salud Pública, Universidad Surcolombiana

***** Hernando Moncaleano Perdomo Teaching Hospital

Corresponding author: José Ramón Mantilla. Mailing address: Carrera 30 No 45-03, Universidad Nacional de Colombia, Bogotá, Colombia
e-mail: jrmantillaa@gmail.com

ron 2 grupos clonales aislados durante diferentes años. En este estudio se propone un método método rápido y fácil para la identificación de *Acinetobacter baumanii*.

Palabras clave: Complejo Acinetobacter baumannii- Acinetobacter calcoaceticus; Región intergénica espaciadora (ITS); RFLP - PCR.

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Introduction

Different Acinetobacter species have been well characterised as being a major public health concern as they have been responsible for well-characterised epidemic outbreaks all around the world (1, 4, 31). Hybridisation studies have shown that the Acinetobacter genus is biochemically and genetically heterogeneous. Thirty-three genomic species (genospecies) have been shown to belong to this genus to date (3, 4, 31). Due to the close phenotypic and genetic relationship between genospecies 1 (A. calcoaceticus), 2 (A. baumannii), 3 and 13TU and the difficulties hampering dividing them by classical biochemical reactions, genospecies1, 2, 3 and 4 have been reported as being A. baumannii-A. calcoaceticus complex or as A. baumannii as the biochemical differences between these four species are subtle and no commercial automated identification systems are capable of discriminating within the A. baumannii-A. calcoaceticus complex. However, A. baumannii remains mainly responsible for outbreaks sensu stricto (1, 4, 13, 31).

Epidemiological studies have demonstrated the usefulness of being able to distinguish the species from the complex (35); accurately identifying species within the *A. baumannii-A. calcoaceticus* complex is therefore important for elucidating these species' ecology, epidemiology and pathology (4, 16, 17, 31). Several genetic methods have been developed for genomic species identification within the *A. baumannii-A. calcoaceticus* complex; these methods include amplified rDNA, restriction analysis (ARDRA), ribosomal operon analysis, *recA* gene and /or *rpoB* gene sequencing and 16S-23S rRNA gene intergenic spacer analysis (4, 6, 11, 19, 25, 27). The latter approach has shown that the intergenic spacer (ITS) region sequence between the 16S and 23S rRNA genes has low intraspecies variation and high levels of interspecies divergence (5). This region could thus lead to identifying species within the same genus due to variability in both length and sequence (10, 18, 26, 32).

Although several epidemiological reports have analysed outbreaks produced by these microorganisms, no attempt has been made to discriminate between these species (28, 30, 36); this study has thus been aimed at discriminating *Acinetobacter baumannii* by restricting intergenic spacer region PCR products.

Materials and Methods

Bacterial strains. A total of 139 isolates were obtained from four Colombian hospitals during 2004, 2005, 2007 and 2009 (the hospitals were designated A-D). They were stored at -70 °C in the Molecular Epidemiology Laboratory's strainbank at the Instituto de Biotecnología, Universidad Nacional de Colombia; eighty-eight of the isolates (63.3%) were related to infection, a further 46 (33.1%) to colonisation and 5 (3.6%) were recovered from the clinical environment. The strains related to infection and colonisation were recovered from blood cultures (58/139), secretions (23/139), catheters (23/139) and urine (30/139). They had previously been identified as being *A. baumannii-A. calcoaceticus* complex by Vitek (Biomerieux, France) and all isolates (except one) were classified as being multi-resistant or resistant (8). Cefotaxime, ceftazidime, cefepime, imipenem, meropenem, ampicillin-sulbactam, piperacillin-tazobactam, ciprofloxacin, amikacin, gentamicin and trimethoprim-sulphametoxazole were the antibiotics evaluated in this study. Multi-resistant strains were considered as being those having exhibited resistance to at least three classes of antimicrobial agent. *A. baumannii* ATCC 19606 was used as RFLP-PCR control.

Amplifying the intergenic spacer region. DNA was obtained by cell lysis in distilled water from colonies grown for 18h at 37 °C (33). The ITS was amplified in an iCycler thermocycler (BioRad, USA), using 1512F (5'GTCGTAACAAGGTAGCCGTA3') and 6R (5'GGGTTYCCCCRTTCRGAAAT3') primers at 62 °C annealing temperature, as previously reported by Chang *et al*. (4). The products were visualised in 1% agarose gel electrophoresis and their sizes were estimated by comparison with a 100 bp DNA ladder (Invitrogen, San Diego, CA).

Restriction fragment length polymorphism (RFLP) PCR. The methodology established by Dolzani *et al.*, was used for identifying *A. baumannii* according to ITS sequence (6). Briefly, in silico analysis led to selecting the Mbo I enzyme to distinguish restriction patterns for *A. baumannii.* The sequences used in the evaluation were those reported by Chang *et al.*, which are available from GenBank (AY601820-AY601848) (4). Once the enzyme had been selected, amplicons from the isolates' ITS were restricted, fragment patterns were analysed by 3.5% agarose gel electrophoresis (NuSieve FMC Bioproducts) and a photographic record was made (Gel-Doc BioRad). The ITS from 26 isolates were sequenced with ABI Prism 3730xl-PE (Applied Biosystems, Macrogen Inc.).

Molecular typing. The genetic structure of populations from hospitals A to D had been obtained in previous studies by repetitive extragenic palindromic PCR (REP-PCR); polyclonal populations and some clone groups were found (28, 29, 30). The genetic relationships amongst isolates identified as being *A. baumannii* were evaluated by REP-PCR typing, using REP IRI (5'IIICGICGICATCIGGC3') and REP 2I (5'ICGICTTATCIGGCCTAC3') primers and 46 °C annealing temperature (34). PCR products were resolved by 2% agarose gel electrophoresis (4.6 V / cm) in 0.5 X TBE buffer for 2 hours and visualised with 1µg/mL ethidium bromide staining; the gels were photographed (Gel-Doc BioRad). The percentage of isolates' electrophoretic profile similarity was estimated by using the Dice coefficient; cluster analysis was performed by using the unweighted pair-group method with arithmetic mean (UPGMA) algorithm and GelCompar II software (version 6.0) (Applied Maths, Sint-Martens-Latem, Belgium). Isolates having ≥75% similarity were considered to be clone groups.

Results

Genospecies identification. The ITS region of 139 clinical isolates and the *A. baumannii* ATCC 19606 reference strain were amplified with 1512F and 6R primers (4). A 786 bp amplification product was obtained for each isolate studied here. All ITS obtained were analysed by **restriction fragment length polymorphism** (RFLP) to discriminate the *Acinetobacter baumannii* specie using the *Mbo*I enzyme which, according to *in silico* results, led to differentiating such specie from the others in the complex. As expected from *in silico* modelling and relative isolation frequency, 122 isolates displayed the 345, 327 49, 36 and 29 bp fragments corresponding to *A. baumannii*. The ITS from 26 isolates sequenced had 100% similarity with those deposited in Genbank for the *A. baumannii* specie. **Genotyping.** The dendrogram obtained with the 120 strains identified as being *A. baumannii* revealed 19 clonal groups, having 75% similarity (data not shown).

Discussion

The genus Acinetobacter consists of 33 named and unnamed species. *A. baumannii*, *A. calcoaceticus*, Acinetobacter 13TU and Acinetobacter genomospecies 3 are phenotypically and genotypically similar, being frequently grouped as the A *baumannii-A. calcoaceticus* complex (ABC); three of this complex's members are frequently found in clinical samples. *A. calcoaceticus* is a soil microorganism which is rarely found in clinical samples. The complex has become important during the last few years due to the increase of outbreaks in hospitals and the fact that the strains involved are resistant to several antibiotics. Grouping the 4 species in the complex is inconvenient as this blurs the variations in each species' biology and epidemiology. Identifying the complex's species is thus important for ascertaining each one's ecology, epidemiology and pathology (4, 16, 17, 31, 35).

Variations being observed in antimicrobial susceptibility, clinical manifestations and the outcome for patients suffering from invasive infections caused by different species from the complex have demonstrated the clinical importance of differentiating the complex's species (35).

Dolzani proposed a method based on RFLP of the ITS in 1995 for identifying species from the complex using ALU1 and Nde 2 enzymes. However, Dolzani considered at the time that, in spite of its simplicity, it still could not be used in routine trials in clinical laboratories (6). This study has described a rapid and easy identification method based on restricting intergenic spacer region PCR products with which Ab can be differentiated from the other members of the complex, using just one restriction enzyme for digesting the ITS region and thereby providing an alternative for identifying A. *baumannii* species from genomic species within the *A. baumannii-A. calcoaceticus* complex which are difficult to identify by phenotypic identification systems (4). Given that many clinical laboratories now have the necessary equipment for using it, this method could be considered as an alternative for identifying *Acinetobacter baumannii* in such institutions.

One hundred and twenty of the 139 isolates previously identified as being *A. baumannii* by Vitek belonged to that species, suggesting that *A. baumannii* is the genospecies being most frequently isolated in hospitals

(7, 20, 15, 22). Eighteen of the 19 isolates which were not identified as being *A. baumannii* by the method being used were identified as being A13 TU and the other one as Acinetobacter genomospecies 3 by ITS sequencing.

Great variability was found amongst isolates identified as *A. baumannii*. Two *A. baumannii* clonal groups were found to be distributed throughout hospitals A, B and D in Bogotá. Such distribution amongst the three hospitals in Bogotá could be explained by patient transfer between hospitals.

Hospital C had the greatest percentage of clonal groups; such *A. baumannii* variability within a single hospital could be explained by epidemic and sporadic clones' coexistence (9, 23).

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