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# Outbreak of extensively drug-resistant *Acinetobacter baumannii* indigo-pigmented strains

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*Acinetobacter baumannii* pigmented strains are not common in clinical settings. In the present work we report an outbreak caused by indigo-pigmented *A. baumannii* strains isolated in an acute hospital in Argentina from March to September 2012. Pan-PCR assays exposed a unique pattern belonging to the recently described regional CC113$^{B}$/CC79$^{P}$ that confirms the relevant relationships among the indigo-pigmented *A. baumannii* strains. All of them were extensively drug-resistant and harbored different genetic elements associated with horizontal genetic transfer as the transposon Tn*2006*, class 2 integrons, AbaR-type islands, IS*125*, IS*26*, *strA*, *strB*, *florR* and the small recombinase IS*CR2* associated to the *sul2* gene proceed by IS*Aba1*.

## INTRODUCTION

*Acinetobacter baumannii* is a well-known significant nosocomial pathogen that causes a variety of diseases (1-3). The ability of this bacterium to survive for long periods on inanimate surfaces and its extensively drug resistance makes *A. baumannii* a successful microorganism able to cause outbreaks (4, 5). Many outbreaks due to *A. baumannii* have been documented in the literature (6-10). However, no outbreaks due to indigo-pigmented *A. baumannii* strains have been documented yet.

The production of indigo pigment in the genus *Acinetobacter* was only previously reported in the environmental *Acinetobacter* sp. ST-550 and in the *A. baumannii* ATCC 19606 strains in the presence of indole as a carbon source (11-13). This production may be attributed to the activity of a monooxygenase or dioxygenase enzyme (11, 12). In the present work we report the molecular characterization of an outbreak of indigo-pigmented *A. baumannii* strains that began in the traumatology service of an acute hospital in Argentina.

## MATERIALS

**Bacterial strains.** A total of thirteen pigmented *A. baumannii* strains were isolated in the traumatology service (n=7), coronary care unit (n=2), plastic surgery unit (n=2), and intensive care unit (n=2) of an acute Hospital from Argentina from March to September 2012 (Table 1). The strains were identified at the species level by using several criteria: a) analyzed on a VITEK 2 Compact (bioMérieux) ; b) amplified ribosomal DNA restriction analysis (ARDRA) using the primers 5'TGGCTCAGATTGAACGCTGGCGGC and 5'TACCTTGTTACGACTTCACCCCA with the following cycling conditions: initial denaturation at 95°C for 5 min, the reaction was run through 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min (14); c) amplification and sequencing of the 16S rRNA with the primers fD2 5'AGAGTTTGATCATGGCTCAG and Rp2 5'ACGGCTACCTTGTTACGACTT described by Weisburg et al. (15), using 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min as cycling conditions; and d) amplification and sequencing of the *ropB* gene using the primers Vic4 5'GGCGAAATGGC(AGT)GA(AG)AACCA and Vic6 GA(AG)TC(CT)TCGAAGTTGTAACC the same cycling conditions as described in c (http:// www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html). Antibiotic susceptibility tests were performed using the VITEK 2 System that uses the panel AST-082 (GNS susceptibility card). The MIC results were interpreted according to CLSI categories (16).

**DNA techniques**. Total DNA was extracted with Master Pure DNA purification kit, following the manufacturer's instructions (Epicentre, Madison, WI, USA). To determine the presence of the most prevalent OXA carbapenemase genes in our region (17), such as *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-58-like}}$, PCR reactions were carried out using the primers and cycling as described in the literature (17). For the *bla*$_{\text{OXA}}$ amplification reactions the strains AB3 (*bla*$_{\text{OXA-23-like}}$) and Ab1 (*bla*$_{\text{OXA-58-like}}$) were used as positive controls (17). The presence of insertion sequences (IS*Aba1*, IS*Aba3*, IS*125*, IS*26*, IS*825*, IS*CR1*, and IS*CR2*), class 1 and class 2 integrons, and the corresponding variable regions of integrons were determined using specific primers as previously described (17-20). To further characterize the strains, the occurrence of AbaR islands using the previously described specific primers (4F, 4R, 2F, 2R) was performed (21, 22). We also searched for the presence of tetracycline resistant genes using specific primers to amplify *tet*(B), *tet*(A), *tet*(M), *tet*(39) and *tet*(H) genes under the conditions described in the literature (21, 22). The amplification of the *iacA* gene (iacAF 5'ATGAATAAGTTGTCTAAAATGGAG and iacAR 5'GCAAAACAACACGCGTAATG), involved in indigo production, was carried out using as positive control the *A. baumannii* strains 19606 and ATCC 17978.

In addition, the relatedness of strains was determined using two different molecular typing techniques, the OD-PCR and the recently described Pan-PCR assay, which consist of a multiplex PCR of 6 genes allowing the identification of relevant relationships among strains (23, 24). To carry out the OD PCR the primer 19 5' GGTCGACYTTNGYNGGRTC was employed, using a low-

stringency amplification protocol (5 min denaturation at 95 °C, 40 cycles in 1 min at 93°C, 1.5 min at 36 °C, 2 min at 72 °C, followed by 10 min at 72 °C) (23, 24). The Pan-PCR assay was performed as described by Yang et al. 2013 using the 6 designed pairs of primers that allow to amplify a group of genes whose variable presence is able to identify the strains of interest (23, 24). The cycling conditions employed for the reaction were an initial denaturation at 95°C for 5 min, followed by 20 cycles of denaturation (95°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 1 min 30 s) and a single final extension at 72°C for 10 min.

**Sequence analysis**. Sequencing was performed on both DNA strands using an ABIPrism 3100 BioAnalyzer and Taq FS Terminator Chemistry (Taq FS, Perkin-Elmer). Sequences were examined and assembled with Sequencher 4.7 software (Gene Codes Corp.) and BLAST (version 2.0) software (http://www.ncbi.nlm.nih.gov/BLAST/).

**RESULTS**

All strain were identify as *A. baumannii* using several methods: a) the bionumber obtained by VITEK compact was 0201010303500352, giving an identification of *A. baumannii* complex with 99 % probability; b) the ARDRA profile obtained was 11123 which is characteristic for *A. baumannii*; c) the sequence analysis of the 16S rRNA gene (Accession number: KF410895) revealed 99% of identity with the sequences corresponding to the 16S rRNA gene of *A. baumannii* (Accession number CP003846), d) the sequence of the *rpoB* gene (Accession number KF410896) was 100 % identical to the *rpoB* gene of *A. baumannii* (Accession number DQ207471).

Although two antibiotic resistance profiles among the 13 indigo-pigmented *A. baumannii* strains were identified (Table I), all strains were categorized as extensively drug-resistant (XDR) according to the recent definitions suggested by Magiorakos et al, 2012 (25).

The clinical outcome of patients involved in the outbreak showed five deaths. However, the association between *A. baumannii* colonization or infection and mortality could not be established because patients were compromised or with underlying diseases.

The indigo-pigmented strains showed a unique pattern by OD-PCR that clustered all of them in a single clone (Figure 1a). Furthermore, we decided to perform the new recently described pan-PCR assay, which demonstrated to be able to distinguish among strains with identical MLST (24). This technique showed a unique amplification pattern, with exception of one strain (33405) in which one

band is missing, confirming the relevant relationships among the indigo-pigmented *A. baumannii* strains (Figure 1b). As this technique is defined as a highly discriminatory PCR assay, we consider that in this particular strain (33405), the pan-PCR assay is showing a distinct variation in the gene content of this strain.

Also, the use of pan-PCR allowed us to identify that the indigo-pigmented *A. baumannii* strains possessed the same amplification pattern as the one obtained in the control strain for the $CC113^{B}/CC79^{P}$ clonal complex, which was shown to be prevalent in clinical *A. baumannii* isolates from our country (26) (Figure 1b). This particular clonal complex, that differ from the International clones I, II and III, was also described in *A. baumannii* isolates from Brazil and Spain (26-28).

The dates when the *A. baumannii* strains were recovered clearly showed that the outbreak began in the traumatology service and then spread to other services (Table 1). Attempts to recover *A. baumannii* strains from environmental sources other than the patients hospitalized yielded no indigo-pigmented *A. baumannii* strains. An XDR non-pigmented *A. baumannii* strain (A179) was recovered from the wound-healing box used in the traumatology service.

The molecular characterization of the outbreak was carried out by PCR reactions and sequence analyses were performed to identify the presence of antibiotic resistant genes and genetic elements associated with antibiotic resistance. The *iacA* gene was also searched giving positive results in all the indigo-pigmented *A. baumannii* strains and also in the XDR non-pigmented *A. baumannii* strain A179.

While all the strains harbored the Tn*2006* transposon, which carries *bla*$_{OXA-23}$, the amplification of *bla*$_{OXA-58}$ and *bla*$_{OXA-143}$ carbapenemase genes gave negative results. All indigo-pigmented strains possessed the IS*125*, IS*26*, *strA*, *strB,* and *florR* genes. Class 2 integrons were also found in all the indigo-pigmented *A. baumannii* strains, whereas no class 1 integrons were found. This result is in accordance with our previous studies, which showed that class 2 integrons are more abundant than class 1 integrons in *A. baumannii* strains from Argentina (20, 29, 30). PCR cartography and sequence analysis exposed that In2-7, which is the common class 2 integron array, was present in the 13 strains studied (20, 29).

In addition, all indigo-pigmented strains were positive for the presence, not only for AbaR-type genomic islands, but also for a cluster containing IS*Aba1-sul2*-IS*CR2*.

Previously, the *A. baumannii* A297 strain harboring a similar cluster, which contains IS*Aba1-sul2*-IS*CR2-strB-strA* genes, has been described (19). However, no positive result to link the *strB* and *strA* genes with IS*CR2* were obtained in our *A. baumannii* indigo-pigmented strains.

The PCR reactions to amplify tetracycline resistant determinants, such as *tet*(A) and *tet*(B) gave negative results. In addition, no evidences of *aadB*, *aac(6')-Ib* and *aphA1* genes were found.

Our first thoughts were that the A179 strain, which was found in a wound-healing box of the traumatology service, maybe the source of the described outbreak and/or the source of the antimicrobial resistant mechanisms found in the indigo-

pigmented strains. To confirm our hypothesis the characterization of A179 was carried out.

The non-pigmented *A. baumannii* A179 strain was only susceptible to minocycline, tigecycline, amikacin and colistin. The same profile was described in nine of the indigo-pigmented strains (Table I). As we found in the indigo-pigmented strains, A179 harbored Tn*2006 and* AbaR-type genomic island. The clonal relationship carried out by OD-PCR and Pan-PCR showed that A179 belongs to a different clone (Figure 1a and 1b). Also, when we compared this strain with the indigo-pigmented strains we found some differences in the content of the genetic determinants analyzed in the present work. Instead of class 2 integrons we found a class 1 integron, which carries the array *aacC1*-orfP-orfQ-*aadA1*. This integron was previously described not only in the wide spread international clone 1, but also in Argentinean isolates (30, 31). As we found in the indigo-pigmented strains, A179 harbors Tn*2006 and* AbaR-type genomic island. However, no evidences of IS*Aba125*, IS*CR2*, *strA*, *strB* and *floR* genes were detected.

**DISCUSSION**

To our knowledge, this is the first report of an outbreak of XDR indigo-pigmented *A. baumannii* strains. The molecular characterization of the strains clearly exposed the wide number on genetic elements present in these strains, thus supporting the general idea that *A. baumannii* has a particular ability to acquire different genetic elements to evolve rapidly to the XDR and PDR.

In all the strains, we observed the presence not only of IS*AbaI* and IS*Aba125,*

218 which are the most prevalent ISs in this microorganism, but also of IS*26* and the
219 IS*CR2* element. Our findings are in agreement with the conception that insertion
220 sequences have a predominant role in the acquisition and dissemination of
221 antibiotic resistance within *A. baumannii*. The virulence associated to indigo-
222 pigmented isolates remains to be established.
223 Our study also highlights the importance of rigorous infection prevention and
224 control measures to control an outbreak of *A. baumannii*. Once the organism is
225 identified, universal hygiene measures should be observed to avoid further
226 spread and outbreaks.
227
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**Legend for Figure 1**

Molecular typing of *A. baumannii* isolates. a) Ethidium bromide-stained 1.5% agarose gel electrophoresis showing OD-PCR patterns obtained in the *A. baumannii* strains. Numbers at the top of each lane correspond to strain numbers. Lane 15 corresponded to 1 kb ladder (O'GeneRuler 1 kb Plus DNA Ladder Fermentas) b) Ethidium bromide-stained 1 % agarose gel electrophoresis showing Pan-PCR amplification patterns obtained in the *A. baumannii* strains. Numbers at the top of each lane correspond to strain numbers. Lanes 2 and 3 corresponded to control strains of $CC109^{B}/CC1^{P}$ and $CC113^{P}/CC79^{P}$, respectively. Lane 17 corresponded to 1 kb ladder (O'GeneRuler 1 kb Plus DNA Ladder Fermentas). The XDR non-pigmented *A. baumannii* strain (A179), recovered from the wound-healing box used in the traumatology service, was also added in both assays.

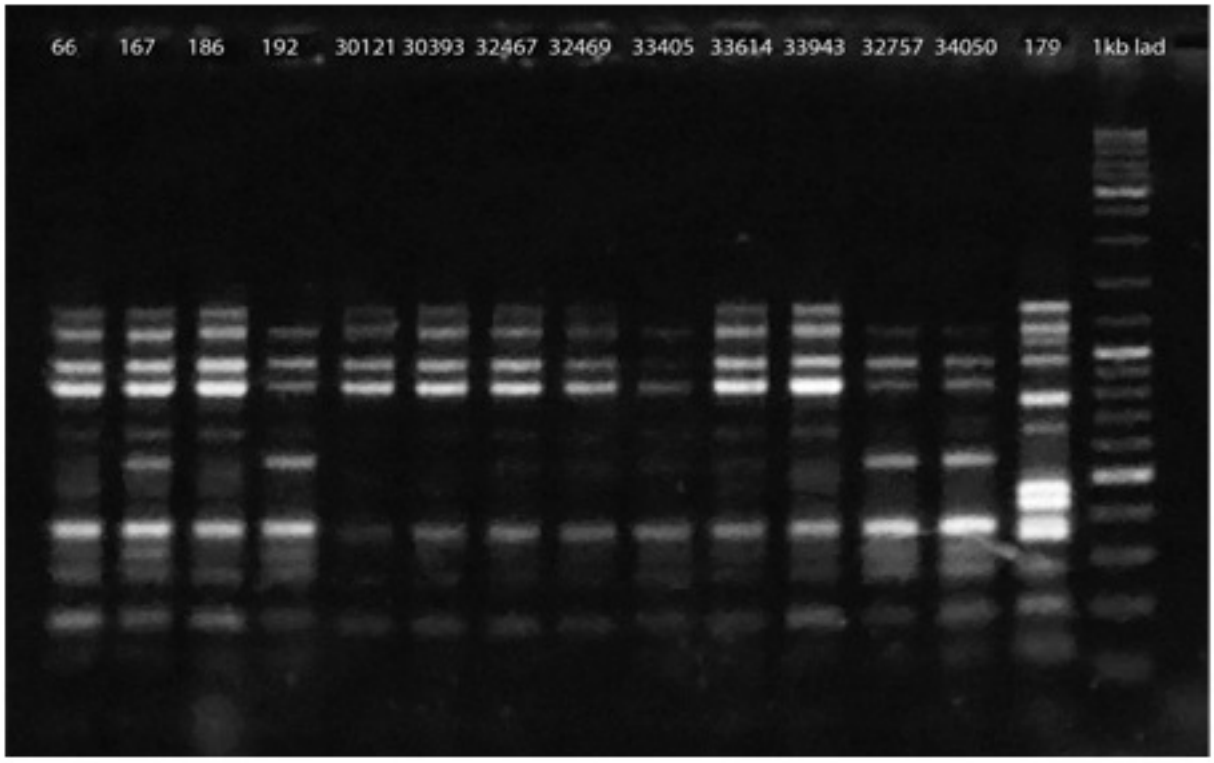
371 **Table 1**. Indigo-pigmented *A. baumannii* strains characteristics and patient´s associated clinical condition.
372

| Strain | Isolation date | Patients age/ sex | Underlying Disease | Diagnosis at admission | Nosocomial diagnostic | Service | Source | Antibiotic resistance profile |
|---|---|---|---|---|---|---|---|---|
| 66 | 3/28/2012 | 47/F[a] | - | ND | Ab surgical site infection | TS | Surgery's wound | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| M30393 | 6/25/2012 | 55/M[b] | DBT | Diabetic foot infection | Ab surgical site infection | TS | SSTI | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| M30121 | 6/19/2012 | 31/F | NUD | Pyomyositis | Ab surgical site infection | TS | SSTI | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| 167 | 6/25/2012 | 65/M | DBT | Diabetic foot infection | Ab surgical site infection | TS | SSTI | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| 192 | 7/17/2012 | 64/M | DBT; CRF | Prosthesis infection | Ab surgical site infection | TS | Mini-BAL | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| 186 | 7/12/2012 | 56/M | - | Diabetic foot infection | Ab surgical site infection | TS | SSTI | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| M32757 | 8/15/2012 | 64/M | DBT; CRF | Prosthesis infection | Ab surgical site infection | TS | SSTI | TMP, CAZ, FEP, CIP, PIP-TZ, IMI, MEM, GEN |
| M32467 | 8/8/2012 | Unkown/F | NUD | Burn infection | Ab surgical site infection | PSU | SSTI | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| M32469 | 8/8/2012 | Unkown/F | NUD | Burn infection | Ab surgical site infection | PSU | SSTI | TMP, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| M33405 | 8/28/2012 | 65/M | - | Scheduled CABG | Ab surgical site infection | CCU | Mini-BAL | TMP, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| M33614 | 9/3/2012 | 45/M | - | Hemorrhagic stroke | Ab VAP | ICU | Mini-BAL | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| M34050 | 9/10/2012 | 60/M | - | Hemorrhagic stroke | Ab VAP | ICU | Tracheal aspirate | TMP, AMK, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |
| F33943 | 9/7/2012 | 82/F | - | Respiratory failure, CAP | Ab VAP | CCU | Mini-BAL | TMP, CAZ, FEP, CIP, PIP-TZ, IMP, MEM, GEN |

373 F, female; M, male; DBT, diabetes; NUD, non-underlying disease; CRF, chronic renal failure; ND, not determined; CAP, community-
374 acquired pneumonia; Ab, Acinetobacter baumannii; VAP, ventilator-associated pneumonia, TS, traumatology service; PSU, plastic
375 surgery unit; CCU, coronary care unit; ICU, intensive care unit; SSTI, skin and soft tissue infection; TMP, trimethoprim-

sulfamethoxazole; AMK, amikacin; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin PIP-TZ, piperacillin-tazobactam; IPM, imipenem; MEM, meropenem; GEN, gentamicin.

1a.




1b.