Comparison of *Acinetobacter baumannii* multidrugs resistant Isolates obtained from French and Tunisian hospitals

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Abstract

**Objectives:** The aim of this study was to assess whether there are differences between the clustering of isolates collected between 2003 and 2005 from two different hospitals in Tunisia and France.

**Methods:** A selection of 62 isolates of *A. baumannii* was studied; 31 from the French Hospital (Poitiers) and 31 from Tunisian Hospital (Rabta). Antibiograms were done using the disc diffusion method. The presence of integrons class 1 and 2 was studied by PCR. Molecular relationship was studied by Random Amplified Polymorphic DNA (RAPD) and Pulsed Field Gel Electrophoresis (PFGE). The sequence typing of adeB gene was determined to identify intraspecific groups.

**Results:** The present study successfully focuses to compare epidemiologic status between two hospitals study. PFGE and RAPD methods were useful to distinguish epidemic and endemic clones in the two collections study. Sequence analysis of an 850-bp internal fragment drug efflux gene adeB revealed 9 novel sequence types (STs).

**Conclusion:** We, statistically, found no significant difference related to the epidemiological situation. This study showed that different genetic types of *A. baumannii* were found in the two collection strains. Epidemics ones were essentially confined in the ICU and were persisting during the two years study. However, more control procedures had to be used on these clones for the Tunisian hospital.

Keywords: *Acinetobacter baumannii*, Epidemiology; nosocomial infections; antibiotic resistance; adeB gene

Introduction

*A. baumannii* is a Gram negative coccobacillus, largely confined in the nosocomial environment that emerged as an important nosocomial pathogen in recent years. Hospital outbreaks caused by this organism have increased worldwide [1,2,3,4]. It was also demonstrated that *A. baumannii* multiresistant strains were essentially isolated in intensive care units (ICU) in critically ill persons [5,6,7]. The aim of this study was to compare the clustering relationship of *A. baumannii* isolates between two different hospitals, located in Tunisia and France. This study was not intended to be a formal assessment of the epidemiological aspects of this pathogen.

Material and methods

Bacterial strains

Our study was performed on a selected collection of 62 isolates of *A. baumannii*: 31 from the French University Hospital (Poitiers) and 31 from the North African University Hospital (Tunisia). These isolates were selected among multidrug-resistant *A. baumannii* isolates recovered during the increased occurrence during (2003–2005) in both hospitals essentially in the intensive care units (ICU). The isolates were selected in relation to their presumptive cases of cross-infection, based on their antibiotic susceptibility profile and their origin in time and space. Identification of *A. baumannii* strains was based on standard biochemical tests and morphologic characteristics by systematic API 32GN (Biomerieux). The growth at 44°C showed that all the strains studied are *baumannii*.

Epidemiological data collection

In order to provide a good comparison between the two contrasting hospital situations, a few epidemiological data were collected. The French teaching hospital had 1579 beds with two units of intensive care (surgery and medical care) that support 58 beds and accept 60 000 patients each year. It has also two intensive paediatric units with 16 beds.

Tunisian hospital has a smaller capacity of acceptance: 960 beds with two units of intensive care (surgical and medical care) that support 34 beds and accept 23000 patients each year. The paediatric intensive care unit supports 40 beds. The two hospitals were available as well for all patients sent by other university hospitals or regional ones that don’t have this unit. But, the number of strains isolated each year was higher in the Tunisian hospital with 100 to 110 strains against 40 to 70 in the European one.

In order to explain this difference of resistance level, the consumption of Imipenem was determined and expressed in defined daily dose (DDD) of imipenem per 1000 hospital days (DDD/1000 hospital days). According to the recommendations of the WHO (World Health Organisation), DDD must be always ≤ 2 g for imipenem.

Antibiotyping

Antibiograms were determined by the disk diffusion method for 8 selected antibiotics shown to be useful to distinguish *Acinetobacter baumannii*.
clusters [8,9]: gentamicin (GM 10UI/ml), imipenem (IPM 10µg/ml), tobramycin (TOB 10µg/ml), amikacin (AN 30µg/ml), tetracycline (TET 30µg/ml), ciprofloxacin (CIP 5µg/ml), ceftazidime (CAZ 30µg/ml) and rifampicin (RIF 30µg/ml). This technique was done in duplicate in the two hospitals study. A Mueller Hinton agar medium (Biorad), was inoculated with a bacterial suspension 0.5 Mc Farland opacity and was incubated 24h at 37°C. Inhibition areas were measured as the diameter of the inhibition zone in mm. Antibiogram similarity coefficients were calculated and analysed by euclidian distance used as a measure of dissimilarity by the "Taxotron Antibiotyping software" (Grimont, Institut Pasteur, Paris, France).

PCR detection of Integrons 1 and 2

Presence of integron class 1 was detected by PCR using the 5’ and 3’ conserved segments. Primers used were 5’CS (GGCATCCAGCGACGAA) and 3’CS (AAGCAGACTTGACCTGA) [10]. Integron class 2 was also performed using 2 primers imAs (ACCTTTTTGTGGCATATCCGTG) and imAcs2 (TACCCTTTGTCCGCATATC) [10].

Random Amplified Polymorphic DNA Analysis (RAPD)

RAPD was performed for all isolates. Isolates were cultured overnight on Nutritive agar and genomic DNA was extracted by phenol – chlorophorm method. Two arbitrarily primers namely; VIL1 (5’ CCGCAGCCAA 3’), VIL5 (5’ AACGCGCAAC 3’) were used according to the procedure described by Johannes et al. [11]. Clusters analysis was performed by the unweighted pair group method with mathematic averaging UPGMA (1% tolerance, 1% Dice coefficient) and the cut off was fixed to 90% of similarity. Dendrograms were performed using Fingerprinting II software (Bio-Rad laboratories, Germany).

Pulsed Field Gel Electrophoresis (PFGE)

PFGE was performed using a consensus protocol for A. baumannii typing with Apa I [12]. Electrophoresis was done in CHEF Mapper Apparatus in run conditions (19H, 14°C, initial and final switch times of 5s and 35s, linear ramp and 6V/cm). Clusters analysis and dendrograms were performed as mentioned below.

AdEB gene detection and sequencing

Detection and partial sequence analysis of adEB was performed with previously published pair primers O3 (5’GTATGAATTGATGCTGC3’) and O4 (5’CATCCTGATGCAATACC3’) that target a 850 pb segment [13, 14]. The PCR product was purified using the QiAquick Purification kit and partial sequencing of adEB was performed by using the Dye-Ex 2.0 Terminator. Sequence alignment and comparison were performed with the Sequencing Analysis software.

Statistical analysis

Data were analyzed using X² test. A p value of < 0.05 was considered to be statistically significant.

Results

Antibiotyping

The levels of antibiotics resistance for the two hospitals are summarised in (Table 2). The results show that the levels had achieved the same degree of resistance except for imipenem which was two times higher for the Tunisian isolates. The difference of imipenem level resistance was statistically significant (p<0.05). (Table 2) illustrates the distribution of antibiotics resistance combinations among strains; French isolates had generated 6 uniformed clusters. Therefore, Tunisian isolates were scattered in 12 clusters by the same classification representing different combinations of antibiotics resistance with 5 clusters containing only one isolate.

Epidemiological data

The epidemiological data collected from the studied hospitals showed a large difference in the imipenem consumption. For the

| Resistance phenotype designationa | No. Isolateb | Antibiogram (disk zone size, mm)c |
|----------------------------------|-------------|----------------------------------|
|                                   |             | GM(21(68)) | TM(22(71)) | AN(17(55)) | CAZ(31(100)) | TE(13(42)) | RA(13(42)) | CIP(4(13)) | IPM(27(67)) |
| French strains                   |             |            |            |            |
| A                                | 14          | 5          | 9          | 6           | 6           | 20          | 6           | 24          |
| B                                | 3           | 8          | 6          | 6           | 7           | 8           | 21          | 6           | 25          |
| C                                | 4           | 16         | 17         | 19          | 19          | 9           | 17          | 6           | 31          |
| D                                | 5           | 20         | 11         | 17          | 15          | 8           | 17          | 6           | 27          |
| E                                | 1           | 24         | 22         | 22          | 12          | 6           | 23          | 6           | 32          |
| F                                | 4           | 9          | 18         | 18          | 8           | 8           | 18          | 6           | 21          |
| Tunisian strains                 |             |            |            |            |
| a                                | 5           | 8          | 20         | 22          | 6           | 6           | 20          | 6           | 28          |
| b                                | 4           | 8          | 18         | 20          | 6           | 6           | 20          | 18          | 28          |
| c                                | 1           | 10         | 20         | 22          | 6           | 10          | 17          | 6           | 15          |
| d                                | 1           | 12         | 20         | 14          | 18          | 18          | 17          | 22          |
| e                                | 7           | 8          | 8          | 10          | 6           | 6           | 18          | 6           | 27          |
| f                                | 4           | 6          | 6          | 6           | 6           | 6           | 18          | 6           | 8           |
| g                                | 2           | 20         | 20         | 6           | 6           | 15          | 22          | 6           | 17          |
| h                                | 2           | 16         | 6          | 22          | 13          | 12          | 6           | 6           | 30          |
| i                                | 1           | 6           | 6          | 20         | 12          | 12          | 18          | 6           | 30          |
| j                                | 1           | 26         | 23         | 25          | 6           | 6           | 6           | 10          |
| k                                | 2           | 16         | 18         | 19          | 6           | 14          | 6           | 23          | 12          |
| l                                | 1           | 25         | 26         | 25          | 21          | 20          | 22          | 32          | 35          |

*aAntibiograms with a similarity coefficient of ≥0.9 were considered indistinguishable and were grouped together in a single resistance phenotype designation. 
**bNumbers of isolates. 
*cAntibiotics designations: GM, gentamicin; TM, tobramycin; AN, amikacin; CAZ, ceftazidim; TE, tetracycline; RA, rifampicin; CIP, ciprofloxacin; IPM, imipenem. Diameter of the disk of antibiotic: 6mm

Table 1: The resistance phenotype patterns and their frequencies in the 2 populations.
French hospital the imipenem DDD, despite the higher frequency of the number of hospitalisation days in Poitiers’ hospital with regard to La Rabta one, we observed a stable and moderate DDD imipenem consumption that passed of 2.22 in 2005 to 2.26 in 2007. In contrast, the DDD of tobramycin in La Rabta hospital was much lower and stable (1.95 in 2005 to 1.98 in 2007).

**Random Amplified Polymorphic DNA Analysis (RAPD)**

All strains were typed by RAPD. Profiles generated 4 and 6 groups for French and Tunisian isolates respectively (Figure 1). The 2 strains collections presented different profiles.

**Pulsed Field Gel Electrophoresis (PFGE)**

PFGE typing method defined respectively 4 and 6 different groups for European and Tunisian isolates which are illustrated in (Figure 2). The 2 strains collections presented different profiles.

**AdeB gene detection and sequencing**

All isolates presented the adeB gene. A selection of sequences representing the 9 adeB sequences types reported in this study has been submitted to EMBL Gene Bank. They all showed new mutations.
which are illustrated in (Table 2) under specific accession numbers (HM002547 to HM002551) in Tunisian strains and (HM046162, HM002552 to HM002554) in French strains.

Combination of typing results

The combined typing results show a good correlation between genomic methods typing (RAPD, PFGE). Based on partial sequencing analysis, the present study has demonstrated that the delineation of adeB STs among genotypically related strains of Tunisia and France matched extremely well the genotypic clustering of these strains with RAPD and PFGE analysis. A positive correlation was observed between antibiotyping and genotyping for French isolates. In contrast, DNA fingerprinting of Tunisian isolates revealed 7 clusters, each one contained isolates with more than one antibiotype indicating that Tunisian strains acquire more rapidly resistance to antibiotics. (Table 3, 4).

Discussion

Acinetobacter, particularly A. baumannii, is implicated in a wide spectrum of nosocomial infections, including primary ventilator-associated pneumonia in patients confined in intensive care unit, secondary meningitis and urinary tract infections.

In our comparative approach, we specified the antibiotic resistance mechanisms and the epidemiology of A. baumannii isolated in two acute- care hospitals; one in Tunis (North Africa), and the second in Poitiers (France).

The two hospitals have achieved a great level of resistance to the use of broad spectrum antibiotics in the hospital had served to eliminate the wide range of antibiotics classes. It seems that extensive and increasing use of broad spectrum antibiotics in the hospital served to eliminate sensitive bacteria and to create a vacant ecological niche to very resistant clones. This multiresistance was observed in several hospitals in the Europe such as the spread of the Oxa-23 clones in England [15, 16], the spread of a VEB-1 ESBL-producing A. baumannii clone in France [17], the dissemination of a multidrug resistant A. baumannii clone in Portugal [18] and the emergence and rapid spread of multiresistant A. baumannii in a Spain hospital [19]. This finding was also reported in Tunisian publications [20, 21]. However another Tunisian report also demonstrated that with anti biotherapy restrictions we could reach a significant reduction of resistance to a large range of antibiotics and permit the decrease of the number of carbapenem resistant isolates [22]. The particularities of our study were in relation with the comparison between the 2 hospitals. We observed a difference in the level of impenem resistance: this resistance was higher in the Tunisian hospital (36%). This finding could be easily explained by the frequent use of this antibiotic in the Rabta hospital because of the high level of Enterobacteriacea and Stenotrophomonas maltophilia β-lactamases. This finding was confirmed by the imipenem consumption in this present study which showed that the defined daily dose (DDD) relative to this antibiotic in the French hospital is always similar to the dose fixed by the WHO (2.22 to 2.26) and which is higher in our hospital (10.1 to 12.4).

The genomic investigation by RAPD and PFGE methods was useful to identify the epidemic strains of A. baumannii. They showed multiple epidemic strains that persisted two years in some cases in the two hospitals study. The analysis of the molecular epidemiology and multidrug resistance of A. baumannii strains in the various parts of the world indicates a considerable degree of geographic diversity in the spread of various strains [23, 24, 25, 26]. It’s also demonstrated in multiple studies in Tunisia [20,21,27] and in France [28,29,30] that the multiresistance is common among A. baumannii giving nosocomial epidemics which were difficult to treat.

The gene adeB codes for the transmembrane protein of the AdeABC...
A multidrug efflux pump has mainly been detected in A. baumannii outbreaks strains [30,31,32]. All MDR isolates in the present study were found to carry the adeB gene. As described by Magnet et al., these genes specifically confer resistance to aminoglycosides and tetracyclines and disruption of this gene leads to the loss of multidrug resistance [14]. In addition, partial sequence analysis of adeB gene encoding the aspecific drug efflux gene showed that it’s a potential tool to identify intraspecific groups among multidrug resistant A. baumannii strains. In fact, the delineation of adeB STs among genotypically related strains of Tunisia and France corroborated extremely well with the genotypic clustering of these strains with RAPD and PFGE analysis. In addition, sequencing of adeB gene had placed the strains of the two collections in distinct groups and with new mutations under specific accession numbers designed by EMBL gene bank. This tool was previously used in a number of related studies [13,30].

In this study, we observed epidemic A. baumannii strains with great antibiotic resistance profiles for both hospitals, but the French clones disappeared more quickly. This finding could be explained by greater control disinfections procedures and strict adherence to infection control policies operated in the French hospital (especially hand and environmental hygiene and use of closed suctioning techniques), and discharge of colonized patients from the hospital as soon as possible. In fact, a systematic procedure of nosocomial infections due to multidrug resistant strains declaration was done in all French hospitals.

Figure 1: Genetic fingerprinting and clusters of French (a) and Tunisian (b) Acinetobacter baumannii isolates using Random amplified Polymorphic DNA.

Figure 2: Genetic fingerprinting and clusters of French (a) and Tunisian (b) Acinetobacter baumannii isolates using Pulsed- field gel electrophoresis technique.
imposed by the CCLIN (centre de coordination de lutte contre les infections nosocomiales).

In the Tunisian hospital, nosocomial infections are not systematically screened and controlled within a national program of health which is still in course of implementation. Deficiencies in the implementation of infection control guidelines in the Tunisian hospital cannot avoid the rapid dissemination of epidemic strains and the evolution of resistance mechanisms as described in previous studies [20,21,27].

Another important factor that can increase the incidence and persistence of nosocomial infections due to A. baumannii in Tunisia could be the tropical climate (warm and humid). This finding was supported by Siau et al on Hong Kong [33] and another report [34,35,36].

A seasonal increase of Acinetobacter infections during summer may be related to the reduced number of the staff assistant due to the holiday’s periods.

This study has demonstrated that there is no statistically significant epidemiological difference between Tunisian and French hospitals. The A. baumannii epidemics that occurred in both hospitals had a significant level of multidrug resistance.

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