The whole genome sequencing of
Acinetobacter-calcoaceticus-baumannii complex strains
involved in suspected outbreak in an Intensive Care Unit of a pediatric hospital

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ABSTRACT

Background: To analyze the genetic characteristics of Acinetobacter-calcoaceticus-baumannii complex strains isolated in suspected outbreak in an Intensive Care Unit of a pediatric hospital in order to promptly stop the dissemination of this dangerous strain.

Methods: This study described the use of whole genome obtained by Next Generation Sequencing to define the clonality of 13 Acinetobacter-calcoaceticus-baumannii complex strains and to study their Resistoma. This was required because Acinetobacter baumannii is known to be resistant to desiccation and to disinfectants and is difficult to treat and eradicate. Thus, this microorganism is a major problem that demands a rigorous outbreak monitoring program to prevent and control the spread of the dangerous strain.

Results: The first result of our analysis has been to describe precisely the characteristics of isolates involved in the nosocomial infection, reduce the dimension of the more problematic isolates sustaining the outbreak and promptly facilitate the control of the strains diffusion. Indeed, our study indicated that among the 13 Acinetobacter baumannii-calcoacteticus complex strains, identified by biochemical and mass-spectrometry assays, 7 were Acinetobacter baumannii, 5 were Acinetobacter calcoaceticus, and one was Acinetobacter haemolyticus. The analysis of clonality of Acinetobacter baumannii indicated that three strains of ST744 were identical for > 99.8% among them and thus have sustained an outbreak in Intensive Care Unit. All personnel and possible environment samples were also monitored and all the procedures aimed to the prevention and control of Hospital Acquired Infections (HAI), have been strictly enforced by the Hospital Infection Control Committee. This gave the possibility to trace a strain from an environmental contamination characterized by high degree of clonality with the one isolated from a patient. On the contrary Acinetobacter calcoaceticus strains were different from each other and thus they were not responsible for any outbreak. The Resistoma analysis indicated a correspondence between phenotypic and genotypic characteristics of analyzed strains.

Conclusions: Next Generation Sequencing is an appropriate technique to trace the circulation of dangerous strains sustaining nosocomial infections. The combination of the high resolution genotyping together with the definition of the antibiotic genetic determinants and the precise species identification make this technique a useful tool employed as standard practice to control HAI.

Key Words: Acinetobacter baumannii, Whole genome sequencing, Pediatric hospital outbreak, Resistoma

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1. INTRODUCTION

The identification of Acinetobacter species by phenotypic methods or simple mass-spectrometry gives ambiguous definition so that these microorganisms are often referred as Acinetobacter-calcoaceticus-baumannii complex.\textsuperscript{[1,2]} Acinetobacter baumannii certainly plays a major role as an opportunistic pathogen mainly involved in nosocomial infections, while Acinetobacter calcoaceticus is often recovered from the environment and is hardly implicated in serious clinical disease. Indeed, Acinetobacter baumannii is recovered in ventilator-associated pneumonia, catheter-related and wound infections particularly in patients admitted in UTI-Intensive Care Unit (UTI).\textsuperscript{[3–6]} Furthermore, it is intrinsically resistant to many antimicrobial agents and frequently acquires genetic elements conferring multi-drug resistance (MDR).\textsuperscript{[7–9]} Acinetobacter baumannii is resistant to desiccation and to disinfectants, it is difficult to be treated and to be eradicated and it requires rigorous monitoring programs to prevent and control hospital outbreaks.\textsuperscript{[10]}

Guidance documents\textsuperscript{[11–13]} on the control of Acinetobacter baumannii nosocomial infections are based on the combined interventions including an active microbiological surveillance, precautions to contain the spread of infections from patient to patient and nursing care protocols for colonized patients. Additional measures regard: antibiotic use restriction, careful hand hygiene, extensive environmental surface decontamination procedures\textsuperscript{[14]} and clinical staff education on how to deal with an outbreak. In particular, a careful examination to search any possible environmental contaminated surfaces around the patient by a precise cultural screening will be performed; adequate cleaning and disinfection of the hospital room, including furniture with special attention to rough handling points such as door handles, faucets and patient units are necessary. All these procedures are required to ensure a proper infection control and in case of suspected outbreaks they must be enforced as soon as possible.\textsuperscript{[15]} Therefore, hospital acquired infection (HAI) investigations are often triggered by laboratory-based surveillance programs to identify an increase of Acinetobacter baumannii infections, a cluster of infected patients, or a new strain with an atypical susceptibility profile.\textsuperscript{[16]}

To trace HAI it is necessary to use the best high resolution typing approach that unequivocally identifies Acinetobacter species and their clonality, in order to search for the index case and to define the strains relatedness to avoid nosocomial cross transmission.\textsuperscript{[17–19]} This study described the use of whole genome sequencing (WGS) by Ion Torrent Personal Genome Machine to analyze Acinetobacter-calcoaceticus-baumannii complex strains isolated in a possible outbreak occurring in a Intensive Care Unit of a pediatric hospital. De novo assembled sequences from 13 strains were analyzed to define strains clonality and Resistoma. The high resolution genotyping, the definition of antibiotic genetic determinants, and the precise species identification make WGS an appropriate technique to be used as standard practice in hospitals in order to trace the circulation of dangerous MDR strains.

2. METHODS

2.1 Bacterial strains

We analyzed 13 non-duplicated bacteria identified according to conventional phenotypic methods as Acinetobacter-calcoaceticus-baumannii complex. They were isolated from 12 different pediatric patients and from one environmental contamination. Eight of these were from patients admitted to UTI-Intensive Care Unit (UTI) from June 24th to July 29th and suspected to have sustained possible outbreak. Two isolates, used as controls, were recovered in the same period from Orthopedic and Pneumology units. The last three strains originated from Neonatal Intensive Care Unit (CNR) in a restricted period close to the previous one (August-September 2015) were also suspected to have originated another outbreak.

Isolates were collected from blood, bronchoalveolar-lavage and upper airways specimens.

Phenotypic identifications were carried out by semi-automated Phoenix system and confirmed by Mass spectrometry microbial identification system Vitek MS (BioMerieux). Antimicrobial susceptibility was made by broth microdilution and Minimum inhibitory concentration (MIC) for Amikacin (AK), Gentamicin (GM), Tobramycin (TO), Ciprofloxacin (CIP), Levofloxacin (Levo), Imipenem (IMP), Meropenem (MEM), Colistin (Coli) and trimethoprim sulfamethoxazole (SXT) were determined following The European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendation and interpretations.\textsuperscript{[7]}

All the procedures aimed at the prevention and control of HAI were strictly enforced by the Hospital Infection Control Committee. The microbiological monitoring in active surveillance was carried out by preventive rectal swab screening of any patients admitted to the hospital in order to know the presence of the dangerous strain and to prevent its spread. During the emerging outbreak we also implemented pro-active measure such as rectal swab screening performed at regular time intervals on all patients and on all their possible contacts in the incriminated ward (UTI). In particular, patients have been screened twice a week for microbiological sentinel events (rectal-, nasal-swabs and broncho-alveolar-lavage) and all personnel (nasal- and throat-swab) and possible environment samples (swabs and/or fluid of various surface) were also
microbiologically monitored in the same period.

2.2 Genome sequencing
DNA of each strain was extracted using the Magtration System 12GC plus (Precision System Science USA, Inc) according to the manufacturer’s instruction and used for library preparation.

DNA libraries were obtained starting from 200 ng of genomic DNA using enzymatic shearing and size-selection (Pippin Prep SAGE SCIENCE) in order to obtain a median library fragments of 470 bp (mean of library size 400 bp + adaptor 70 bp). The libraries size and quality were checked using DNA High-Sensitivity Kit and the Bioanalyzer-2100 (Agilent Technologies, CA, USA) and the mean size of the library was 491 bp with a coefficient of variation of 9%, while concentrations were evaluated by Qubit dsDNA HS-Assay Kit (Invitrogen). The templates preparation were carried out using the Ion PGM HiQ-OT2 following the manufacturer’s instruction. DNA sequences were obtained by Ion Torrent PGM (Thermo Fisher Scientific) using Ion PGM HiQ Sequencing Kit (Thermo Fisher Scientific) and Ion 316v2 chips (Thermo Fisher Scientific). The sequenced samples give a mean of 413,517 reads (from 689,863 to 208,895 reads) with a standard deviation of 140,946 reads.

2.3 Bioinformatic analyses
The obtained WGS reads have been de novo assembled using SPAdes v3.1.0. After the assembly procedure, we got a mean of 307 contigs (from 455 to 152) with 100 as standard deviation. The mean size of them was 14,452 base pairs (from 26,073 to 8,861). More in detail, the mean size of the higher assembled fragment was 336,625 bp (from 50,885 to 201,190) with a Standard deviation of 96,532, while the mean of the lower length fragment size was 103 (from 455 to 152) with 100 as standard deviation. The assembled contigs for each bacterial genome were blasted against the listed databases: Resistance Determinants DataBase (RED-DB) (http://www.fibim.unisi.it/REDDB/). Center for Genomic Epidemiology ResFinder2.1 (https://cge.cbs.dtu.dk/services/ResFinder/), CARD Resistance Gene Identifier-v2.0 (http://argard.mcmaster.ca/), ARG-ANNOT Mutational Gene DB (http://en.mediterranee-infection.com/article.php?laref=283%26titre=arg-annot-),[21] More in detail, RED-DB, ARG-ANNOT and PmrA, PmrB, mcr-1 (Polymixin antibiotic resistance) gene sequences have been used to prepare in-house databases. The first three databases have been used to search for genes conferring resistance, while the latter was used to look for point mutations.

3. RESULTS
3.1 Epidemiology
This is the first observed outbreak sustained by Acinetobacter baumannii observed in our hospital. During the period in observation, we found an increase of colonized/infected patients by Acinetobacter-baumannii-calcoaceticus complex admitted in UTI. Indeed the rate of colonized/infected patients in that ward respect to the all the hospital was of 29.4% (5 UTI patients out of 17 hospital patients) in 2013 and 33% (4 out of 12) in 2014. The rate increased up to 56.2% (9 out of 16) in 2015 and went back to the registered ward/hospital average, since in the first trimester 2016 we observed a rate of 33% colonized/infected patients (1 out of 3). It is to be stressed that the majority of Acinetobacter baumannii colonized/infected patients in 2015 have been concentrated in a one month period (June-July 2015).

3.2 Clonal analysis
The first result of our WGS analysis was a correct species identification of 13 Acinetobacter-baumannii-calcoaceticus complex isolates suspected to sustain nosocomial outbreaks in a one month period of time in the summer of 2015. We defined the strains as suspected outbreak, because isolated frequently in a short period of time (one month) in the same ward.

Thus, the assembled genomes were compared to the microbial sequences database by Basic Local Alignment Search Tool (blast), in order to define Acinetobacter species, since the phenotypic analysis identified only the complex.
study indicated that among the 13 Acinetobacter-baumannii-calcoaceticus complex only 7 belong to the Acinetobacter baumannii specie, while the other 5 were classified Acinetobacter calcoaceticus, and one Acinetobacter haemolyticus.

Indeed the precise species identification gave us the possibility to divide our outbreak analysis between the Acinetobacter baumannii and Acinetobacter calcoaceticus, decreasing the alarm about the possible spread of the more virulent dangerous MDR-resistant Acinetobacter baumannii isolates. We analyzed strain clonality using extended-MLST the Neighbor-Joining phylogenetic tree and Minimum Spanning Tree for Acinetobacter baumannii/Acinetobacter calcoaceticus were obtained analyzing 1,701 and 985 gene respectively, as reported in Figures 1 and 2. Using the above reported data we could also define the MLST for Acinetobacter baumannii revealing that three isolates were ST744, two ST195 and one ST281, while the last strain (contig_47) was not yet definable on the basis of available MLST databases (ST-ND). For Acinetobacter calcoaceticus the ST could not be defined since alleles for some gene loci have not been reported, yet.

Our analysis using extended-MLST indicated that three strains (contig_34, _35 and _48) were clonal. Indeed the ST744 Acinetobacter baumannii differed for one or two alleles on 1,701 studied genes (> 99.8% of identity) and sustained an outbreak in UTI (see Figure 1).

In addition, we indentified an environment contamination since the two ST195 strains, isolated from a patient (contig_36) and from the room door handle (contig_38), resulted 99.8% identical (see Figure 1). The ST281 (contig_37) recovered from UTI was not be related to the other Acinetobacter baumannii UTI-strains.

The molecular analysis of suspected CNR strains indicated that only one was Acinetobacter baumannii (ST-ND, Contig_47) unrelated to all the other and thus not relevant. In addition, the other two CNR strains resulted Acinetobacter calcoaceticus (contig_45 and _46), they were not related each other and were different from the other UTI Acinetobacter calcoaceticus strains showing allelic differences higher than 31% (see Figure 2).
Figure 2. Phylogenetic tree and comparison tables of *Acinetobacter calcoaceticus* evaluated on 985 genes

A: Neighbor-Joining tree shows the phylogenetic distance among the 5 strains; B: Minimum Spanning Tree is drawn for the analyzed strains indicating the distances in term of number of different alleles separating them. C-E: Comparison table with allelic distance as number of allelic differences or % was calculated using as reference sequence from the Contig_41 (panel C), Contig_33 (Panel D) and Contig_39 (Panel E), respectively.

Table 1. Acinetobacter baumannii antibiotic susceptibility

<table>
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<tr>
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<th>ST195</th>
<th>ST281</th>
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*Preliminary Stop codon; AK = amikacin; GM = Gentamicin; TO = Tobramycin; IMP = Imipenem; MEM = Meropenem; CIP = Ciprofloxacin; LEVO = Levofloxacin; COLI = Colistin; and SXT = trimethoprim sulfamethoxazole; The phenotypic antimicrobial susceptibility: S = sensitive; R = Resistant"
3.3 Antimicrobial susceptibility
The phenotypic antimicrobial susceptibility is shown in Table 1.

3.4 Resistoma
The analysis of aminoglycosides resistance in our survey indicated that ST195 isolates carry the genetic determinants aph(3’)-Ib, aph(3’)-Ic, aph(6)-Id[22] and armA 16S-RNA-methylase[23] and ST281 presented the same genes showed above with the addition of aadA1. These genetic determinants explained the resistance to all the aminoglycosides tested AK, GM, TO. Finally the ST744 isolates do not present any gene coding for aminoglycosides resistance in accordance with the phenotypic test.

The beta lactams analysis revealed that, Acinetobacter baumannii ST195 and ST281 strains resulted phenotypically carbapenem resistant. Indeed, the genotype of these strains is characterized by the presence of blaADC, blaOXA-66 and blaOXA-23 all controlled by the upstream ISAba1 genetic element. Moreover, ST195 isolates have also blaTEM-19. The ST744 isolates are all sensitive to carbapenems and presented blaOXA-51-like gene and blaADC gene, but without the regulatory ISAba1 upstream sequences.

Resistance to quinolones is due to chromosomal mutation of GyrA and ParC gene[24, 25] In our analysis only the strains ST195 and ST281 are characterized by these mutations and resulted ciprofloxacin and levofloxacin resistant. To analyze the genes responsible for Polymyxins resistance we investigated the presence of mutations in PmrA, PmrB and the lpxA, lpxC, lpxD genes.[26–31] Acinetobacter baumannii strains characterized by ST281, ST195, ST744 do not present any mutations for the PmrA, lpxA, lpxC genes. The analysis of PmrB gene showed that the ST281 presented 3 mutations (P188T, N440H, A444V), the ST744 had 2 mutations (L237M, N440H) and finally the ST195 displayed 3 mutations (P170L, N440H, A444V). Moreover, lpxD gene in ST744 showed R57C, K117E, K356E mutations. It is important to stress that these mutations have never been reported to induce polymyxins resistance in accordance to the phenotypic susceptibility tests (MIC ≤ 1 mg/ml).[18, 19]

Sulphonamide resistance in gram-negative bacteria is dependent on genes encoding dihydropteroate-synthase sul1 and sul2.[32] In our analysis we found that both ST195 isolates are characterized by the sul2 sulphonamide resistance determinant, while the ST281 strain presented the sul1 gene. These data explain the Trimethoprim/sulfamethoxazole (SXT) phenotypical resistance present only in MDR strains (see Table 1).

Regarding the last Acinetobacter baumannii strain (ST-ND) studied, no genetic determinants have been found for any antibiotic as expected by phenotypic assays. Genetic analysis of quinolones resistance gene has only revealed mutations not associated, until now, with antibiotic resistance thus in accordance to phenotypic data[24, 25]. In addition, the presence of beta-lactams resistance gene (see Table 1), lacking for ISAb1 elements involved in the transcriptional regulation, explained the phenotypic sensitivity.

4. DISCUSSION AND CONCLUSIONS
Our study described the monitoring of suspected outbreak sustained by Acinetobacter baumannii-calcoacteticus complex occurred in a pediatric setting analyzing whole bacterial genome by NGS technology (Ion-Torrent platform). The use of this technology has been previously validated in a case of MRSA pediatric outbreaks making NGS a useful tool in HAI investigation.[33] The need to perform a precise genetic strain characterization was determined by the observation of the doubling of patients infected with Acinetobacter baumannii-calcoacteticus complex in a short observation period. Such necessity was burdened by the fact that this was the first case of suspected outbreak supported by Acinetobacter baumannii-calcoacteticus complex in our hospital. The first important result obtained by NGS-analysis revealed that among the 13 Acinetobacter baumannii-calcoacteticus complex isolates only 7 were Acinetobacter baumannii, thus reducing the dimension of the hypothetical outbreak. Indeed, the correct species identification obtained by sequence analysis revealed and overcame the deficiencies of phenotypic identification methods. It is to be stressed that among the Acinetobacter baumannii, 5 strains could be defined as MDR, in accordance with the criteria of the Centers for Disease Control and Prevention (CDC), since they showed resistance to fluoroquinolones, aminoglycosides, carbapenems.[34] In addition, one of these MDR strains was isolated from an environmental specimen in ICU. The study of the Acinetobacter baumannii clonality revealed that three of them (ST744) sustained an outbreak because they were at least 99.8% identical, analyzing 1,701 gene loci. Fortunately, these isolates resulted to be sensitive to all the tested antibiotics (aminoglycosides, carbapenems, quinolones, polymyxins, Trimethoprim/sulfamethoxazole) and thus they could easily be treated avoiding any further outbreak expansion.

Finally, the analysis of MDR ST195 strains (Contig_36 and _38, coming from a patient and a door-handle respectively), underlining that they were 99.8% identical based on 1,701 genes analyzed, showed how an environmental contamination can be a possible source of nosocomial infection spread. This prompt information, strictly enforced by the Hospital Infection Control Committee, was important to begin in real
time the successive strategy of containment and to block dangerous strain circulation. Indeed, thank to this survey in the first trimester 2016 the percentage of infected patients has been lowered to 30% that has been the normal rate registered in the previous years.

The strain ST281 despite its MDR resulted to be different for 317 out of 1,701 (18.9%) of the gene loci analyzed and did not spread to other patients. Thus, our data indicate that NGS used for the microbial whole gene sequencing allowed us to understand at a finer level, the dynamic of strain transmission within the hospital in a restricted period of time giving us the opportunity to define a real single outbreak.

It is known that today WGS gives more information than any other assay for molecular characterization of strains involved in an outbreak making its use ideal in local epidemiological analysis. In addition, the sequencing of the whole microbial genome is used to study not only the presence of resistance genes, but also mutations involved in the mechanism of antimicrobial resistance and it gives information regarding the regulation of gene expression Indeed, by our analysis we found a strict correlation between phenotypic and genotypic data for antibiotic resistance, emphasizing the role of the ISAb1 sequences, which are strong promoters necessary for gene expression of beta-lactamases. All Acinetobacter baumannii have the blaADC gene, but only the ST195 and ST281 strains are characterized by the upstream Insertion Sequences (IS) genetic element ISAba1, that determines the overexpression of blaADC gene responsible for resistance to extended spectrum cephalosporin (ESBL). In addition among the ESBLs identified in Acinetobacter baumannii we have to consider the class A beta-lactamases encoded by the blatem1 gene and more importantly, the class D beta-lactamases encoded by blaOxa genes. Also the latter genes are regulated by upstream IS genetic (ISAb1a) elements decisive for the resistance to carbapenems. It is of note that only in ST195 and ST281 isolates we have found blaOxa-66 and blaOxa-23 regulated by IS genetic elements and also the blatem1-19. It is to be stressed that blaOXA-23 gene encodes the most prevalent and endemic carbapenemase in Italy. Regarding the analysis of polymixins, all the strains analyzed had a phenotypic susceptibility that correlate with the absence of a specific genetic determinant except PmrB-P170L in ST195 strain, that in a single report was indicated as responsible for a possible resistant mechanism. In general, our genetic data confirmed the phenotypic susceptibility test objectively supporting the antibiotic therapies adopted by clinicians.

In conclusion, WGS is a fundamental tool in the active surveillance of an outbreak. It will be included in standard prevention and control of infection spread in order to help Hospital Infection Control Committees to trace circulation of dangerous strains and to study their epidemiological aspect.

CONFLICTS OF INTEREST DISCLOSURE
The authors declare no conflict of interest.

REFERENCES


