Epidemiologal investigation of an *Acinetobacter baumannii* outbreak using Core Genome Multilocus Sequence Typing

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Running Head: cgMLST typing *Acinetobacter baumannii* outbreak

Keywords: *Acinetobacter baumannii*, cgMLST, carbapenem-resistance

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Word Count

Abstract: 242

Text: 1659

ABSTRACT

Carbapenem-resistant (CR) *Acinetobacter baumannii* is a serious nosocomial pathogen able to cause a variety of serious, often life-threatening infections and outbreaks. We aimed to investigate the molecular epidemiology of clinical isolates of CR-*A. baumannii* from an outbreak occurred in the intensive care unit (ICU) of our hospital.

From January to April 2017, 13 CR-*A. baumannii* isolates were collected at the "L. Spallanzani" hospital, Rome, Italy; typing was performed by repetitive extragenetic palindromic- based (rep)-PCR-based DiversiLab system and WGS data were used for in *silico* analysis of traditional MLST types, for identifying resistance genes and for core genome multi locus sequence typing (cgMLST) analysis. Epidemiological data were obtained from hospital records.

Strains were cultured from 7 patients treated in the ICU of our hospital; all isolates showed a multi-drug resistant (MDR) profile, carrying the $bla_{\text{OXA-23}}$ carbapenemase. Typing performed by rep-PCR and MLST showed that the isolates clustered into one group while the cgMLST approach, which uses 2690 gene targets to characterize the gene-by-gene allelic profile of *A. baumannii,* highlighted the presence of two cluster types. These results allowed us to identify two patients who entered the ICU already colonized by two different strains of CR-*A. baumannii*; we hypothesize that these two patients could be the source of two separate transmission chains.

Our results show that whole-genome-DNA sequencing by cgMLST is a valuable tool, better suited for prompt epidemiological investigations than traditional typing methods because of its higher discriminatory ability in determining clonal relatedness.

Introduction

Adapted to persist in healthcare settings, *Acinetobacter baumannii* is one of the most successful pathogens in causing nosocomial infections, from respiratory diseases to bacteraemia and meningitis (1). It is often isolated in Intensive Care Units (ICUs) where its frequent resistance to carbapenems limits treatment options, increasing the risk of adverse outcomes for patients (2). The ability to survive in the environment and to acquire resistance determinants also allowed *A. baumannii* to become one of the most frequent causes of hospital outbreaks among Gram-negative pathogens (3, 4).

In recent years, several typing methods have been used to recognise distinct clonal lineages of *A. baumannii* during hospital outbreaks. The newer WGS-based methods, characterized by a higher discriminatory power, are now replacing standard approaches such as the traditional MLST and semi-automated repetitive extragenic palindromic-PCR (rep-PCR, DiversiLab) (5-7). Some studies carried out to investigate the epidemiology of Carbapenem-Resistant (CR)-*A. baumannii* outbreaks have shown that the WGS-based method known as core genome MLST (cgMLST) demonstrated good typing ability by extending the traditional MLST concept to the entire genome, thus offering additional information on genetic diversity (8-10). This method has been used for several bacteria causing outbreaks including, among others, *Klebsiella pneumoniae*, *Neisseria meningitis and Listeria monocytogenes* (11-13).

In this paper, we describe the use of the cgMLST technique to investigate an outbreak of CR-*A. baumannii* occurred in the intensive care unit (ICU) of our hospital.

Methods

Study design

Between December 2016 and May 2017, we analysed strains of CR-*A. baumannii* isolated from 7 critically ill patients treated in the ICU of the "L. Spallanzani" hospital in Rome, Italy, to investigate a potential clonal spread. According to the hospital's surveillance programme aimed at

detecting carbapenem-resistant bacteria, rectal swabs were taken from all patients upon admission, and then once weekly for the entire duration of their stay. The isolates included in this study were obtained from these surveillance samples as well as from infection sites. The strains were characterized using the typing methods described below, and their profiles were compared with those belonging to the Global Clone 1 and 2 (GC1 and GC2), which are dominant in Europe (14). Epidemiological data, including time of infection and transfer to other wards, movements of patients within the ICU and rooms occupied, were extracted from hospital records.

Identification and Susceptibility testing

Samples were cultured on a selective medium designed to screen for carbapenemase production (chromID CARBA, bioMèrieux, Marcy l'Etoile, France); species identification and antimicrobial susceptibility were obtained by the Vitek-2 system (BioMérieux). As per EUCAST recommendations, MICs for tigecycline and colistin were confirmed by Etest (BioMérieux) and microbroth dilution methods (UMIC, Arnika Biocentric, Bandol, France), respectively (15).

Rep-PCR

The UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) was used for genomic DNA extraction, and molecular typing by rep-PCR was performed as previously described; (16) results were analysed using the 2100 expert software. The presence of clusters was investigated by Pearson's correlation coefficient and the unweighted pair-group method using average linkages (UPGMA). The guidelines followed for strain-level discrimination were those provided by the manufacturer: strains were considered as indistinguishable in case of a >97% similarity (no differences in fingerprints), as similar for a >95% similarity (1-2 band difference in fingerprints) and different if the similarity was <95%. The optimal cut-off chosen for cluster definition was 95% (6 Higg12). The Global Clones 1 and 2 were included in the analysis.

105 *WGS, assembly and cgMLST*

106 DNA was quantified using the Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA).
107 Sequence data for all strains were obtained by the Illumina MiSeq System with 250-bp paried-end
108 reads; DNA libraries were prepared using a Nextera XT DNA sample preparation kit (Illumina, San
109 Diego, CA, USA), according to manufacturer's instructions. Raw sequence data were submitted to
110 the Sequence Read Archive (SRA) (Table 1). Sequence quality trimming was carried out as
111 described by Bletz (17); d*e novo* assembly was performed by use of the Velvet assembler software
112 (v1.1.04), integrated in the RIDOM SEQSPHERE^{+} software (version 2.1, Ridom GmbH, Münster,
113 Germany).
114 In-depth phylogenetic analysis was achieved by cgMLST, using the software with default
115 parameters (17). To generate an *ad hoc* scheme, Open Reading Frames (ORFs) in the genome of
116 each isolate were identified by SeqSphere, using the whole-genome AB042 *A. baumannii* strain as
117 reference (GenBank Accession number CP019034.1). Briefly, 2690 targets genes were used to
118 characterize the gene-by-gene allelic profile of the *A. baumannii* strains, under the assumption that a
119 well-defined cgMLST scheme should cover at least 95% of the genes present in all analysed
120 isolates, compared to the reference strain. The resulting set of target genes was then used for
121 interpreting the clonal relationship displayed in a minimum spanning tree (MST) using the
122 "pairwise ignore missing values" parameter during distance calculations.
123 In accordance with other studies, we decided to set the cluster definition threshold at a maximum
124 difference of 12 alleles in a pairwise comparison (8, 9).
125
126 *MLST and resistance genes identification*
127 The identified ORF were uploaded to the ResFinder v3.0 web server for identifying resistance
128 genes and the MLST web server for the Pasteur and Oxford scheme MLST analysis
129 (www.genomicepidemiology.org) (18).
130

Results and discussion

Of the 13 isolates studied, nine were cultured from clinically relevant samples, and the remaining four were obtained from rectal swabs. Only the first two isolates from each patient were included in the analysis, with one exception (Patient 1) for whom only one strain (isolated from a bronchoaspirate 17 days after admission) was available. For patients 3, 5 and 6, the second isolate was collected once the patient had been transferred from the ICU to a clinical ward. Two of the 7 patients, (1 and 4) were colonised prior to ICU admission, as determined by their positive rectal swabs (Table 1).

All strains were resistant to amoxicillin/clavulanic acid (MIC ≥ 32 mg/L), cefotaxime (MIC ≥ 64 mg/L), ciprofloxacin (MIC ≥ 4 mg/L), ertapenem (MIC ≥ 8 mg/L), imipenem (MIC ≥ 16 mg/L), gentamicin (MIC ≥ 16 mg/L) and trimethoprim/sulfamethoxazole (MIC ≥ 320 mg/L); colistin MICs ranged from 0.5-0.75 mg/L (all strains were susceptible), while the MICs for tigecycline were between 0.5 and 1.5 mg/L. Also the resistance profile obtained from the i*n silico* ResFinder analysis showed identical resistance gene profiles for all isolates, as well as the presence of the following genes: $bla_{\text{OXA-66}}$ β-lactamase (an OXA enzyme belonging to the intrinsic OXA-51-like enzymes) (19), $bla_{\text{OXA-23}}$ carbapenemase, and the cephalosporinase-encoding $bla_{\text{ADC-25}}$ (Table 1). In addition, all isolates were shown to belong to the Sequence Type (ST)-2 and ST451using the Pasteur and Oxford scheme respectively. Typing performed by rep-PCR produced a similar result; all clinical isolates clustered into one group (Group-A) displaying a > 95% similarity with GC2, and only 70% with GC1, as expected since GC2 is the most prevalent clone in Europe and has been involved in the majority of outbreaks (20) (see Fig. S1 in the supplemental material).

For a more in-depth analysis, genotyping results obtained by MLST schemes and rep-PCR were compared with those of the cgMLST method. Sequences were obtained with a median mean coverage of 108 (range: 67-115) for all strains. The final cgMLST scheme consisted of 2690 target genes; of these, 2499 were consistently present. The percentage of good targets, based on the core genome, ranged between 96.9% and 98.4% (median 98.3%). These results show the presence of

two clusters types (CT-1 and CT-2) differentiated by only 17 allelic differences, where the traditional methods described above had highlighted only one (Figure 1). Nine isolates grouped within CT-1, (AC1 from P-1, AC2 and AC3 from P-2, AC4 and AC5 from P-3, AC8 and AC9 from P-5 and AC10 and AC11 from P-6), while 4 strains isolated from 2 patients (P-4 and P-7) gave rise to CT-2 (AC6, AC7, AC12 and AC13). The strains within each cluster showed a very high level of correlation; both clusters displayed a good similarity with GC2 (up to 252 differences), and only a distant correlation with GC1 (>2000 differences) (Figure 1).

The presence in each cluster of a patient colonised prior to admission (patients 1 and 4), and the fact that the same clones were later found in other patients treated in the ICU, suggested that two distinct transmission events occurred. To support this hypothesis, hospital records were studied to determine how the patients were treated and moved within the ICU. The ward is composed of nine single rooms; the 7 patients occupied rooms 2, 3, 4, and 5, which are next to each other. Figure 2 shows the timeline of patients and room occupancy during the five-month study period, and the days in which the patients were negative or positive for CR-*A. baumannii*. For cluster-type 1, we hypothesize a chain transmission involving patients 1, 2, 3, 5, and 6, as suggested by the brief overlap between the end of one patient's stay and the beginning of the next (Figure 2). Cluster-type 2 could have originated from a separate transmission from patient 4 to patient 7, both hosted in room 5.

Our study did not include environmental samples, which could have been useful in identifying a possible role of the environment in the transmission chain (21). However the genotyping results and the epidemiological data support the hypothesis that the origin of the two distinct clusters could be traced back to patients 1 and 4, who had been admitted to the ICU with rectal swabs already positive for CR-*A. baumannii*.

Investigations were undertaken to highlight possible breaches in the isolation precautions employed, but nothing was found that justified any changes in the hospital's infection control procedures.

Every effort was made to contain the outbreak and our continued surveillance showed, to this day, no further cases either in the ICU or in the clinical wards where the patients were later transferred. In conclusion, our data are in agreement with other recent reports (8, 9) that highlight how WGS-based methods such as the cgMLST represent reliable techniques that will likely become the gold standard for strain subtyping in support of epidemiological investigations.

208 **Legends to figures**

209 **Figure 1.** Minimum spanning tree based on cgMLST analysis of 13 CR-*A. baumannii* isolates
210 (AC1 to AC13) and Global Clones 1 and 2 strains (GC1 and GC2). Each circle represents an allelic
211 profile, i.e. genotype, based on sequence analysis of up to 2,690 target genes. The numbers on the
212 connecting lines illustrate the numbers of target genes with different alleles. Cluster types (grouped
213 together in the dotted ellipses) consist of closely related genotypes (≤12 allele differences) and are
214 numbered consecutively (I and II).

215

216 **Figure 2.** Timeline and room occupancy of all 7 patients (P-1 to P-7) positive for CR- *A. baumannii*
217 during December 2016 and May 2017. All patients occupied the same room for the entire duration
218 of their stay, except patient 4 who was moved from room 2 to 5 after 18 days.
219 Grey boxes: days in which patients were negative for CR-*A. baumannii*
220 Black boxes: days in which patients were positive for CR-*A. baumannii*
221 CT-1: Cluster type 1
222 CT-2: Cluster type 2

223

224 **Supplemental material**

225 **Figure S1.** Dendrogram generated by the DiversiLab software of rep-PCR-banding patterns for
226 the13 CR-*A. baumannii* isolates (AC1 to AC13) and Global Clones 1 and 2 strains (GC1 and GC2)
227 included for comparison. Using a similarity threshold of 95% to define a cluster, the resulting rep-
228 PCR cluster is named A.

229

230 Raw sequence data were submitted on Sequence Read Archive (SRA) under the BioProject
231 PRJNA427128, SRA Study: SRP127890, Accession numbers Sequences see Table1.

232

Funding Information

This work was supported by "Ricerca corrente" and "5X1.000" research funds from the Italian Ministry of Health.

Transparency declarations

None of the authors declared any conflicts of interest.

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Table 1. Summary of the clinical data and molecular characterization of CR-*A. baumannii* isolates.

Patient	Date of admission	Date of isolation	Date of discharge from ICU	Strain	Ward	Sample	β-lactamase	Genes encoding non-β-lactam resistance	ST 'Pasteur'	ST 'Oxford'	DiversiLab Group-Type	cgMLST Cluster-Type	SRA Accession Number
P-1*	24/12/2016	24/12/2016	19/01/2017	AC0•	ICU	Rectal swab	ND	ND	ND	ND	A	ND	ND
		10/01/2017		AC1	ICU	Bronchoaspirate	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6436005
P-2	20/01/2017	02/02/2017	13/02/2017	AC2	ICU	Rectal swab	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6436006
		09/02/2017		AC3	ICU	Blood	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6436007
P-3	10/02/2017	25/02/2017	07/03/2017	AC4	ICU	Blood	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6436008
		15/03/2017		AC10	CW	Bronchoaspirate	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6436000
P-4*	11/03/2017	11/03/2017	09/04/2017	AC7	ICU	Rectal swab	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	2	SRR6436003
		13/03/2017		AC8	ICU	Bronchoaspirate	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	2	SRR6436004
P-5	05/03/2017	16/03/2017	21/03/2017	AC9	ICU	Rectal swab	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6435999
		24/03/2017		AC11	CW	Blood	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6435994
P-6	16/03/2017	23/03/2017	27/03/2017	AC12	ICU	Rectal swab	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6435995
		28/03/2017		AC13	CW	Blood	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	1	SRR6435996
P-7	10/04/2017	20/04/2017	11/05/2017	AC15	ICU	Bronchoaspirate	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	2	SRR6435997
		30/04/2017		AC16	ICU	Blood	bla_{OXA-66}, bla_{OXA-23}, bla_{ADC-25}	*aph(3')-VI-a, aph(6')-Id, armA, strA, mph(E), mrs(E), sul2, tet(B)*	2	451	A	2	SRR6435998

* Patients colonised prior to admission.

• Molecular characterization and typing was not performed on rectal swab Patient 1 (study code AC0).

Abbreviation: ICU, Intensive Care Unit; CW, Clinical ward; ND, not done; SRA, Sequence Read Archive.

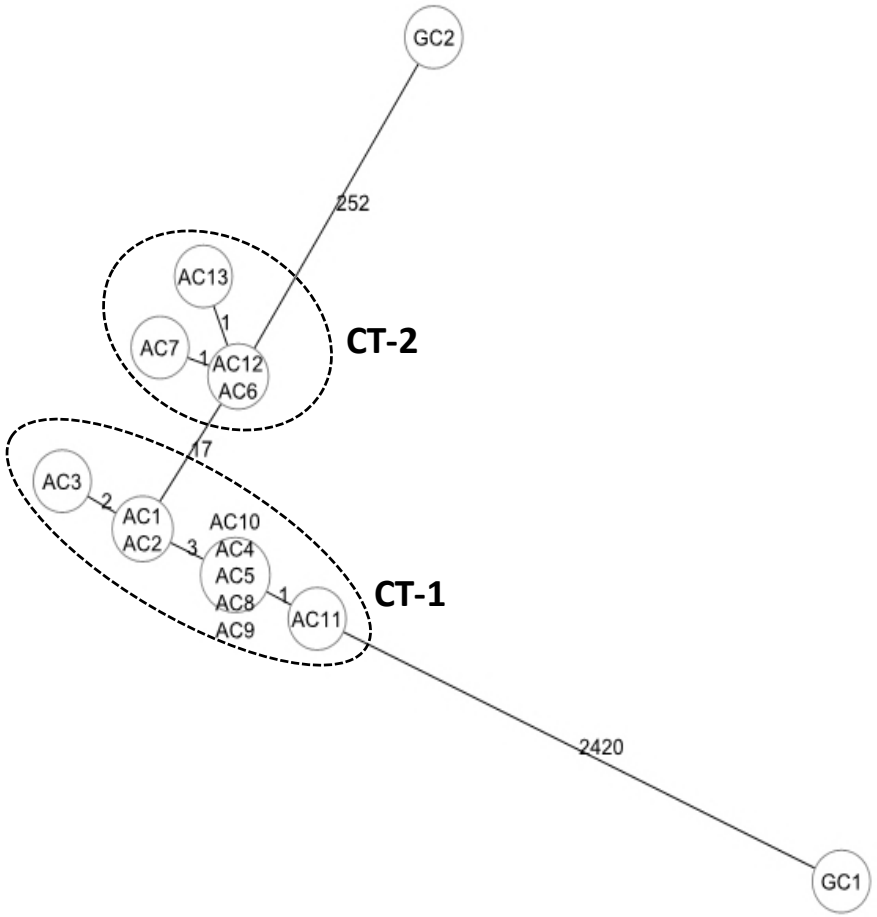

Figure 1. Minimum spanning tree based on cgMLST analysis of 13 CR-*A. baumannii* isolates (AC1 to AC13) and Global Clones 1 and 2 strains (GC1 and GC2). Each circle represents an allelic profile, i.e. genotype, based on sequence analysis of up to 2,690 target genes. The numbers on the connecting lines illustrate the numbers of target genes with different alleles. Cluster types (grouped together in the dotted ellipses) consist of closely related genotypes (≤12 allele differences) and are numbered consecutively (I and II).

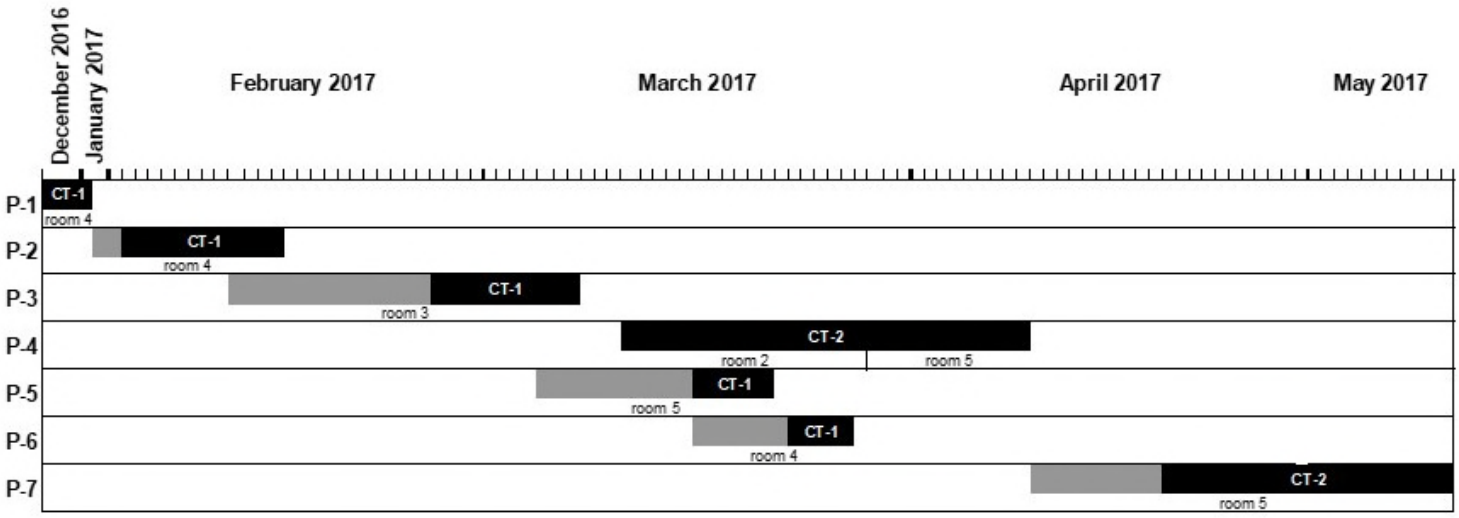

Figure 2. Timeline and room occupancy of all 7 patients (P-1 to P-7) positive for CR-*A. baumannii* during December 2016 and May 2017. All patients occupied the same room for the entire duration of their stay, except patient 4 who was moved from room 2 to 5 after 18 days.

Grey boxes: days in which patients were negative for CR-*A. baumannii*

Black boxes: days in which patients were positive for CR-*A. baumannii*

CT-1: Cluster type 1

CT-2: Cluster type 2