

Diversity of *Acinetobacter baumannii* in Four French Military Hospitals, as Assessed by Multiple Locus Variable Number of Tandem Repeats Analysis

Yolande Hauck^{1,2}, Charles Soler³, Patrick Jault³, Audrey Mérens⁴, Patrick Gérome⁵, Christine Mac Nab³, François Trueba⁶, Laurent Barges³, Hoang Vu Thien⁷, Gilles Vergnaud^{1,2,8}, Christine Pourcel^{1,2*}

1 Univ Paris-Sud, Institut de Génétique et Microbiologie, UMR8621, Orsay, France, **2** CNRS, Orsay, France, **3** Laboratoire de biologie clinique, HIA Percy, Clamart, France, **4** Laboratoire de biologie clinique, HIA Bégin, Saint Mandé, France, **5** Service de biologie médicale, HIA Desgenettes, Lyon, France, **6** Service de biologie médicale, HIA Val de Grâce, Paris, France, **7** Bactériologie, Hôpital Armand Trousseau, Assistance Publique-Hôpitaux de Paris (APHP), Paris, France, **8** DGA/MRIS- Mission pour la Recherche et l'Innovation Scientifique, Bagneux, France

Abstract

Background: Infections by *A. calcoaceticus*-*A. baumannii* (ACB) complex isolates represent a serious threat for wounded and burn patients. Three international multidrug-resistant (MDR) clones (EU clone I-III) are responsible for a large proportion of nosocomial infections with *A. baumannii* but other emerging strains with high epidemic potential also occur.

Methodology/Principal Findings: We automatized a Multiple locus variable number of tandem repeats (VNTR) analysis (MLVA) protocol and used it to investigate the genetic diversity of 136 ACB isolates from four military hospitals and one childrens hospital. *Acinetobacter* sp other than *baumannii* isolates represented 22.6% (31/137) with a majority being *A. pittii*. The genotyping protocol designed for *A. baumannii* was also efficient to cluster *A. pittii* isolates. Fifty-five percent of *A. baumannii* isolates belonged to the two international clones I and II, and we identified new clones which members were found in the different hospitals. Analysis of two CRISPR-cas systems helped define two clonal complexes and provided phylogenetic information to help trace back their emergence.

Conclusions/Significance: The increasing occurrence of *A. baumannii* infections in the hospital calls for measures to rapidly characterize the isolates and identify emerging clones. The automatized MLVA protocol can be the instrument for such surveys. In addition, the investigation of CRISPR/cas systems may give important keys to understand the evolution of some highly successful clonal complexes.

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* E-mail: christine.pourcel@u-psud.fr

Introduction

The genus *Acinetobacter* currently counts 27 validly named species (<http://www.bacterio.cict.fr/a/acinetobacter.html>) and several unnamed provisional species and genomic species [1,2]. *Acinetobacter baumannii*, *Acinetobacter pittii* sp. nov (formally *Acinetobacter* gen. sp. 3) and *Acinetobacter nosocomialis* sp. nov (formally *Acinetobacter* gen. sp. 13 TU) [3] are mostly associated with clinical issues [4]. These species and the closely related species *A. calcoaceticus*, an environmental species, are difficult to distinguish phenotypically, which has led to the proposal to lump them together into the *A. calcoaceticus*-*A. baumannii* (ACB) complex [3,5]. *A. baumannii* infection has almost exclusively been observed in critically ill hospitalized patients [6]. The emergence of resistance to multiple antibiotics including carbapenems in *A. baumannii* is worrying [7,8,9]. Three clonal lineages (European (EU) clones I-III) of multidrug resistant (MDR) and extensively-drug resistant (XDR) *A. baumannii* strains have been found to be associated with outbreaks in hospitals at different locations [10,11]. EU clones I

and II are now known to occur worldwide [8]. Antimicrobial resistance and epidemic spread of strains of *A. pittii* and *A. nosocomialis* is still limited compared to *A. baumannii* [12,13].

The natural reservoir of *A. baumannii* clinical isolates is unknown although *A. baumannii* was isolated from soil, meat, fish and vegetables [14,15]. Different *Acinetobacter* species including *A. baumannii* were found to grow in the rhizosphere of wheat [16]. The coexistence of different *Acinetobacter* species in a single environment might favor genomic transfer, a major source of genetic diversity [17,18]. The implication of *A. baumannii* in combat casualties was investigated in several studies, as these patients often suffer from severe burns and wounds and are initially treated in field hospitals [19,20]. In a recent study, 65 isolates from 48 patients were investigated showing the presence of European clones I to III genotypes and of other emerging genotypes defined by amplified fragment length polymorphism (AFLP) typing [19]. The difficulty in evaluating the genetic diversity of isolates infecting these patients worldwide is the use of different genotyping techniques by different laboratories. AFLP

and other pattern-based genotyping methods such as pulse field gel electrophoresis (PFGE) [21] or rep-PCR typing [22] are currently used but the results cannot easily be compared between laboratories or stored into databases. By contrast, Multilocus sequence typing (MLST) schemes [23,24,25,26] and multiple locus variable number of tandem repeats (VNTR) analysis (MLVA) schemes [27,28,29] provide a genotype in the form of a code that can be easily shared. Both approaches allow the identification of clonal lineages and the investigation of population structure [26,30,31]. In addition, one proposed MLVA-8 scheme was capable of high resolution sub-typing in a study of international clonal lineage II [32].

Clustered Regularly Interspaced (CRISPR) elements are DNA structures made of short repeats separated by stretches of DNA called spacers derived from invading nucleic acids and which number can vary from one strain to another [33]. Together with genes called *cas* for “CRISPR-associated”, they constitute a widespread adaptive immune system in prokaryotes [34]. They appear to be transferable in an intra and interspecies manner [35]. CRISPR polymorphism has been used to genotype strains and to perform phylogenetic analyses in a number of bacterial species [36]. Two CRISPR-*cas* systems have been found in the genome of several *A. baumannii* strains and in the *A. bayli* ADP1 strain [37].

In the present investigation we automatized the previously published MLVA-8 scheme and applied it to the typing of a collection of ACB complex isolates from four military and one civilian hospitals. Presence of the CRISPR-*cas* systems was also investigated to evaluate their distribution and further define some clones.

Results

Automatization of the MLVA Assay

We previously described the identification of 10 VNTRs in the genome of three *A. baumannii* strains available at that time, and the selection of 8 of those for an efficient MLVA-8 genotyping scheme (which will be called MLVA-8_{Orsay} to unambiguously identify this selection of 8 loci). The protocol involved individual PCR amplifications and agarose gel electrophoresis [29]. In the present study we included the 10 VNTRs (MLVA-10_{Orsay}) and automatized the assay in order to accelerate the procedure and to obtain a more accurate measurement of alleles for the 5 microsatellites loci. The VNTRs were amplified in two multiplex PCRs (Table 1) and the products were analysed on a capillary electrophoresis device. The conversion of amplicon size to repeat number was performed automatically by the CEQ8000 Genetic Analysis System software. The new protocol was first successfully tested on six reference strains (ATCC 17978, ACICU, AYE, RUH 134, RUH 875, RUH 5875 listed in Table S1) previously typed manually using agarose gel electrophoresis, showing identical results (the values are given in Table S2). In order to test whether MLVA-10_{Orsay} could be used for typing the different members of the ACB complex, we genotyped five *A. pittii* and five *A. nosocomialis* reference strains (Table S2). Polymorphism was observed in the different samples and at least 8 out of 10 VNTRs could be amplified. Three loci, Abaum3002, Abaum3530 and in particular Abaum0826 were responsible for amplification failures. VNTR amplification was not observed when DNA of species not belonging to the ACB complex was analysed.

Investigation of ACB Complex Isolates in Five French Hospitals

We wished to investigate the diversity of isolates recovered from military hospitals which mostly care for wounded and

burn patients, soldiers and civilians (Table S1). We also included in the analysis twelve isolates from the A. Trousseau hospital in Paris which cares for children with different pathologies. Among 136 ACB complex isolates, *rpoB* sequencing identified 27 *A. pittii* (genomic species 3) isolates, 2 *A. nosocomialis* isolates (genomic species 13TU), 1 *Acinetobacter* sp isolate (98% identity with *A. oleivorans*) and 106 *A. baumannii* isolates. The calculated typeability of MLVA-10_{Orsay} on *A. baumannii* samples was 98%, as missing values occurred for Abaum0826, Abaum3406 and Abaum3002. The absence of amplification could be due to mismatches in the target region of primers or to complete or partial deletion of the locus, but this was not confirmed. Therefore absent data were not considered as a character in the clustering analysis. Figure 1 and Figure 2 show a clustering analysis of *A. baumannii* isolates only. With a cut-off value of 40% corresponding to the existence of identical size alleles for at least 4 VNTRs, 11 clusters (two isolates or more) and 13 singletons were observed. As shown with green and red colors on Figure 1 and Figure 2, 53% (56/106) of the isolates fell into the two larger clusters respectively including RUH 134 the reference strains for EU clone I (32 isolates) and RUH 875 the reference strain for EU clone II (24 isolates). The majority of isolates in these clusters were multidrug resistant whereas those in smaller clusters, and non-epidemic isolates (singletons), were generally sensitive to several classes of antibiotics. Interestingly 7 out of 8 isolates in a small cluster from 6 patients in the Percy hospital (shown in yellow color and called here the Percy cluster) were XDR or MDR. Only one isolate (L06) clustered with RUH 5875, the reference strain for EU clone III. Abaum0826 was not amplified in 13% (15/116) of samples which do not belong to the EU clone I and EU clone II, and our efforts to design new primers that amplify all the isolates failed.

Calculated typeability of MLVA-10_{Orsay} on other members of the ACB complex was 86.5% due to missing data for VNTRs Abaum3002, Abaum3530 and Abaum0826. The dendrogram on Figure S1 reveals the existence of some groups (shown with different colors) sharing 4 VNTR alleles and possibly reflecting an epidemic situation. All isolates except B20 were susceptible to antibiotics.

A clustering analysis was performed with a total of 173 ACB complex isolates including those previously typed by MLVA [29], and the result showed that although most strains clustered according to the species, there was some inter-species clustering as shown on the minimum spanning tree in Figure S2.

CRISPR Analysis

We wished to investigate the presence of CRISPR-*cas* systems in members of the ACB complex and evaluate their potential use for epidemiological and phylogenetic studies. We searched by PCR for the presence of the *casI* gene from the two known CRISPR-*cas* systems. The first one is present in genome sequence data from *A. baumannii* AYE, AB0057 and AB307 (called here the AYE system), and the second is found in *A. baylii* ADP1 and in the *A. baumannii* type strain ATCC 19606^T (called here the ADP system). The AYE *casI* gene was present in all the EU clone I isolates as defined by MLVA-10_{Orsay} except in isolate L03 placed in an outgroup position (Figure 2). All the isolates in the Percy cluster possessed an AYE *casI* gene. The sequence of the A28 (EU clone I) and P16 (Percy cluster) *casI* amplicons was identical to that of the AYE strain. The ADP *casI* gene was found in four isolates dispersed throughout the tree; A22, L21, V02 and L03 (Figures 1 and 2).

Table 1. Oligonucleotides used for the multiplex PCR reactions.

| | VNTRa | Oligonucleotide | TM °C | Repeat size (bp) | Size of flanking regions (bp) ^b | Allele range (bp) ^c | | |
|---------------------|---------------------|-----------------------------|--------------------------|------------------|--|--------------------------------|-----|-----|
| Multiplexe 1 | Abaum_3406_L_D4 | CACTATATTGAAGTGCTTTTA | 48 | 30 | 231 | 342 | 669 | |
| | Abaum_3406_R | GTGGTTTTCTATTGGTACATTAC | 58 | | | | | |
| | Abaum_826_L_D3 | TGACTACTGAAACAGTTTTTG | 56 | 9 | 361 | 415 | 649 | |
| | Abaum_826b-R | ACTTGTTTGAGCTATAGAA | 54 | | | | | |
| | Abaum_17_L_D3 | GTGAGGGTAGAGTATTTGCTC | 62 | 9 | 80 | 85 | 346 | |
| | Abaum_17_R | GAGTTAGGGAGCTTTTATATGG | 64 | | | | | |
| | Abaum_845b_L_D2 | CCAAATTGCTCCAATATCTGAACT | 66 | 7 | 115 | 129 | 346 | |
| | Abaum_845b_R | GCAAATTTATACAGCTCAAGAAAATGG | 72 | | | | | |
| | Abaum_2396_L_D4 | CAAGTCCAATCAACTCATGATG | 62 | 6 | 105 | 147 | 315 | |
| | Abaum_2396_R | CTCCTGTAAGTGCTGTCAGCC | 68 | | | | | |
| | Abaum_3468_Lb_D2 | AAGACCACCTTTCACAACTAAA | 58 | 6 | 308 | 350 | 429 | |
| | Abaum_3468_Rb | TTTGCTGATGGTTGGGCTA | 58 | | | | | |
| | Multiplexe 2 | Abaum_3530_L_D2 | TGCAACCCGATTCTAGGAAC | 62 | 60 | 121 | 162 | 642 |
| | | Abaum_3530_R | CCTTGAACAACATCGATTACTGGA | 66 | | | | |
| Abaum_3002_L_D3 | | GACTGAAGCAAGACTAAAACGT | 62 | 57 | 121 | 428 | 596 | |
| Abaum_3002_R | | TCTGGCAGCTTCTTCTGAGC | 68 | | | | | |
| Abaum_2240b_L_D4 | | AAGGTTCCGTGTGAACTGATGATT | 68 | 99 | 209 | 204 | 538 | |
| Abaum_2240b_R | | CAGGTGTCAAATCGTAAACAAA | 60 | | | | | |
| Abaum_1988_L_D3 | | GGCAAGCATGCTCAAGGGCC | 70 | 26 | 77 | 136 | 447 | |
| Abaum_1988_R | | CAGTAGACTGCTGGTTAATGAG | 64 | | | | | |

^aThe forward primer is labeled with one of three dyes D2, D3 and D4. The allele range for each reaction is indicated showing that amplicons with the same dye cannot overlap.

^bValues referred to the genome of strain ATCC 17978 and considered for allele assignment.

^cThe allele range for each reaction is indicated showing that amplicons with the same dye cannot overlap. Values obtained with *A. baumannii* isolates.

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We then studied whether the AYE CRISPR was polymorphic, ie. whether the spacer composition was different in the different isolates. The number of spacers in the CRISPR locus of strains AYE, AB057 and AB307 are respectively 59 (3569bp long), 52 (3149 bp long) and 45 (2729 bp long) (Figure 3A). They share the majority of their spacers except those present at the growing end, as initially described in *Yersinia pestis* [38]. To facilitate the analysis of the CRISPR polymorphism in the different isolates, we amplified only the portion where new spacers are added. The PCR forward primer was selected inside spacer 49 of strain AYE as shown on Figure 3A and the reverse primer inside the sequence flanking the last motif. Amplification of strains AYE, AB0057 and AB307 were expected to provide amplicons of respectively 729 bp, 369 bp and 309 bp according to sequenced genome data, and this was confirmed experimentally (Figure S3 and data not shown). We then tested all the isolates possessing the AYE *cas 1* gene and others closely related by genotyping (part of the result is shown in Figure S3). Isolates in Percy cluster all produced a 309 bp fragment but the amplification was weak suggesting that the primers did not match perfectly. The majority of isolates in the EU clone I produced a 369 bp amplicon, and interestingly larger amplicons were observed for isolates placed in an outgroup position: P50, P54, P45, P65 and A28 produced fragments of respectively 1700 bp, 1700 bp, 1800 bp, 2300 bp and 490 bp. Complete (A28) or partial sequencing of these amplicons revealed the presence of new unique spacers, some of them matching chromosomal sequences or plasmids (Figure 3B and Table S3).

Discussion

MLVA

We describe an improved automatised MLVA assay which allows the rapid and efficient genotyping of *A. baumannii* isolates and may also be helpful to genotype isolates of other members of the ACB complex. MLVA-10_{Orsay} allows the definition of clusters of isolates, some of which correspond to known clonal lineages and others apparently emerging locally. The four minisatellites also present in the MLVA-8_{Orsay} scheme (Abaum3530, Abaum3002, Abaum2240, Abaum1988) are very informative to distribute isolates into majors clones, such as EU clone I and EU clone II. Inside these clones polymorphism is mostly provided by the fifth minisatellite, Abaum3406 and by the 5 microsatellites (Abaum0826, Abaum0017, Abaum0845, Abaum2396, Abaum3468) which bring a high level of discrimination. Abaum3406, a 30 bp repeat unit VNTR, is very polymorphic even inside lineages, as compared to the four other large-repeat units VNTRs. It encodes a portion of the copper resistance protein A precursor (also called multicopper oxydase), characterized by the presence of repeated amino-acids, with a high degree of variability. Copper-based biocides are used to fight health-care associated organisms and might exert a selective pressure for resistance [39]. The possible role of repeat number variation in the gene bearing Abaum3406 needs to be further investigated.

Persistence and Evolution of MDR Strains

The MDR and XDR isolates are mostly distributed in three MLVA clones, two of which correspond to EU clones I and II and

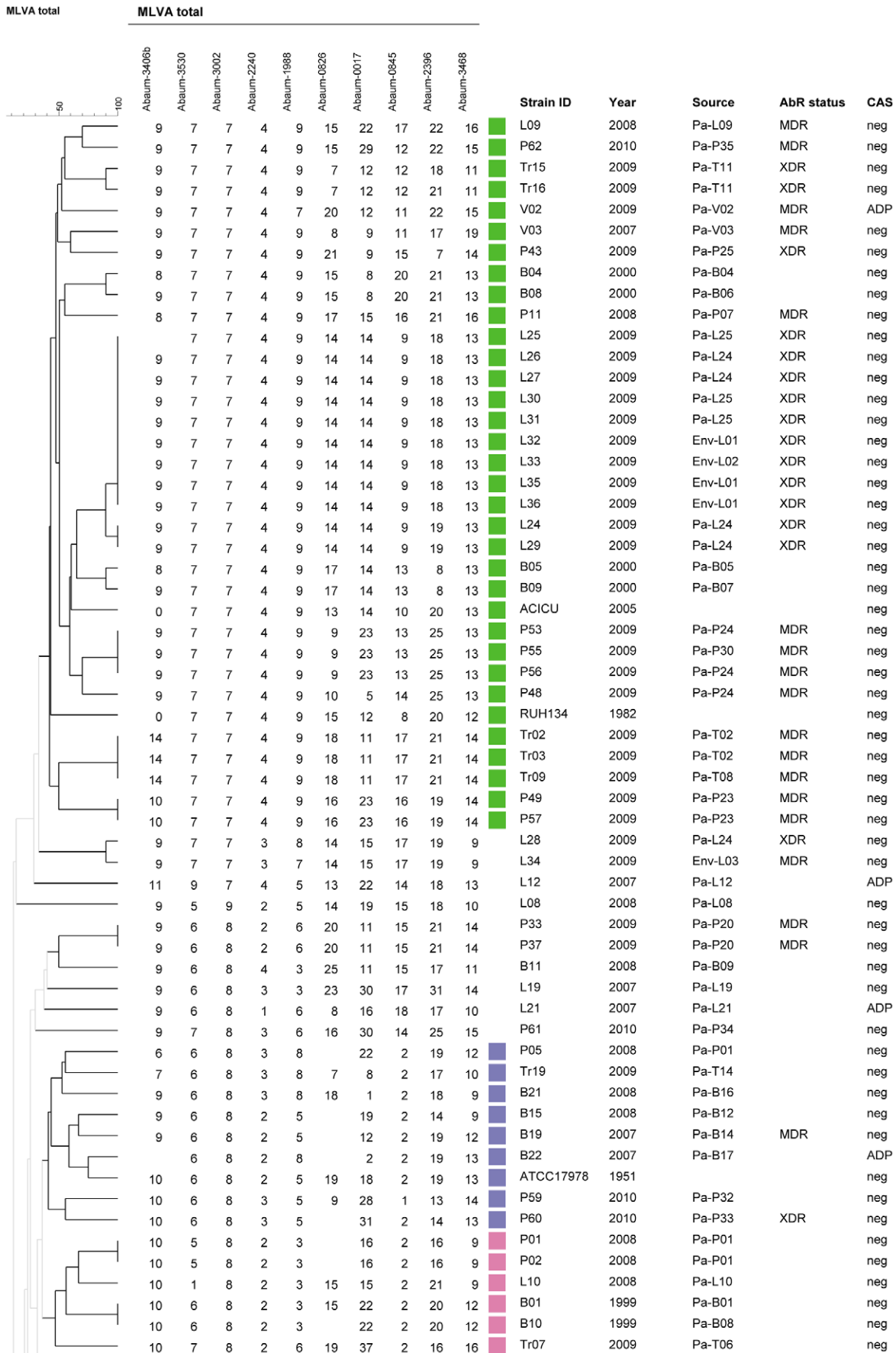


Figure 1. Genetic diversity of 59 *A. baumannii* isolates. A 40% cut-off value was used to define MLVA clusters, shown with different colors when containing 5 isolates or more. On the side are indicated the year of isolation, the source, the antibiotics resistance status and the existence of a CRISPR-cas system.

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the third being constituted of 7 isolates from 6 patients of Percy hospital. The definition of this local clone is further sustained by the presence of the AYE CRISPR-cas system in the 7 isolates (see dedicated paragraph below). Two other clones with mostly antibiotics-susceptible isolates from different hospitals were observed, one of which include the ATCC 17978 strains. The distribution of isolates from Trousseau hospital was similar to that of the military hospitals, although their small number preclude statistical analyses. It would be interesting to genotype by MLVA isolates from additional reference clones previously analysed by different genotyping techniques in order to better compare the bacterial populations [26]. This will be the subject of future collaborative studies.

Multiple isolates were recovered from 12 patients (2 to 10) and in 5 of them they showed the same genotype. In the other patients, variants of the same genotype were observed, differing at one or two small-size-repeat VNTRs, and in addition, 4 patients (P01, P23, P24, L24) harbored isolates of unrelated genotypes. In the Percy cluster, the first isolate recovered in 2008 was susceptible to all antibiotics except cotrimoxazol (SXT). Isolates of the same clonal lineage were found in another patient later in 2008, then in 2009 and 2010 and they were all MDR or XDR. The patients were in different wards. These observations suggest that a complex population of variants of this clone persists in the hospital. Some patients stay for long periods of time in the hospital and may be reservoir for *A. baumannii*. During a period of one year, 10 EU clone I isolates were recovered from burn patient Pa-P02 with 6 genotypes due to differences at VNTRs Abaum0826, Abaum0017 and Abaum0845. Although they were always susceptible to at least two classes of antibiotics these strains could not be eliminated. In 2009 two patients from hospital Desgenettes were infected with a XDR strain of EU clone II (Pa-L24 and Pa-L25) and the strain could be found on different environmental sources, including a door handle and a bench (Env-L01 and Env-L02). Over a period of three months, 3 genotype variants could be observed. It is possible that different genotypes coexist in a single source and in the future it will be interesting to systematically analyze several isolates from the same culture to test this hypothesis. The automated MLVA method will make such investigations easier.

Some of the military patients were wounded in other countries and were first treated in field hospitals before being transferred to France. We did not find any relationship between the isolates' genotype and the localization of the field hospital. Therefore we have no indication on the source of infection.

Clinical Outcome

A. pittii isolates represent 20% of the *Acinetobacter* isolates and a third of them form a cluster. This observation is interesting because it suggests the existence of clonal lineages spreading to different locations, similarly to what is observed with *A. baumannii*. These strains are susceptible to antibiotics except fosfomycin as previously reported [40,41]. They were found mostly in blood from patients having a deep vein catheter whereas infections of wounds and skin were mostly due to *A. baumannii*. Difference in clinical outcome depending on the genome species has been previously investigated [42], showing that *A. baumannii* was more frequently associated with pneumonia. Wisplinghoff et al. investigated 295 isolates from patients in the US with

bloodstream infection and found that one third was due to *A. nosocomialis* and *A. pittii* [43]. In other studies *A. pittii* were found at similar or even higher levels than *A. baumannii*, independently of the source [12,44]. In the present work and in others, *A. baumannii* was less susceptible to antibiotics than *A. nosocomialis* and *A. pittii* [45,46].

The CRISPR-cas Systems

We searched for the presence of the CRISPR-cas systems observed in some sequenced genomes to evaluate their potential for genotyping and to help in defining clonal complexes. The majority of isolates do not possess either one of the described systems which prevent their use as a tool for genotyping. However, the AYE CRISPR-cas system was very informative to investigate the diversity of the EU clone I lineage. Five isolates possess longer CRISPRs and this may suggest that they have further evolved in the environment as compared to the rest of the clone. Isolate L03 placed in an outgroup position, does not possess the AYE CRISPR-cas system and may represent an ancestral genotype for this clone. All isolates in the Percy cluster possess an EU clone I CRISPR-cas system indicating that they may be phylogenetically related to this clone but this requires further investigation by whole genome draft sequencing analysis. The five isolates with the ADP CRISPR-cas system are distributed in different clusters suggesting that the system may have been acquired by horizontal transfer at different time during evolution. Therefore we believe that investigation of these elements, although restricted to a subpopulation of isolates, may provide interesting phylogenetic information to understand the emergence of major lineages.

Materials and Methods

Ethics Statement

Strains were collected from different specimens as part of the patients' usual care, without any additional sampling. A code was used to refer to each patient without offering any possibility to trace these patients, and no information was reported except the sample source. Ethic committees in each hospital providing bacterial isolates were consulted and they declared that patient informed consent was not needed: for the military hospitals the "Comité d'éthique et des expérimentations" and for hospital Armand Trousseau the "Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédicale (CCPPRB) Ile-De-France – Paris – Saint Antoine".

Bacterial Samples

The isolates were obtained from specimens of patients from different wards or from hospital environment (Table S1). A total of 124 ACB complex isolates were collected in four military hospitals (from soldiers and civilian patients) from 2007 to 2010: 60 were from Hôpital d'Instruction des Armées (HIA) Percy, 35 isolates from HIA Desgenettes, 6 from HIA du Val de Grâce, 23 from HIA Begin. In addition 12 ACB complex isolates were collected from Armand Trousseau children hospital in Paris. In some cases several isolates were recovered from a single patient (up to ten for patient Pa-P02). Samples were streaked across Mueller-Hinton agar and incubated at 37°C for 24 hours. Bacteria were identified as belonging to ACB complex using standard automated biochemical testing methods. *Acinetobacter baumannii* were tested

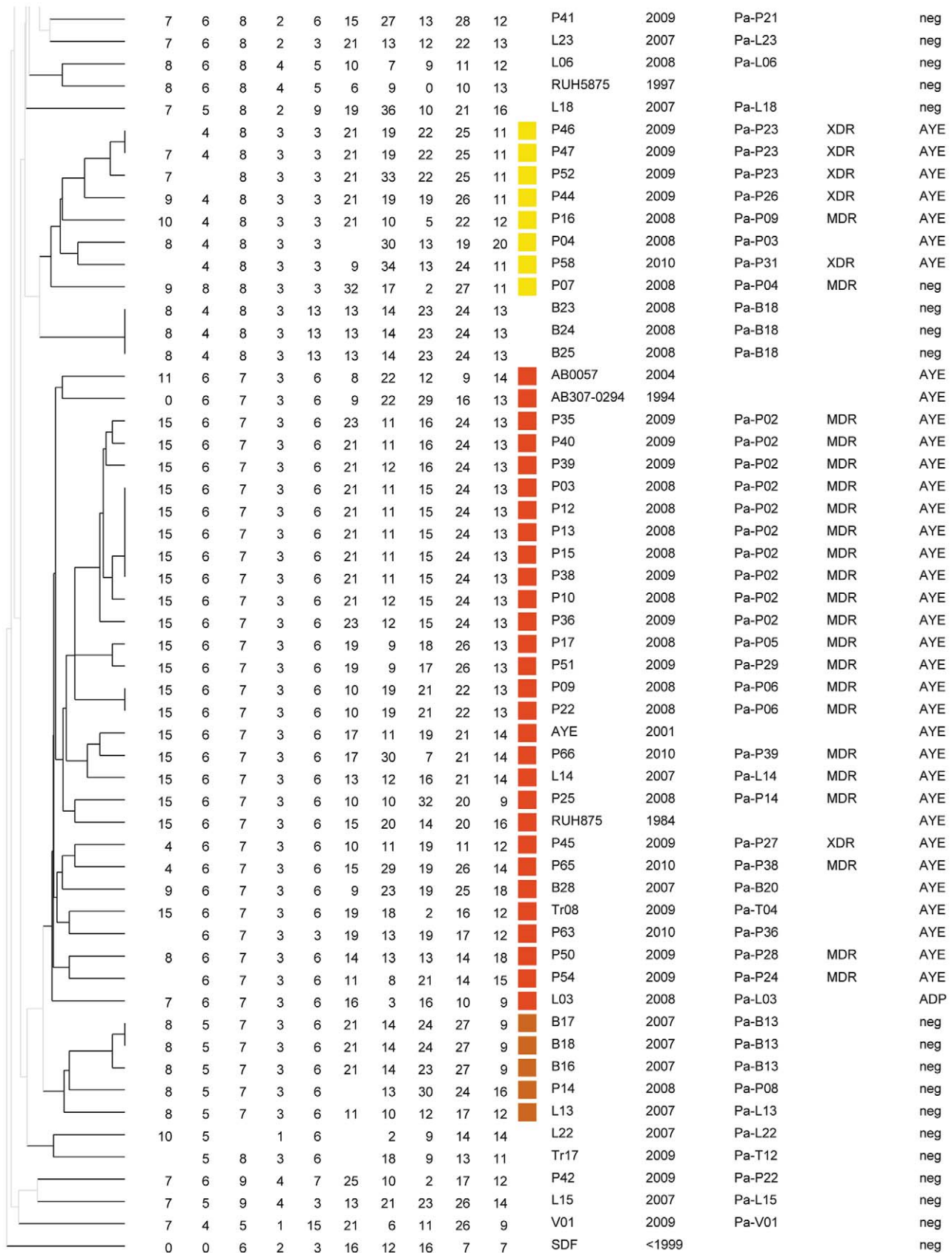


Figure 2. Genetic diversity of 56 *A. baumannii* isolates. The legend is that of Figure 1. doi:10.1371/journal.pone.0044597.g002

(Beckman Coulter Genomics, UK). All new sequence data has been deposited in GenBank under the accession numbers HE801220 to HE801224 and HE803768 to HE803825.

Complete genome sequences used in this study were: *A. baumannii* strains AB0057 (GenBank: <http://www.ncbi.nlm.nih.gov/gate1.inist.fr/nucleotide/213155370/CP001182-3>) and AB307 (Genbank: CP001172.1) [49], ACICU (GenBank: NC_010611) [50], ATCC17978 (GenBank: NC_009085) [51], AYE (GenBank: NC_010410), SDF (Genbank: CU468230.2) [52] and ADP1 (Genbank CR543861) [53]. The sequence of *A. baumannii* ATCC 19606^T was recovered from the Acinetobacter group sequencing project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>).

Supporting Information

Figure S1 Genetic diversity of 40 non-*A. baumannii* ACB complex isolates. A 40% cut-off value was used to define MLVA clusters, shown with different colors when containing 3 isolates or more. On the side are indicated the year of isolation, the patient code (source), the site of isolation (removal) and the Acinetobacter species. (TIF)

Figure S2 Minimum spanning analysis of MLVA data for 173 ACB complex isolates. Isolates from the present study belonging to the two larger *A. baumannii* clonal complexes and to the *pittii* and *nosocomialis* species are shown with colors. (TIF)

Figure S3 Polymorphism of the AYE CRISPR locus. Agarose gel electrophoresis of amplicons corresponding to the

CRISPR growing end. The analysed samples are A) P03, P04, P09, P12, P15, P16, P17, P22, P25, P35, P36, P39, P40, L04, AYE, A28, R12, R17, P44, P07, P46, P47. B) P66, P65, P63, P54, P14, Tr08, P51, P50. (TIF)

Table S1 List of isolates used in the present study. An initial is used for the different hospitals: P Percy, L Lyon, V Val de Grâce, B Béjin, Tr Trousseau. (DOC)

Table S2 Result of MLVA genotyping of reference strains. (DOCX)

Table S3 List of spacers identified in sequenced AYE CRISPR loci. ¹ The table corresponds to the dictionary produced by CRISPRcompar [Grissa, 2008 #893] where spacers are annotated in each analysed sample as shown in the second column. (DOCX)

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Author Contributions

Conceived and designed the experiments: CS GV CP. Performed the experiments: YH AM CMN CP. Analyzed the data: CS AM PG HVT. Contributed reagents/materials/analysis tools: PJ FT LB AM PG HVT. Wrote the paper: CP GV.

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