title

Genomic analysis of antibiotic resistance for *Acinetobacter baumannii* in a critical care center

short running title

Genomic analysis for *A. baumannii*

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ICMJE Statement

All authors meet the ICMJE authorship criteria.

Abstract

Introduction: *Acinetobacter baumannii* (*A. baumannii*) is commonly associated with outbreaks and antibiotic-resistant nosocomial infection. This study aimed to determine the relationship between antibiotic resistance and genotypes of *A. baumannii*.

Methods: A study was conducted in the critical care center (CCC) of Juntendo University Urayasu Hospital between January 2012 and September 2015. Antimicrobial susceptibility tests were performed according CLSI guidelines. All *A. baumannii* isolates were verified to carry carbapenemase genes and the IS*Aba1* element using PCR. The genetic relationship of all *A. baumannii* isolates was determined by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Results: During the study period, 1634 patients were admitted to the CCC. *A. baumannii* was detected in 43 patients (average age, 58 ± 19 years; 67.4% male). Six patients were determined to be extensively drug-resistant *A. baumannii* (XDR-AB) and 21 patients determined to be multidrug-resistant *A. baumannii* (MDR-AB). Antimicrobial susceptibility linked genotypes of *A. baumannii*. Molecular characterization by PFGE and MLST showed that closely-related clones of *A. baumannii* had spread in the CCC.

Conclusion: Resistance to antimicrobial drugs was significantly associated with certain *A. baumannii* genotypic types and molecular types. Thus we probably predict whether the genotype spread in the CCC or not when the susceptibility is examined, facilitating the

appropriate isolation of patients.

Keywords:

carbapenemase; outbreak; OXA-51-like; IS*Aba1*; IMP genes

Introduction

Acinetobacter baumannii (*A. baumannii*) is an aerobic gram-negative bacillus that is ubiquitous in the natural environment. Although *A. baumannii* is typically harmless and colonizes the skin and respiratory tracts of patients as well as medical instruments [1], it is an opportunistic pathogen that can cause bacteremia, ventilator-associated pneumonia, and urinary tract infection in hospital setting [2]. Since the early 1980s, it has been associated with antibiotic-resistant nosocomial infections, especially in intensive care units (ICUs) in Europe.

The intercontinental spread of multidrug-resistant *A. baumannii* (MDR-AB) between Europe and other continents has been described since the 1990s [2]. Molecular epidemiological typing has been employed to investigate intercontinental outbreaks of *A. baumannii*, and to determine the genotypes of *A. baumannii* isolates from various locations worldwide, mainly in Europe. In addition to clones identified from these international outbreaks, two *A. baumannii* clones from Europe that have spread globally were identified. These have been designated as global clone (GC) I and II (previously known as the European clone or international clone I and II). The GCI and II lineages were first reported 23 years ago [3].

In 2008, a university hospital in Japan reported that 23 patients in the critical care center (CCC) were infected with MDR-AB, and four patients died [4]. Subsequently,

several CCCs in Japan also reported continuous MDR-AB outbreaks in 2009, 2010, and 2011. Each CCC serves a pre-determined population and accepts ambulances from the surrounding area. Thus, a CCC closure due to an outbreak can disrupt the local emergency care system. In 2010, four academic associations related to infectious diseases in Japan suggested the necessity of effective *Acinetobacter* surveillance, promotion of the development of new antibiotics, and their accelerated approval for domestic use (http://www.kansensho.or.jp/modules/guidelines/index.php?content_id=26).

A. baumannii can easily acquire multi-drug resistance, including resistance to carbapenem [5]. The most common mechanism of carbapenem resistance in *A. baumannii* is through the action of carbapenem-hydrolyzing class D β-lactamase (CHDL), also known as an oxacillinase (OXA). To date, four main phylogenetic subgroups of OXA-type carbapenemase (OTC) have been identified in *A. baumannii*: OXA-23-like, OXA-40-like, OXA-51-like, and OXA-58-like. OXA-51-like enzymes are chromosomally-encoded naturally occurring OTCs in *A. baumannii*. However, OXA-51-like enzymes are not typically potent against antibiotics and were demonstrated to confer carbapenem resistance when an IS*Aba1* element is inserted upstream of the gene. Genes encoding acquired OXAs, including OXA-23-like, OXA-40-like, and OXA-58-like, have been found mostly on plasmids. An insertion sequence linked to OXA-23 and OXA-58 genes were also demonstrated to regulate the expression of the carbapenemase [2].

The expression of carbapenem-hydrolyzing class B β-lactamases, also known as metallo-β-lactamases (MBLs), is a powerful mechanism for carbapenem resistance carried by plasmids. To date, three groups of acquired MBLs have been identified in *A. baumannii*: IMP-like, VIM-like, and SIM-1. However, MBL rarely mediates carbapenem resistance in *A. baumannii* [6].

The CCC at Juntendo University Urayasu Hospital has been experiencing an MDR-AB outbreak since 2012. The majority of MDR-AB outbreak was colonization in our hospital and there was no transfer around another hospital in same medical area. On average, 51 cases of *Acinetobacter* spp. including *A. baumannii* colonization were detected yearly from 2003 to 2011 prior to the outbreak. However, the number of *Acinetobacter* spp. cases increased to 115 in 2012, 80 in 2013, 97 in 2014, and 100 in 2015. Since 2012, significant efforts included clarification of the clinical features between antibiograms and PCR patterns of *A. baumannii* isolates. The present study investigated the relationship between *A. baumannii* genotypes and their resistance to antibiotics.

Patients and methods

Study design, setting, and participants

This study was conducted at Juntendo University Urayasu Hospital, a 653-bed facility that includes 15 and in the CCC. The CCC serves approximately 1.6 million people residing in the east side of Chiba prefecture, which is located adjacent to Tokyo, Japan.

Approximately 22,000 patients, including around 5,000 patients in critical condition transported by ambulance, are treated in the emergency center annually. These include trauma, burn, poisoning, cardiopulmonary arrest, shock, and other critical internal medical conditions.

We screened a sputum or throat swab using selective medium for isolation of *Acinetobacter* spp. at the time of admission to the CCC and weekly thereafter as a surveillance. Bacterial cultures obtained from the surveillance conducted in the CCC between January 2012 and September 2015 including *A. baumannii*. Exclusion criteria were death within 24 hours of admission, and pediatric patients <15 years old.

Definitions of MDR and XDR A. baumannii

According to the new definition for drug-resistant *A. baumannii* provided by the European Centre for Disease Prevention and Control and the Centers for Disease Control and Prevention, MDR-AB is defined as *A. baumannii* with non-susceptibility to ≥1 agent in at least three or more of the following categories: combinations of penicillins and β-lactam inhibitors, extended-spectrum cephalosporins, aminoglycosides, fluoroquinolones, carbapenems, polymyxins, and tigecycline. Extensively drug-resistant *A. baumannii* (XDR-AB) is defined as non-susceptibility to ≥1 agent in the standard categories except polymyxins or tigecycline [7].

Antimicrobial susceptibility testing

Once bacteria were cultured from the sputum or throat swab sample, antimicrobial susceptibility tests for common bacteria were performed to identify the causative agent according to the routine protocol of our bacteriology laboratory. Minimal inhibitory concentrations (MICs) against piperacillin (PIPC), ceftazidime (CAZ), meropenem (MEPM), levofloxacin (LVFX), and amikacin (AMK) were determined using broth microdilution method according to guidelines from the Clinical and Laboratory Standards Institute (CLSI) [8].

Amplification of carbapenem resistance genes and IS by PCR

For samples with successful *A. baumannii* culture and isolation, the presence of carbapenemase genes (OXA-23-like, OXA-24-like, OXA-51-like, and OXA-58-like) were evaluated by multiplex PCR using four pairs of previously described primers [9,10] using the LightCycler 96 system (Roche, Switzerland). OXA-51 was naturally occurring OTCs in *A. baumannii* that was used as marker gene of *A. baumannii.* Additionally, two MBL genes (IMP- and VIM-like) were also evaluated by PCR as previously described [11]. The presence of IS*Aba1* element in all *A. baumannii* isolates was assessed by PCR, using the HFR and HRR primers [12] and the LightCycler 96 system.

Pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

The genetic relationship of all *A. baumannii* isolate was determined by PFGE using the Sma1 restriction enzyme (Takara Bio, Japan). PFGE was performed using a

Fingerprinting II Software Version 3.0 with support from SRL, Inc (Japan). Relatedness determinations were done as previously described [13]. Sequence types (STs) for *A. baumannii* were determined by MLST, specifically for clonal lineages using the Pasteur scheme [14,15], by amplification and sequence analysis of fragments of seven internal house-keeping genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*). Determination of the STs was carried out using the Pasteur MLST database website (http://pubmlst.org/abaumannii/).

Results

Patient background and bacteria isolation

The enrollment criteria for the study are provided in Figure 1. During the observation period, 1634 patients were admitted to the CCC. Of these, 281 patients had positive cultures from sputum or throat swab. Nine patients were excluded: five were pediatric patients (<15 years old), and four patients died within 24 hours of admission. After the exclusion, samples from 272 patients were further studied.

A. baumannii was detected in 43 patients with an average age of 58 ± 19 years. The majority were male (29/43, 67.4%). Three patients were identified as having acquired nosocomial infection. Because two patients were diagnosed with VAP due to *A. baumannii* and a patient was with positive *A. baumannii* from blood cultures. Forty patients were colonized with *A. baumannii*.

Genotypic determination and antimicrobial susceptibility testing

The carbapenemase genotypes of *A. baumannii* are listed in the Table 1. Multiplex PCR identified the same *A. baumannii* genotype (possessing OXA-51-like, IS*Aba1*, and IMP, but not OXA-23-like, OXA-40-like, OXA-58-like, or VIM) in six of the 43 patients. The average age of patients with this genotype of *A. baumannii* was 51 ± 24 years, and 33.3% (2/6) were male. Their average length of stay in the CCC was 26 ± 5 days (Table 2). *A. baumannii* isolated from these patients were resistant to PIPC, CAZ, MEPM, LVFX, and AMK (Fig. 2A). These isolates were determined to be XDR-AB.

A. baumannii from 21 of the 43 patients had OXA-51-like and IS*Aba1*, but not IMP, OXA-23-like, OXA-40-like, OXA-58-like, or VIM. The average age of these patients was 53 ± 16 years, and 76.2% (16/21) were male. Their average length of stay at the CCC was 25 ± 13 days (Table 2). These isolates were not susceptible to PIPC, CAZ, LVFX or AMK and were susceptible to MEPM (Fig. 2B). They were determined to be MDR-AB.

The other 16 patients had *A. baumannii* possessing only the OXA-51-like genotype, and not IS*Aba1*, OXA-23-like, OXA-40-like, OXA-58-like, IMP, or VIM. The average age of these patients was 68 ± 13 years and 68.7% (11/16) were male. Their average CCC stay was 14 ± 10 days (Table 2). These isolates were sensitive to virtually all drugs: PIPC (6.3%), CAZ (0%), MEPM (0%), LVFX (6.3%), and AMK (6.3%) (Fig. 2C).

Based on these results, the antibiotic susceptibility or resistance was significantly

associated with certain *A. baumannii* genotypes. Therefore, antimicrobial susceptibility could indicate genotype of *A. baumannii.*

PFGE and MLST findings

PFGE was conducted for samples from all 43 patients with *A. baumannii* including those with XDR-AB and MDR-AB. Isolates from three patients (No.10, 33, and 40) were excluded due to the potential for contamination with other bacteria during long-term sample storage at −80°C. The PFGE results showed that isolates from 26 patients with *A. baumannii* genotypes carrying OXA-51-like and IS*Aba1* regardless of the presence of the IMP gene belonged to two closely-related types: PFGE type X-1 and X-2 (Fig. 3). The similarity coefficient for isolates from these two PFGE types was 93%. Based on this similarity, we hypothesized that closely-related *A. baumannii* genotypes had been spreading in the CCC for 4 years.

In contrast, *A. baumannii* isolated from the other 14 patients (OXA-51-like(+) IS*Aba1*(−) IMP(−) genotype) were detected as various clones belonging to PFGE type Y-1 to Y-14 (Fig. 3). The lineage clonality of these *A. baumannii* isolates was low, and the similarity coefficient was approximately 55 to 75%.

Results of the MLST showed that the PFGE type X-1 and X-2 isolates carrying OXA-51-like and IS*Aba1* belonged to ST2 according to the protocol of the Pasteur Institute (two alleles each for *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*). Those belonging to

GCII were the only strains that could cause outbreaks in our CCC, and potentially could have spread from the CCC throughout the hospital.

Discussion

To date, most of the previous studies about MDR-AB were international spread or multicenter study because MDR-AB is rarely detected [2]. The present study shows the relationship of *A. baumannii* from 43 patients about the susceptibility, the genotype and the molecular type at a single center. This study shows that genotypic determination of *A. baumannii* can predict the susceptibility to antimicrobial drugs. We found that two closely related strains, as determined by molecular characterization by PFGE and MLST, had spread in our CCC. Therefore, we probably predict whether the genotype spread in the CCC or not when the susceptibility is examined, facilitating the appropriate isolation of patients. This isolation method can be applied not only to *A. baumannii* but also to gram negative bacillus when susceptibility is consistent with genotype of bacteria.

Specifically, *A. baumannii* genotypes carrying OXA-51-like and IS*Aba1* had spread in our CCC. Four OXA enzymes of *A. baumannii* have been reported to cause outbreaks globally, with OXA-23-like and OXA-51-like as the major oxacillinases associated with outbreaks in Asia [2]. The strains isolated in our CCC also carried OXA-51-like; however, they did not possess the OXA-23-like gene. Based on the MLST results, the strains found to be spreading in the CCC belonged to GCII. GCII has been associated with the presence

of OXA-51 and IS*Aba1*, OXA-23, OXA-40, or OXA-58[3]. In our CCC, the GCII strains possessed OXA-51-like and IS*Aba1*.

To date, the IMP gene has been identified in *A. baumannii* in a variety of geographic regions, including Italy, Spain, Portugal, Greece, Australia, Japan, Singapore, South Korea and Hong Kong [2,6,16]. The present PCR and PFGE analyses revealed that six patients harbored *A. baumannii* with the IMP gene and the PFGE type X-1 detected two genotype strains: IMP(+) and IMP(-). Plasmids carrying the IMP gene were more than 60 kbp [17]. The PFGE analysis we used was capable of detecting differences of approximately 50 to 300 kbp. However, plasmids containing the IMP gene could not be detected by PFGE, and both the IMP(+) and IMP(-) genotypes would be assigned to the same molecular type.

Some limitations exist in this study. First, the present outbreak of *A. baumannii* is characterized by the specific genotype this is OXA-51-like(+) and IS*Aba1*(+) in the CCC. However, other molecularly characterized strains may be spread in other hospitals and must be detected independently by PCR and PFGE. Second, *A. baumannii* can easily acquire multi-drug resistance owing to plasmid-mediated gene transformation [5]. However, as has been stated, the PFGE type X-1 belonged to OXA-51-like(+) and IS*Aba1*(+) regardless of the presence of the IMP gene, and the PFGE technique was unable to detect minor genetic differences, such as the IMP gene.

In conclusion, susceptibility to antimicrobial drugs can predict *A. baumannii* genotypic types in the CCC.

Acknowledgments

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Disclosure

Approval of the research protocol: This study was approved by the institutional review board of the National Human Genome Research Institute, and was conducted according to the principles of the Declaration of Helsinki. This study was also approved by the Ethics Committee of the Faculty of Medicine, Juntendo University Urayasu Hospital (approval No.30-004).

Informed Consent: Informed consent was obtained from the guardians by opt-out in publicity documents.

Registry and the Registration No. of the study/Trial: N/A

Animal Studies: N/A

Conflict of Interest: This study was supported by a grant from the Ministry of Health,

Labor and Welfare of Japan [H27 Kiban Ippan 15H04795]. There are no conflicts of interest to declare.

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Table 1. Genotypic determination of carbapenemase genes of *Acinetobacter baumannii*

Patient No.	Carbapenemase genotype						
	OXA-23	OXA-40	OXA-51	OXA-58	IS*Aba1*	IMP	VIM
25, 34, 36, 37, 38, 39	—	—	+	—	+	+	—
1, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 15, 22, 23, 24, 26, 29, 30, 31, 35, 41	—	—	+	—	+	—	—
2, 7, 9, 16, 17, 18, 19, 20, 21, 27, 28, 32, 33, 40, 42, 43	—	—	+	—	—	—	—

Table 2 . Patient background and *Acinetobacter baumannii* genotypes

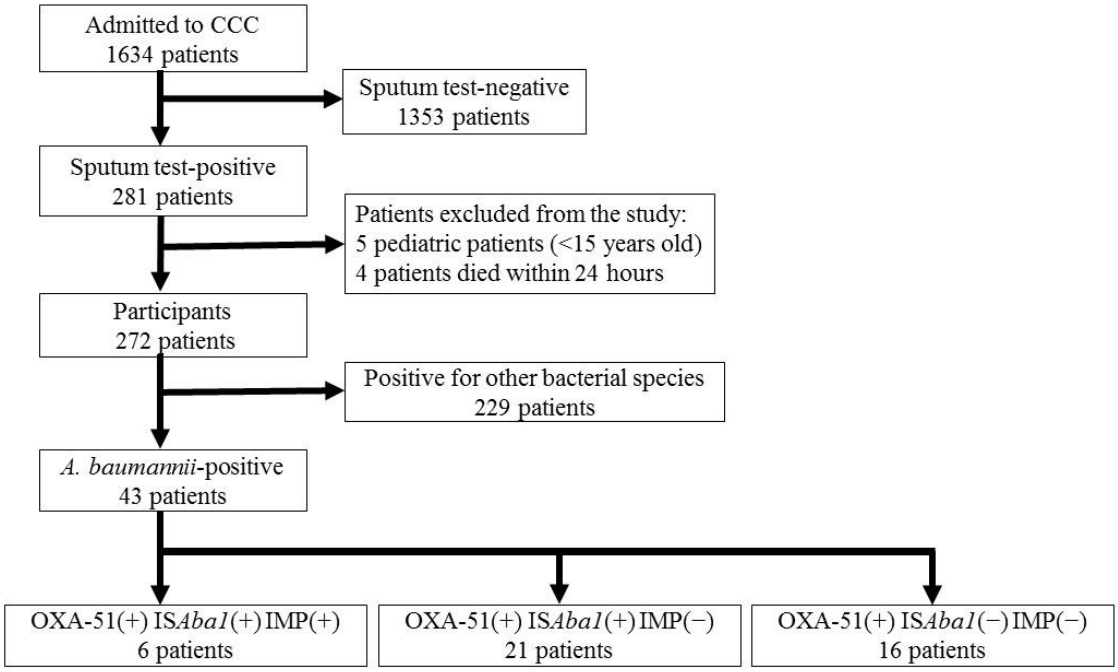
Genotype	OXA-51-like(+) IS*A-ba1*(+) IMP(+)	OXA-51-like(+) IS*A-ba1*(+) IMP(−)	OXA-51-like(+) IS*A-ba1*(−) IMP(−)
Number of patients	6	21	16
Age	51 ± 24	53 ± 16	68 ± 13
Sex (% male)	33.3	76.2	68.7
Days in CCC (days)	26 ± 5	25 ± 13	14 ± 10
Drug resistance	Fig. 2A (XDR-AB)	Fig. 2B (MDR-AB)	Fig. 2C
PFGE type	X-1	X-1 or X-2	Y1-14
Spreading in CCC	Yes	Yes	No

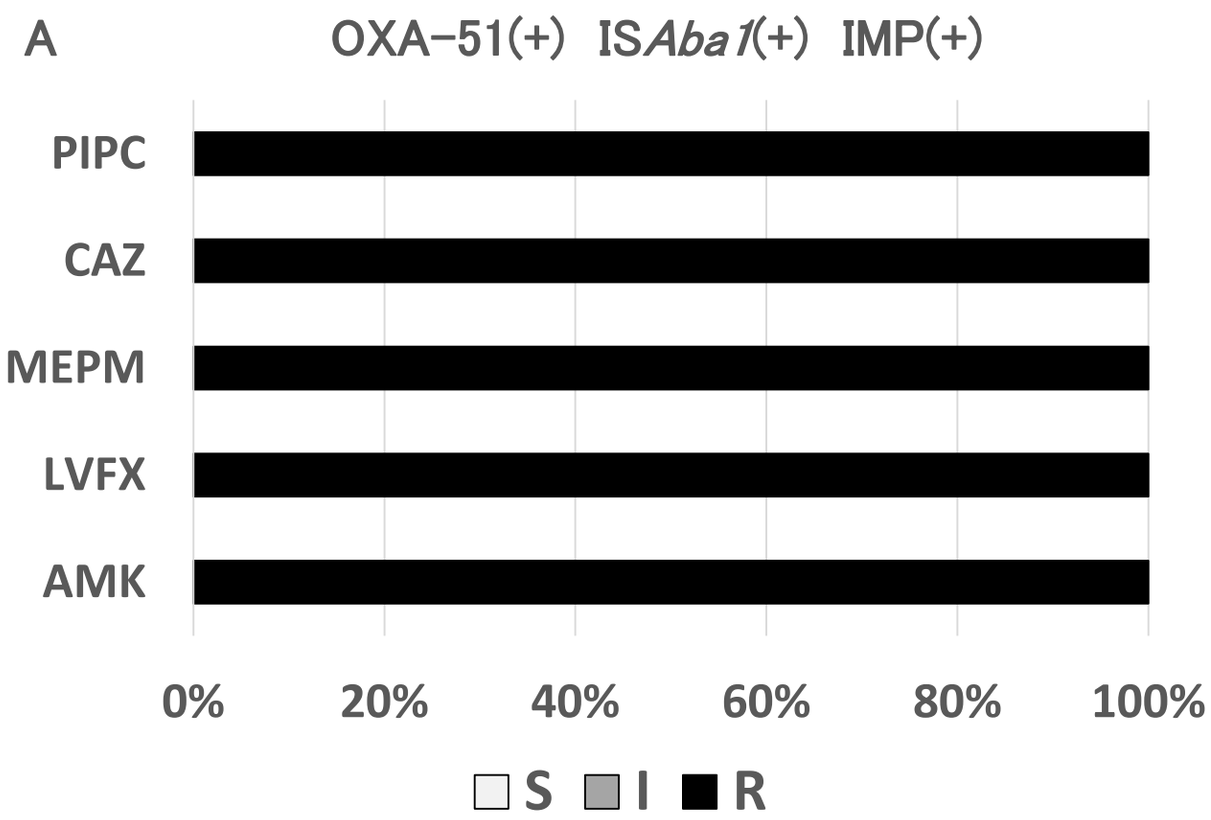
Legends to figures

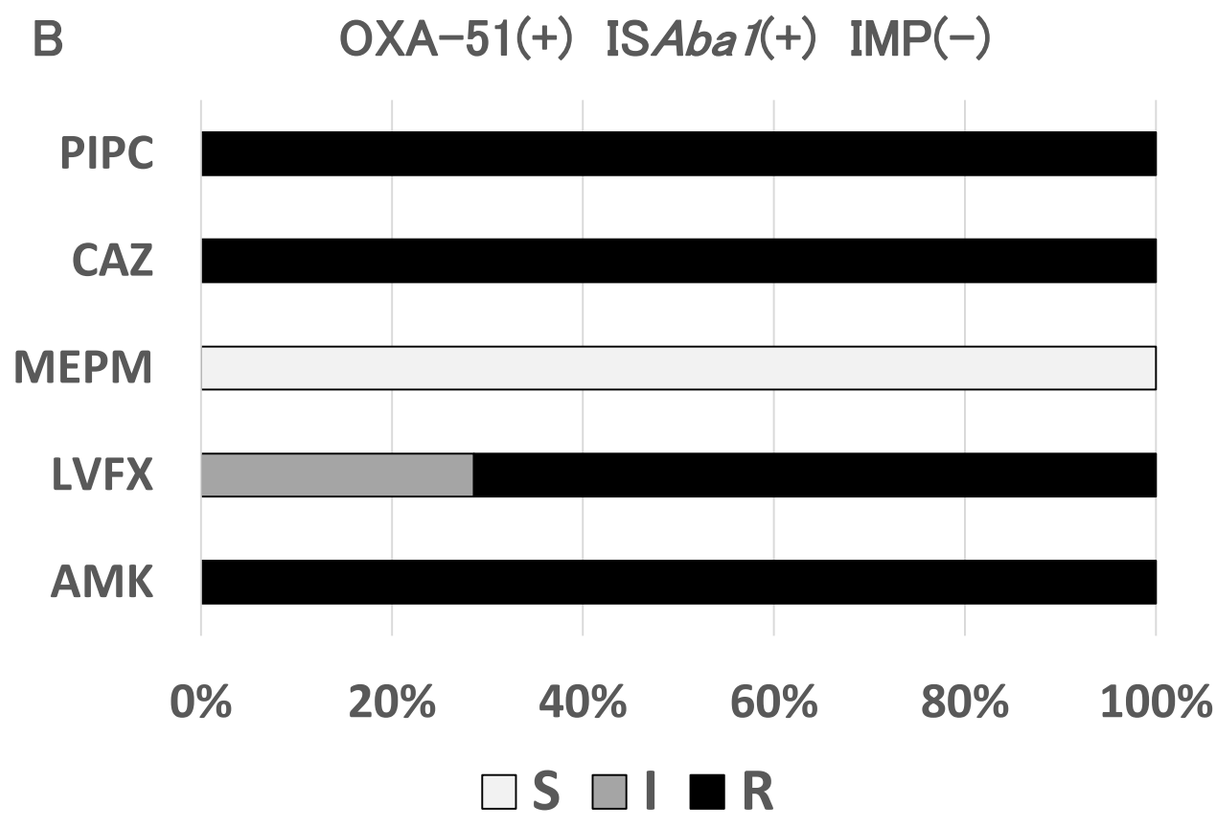
Fig. 1. Summary of study design and participants After implementing the exclusion criteria, *Acinetobacter baumannii* was detected in 43 patients.

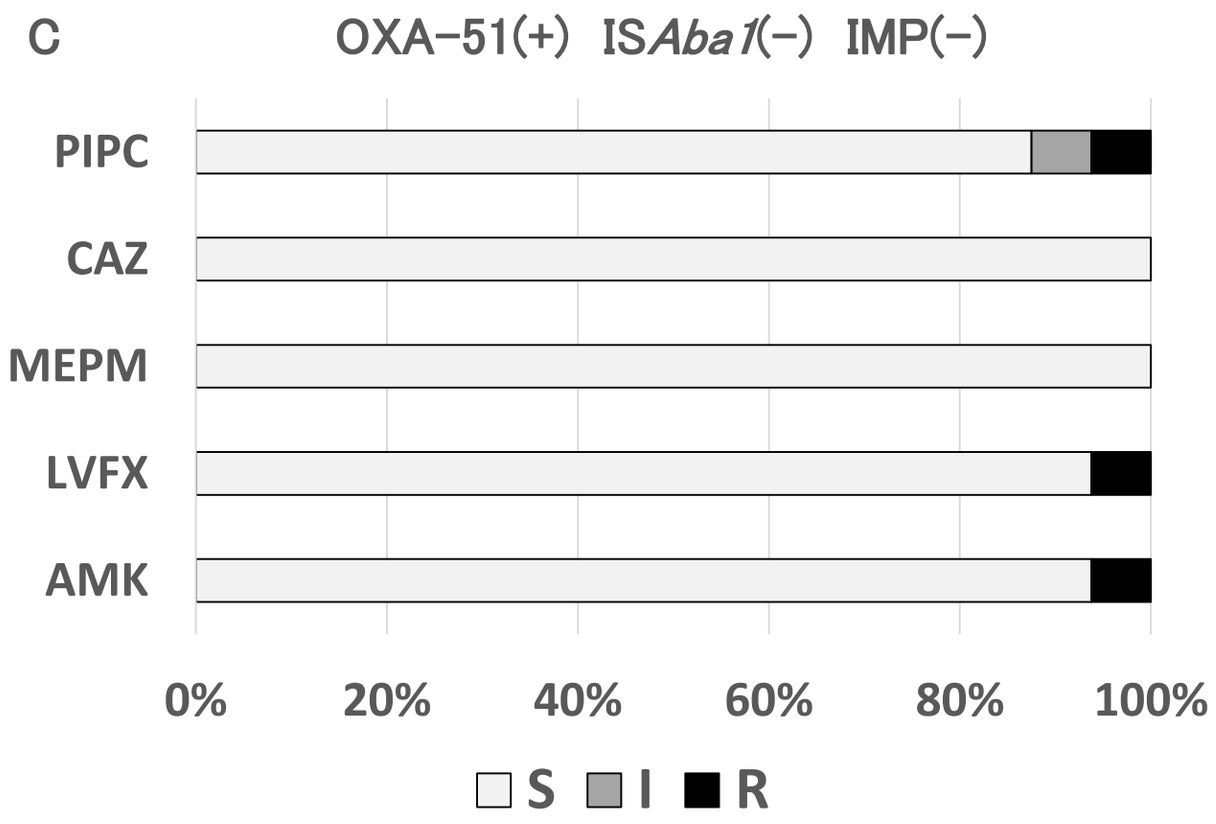
Fig. 2. Antimicrobial susceptibility of *Acinetobacter baumannii* according to the genotype Susceptibility to piperacillin (PIPC), ceftazidime (CAZ), meropenem (MEPM), levofloxacin (LVFX), and amikacin (AMK) was evaluated according to CLSI guidelines. (A) Antimicrobial susceptibility of the OXA-51-like(+) IS*Aba1*(+) IMP(+) genotype. (B) Antimicrobial susceptibility of the OXA-51-like(+) IS*Aba1*(+) IMP(−) genotype. (C) Antimicrobial susceptibility of the OXA-51-like(+) IS*Aba1*(−) IMP(−) genotype. White bars, sensitive (S); gray bars, intermediate-sensitive (I); black bars, resistant (R).

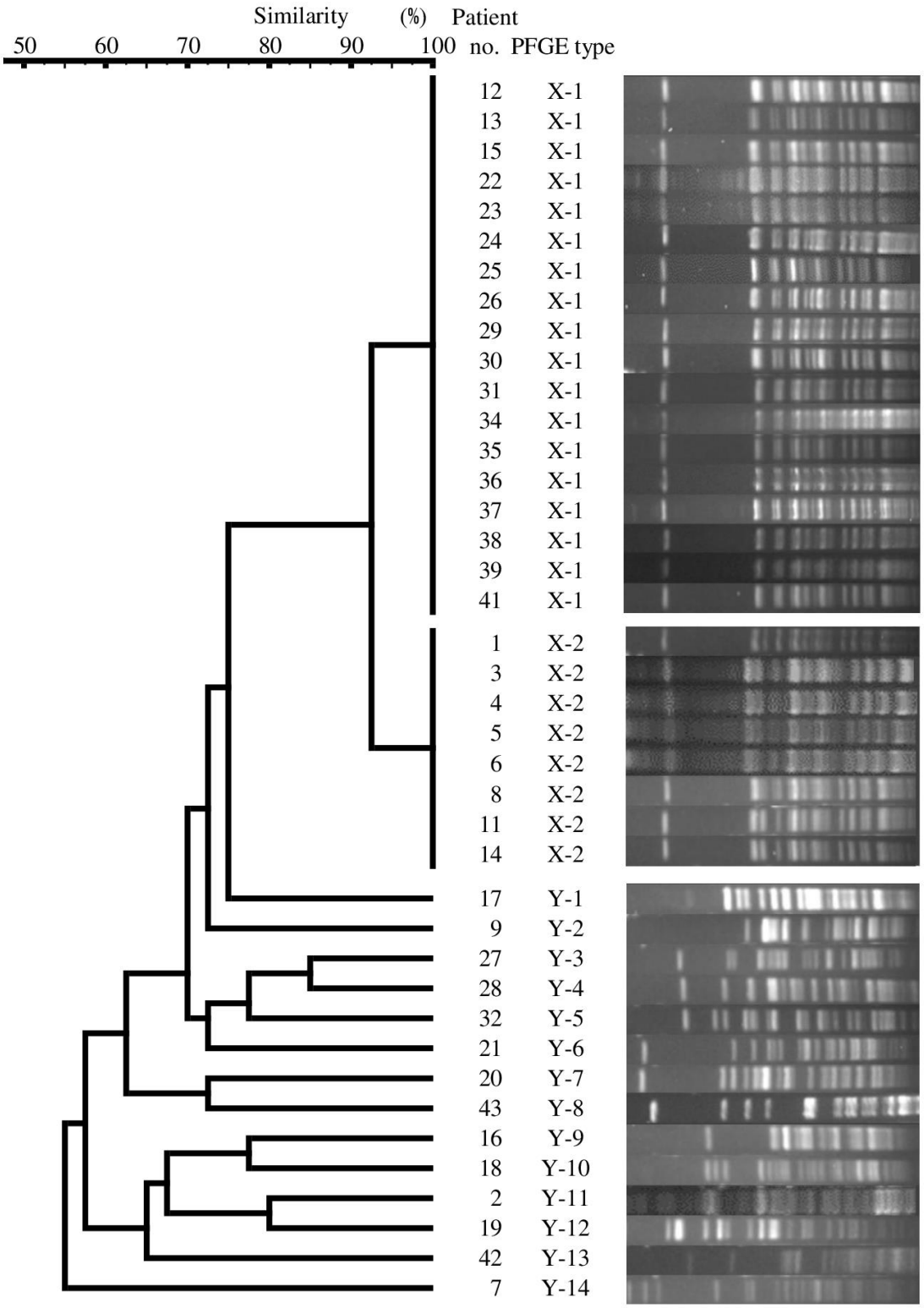
Fig. 3. Results of the pulsed field gel electrophoresis (PFGE) analysis *Acinetobacter baumannii* carrying OXA-51-like and IS*Aba1* from 26 patients belong to two closely related PFGE types.


Admitted to CCC
1634 patients
Sputum test-negative
1353 patients
Sputum test-positive
281 patients
Patients excluded from the study:
5 pediatric patients (<15 years old)
4 patients died within 24 hours
Participants
272 patients
Positive for other bacterial species
229 patients
A. baumannii-positive
43 patients
OXA-51(+) ISAba1(+) IMP(+)
6 patients
OXA-51(+) ISAba1(+) IMP(−)
21 patients
OXA-51(+) ISAba1(−) IMP(−)
16 patients


A
OXA-51(+) ISAba1(+) IMP(+)
PIPC
CAZ
MEPM
LVFX
AMK
0%
20%
40%
60%
80%
100%
S
I
R


B
OXA-51(+) ISAba1(+) IMP(−)
PIPC
CAZ
MEPM
LVFX
AMK
0%
20%
40%
60%
80%
100%
S
I
R


C
OXA-51(+) ISAba1(−) IMP(−)
PIPC
CAZ
MEPM
LVFX
AMK
0%
20%
40%
60%
80%
100%
S
I
R


Similarity (%)
50
60
70
80
90
100
Patient no.
PFGE type
12 X-1
13 X-1
15 X-1
22 X-1
23 X-1
24 X-1
25 X-1
26 X-1
29 X-1
30 X-1
31 X-1
34 X-1
35 X-1
36 X-1
37 X-1
38 X-1
39 X-1
41 X-1
1 X-2
3 X-2
4 X-2
5 X-2
6 X-2
8 X-2
11 X-2
14 X-2
17 Y-1
9 Y-2
27 Y-3
28 Y-4
32 Y-5
21 Y-6
20 Y-7
43 Y-8
16 Y-9
18 Y-10
2 Y-11
19 Y-12
42 Y-13
7 Y-14