

Carbapenem-non-susceptible *Acinetobacter baumannii* of sequence type 92 or its single-locus variants with a G428T substitution in zone 2 of the *rpoB* gene

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Objectives: To investigate the epidemiological traits of carbapenem-non-susceptible *Acinetobacter baumannii* (CNSAB) and the usefulness of phylogenetic grouping based on partial *rpoB* gene sequencing in defining the epidemiological traits of CNSAB.

Methods: A total of 547 non-duplicate clinical isolates of *Acinetobacter* spp. were collected from 19 hospitals in Korea in 2008. Detection of genes encoding OXA carbapenemases and metallo- β -lactamases was performed by PCR. The epidemiological relationships of the isolates were investigated by multilocus sequence typing and repetitive-sequence-based PCR. The 450 bp sequence (zone 2) of the *rpoB* gene was amplified and sequenced.

Results: Molecular characterization of the 272 CNSAB isolates identified five sequence types (STs): ST92, ST75, ST137, ST138 and ST69. The first four of these STs were clustered into clonal complex (CC) 92, sharing alleles at six of seven housekeeping gene loci; ST69 shared alleles at five of seven loci. CNSAB of CC92 carried the *bla*_{OXA-23} gene ($n=169$), the *bla*_{OXA-51}-like gene preceded by IS*Aba1* ($n=89$) or both ($n=14$). Notably, all CNSAB isolates carried a G428T substitution in zone 2 of the *rpoB* gene.

Conclusions: CNSAB isolates of CC92 with the G428T substitution in zone 2 of the *rpoB* gene are disseminated nationwide in Korea. *A. baumannii* with the single nucleotide substitution may be more likely to acquire carbapenem resistance than are other isolates.

Keywords: clonal complex 92, European clone II, multilocus sequence typing

Introduction

The genus *Acinetobacter* consists of 22 nomenclature species and at least 11 additional putative species.¹ *Acinetobacter baumannii* is an opportunistic pathogen and is the most common cause of serious nosocomial infections within the genus *Acinetobacter*, especially those found in intensive care units (ICUs).¹ Carbapenems are recommended as first-line therapy in the treatment of serious infections caused by *A. baumannii* resistant to oxyimino-cephalosporins. The recent dissemination of carbapenem-non-susceptible *A. baumannii* (CNSAB) in many parts of the world is therefore very worrisome, resulting in limited antimicrobial treatment options.

Worldwide dissemination of carbapenem-resistant European clones I and II (EU-I and EU-II) has been reported by several authors. Mugnier *et al.*² identified 8 EU-I isolates of sequence type (ST) 25, ST44 or two novel STs, as well as 10 EU-II isolates of ST92 (ST22 was moved to ST92 after an update of allelic

profiles at the web site <http://pubmlst.org>) or ST118 (moved from ST53) among 20 *A. baumannii* isolates carrying the *bla*_{OXA-23} gene originating from 15 countries. Wide dissemination of CNSAB isolates of ST92 and its single-locus variants has also been described in China and Korea.^{3,4} Nemec *et al.*⁵ found that 20 isolates carrying *bla*_{OXA-58}-like, *bla*_{OXA-24}-like or *bla*_{OXA-51}-like genes preceded by IS*Aba1* from 23 *A. baumannii* isolates for which carbapenem MICs were ≥ 8 mg/L isolated from the Czech Republic belonged to EU-II. Dissemination of EU-II isolates carrying the *bla*_{OXA-58} gene in ICUs in Rome has also been reported.⁶ In contrast, Kulah *et al.*⁷ reported that multiple clones of *A. baumannii* carrying the *bla*_{OXA-58} gene were responsible for a sustained outbreak that occurred in a hospital in Turkey; however, those isolates were not associated with the *A. baumannii* EU-I, EU-II or EU-III strains.

Scola *et al.*⁸ demonstrated the usefulness of RNA polymerase β -subunit (*rpoB*) gene sequencing in the differentiation and identification of *Acinetobacter* spp. Ko *et al.*⁹ reported that

A. baumannii isolates could be divided into three subgroups (I, II or III) based on phylogenetic grouping inferred from the sequence of a variable region, zone 2, in the *rpoB* gene. Furthermore, they reported that the grouping correlated with antimicrobial resistance profiles. While most isolates of subgroup I were susceptible to colistin and polymyxin B, many isolates of subgroups II and III exhibited resistance to these drugs. However, they did not focus on correlations between the grouping and carbapenem resistance profiles in *A. baumannii*.

The aims of this study were to define the epidemiological traits of CNSAB isolates from clinical specimens from patients hospitalized at 19 different hospitals in Korea. We also aimed to investigate the usefulness of phylogenetic grouping based on partial *rpoB* gene sequencing in defining the epidemiological traits of CNSAB.

Materials and methods

Bacterial isolates

A total of 547 clinical isolates of *Acinetobacter* spp. were collected from 19 different hospitals in six provinces of Korea in 2008 (Figure S1, available as Supplementary data at JAC Online). During those periods, the isolates were consecutively collected at each hospital and recovered as one isolate per patient. The strains were isolated from blood, sputum, wound and urine specimens. The isolates were identified using the ATB 32 GN system (bioMérieux, Marcy l'Étoile, France) and 16S–23S rRNA intergenic spacer region sequencing.¹⁰

Antimicrobial susceptibility testing

Antibiotic-containing discs (Becton Dickinson, Sparks, MD, USA) were used for routine antibiograms in a disc diffusion assay. The modified Hodge test and the imipenem and EDTA–sodium mercaptoacetic acid double-disc synergy (IEDDS) test were performed on MacConkey agar plates as described previously for the screening of carbapenemases and metallo- β -lactamases (MBLs), respectively.¹¹ MICs of colistin were determined using the agar dilution method according to CLSI guidelines.¹² *A. baumannii* ATCC 19606 and *Acinetobacter* genomospecies 14TU ATCC 17905 were used as control strains.

Identification of carbapenemase genes

Whole-cell lysates of the clinical isolates were used as templates for PCR amplification. PCR experiments were carried out to detect the genes encoding MBLs (IMP-1 variants, VIM-2 variants and SIM-1) and OXA carbapenemases (OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like) as described previously.¹³ A simple two-step PCR experiment was performed to sequence the upstream region of the *bla*_{OXA-51}-like gene as described previously.¹⁴ PCR products were subjected to direct sequencing with an automatic sequencer (Model 3730xl; Applied Biosystems, Weiterstadt, Germany).

PFGE and Southern blotting

Plugs containing whole genomic DNA of the isolates were digested with S1 nuclease or I-CeuI. DNA fragments were separated using PFGE with a CHEF-DRII device (Bio-Rad, Hercules, CA, USA). PFGE was performed at 6 V/cm for 20 h with pulse times ranging from 9 to 90 s at a temperature of 14°C. The gels with S1 nuclease-treated linearized plasmids and I-CeuI-digested chromosomal DNA were blotted onto nylon membranes (Bio-Rad) and hybridized with probes specific for the *bla*_{OXA-23} gene or 16S rDNA. The probes were obtained via PCR experiments as described above.

Probe labelling, hybridization and detection were performed with the DIG DNA Labeling and Detection Kit (Roche Diagnostics, Indianapolis, IN, USA).

Repetitive-sequence-based PCR (Rep-PCR)

Rep-PCR experiments were performed using the DiversiLab system (bioMérieux, Grenoble, France) according to the manufacturer's instructions, to investigate the clonal relatedness of *A. baumannii* isolates. Results were analysed with DiversiLab software using the Kullback–Leibler method to determine distance matrices and the unweighted pair group method with arithmetic averages to create a dendrogram. Isolates showing >95% similarity were considered to be related.¹⁵

Phylogenetic grouping based on partial *rpoB* gene sequencing

The 450 bp sequence (zone 2) of the *rpoB* gene was amplified using the primers Ac1005F (5'-GTGATAARATGGCGGGCTGT-3') and Ac1598R (5'-CGBGCRTGCATYTTGTCRT-3') as previously described.⁸ The phylogenetic relationship based on the *rpoB* zone 2 sequence was estimated using the neighbour-joining method in molecular evolutionary genetic analysis software.

Multilocus sequence typing (MLST)

MLST was performed using the method of Bartual *et al.*¹⁶ Fragments of seven housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi* and *rpoD*) were amplified by PCR and sequenced. Two primer sets were redesigned: *gpi*2-F (5'-AATACCGTGGTGCTACG-3') and *gpi*2-R (5'-TTCAGGAGCAATCC CCCACT-3'); *rpoD*2-F (5'-CGAATYGCATTGCAAAACG-3') and *rpoD*2-R (5'-CNGCAATYTTTTGYTGAA-3'). Allele numbers were assigned an ST after the distinct allele sequences were submitted to a dedicated database (<http://pubmlst.org>). A clonal complex (CC) was used to assess the genetic relatedness of the STs; the most stringent definition—sharing alleles at six of seven loci—was used.¹⁷ MLST described by Diancourt *et al.*¹⁸ was performed for representative strains allocated to the STs associated with CNSAB. Allelic profiles of seven housekeeping genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB* and *rpoB*) were analysed using *Acinetobacter* MLST database (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/>).

Results

Acinetobacter spp.

The clinical isolates of *Acinetobacter* spp. ($n=547$) were identified as *A. baumannii* ($n=388$), *Acinetobacter* genomospecies 13TU ($n=82$), *Acinetobacter* genomospecies 3 ($n=62$), *Acinetobacter bereziniae* ($n=13$) and *Acinetobacter* genomospecies 14TU ($n=2$) (Table 1).

Grouping of *A. baumannii* isolates based on partial *rpoB* gene sequencing

A phylogenetic tree clustered the 388 *A. baumannii* isolates into five groups according to the *rpoB* zone 2 sequence using the neighbour-joining method (Figure 1). The group A isolate was defined as that carrying the same nucleotide sequence in zone 2 of the *rpoB* gene as that carried by the *A. baumannii* ATCC 19606 reference strain. The group B isolate was defined as that with a G-to-T substitution at the 428th nucleotide in zone 2

Table 1. Antimicrobial susceptibilities and *A. baumannii* groups according to substitution in zone 2 of the *rpoB* gene sequence

Species (no. of isolates)	Substitution ^b	IPM (breakpoints, mm) ^a			MEM (breakpoints, mm)			CST (MIC, mg/L)	
		R (≤13)	I (14–15)	S (≥16)	R (≤13)	I (14–15)	S (≥16)	R (≤2)	S (≥4)
<i>A. baumannii</i> , group (388)									
B (320)									
B-0 ^c	G428T	221	23	65	270	2	37	0	309
B-1	G428T, C292T	0	0	4	0	0	4	0	4
B-2	G428T, C292T, G89C	0	0	5	0	0	5	0	5
B-3	G428T, T431A	0	0	2	0	0	2	0	2
C (36)									
C-0	C449T	0	0	8	0	0	8	0	8
C-1	C449T, C269A	0	0	23	0	0	23	0	23
C-2	C449T, C239T	0	0	3	0	0	3	0	3
C-3	C449T, C114T	0	0	2	0	0	2	0	2
E (13)									
E-0	G116A	0	0	12	0	0	12	0	12
E-1	G116A, C449T	0	0	1	0	0	1	0	1
D (5)	A407G	0	0	5	0	0	5	0	5
A (14)		0	0	14	0	0	14	1	13
<i>Acinetobacter</i> genomospecies 13TU (82)		2	2	78	4	0	78	0	82
<i>Acinetobacter</i> genomospecies 3 (62)		8	0	54	8	0	54	0	62
<i>A. bereziniae</i> (13)		0	0	13	0	0	13	1	12
<i>Acinetobacter</i> genomospecies 14TU (2)		0	0	2	0	0	2	2	0

IPM, imipenem; MEM, meropenem; CST, colistin; R, resistant; I, intermediate; S, susceptible.

^aCLSI breakpoints were applied for the interpretation of the results.

^bThe reference sequence of *A. baumannii* ATCC 19606 was defined as group A.

^cThere was a significant difference ($P < 0.001$) in carbapenem susceptibility between the B-0 group (88.0%) and non-B-0 groups (0%) by χ^2 test (Fisher's exact test) using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA).

compared with the *A. baumannii* ATCC 19606 reference strain sequence. Isolates belonging to subgroup B-0 had only one G428T substitution. Group C, D and E isolates were defined as those with a C449T, A407G or G116A substitution, respectively, in zone 2 compared with the *A. baumannii* ATCC 19606 reference strain sequence (Table 1).

Carbapenem resistance

While 70% (272/388) of *A. baumannii* isolates exhibited intermediate susceptibility or resistance to imipenem and/or meropenem, the majority of non-*baumannii* *Acinetobacter* (NBA) isolates were susceptible to these drugs (Table 1). Most CNSAB (266/272) showed positive results on the modified Hodge test; however, none had positive results on the IEDDS test. The *bla*_{OXA-23} gene was detected in 169/272 CNSAB isolates, while the ISAb₁ element associated with the *bla*_{OXA-51}-like gene was detected in 89/272 isolates. Fourteen isolates, all of which were collected from the same hospital, carried both genes. Genes encoding OXA-24-like and OXA-58-like carbapenemases or MBLs were not detected in the *A. baumannii* isolates. Interestingly, all 272 CNSAB isolates belonged to subgroup B-0. However, some (37/309) isolates belonging to subgroup B-0 were susceptible to carbapenems.

Most (10/12) of the carbapenem-non-susceptible NBA isolates showed positive results on the IEDDS test, suggesting the production of MBLs. The *bla*_{IMP-1} and the *bla*_{VIM-2} genes were detected in six and four of these NBA isolates, respectively. With the exception of an *Acinetobacter* genomospecies 3 isolate carrying the *bla*_{OXA-23} gene, no genes encoding OXA carbapenemases were detected in any of the NBA isolates.

Location of the *bla*_{OXA-23} gene

A probe specific for the *bla*_{OXA-23} gene hybridized with 100–150 kb I-CeuI chromosomal fragments from *A. baumannii* and *Acinetobacter* genomospecies 3 isolates, but not with S1 nuclease-treated linearized plasmids. The 16S rDNA probe also hybridized with the same chromosomal fragments, suggesting the presence of the *bla*_{OXA-23} gene on the chromosome rather than on a plasmid (data not shown).

Clonal relationship

Results of rep-PCR experiments on the 388 *A. baumannii* isolates are shown in Figure 2, illustrating that the *A. baumannii* isolates were clustered into 57 distinct band patterns, using a clonal relationship index of >95% as a threshold. All 272 CNSAB isolates belonged to one of three band patterns (17, 18 or 19).

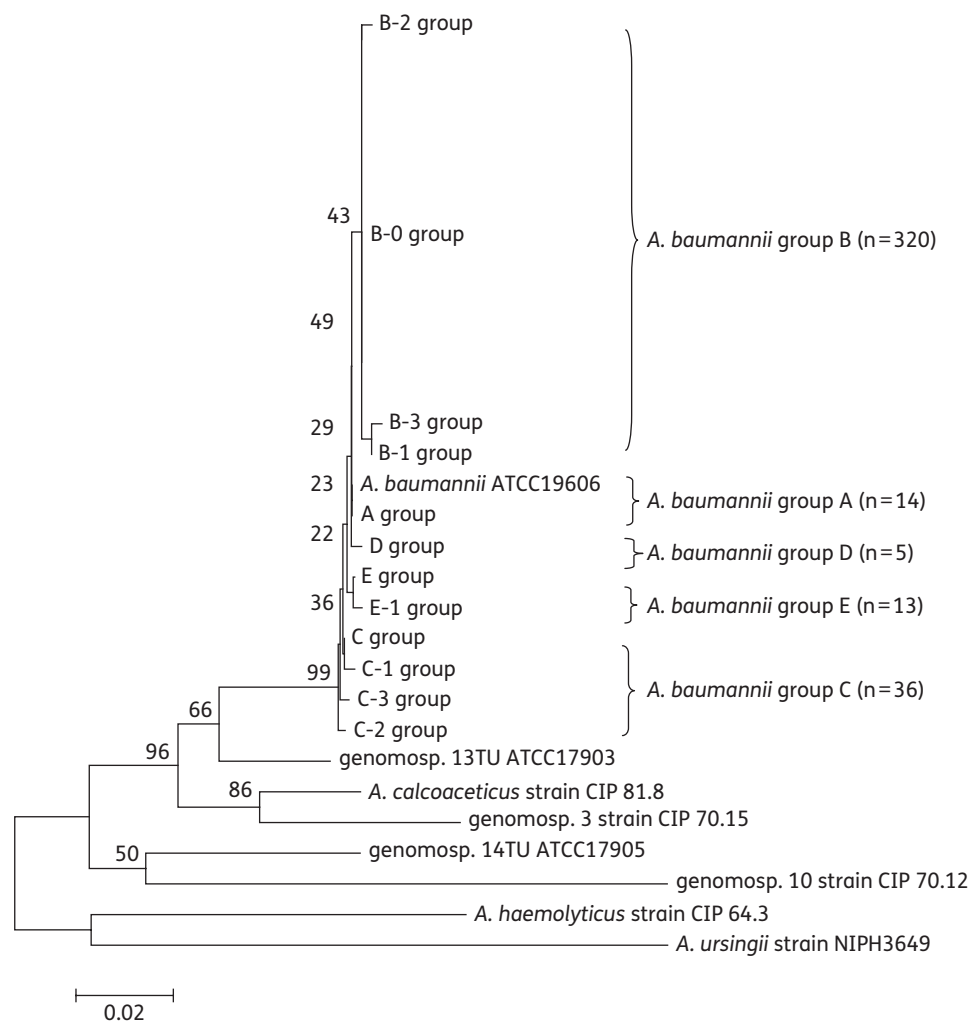


Figure 1. Phylogenetic tree of 388 isolates of *A. baumannii* inferred from zone 2 sequencing of the *rpoB* gene. This tree was grouped using the neighbour-joining method in molecular evolutionary genetic analysis software. Values on the branches of the clusters represent the results of bootstrapping analysis.

These band patterns were similar to those of the EU-II control strain described by Higgins *et al.*¹⁹ A total of 57 different STs were identified in 388 *A. baumannii* isolates using MLST experiments by the method of Bartual *et al.*,¹⁶ and the 272 CNSAB isolates were classified into five STs (ST92, ST75, ST138, ST137 and ST69; Figure 2). However, the isolates of the aforementioned five STs were identified as an identical ST, ST2 (2-2-2-2-2-2), by the MLST method described by Diancourt *et al.*¹⁸

Many (159/272) CNSAB isolates were identified as ST92 including 76 isolates carrying the *bla*_{OXA-23} gene, 69 isolates carrying the ISAb₁ element associated with the *bla*_{OXA-51}-like gene and 14 isolates carrying both. One hundred and five isolates carrying the *bla*_{OXA-23} gene ($n=93$) or the ISAb₁ element associated with the *bla*_{OXA-51}-like gene ($n=12$) were identified as ST75 ($n=64$), ST137 ($n=3$) or ST138 ($n=38$) single-locus variants of ST92. The remaining eight isolates carrying the ISAb₁ element associated with the *bla*_{OXA-51}-like gene were identified as ST69, a double-locus variant of ST92.

Susceptibility to colistin

All but four *Acinetobacter* spp. isolates exhibited susceptibility to colistin. Only one of 403 *A. baumannii* isolates (MIC 8 mg/L) and one of 13 *A. bereziniae* isolates (MIC 8 mg/L) showed low-level resistance to colistin. None of the 82 *Acinetobacter* genomospecies 13TU isolates or of the 62 *Acinetobacter* genomospecies 3 isolates showed resistance to colistin (Table 1). However, both *Acinetobacter* genomospecies 14TU isolates showed high-level resistance to colistin (MIC 32 mg/L). Interestingly, the *Acinetobacter* genomospecies 14TU ATCC 17905 reference strain also showed high-level resistance to the drug (MIC 64 mg/L).

Discussion

The 272 CNSAB isolates were identified as ST92, one of its single-locus variants (ST75, ST138 or ST137) or its double-locus variant (ST69). ST92 and its single-locus variants can be clustered into a

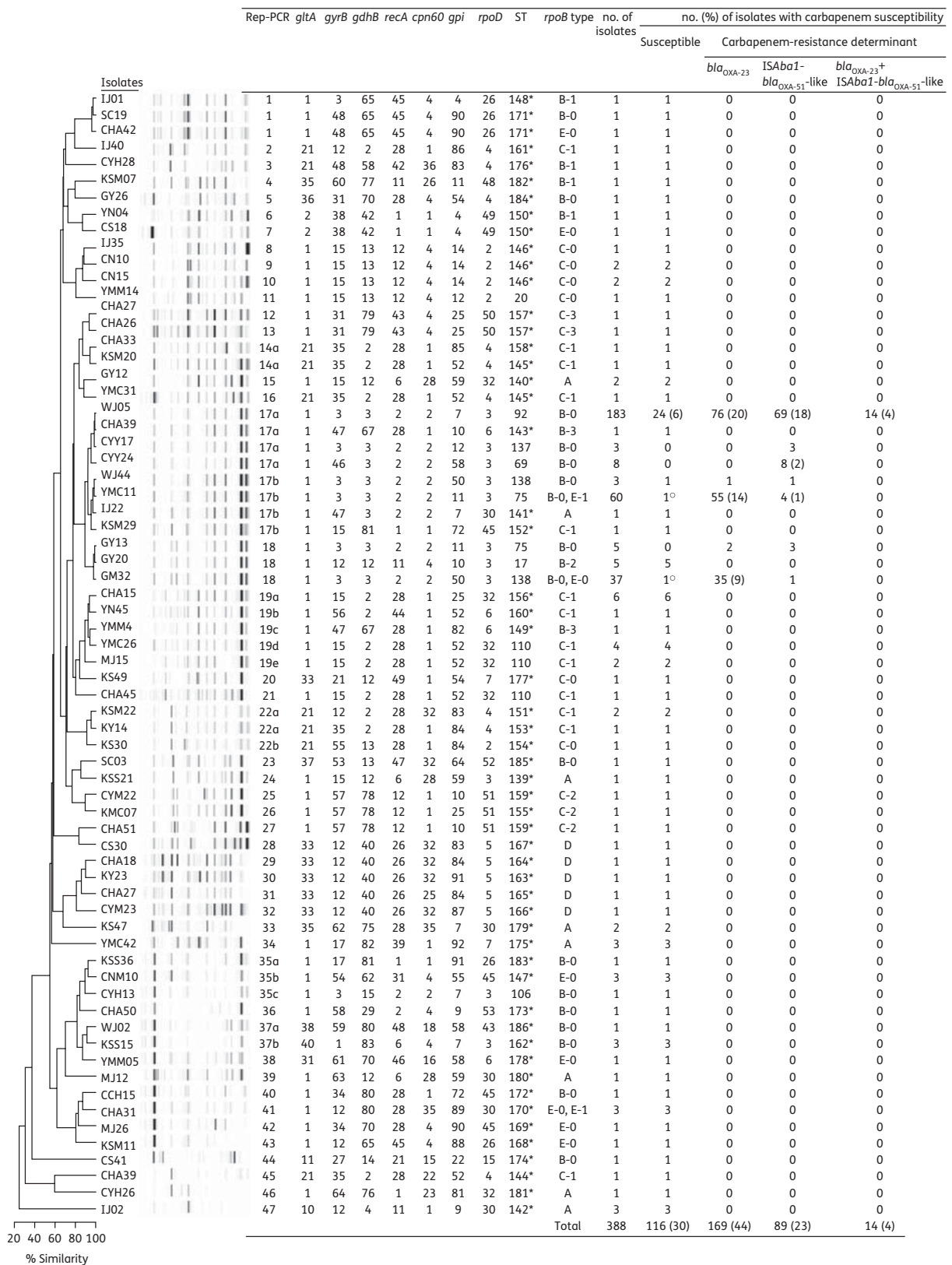


Figure 2. Rep-PCR banding patterns and STs of 388 isolates of *A. baumannii*. Isolates that showed >95% similarity were assigned the same banding pattern number, and a small letter (a–e) was given to isolates with >90% similarity. The asterisks indicate novel STs found in this study. Open circles indicate the number of isolates that belonged to group E.

single clonal complex (CC92) because they share six of seven housekeeping gene loci. The results of the Institute Pasteur MLST scheme also supported the clonal relatedness of the isolates in a broader sense, in that they share an identical ST, ST2. CC92 also includes other single-locus variants of ST92, including ST118 (1-3-3-2-2-3-3) identified from an isolate from France, ST88 (1-3-3-2-2-10-3) and ST90 (1-3-3-2-2-62-3) from China, and ST4 (1-12-3-2-2-7-3) from the UK. Therefore, clonal expansion of *A. baumannii* CC92 may be the main cause of the recent worldwide dissemination of CNSAB.

A. baumannii isolates clustered into five groups according to the sequence of zone 2 of the *rpoB* gene. Grouping correlated well with the carbapenem resistance profiles of the *A. baumannii* isolates. All 272 CNSAB isolates belonged to subgroup B-0, while all of the isolates belonging to the other groups were susceptible to carbapenems. Subgroup B-0 comprised 37 carbapenem-susceptible isolates and 272 CNSAB isolates, including 169 isolates carrying the *bla*_{OXA-23} gene, 89 isolates carrying the IS*Aba1* element upstream of the *bla*_{OXA-51}-like gene and 14 isolates carrying both (Figure 2). It was interesting that only *A. baumannii* isolates with the single G428T substitution in zone 2 of the *rpoB* gene acquired carbapenem-resistance determinants. Further studies are needed to investigate the reasons for these phenomena.

While the *bla*_{OXA-23} gene has mainly been detected in *A. baumannii*, reports of NBA isolates harbouring the OXA carbapenemase are rare. Recently, two *Acinetobacter* genomospecies 3 isolates carrying the *bla*_{OXA-23} gene were reported in the Irish Republic.²⁰ One *Acinetobacter* genomospecies 3 isolate harbouring chromosome-borne OXA-23 was identified in this study. To our knowledge, this is the second report of an OXA-23-producing NBA isolate.

In this study, only 1 of 403 *A. baumannii* isolates was identified as colistin resistant, in contrast to results reported by Ko *et al.*⁹ Interestingly, both *Acinetobacter* genomospecies 14TU isolates showed resistance to colistin. Furthermore, the *Acinetobacter* genomospecies 14TU ATCC 17905 reference strain also showed resistance to colistin. These results suggest that the *Acinetobacter* genomospecies 14TU may be intrinsically resistant to colistin, as described by Nemeč *et al.*²¹

In conclusion, CNSAB isolates of CC92 with a G428T substitution in zone 2 of the *rpoB* gene were widely disseminated in Korea, as has been reported in European countries and China.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org/>).

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