1   Molecular characterization of carbapenem-non-susceptible *Acinetobacter* spp. in

2   Japan; Predominance of multidrug-resistant *Acinetobacter baumannii* clonal

3   complex 92 and IMP-type metallo-β-lactamase-producing non-*baumannii*

4   *Acinetobacter* species

5

6

7   Yuichi Kouyama, Sohei Harada, Yoshikazu Ishii*, Tomoo Saga, Ayumi Yoshizumi,

8   Kazuhiro Tateda and Keizo Yamaguchi

9

10 Department of Microbiology and Infectious Diseases, Toho University School of

11 Medicine, Tokyo, Japan

12

13 **Running title**: Carbapenem-non susceptible *Acinetobacter* spp. in Japan

14

17

18 ***Corresponding author**: Yoshikazu Ishii, Ph. D.

19 Department of Microbiology and Infectious Diseases, Toho University School of

20   Medicine

21   Omori-nishi, Ota-ku, 1438540 Tokyo, Japan

22   TEL: +81-3-3762-4151

23   FAX: +81-3-5493-5415

24   E-mail: yishii@med.toho-u.ac.jp

25   **Abstract**

26   We conducted an epidemiological study concerning carbapenem-non-susceptible

27   clinical isolates of *Acinetobacter* spp. in Japan by molecular procedures including

28   carbapenemase gene identification and amplified ribosomal DNA restriction

29   analysis. Among 598 clinically isolated *Acinetobacter* spp. in 2007, 27 (4.5%) were

30   non-susceptible to carbapenems. Most carbapenem-non-susceptible *Acinetobacter*

31   *baumannii* (13/14) belonged to clonal complex (CC) 92, harbored $bla_{\text{OXA-51-like}}$

32   genes, including novel $bla_{\text{OXA-206}}$, downstream of IS*Aba*1, and were recovered

33   mainly from the Kanto region. Carbapenem-non-susceptible *A. baumannii* CC92

34   isolates were further divided by pulsed-field gel electrophoresis into two groups,

35   one of which was characterized by the presence of $bla_{\text{OXA-23}}$. One *A. baumannii*

36   CC276 isolate carried $bla_{\text{IMP-1}}$ and $bla_{\text{OXA-58}}$. Almost all non-*baumannii*

37   *Acinetobacter* isolates (12/13), including *Acinetobacter pittii* (formerly

38   *Acinetobacter* genomic species 3) and *Acintobacter nosocomialis* (formerly

39   *Acinetobacter* genomic species 13TU), produced IMP-type metallo-ß-lactamases,

40   and were recovered from various regions in Japan. This is the first report

41   describing the nationwide molecular epidemiology of carbapenem-non-susceptible

42   *Acinetobacter* spp. with genomic species level identification in Japan.

Introduction

Among *Acinetobacter* species, *Acinetobacter baumannii* is the most important nosocomial pathogens, frequently resistant to multiple antimicrobials. Carbapenems play an important role for treatment of *Acinetobacter* infections, but carbapenem-resistant *A. baumannii* have spread worldwide rapidly in these two decades with multiple resistant means [1]; OXA-type class D carbapenemases, which are intrinsic or acquired, could be potentiated by promoter sequences located within IS*Aba* insertion sequences [2, 3]. Metallo-β-lactamases (MBLs) also confer carbapenem resistance on this pathogen. β-lactamases are shown to work synergistically with other mechanisms including alteration of drug permeability and efflux pumps [1].

More generally, it is apparent that the population structure of *A. baumannii* comprises three major international lineages, named European clones I, II, and III [4, 5]. A subgroup of European clone II involving clonal complex (CC) 92, has spread globally [6], and is wide spread in China [7] and Korea [8]; it is also recorded in Australia [9].

While these strains remain relatively rare in Japan, it is a matter of concern [10]. In the past reports about *Acinetobacter* spp. in Japan, *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (ACB complex) including

62   *Acinetobacter pittii* (formerly *Acinetobacter* genomic species 3) and *Acinetobacter*

63   *nosocomialis* (formerly *Acinetobacter* genomic species 13 TU) had been mentioned

64   as "*A. baumannii*" and its mechanism of carbapenem resistance was explained by

65   MBLs; epidemiological information including genetic species identification and

66   class D carbapenemase is lacking [11, 12].

67   Here, we characterized the genetic mechanisms of carbapenem-

68   non-susceptibility in clinical *Acinetobacter* spp. from a nationwide surveillance

69   study in Japan, and analyzed the molecular epidemiology of

70   carbapenem-non-susceptible isolates.

71

72   **Materials and methods**

73

74   *Bacterial strains*

75   A total of 598 clinical isolates of *Acinetobacter* spp. were collected from 72

76   hospitals and other healthcare institutions as part of a nationwide survey

77   between January and December 2007 by the Levofloxacin Surveillance Group

78   [13]. The maximum number of isolates collected per institution was 10. Only one

79   isolate was accepted from each patient. Isolates with resistance to imipenem or

80   panipenem (MICs $\geq$8 mg/L) in the survey [13] were investigated further.

81

Identification at the level of species/genomic species

83   Biochemical identification was performed in 27 carbapenems

84 non-susceptible isolates by the BD Phoenix automated system (BD Diagnostic

85 Systems, Sparks, MD, USA). Amplified ribosomal DNA restriction analysis

86 (ARDRA) was performed to identify species and genomic species [14]. Reference of

87 ARDRA patterns were obtained from website

88 (http://users.ugent.be/~mvaneech/ARDRA/Acinetobacter.html). PCR

89 amplification and nucleotide sequencing of intrinsic $bla_{\text{OXA-51-like}}$ gene were

90 performed to confirm identification of *A. baumannii* (Table 1). Nucleotide

91 sequences of the amplified products were determined using an ABI 310 genetic

92 analyzer with Big Dye terminator Ver.3.1 cycle Sequencing kit (Applied

93 Biosystems, Foster City, CA, USA).

94

Antimicrobial susceptibility testing

96   Minimum inhibitory concentrations were measured by the broth

97 microdilution method of the Clinical and Laboratory Standards Institute [15].

98 Antimicrobial agents tested were as follows: ampicillin, ceftazidime, imipenem,

99 meropenem and minocycline (Sigma Chemical Co. St Louis, MO, USA),

6

100   gentamicin and sulbactam (Wako Pure Chemical, Osaka, Japan), ciprofloxacin

101   (MP Biomedicals, Solon, OH, USA), and cefepime (Bristol-Myers Squibb, Tokyo,

102   Japan).

103

104   *Phenotypic and genotypic tests for MBLs*

105   Production of MBLs was screened by the double-disk synergy test using

106   ceftazidime (30 μg) and sodium mercaptoacetate (30 μg) discs (Eiken Chemical,

107   Inc., Tokyo, Japan) [16]. Genes for IMP-1, IMP-2, VIM-1, and VIM-2

108   carbapenemases were sought by PCR (Table 1) [12].

109

110   *Detection of class D carbapenemase genes and associated ISAba*

111   bla_{OXA} genes were sought by multiplex PCR (Table 1) [17]. The presence of

112   an IS*Aba*-type insertion sequence upstream of bla_{OXA} genes was investigated by

113   PCR and nucleotide sequencing, using combinations of the forward primers for

114   IS*Aba* and the reverse primer for the relevant bla_{OXA} gene.

115

116   *Molecular typing by pulsed-field gel electrophoresis (PFGE)*

117   Agarose gel plugs containing *Apa* I-digested genomic DNA were prepared

118   with the CHEF Bacterial Genomic DNA Plug Kit (Bio-Rad, Hercules, CA). The

119   DNA fragments were separated with a CHEF MAPPER (Bio-Rad) for 18.5 h at

120   14 °C with a 1 to 17s linear ramp of 6V/cm. Restriction patterns were analyzed

121   with Fingerprinting II software (Bio-Rad) and cluster analysis was performed by

122   the unweighted pair-group method with mathematical averaging. Position

123   tolerance and optimization were set at 1.5% and 1.5%, respectively. Only

124   restriction fragments larger than 50 kb were used for analysis. Isolates with

125   >85% similarity were assigned to the same strain subgroup.

126

127   *Multilocus sequence typing (MLST)*

128   Sequence types (STs) of *A. baumannii* isolates were determined according

129   to the MLST scheme (http://pubmlst.org/abaumannii/) [18, 19]. Clonal complexes

130   (CCs) were determined by eBURST version 3 (http://eburst.mlst.net/) with

131   definition of the groups by sharing alleles at ≥6 of 7 loci and bootstrap values of

132   1000 [20].

133

134   *Statistical analysis*

135   Distribution of drug resistance, as well as resistance determinants, was

136   estimated by Fisher's exact test. A p value <0.05 was considered as a statistically

137   significant difference.

138

139   *Nucleotide sequence accession number*

140   The nucleotide sequence of $bla_{\text{OXA-206}}$ was assigned accession number

141   AB634250.

Results

Isolates with reduced susceptibilities to carbapenems

Acinetobacter spp. were isolated as follows; 182 isolates were from Kanto region, 26 isolates were from Hokkaido, 77 isolates were from Tohoku region, 86 isolates were from Tokai and Hokuriku region, 134 isolates were from Kansai and Chugoku region, and 93 isolates were from Kyusyu region.

Among the 598 clinical isolates of *Acinetobacter* spp. (333 from respiratory tract, 45 from urinary tract, 79 from blood, 74 from pus, and 67 from other sites), 27 isolates (4.5%) were resistant to either or both imipenem and panipenem (17 from respiratory tract, 2 from urinary tract, 2 from blood, 2 from pus, and 4 from other sites).

These 27 carbapenem-non-susceptible isolates were further identified at species/genomic species level by ARDRA identification; 51.9% (14/27) of tested *Acinetobacter* species was identified as *A. baumannii*. In agreement with ARDRA-based idenitification, all 14 *A. baumannii* isolates were detected bla_{OXA-51} $_{like}$ gene by PCR, a hallmark of this species.

Carbapenem-non-susceptible *A. baumannii* were isolated in two areas. Of the 14 *A. baumannii* isolates, 13 were from five different hospitals in Kanto region (13/182), and one was from Kyushu (1/93). In contrast, the non-*baumannii*

161   isolates were from more diverse areas; Kanto, Tokai, Kyushu, Hokkaido and

162   Hokuriku (Table 2).

163

164   *Antimicrobial susceptibility*

165   Antibiogram data are described in Table 2. Compared with

166   non-*baumannii Acinetobacter* spp., *A. baumannii* was more frequently

167   non-susceptible to other classes of drug, namely, ampicillin/sulbactam (71% in *A.*

168   *baumannii* vs. 0% in non-*baumannii*, $p<0.01$) and ciprofloxacin (93% vs. 31%,

169    $p<0.01$).

170

171   *MBL-producing isolates*

172   Thirteen of the 27 isolates were determined to have MBLs by phenotypic

173   testing and PCR showed 12 of these to carry bla_{IMP-1}. All of them except one were

174   non-baumannii *Acitetobacter* isolates.　The bla_{IMP-2} was detected in one *A.*

175   *nosocomialis* isolate. No MBL genes were found in isolates negative by the

176   phenotypic test.

177

178   *Class D carbapenemases and ISAba*

179   Sequencing of the PCR products revealed that the $bla_{OXA-51-like}$ gene harbored by

180   12/14 *A. baumannii* isolates was bla_{OXA-66} and one isolate harbored bla_{OXA-64},

181   while one isolate harbored $bla_{OXA-206}$, a new variant gene. The isolate with

182   bla_{OXA-64} also had IS*Aba3*-like-bla_{OXA-58} and the isolate with $bla_{OXA-206}$, a single

183   amino acid variant of OXA-66 carried IS*Aba1*-bla_{OXA-23}. Of these 14, six carried

184   bla_{OXA-23} and one had bla_{OXA-58}. One *A. lwoffii* isolate and the three *A. pittii*

185   isolates harbored bla_{OXA-58}. IS*Aba1* was located 34-bp upstream of the bla_{OXA-66}

186   and $bla_{OXA-206}$ genes, or 8-bp upstream of the bla_{OXA-23} gene. IS*Aba3*-like was

187   located 17-bp upstream of bla_{OXA-58}. IS*Aba1* was not detected upstream of

188   bla_{OXA-66} in TUM10629 and upstream of bla_{OXA-64} in TUM 10635, and IS*Aba3*-like

189   was not found upstream of bla_{OXA-58} in *A. lwoffii* TUM 10655.

190   *Genetic relatedness of A. baumannii isolates*

191   The *A. baumannii* isolates were classified into 3 subgroups by PFGE

192   (Figure). PFGE subgroups A and B comprised 7 and 6 isolates, respectively,

193   whereas one isolate was unique. PFGE subgroup A consisted of five isolates,

194   TUM10629 to TUM10633 (Table 2), carrying both bla_{OXA-66} and bla_{OXA-23} from one

195   hospital in the Kanto region, TUM10641 carrying $bla_{OXA-206}$ and bla_{OXA-23}, and

196   TUM10642 from outside Kanto region. All isolates from Kanto region with only

197   bla_{OXA-66} were classified into the PFGE subgroup B. The unique isolate, allocated

198   to subgroup C, was TUM10635, harboring bla_{OXA-64}, bla_{OXA-58}, and bla_{IMP-1}.

199   The isolates of PFGE subgroup A and B were determined to belong to CC92

200 (i.e. ST208 and ST219). TUM10635, the *A. baumannii* isolate with IMP-1, had

201 novel sequences in *gdhB* and *gpi* and was assigned to ST276.

202

203 **Discussions**

204   The prevalence of carbapenem-non-susceptibility among *Acinecobacter*

205 spp. in Japan (4.5%) was at lower level than reports in other regions: 26.9% in

206 Korea [21], 49% in Taiwan [22], 50-52.4% in China [23], and 22-26% in Europe

207 [24]. Moreover, as observed in Korea [25], carbapenem-non-susceptible *A.*

208 *baumannii* was more frequently resistant to ampicillin/sulbactam and

209 ciprofloxacin than for non-*baumannii* isolates.

210   Geographically, carbapenem-non-susceptible *A. baumannii* isolates were

211 recovered mainly from the Kanto region, while non-*baumannii* isolates were

212 distributed in various regions.

213   While ST92 has been the global epidemic clone among

214 carbapenem-non-susceptible *A. baumannii*,[26] 13 out of 14

215 carbapenem-non-susceptible *A. baumannii* isolates belonged to ST208 or its

216 single variant ST219, the member of CC92 in our study. The fact that the isolates

217 carrying *bla*OXA-66 belonged to CC92 is compatible with the finding that the

218   isolates carrying bla_{OXA-66} often belonged to STs such as ST98 (formerly ST34)

219   included in CC92 demonstrated in a recent report [27] (Table 2). CC92 has

220   increasingly been documented as a globally disseminated linage included in

221   European clone II, often with multidrug resistance [6, 9]. $bla_{OXA-51-like}$ gene can

222   confer carbapenem-non-susceptibility to the bacteria if IS$Aba1$ providing

223   promoter sequences for overexpression is located adjacent to $bla_{OXA-51-like}$[2].

224   PFGE subgroup A was characterized by the presence of bla_{OXA-23}, an

225   important determinant for carbapenem-resistance. Five isolates of PFGE

226   subgroup A were recovered from a single hospital in Kanto region, suggesting

227   possible clonal spread due to nosocomial transmission or local endemicity.

228   Nevertheless, one isolate (TUM10642) of the same PFGE subgroup was recovered

229   from Kyushu, a region very distant from Kanto. In contrast to PFGE subgroup A,

230   bla_{OXA-23} was absent among isolates of PFGE subgroup B; these strains seemed to

231   owe their carbapenem-low-susceptibility to bla_{OXA-66} with a promoter within

232   IS$Aba1$ [2].

233   In our results, PFGE revealed that ST208 contained at least two clones, PFGE

234   subgroup A and B, and most of the isolates belonging to subgroup A harbored

235   bla_{OXA-23}, while the isolates belonging to subgroup B harbored only $bla_{OXA-51\ like}$

236   gene. These result demonstrated that PFGE has a greater discriminatory power

14

than MLST. While CC92 carrying bla_{OAX-23} has been documented worldwide [6, 9],

CC92 with and without bla_{OXA-23} were observed as carbapenem-non-susceptible

strains in this study.

One *A. baumannii*, TUM10635, showed several distinctive aspects: the

presence of both the bla_{OXA-58} and bla_{IMP-1} gene, a novel ST 276, the foundation of

CC 276, and a unique PFGE band pattern (Figure). Interestingly, its intrinsic

$bla_{OXA-51-like}$ gene, OXA-64, was also reported in NDM-1 type

metallo-ß-lactamase-producing *A. baumannii* from Germany [28].

In contrast to *A. baumannii*, almost all isolates of non-*baumannii*

Acintetobacter isolates proved to produce MBLs (Table 2; 92% in non-*baumannii*

vs 7.1% in *A. baumannii*, p<0.01). PCR and sequencing revealed bla_{IMP-1} in all but

one MBL-producing isolates. Previous studies in East Asia have demonstrated a

similar, but not identical, trend: in Korea, carbapenem-resistant *A. nosocomialis*

and *A. calcoaceticus*, harbored the VIM-2 type MBL gene [25, 29]. In Taiwan,

MBL genes of bla_{IMP-1} and bla_{VIM-11} were detected in *A. pittii* and *A. nosocomialis*

[22]. In these reports, MBLs were not detected in *A. baumannnii*. The difference

of MBL gene might reflect the predominant MBL genes among other pathogens;

in Japan, the IMP-1-type MBL is most prevalent among *Pseudomonas aeruginosa*

[30]. While previous study in Japan explained carbapenem-resistant *"A.*

256   *baumannii*" due mainly to production of MBLs [11], however their data did not

257   analyze species/ genomic species identification by ARDRA. It will be necessary to

258   identify more detailed species for the antibiotic susceptibility surveillance of

259   *Acinetobacter* species. Meanwhile, *bla*OXA-58 located 17-bp downstream of

260   IS*Aba3*-like elements was detected mainly in isolates of *A. pittii*, similar to the

261   situation in Taiwan [22].

262   Our study revealed that most carbapenem-non-susceptible *A. baumannii*

263   belonged to CC 92 known as a worldwide disseminated clone, and consisted of at

264   least two lineages with or without *bla*OXA-23 gene. The MBL producing *A.*

265   *baumannii* was rare; only one isolate belonging to CC276 and containing *bla*OXA-64

266   and *bla*OXA-58 also harbored *bla*IMP-1. This is the first report showing the

267   differences of ß-lactamases among carbapenem-non-susceptible *Acinetobacter* spp.

268   with genomic species level identification in Japan. These observations enhance

269   our understanding of the epidemiology of carbapenem-non-susceptible

270   *Acinetobacter* spp..

271

16

275 appreciation to David M. Livermore, Yohei Doi and Tse Hsien Koh for their

276 critical comments and careful reviewing of the manuscript.

277 **FUNDING**

REFERENCES

[1] Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: emergence of a successful pathogen. Clin Microbiol Rev. 2008;21:538-82.

[2] Turton JF, Ward ME, Woodford N, Kaufmann ME, Pike R, Livermore DM, et al. The role of IS*Aba1* in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. FEMS Microbiol Lett. 2006;258:72-7.

[3] Poirel L, Nordmann P. Genetic structures at the origin of acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene bla_{OXA-58} in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2006;50:1442-8.

[4] Dijkshoorn L, Aucken H, Gerner-Smidt P, Janssen P, Kaufmann ME, Garaizar J, et al. Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. J Clin Microbiol. 1996;34:1519-25.

[5] van Dessel H, Dijkshoorn L, van der Reijden T, Bakker N, Paauw A, van den Broek P, et al. Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals. Res Microbiol. 2004;155:105-12.

[6] Mugnier PD, Poirel L, Naas T, Nordmann P. Worldwide dissemination of the bla_{OXA-23} carbapenemase gene of *Acinetobacter baumannii*. Emerg Infect Dis. 2010;16:35-40.

302 [7] Fu Y, Zhou J, Zhou H, Yang Q, Wei Z, Yu Y, et al. Wide dissemination of

303 OXA-23-producing carbapenem-resistant *Acinetobacter baumannii* clonal

304 complex 22 in multiple cities of China. J Antimicrob Chemother. 2010;65:644-50.

305 [8] Park YK, Choi JY, Jung SI, Park KH, Lee H, Jung DS, et al. Two distinct

306 clones of carbapenem-resistant *Acinetobacter baumannii* isolates from Korean

307 hospitals. Diagn Microbiol Infect Dis. 2009;64:389-95.

308 [9] Runnegar N, Sidjabat H, Goh HM, Nimmo GR, Schembri MA, Paterson DL.

309 Molecular epidemiology of multidrug-resistant *Acinetobacter baumannii* in a

310 single institution over a 10-year period. J Clin Microbiol. 2010;48:4051-6.

311 [10] Ishii Y, Yamaguchi K. Evaluation of the susceptibility trends to meropenem

312 in a nationwide collection of clinical isolates in Japan: a longitudinal analysis

313 from 2002 to 2006. Diagn Microbiol Infect Dis. 2008;61:346-50.

314 [11] Takahashi A, Yomoda S, Kobayashi I, Okubo T, Tsunoda M, Iyobe S.

315 Detection of carbapenemase-producing *Acinetobacter baumannii* in a hospital. J

316 Clin Microbiol. 2000;38:526-9.

317 [12] Shibata N, Doi Y, Yamane K, Yagi T, Kurokawa H, Shibayama K, et al. PCR

318 typing of genetic determinants for metallo-β-lactamases and integrases carried by

319 gram-negative bacteria isolated in Japan, with focus on the class 3 integron. J

320 Clin Microbiol. 2003;41:5407-13.

321 [13] Yamaguchi K, Ohno A, Ishii Y, Tateda K, Iwata M, Kanda M, et al. [In vitro

322 susceptibilities to levofloxacin and various antibacterial agents of 12,919 clinical

323 isolates obtained from 72 centers in 2007]. Jpn J Antibiot. 2009;62:346-70.

324 [14] Vaneechoutte M, Dijkshoorn L, Tjernberg I, Elaichouni A, de Vos P, Claeys G,

325 et al. Identification of *Acinetobacter* genomic species by amplified ribosomal DNA

326 restriction analysis. J Clin Microbiol. 1995;33:11-5.

327 [15] Clinical and Laboratory Standards Institute. Methods for Dilution

328 Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically- Eighth

329 edition: Approved standard M107-A8. CLSI, Wayne, PA, USA, 2009.

330 [16] Arakawa Y, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H, et al.

331 Convenient test for screening metallo-β-lactamase-producing gram-negative

332 bacteria by using thiol compounds. J Clin Microbiol. 2000;38:40-3.

333 [17] Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, et al.

334 Multiplex PCR for genes encoding prevalent OXA carbapenemases in

335 *Acinetobacter* spp. Int J Antimicrob Agents. 2006;27:351-3.

336 [18] Bartual SG, Seifert H, Hippler C, Luzon MA, Wisplinghoff H,

337 Rodriguez-Valera F. Development of a multilocus sequence typing scheme for

338 characterization of clinical isolates of *Acinetobacter baumannii*. J Clin Microbiol.

339 2005;43:4382-90.

340   [19] Martinovich A, Edelstein M, Kozlov R. Multilocus Sequence Typing of

341   *Acinetobacter* Strains from Russia and Belarus That Produce Acquired OXA

342   Carbapenemases. In: Abstracts of the Forty-ninth Interscience Conference on

343   Antimicrobial Agents and Chemotherapy, San Francisco, CA, 2009. Abstract

344   C2-626. American Society for Microbiology, Washington, DC, USA.

345   [20] Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring

346   patterns of evolutionary descent among clusters of related bacterial genotypes

347   from multilocus sequence typing data. J Bacteriol. 2004;186:1518-30.

348   [21] Jeon BC, Jeong SH, Bae IK, Kwon SB, Lee K, Young D, et al. Investigation of

349   a nosocomial outbreak of imipenem-resistant *Acinetobacter baumannii* producing

350   the OXA-23 β-lactamase in korea. J Clin Microbiol. 2005;43:2241-5.

351   [22] Lin YC, Sheng WH, Chen YC, Chang SC, Hsia KC, Li SY. Differences in

352   carbapenem resistance genes among *Acinetobacter baumannii*, *Acinetobacter*

353   genospecies 3 and *Acinetobacter* genospecies 13TU in Taiwan. Int J Antimicrob

354   Agents. 2010;35:439-43.

355   [23] Xiao YH, Giske CG, Wei ZQ, Shen P, Heddini A, Li LJ. Epidemiology and

356   characteristics of antimicrobial resistance in China. Drug Resistance Updates :

357   2011;14:236-50.

358   [24] Turner PJ. MYSTIC Europe 2007: activity of meropenem and other

359　broad-spectrum agents against nosocomial isolates. Diagn Microbiol Infect Dis.

360　2009;63:217-22.

361　[25] Lee JH, Choi CH, Kang HY, Lee JY, Kim J, Lee YC, et al. Differences in

362　phenotypic and genotypic traits against antimicrobial agents between

363　*Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU. J Antimicrob

364　Chemother. 2007;59:633-9.

365　[26] Adams-Haduch JM, Onuoha EO, Bogdanovich T, Tian G, Marchall J, Urban

366　CM, Spellberg BJ et al. Molecular epidemiology of carbapenem-nonsusceptible

367　*Acinetobacter baumannii* in the United States. J Clin Microbiol. 2011;49:3849-54.

368　[27] Hamouda A, Evans BA, Towner KJ, Amyes SG. Characterization of

369　epidemiologically unrelated *Acinetobacter baumannii* isolates from four

370　continents by use of multilocus sequence typing, pulsed-field gel electrophoresis,

371　and sequence-based typing of *bla*$_{OXA-51-like}$ genes. J Clin Microbiol.

372　2010;48:2476-83.

373　[28] Pfeifer Y, Wilharm G, Zander E, Wichelhaus TA, Gottig S, Hunfeld KP, et al.

374　Molecular characterization of *bla*$_{NDM-1}$ in an *Acinetobacter baumannii* strain

375　isolated in Germany in 2007. J Antimicrob Chemother. 2011;66:1998-2001.

376　[29] Lee K, Kim MN, Choi TY, Cho SE, Lee S, Whang DH, et al. Wide

377　dissemination of OXA-type carbapenemases in clinical *Acinetobacter* spp. isolates

378   from South Korea. Int J Antimicrob Agents. 2009;33:520-4.

379   [30] Kimura S, Alba J, Shiroto K, Sano R, Niki Y, Maesaki S, et al. Clonal

380   diversity of metallo-β-lactamase-possessing *Pseudomonas aeruginosa* in

381   geographically diverse regions of Japan. J Clin Microbiol. 2005;43:458-61.

382

383

384 **FIGURE LEGEND**

385 **Figure.** Pulsed-field gel electrophoresis (PFGE) of 14 carbapenem-non-susceptible

386 *Acinetobacter baumannii* isolates in Japan. *A. baumannii* comprised PFGE

387 subgroup A, B and C at a level 85% (indicated by the broken line). While PFGE

388 subgroup A and B belonged to CC92, PFGE subgroup C with unique band pattern

389 belonged to CC 276 (Table 2).

Table 1. Primers used in this study

Primers	Sequence(5' to 3')	Aim	Ref
OXA-51 like Fw	TAA TGC TTT GAT CGC CCT TG		
OXA-51 like Rv	TGG ATT GCA CTT CAT CTT GG		
OXA-23 like Fw	GAT CGG ATT GGA CCA GA		
OXA-23 like Rv	ATT TCT GAC CGC ATT TCC AT	multiplex PCR	[17]
OXA-24 like Fw	GGT TAG TTG GCC CCC TTA AA		
OXA-24 like Rv	AGT TGA GCG AAA AGG GGA TT		
OXA-58 like Fw	AAG TAT TGG GGC TTG TGC TG		
OXA-58 like Rv	CCC CTC TGC GCT CTA CAT AC		
ISAba-1Fw	CAC GAA TGC AGA AGT TG		[2]
ISAba-1Rv	CGA CGA ATA CTA TGA CAC	ISAba family	
ISAba-3 like	AGC AAT ATC TCG TAT ACC GC		[3]
OXA-23 W F	GGG CAT ATG AAT AAA TAT TTT ACT TGC TAT GTG G		
OXA-23 W R	GGG GGA TCC TTA AAT AAT ATT CAG CTG TTT TAA TGA TTT C		
OXA-51 W F	GGG GGC ATA TGA ACA TTA AAG CAC TCT TAC	simplex PCR	This study
OXA-51 W R	CCC GGA TCC TGC TAT AAA ATA CCT AAT TG		
OXA-58 W F	GGG CCA TGG GTA TGA AAT TAT TAA AAA TAT TGA GTT TAG TT		
OXA-58 W R	CCG GAT CCT GTT ATA AAT AAT GAA AAA C		
OXA-51 likeS F	AAA GCT TCC GCT ATT CC	Sequence	This study
OXA-51like S R	GGA GTA ATT TTT AGA GGA CC		
IMP-1 F1	ACC GCA GCAGAG TCT TTG		
IMP-1 R1	ACA ACC AGT TTT GCC TTA CC		
IMP-2 F2	GTT TTA TGT GTA TGC TTC C		
IMP-2 R2	AGC CTG TTC CCA TGT AC	MBLs	[12]
VIM-1 F3	AGT GGT GAG TAT CCG ACA G		
VIM-1 R3	ATG AAA GTG CGT GGA GAC		
VIM-2 F4	ATG TTC AAA CTT TTG AGT AAG		
VIM-2 R4	CTA CTC AAC GAC TGA GCG		

Table 2. Characterization of carbapenem-non-susuceptible *Acinetobacter* species in Japan.

isolate No.	Species	β-lactamase				PFGE	MLST		Hp	Location of hospitals	Sample	MIC (mg/L)							
		OXA-51	OXA-23	OXA-58	MBL		ST	CC				SAM	CAZ	FEP	IPM	MEM	GEN	MIN	CIP
10629	*A.baumannii*	66	23**			A	208	92	1	Kanto	Pus	32/16	>256	128	64	64	>512	8	64
10630	*A.baumannii*	66*	23**			A	208	92	1	Kanto	Sputum	128/64	256	256	64	128	8	16	128
10631	*A.baumannii*	66*	23**			A	208	92	1	Kanto	Blood	64/32	128	128	64	128	4	8	64
10632	*A.baumannii*	66*	23**			A	208	92	1	Kanto	Sputum	32/16	128	128	64	128	2	8	32
10633	*A.baumannii*	66*	23**			A	208	92	1	Kanto	Pus	64/32	128	128	64	128	4	8	32
10634	*A.baumannii*	66*				B	208	92	1	Kanto	Sputum	4/2	256	64	8	16	8	≤0.25	≤0.06
10635	*A.baumannii*	64		58†	IMP-1	C	276	276	1	Kanto	Sputum	4/2	128	16	2	8	128	4	64
10636	*A.baumannii*	66*				B	219	92	2	Kanto	Sputum	8/4	128	64	4	8	>512	2	128
10637	*A.baumannii*	66*				B	219	92	2	Kanto	Sputum	32/16	256	128	4	16	>512	2	>128
10638	*A.baumannii*	66*				B	219	92	2	Kanto	Sputum	32/16	256	128	4	16	>512	4	>128
10639	*A.baumannii*	66*				B	208	92	3	Kanto	Other	32/16	256	64	16	32	>512	2	32
10640	*A.baumannii*	66*				B	208	92	4	Kanto	Sputum	64/32	128	32	2	8	256	2	64
10641	*A.baumannii*	206*	23**			A	208	92	5	Kanto	Other	128/64	256	256	32	128	4	8	128
10642	*A.baumannii*	66*				A	208	92	6	Kyusyu	Urine	4/2	128	16	8	16	256	2	64
10643	*A. pittii*			58†	IMP-1				7	Hokkaido	Sputum	8/4	8	8	16	8	4	≤0.25	1
10644	*A. pittii*			58†					8	Kanto	Sputum	4/2	256	256	32	32	8	≤0.25	16
10645	*A. pittii*			58†	IMP-1				9	Kanto	Urine	4/2	256	128	64	64	1	≤0.25	64
10646	*A.calcoaceticus*				IMP-1				10	Tokai	Blood	2/1	>512	512	32	128	>512	≤0.25	2
10647	*A.calcoaceticus*				IMP-1				10	Tokai	Sputum	4/2	>512	256	32	64	4	≤0.25	0.13
10648	*A.calcoaceticus*				IMP-1				10	Tokai	Sputum	2/1	512	64	16	64	16	≤0.25	0.13
10649	*A.calcoaceticus*				IMP-1				10	Tokai	Sputum	4/2	>512	512	64	128	64	≤0.25	0.25

10650	*A. nosocomialis*		IMP-1	10	Tokai	Sputum	2/1	512	256	64	128	>512	≤0.25	32
10651	*A. nosocomialis*		IMP-2	11	Kyusyu	Sputum	4/2	512	256	16	32	>512	≤0.25	≤0.06
10652	*A. nosocomialis*		IMP-1	12	Kyusyu	Sputum	2/1	512	128	16	32	256	≤0.25	≤0.06
10653	*A. nosocomialis*		IMP-1	12	Kyusyu	Sputum	4/2	512	256	32	16	2	≤0.25	4
10654	*A.lwoffii*		IMP-1	10	Tokai	blood	4/2	512	256	32	64	0.5	≤0.25	0.125
10655	*A.lwoffii*	58	IMP-1	13	Hokuriku	Other	1/0.5	256	32	8	16	1	≤0.25	≤0.06

SAM, ampicillin/sulbactam; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; MEM, meropenem; GEN, gentamicin; MIN, minocyclin; CIP,

ciprofloxacin; PFGE, pulsed-fielded gel electrophoresis; MLST, multilocus sequencing type; ST, sequence type; CC, clonal complex.
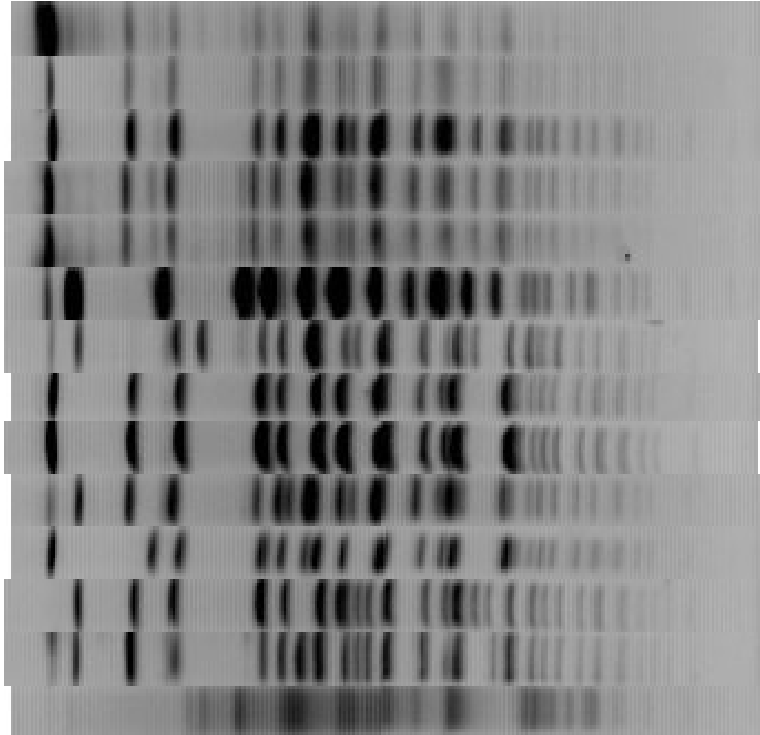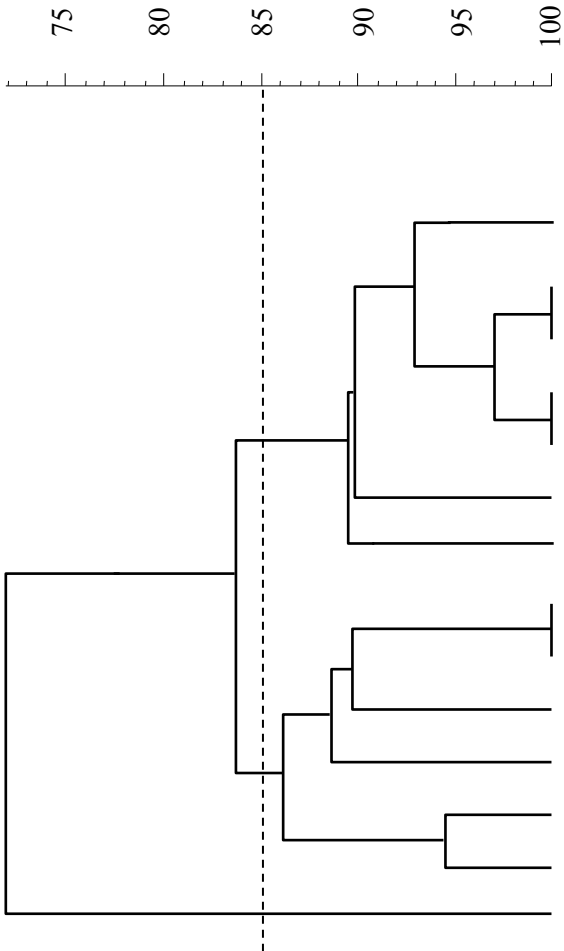
‡ Hp means hospital number.

*Detection of IS*Aba1* at 34-bp upstream from *bla*$_{\text{OXA-51 like}}$ gene.

** Detection of IS*Aba1* at 8-bp upstream from *bla*$_{\text{OXA-23}}$ gene.

†Detection of IS*Aba3*-like at 17-bp upstream from *bla*$_{\text{OXA-58}}$ gene.

Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]

Isolate name	PFGE subgroup	Clonal Complex
TUM10642	A	92
TUM10632	A	92
TUM10633	A	92
TUM10630	A	92
TUM10631	A	92
TUM10629	A	92
TUM10641	A	92
TUM10637	B	92
TUM10638	B	92
TUM10634	B	92
TUM10636	B	92
TUM10639	B	92
TUM10640	B	92
TUM10635	C	276