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EMERGENCE OF DIFFERENT *ACINETOBACTER BAUMANNII* CLONES IN A CROATIAN HOSPITAL AND CORRELATION WITH ANTIBIOTIC SUSCEPTIBILITY

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Key words: *Acinetobacter baumannii*, carbapenems, carbapenem-hydrolyzing oxacillinase, resistance

ABSTRACT

Objectives: During routine diagnostic laboratory work, the clinical microbiologist observed an increase of *Acinetobacter baumannii* isolates with three different carbapenem susceptibility patterns : susceptible, intermediate and resistant. Isolates belonging to the same carbapenem susceptibility phenotype exhibited identical susceptibility/resistance patterns to non- β -lactam antibiotics. This prompted us to analyze the mechanisms of carbapenem-resistance and the molecular epidemiology of the isolates. A total of 59 *A. baumannii* isolates were analyzed and grouped according to their susceptibility to imipenem: group 1 were susceptible (N=24), group 2 were intermediate (N=8) and group 3 were resistant (N=27) to imipenem.

Material and methods: PCR and sequencing was used to detect resistance genes. Genotyping of the isolates was performed by PFGE and MLST.

Results : Out of 27 resistant isolates, 20 harboured *bla*_{OXA-40-like} and 7 *bla*_{OXA-23-like} genes. *ISAbal1* was found upstream of *bla*_{OXA-51} and *bla*_{OXA-23} genes. PFGE genotyping demonstrated the existence of three major *A. baumannii* clones in GH Pula and determination of sequence groups showed that the isolates belonged to international clones commonly associated with multidrug-resistance. MLST (performed on six isolates) showed diverse population structure of isolates belonging to the same cluster, including ST 195, ST 231, ST 775 and ST1095.

Conclusions: A previous study conducted in 2009-2010 showed that reduced susceptibility to carbapenems in GH Pula was only associated with upregulation of the intrinsic OXA-51 β -lactamase. In this study a shift to isolates with acquired oxacillinases, belonging to two major clones was reported.

1. INTRODUCTION

Acinetobacter baumannii is a Gram-negative opportunistic pathogen causing infections in health care settings globally [1]. As an emerging pathogen it represents a serious public health concern owing to its increasingly multidrug-resistant capacity [2]. Carbapenem-resistance can develop due to hyperproduction of chromosomally encoded OXA-51-like β -lactamase mediated by *ISAbal* upstream of the *bla*_{OXA-51} genes. Rarely, resistance can occur because of porin loss (CarO) or overexpression of efflux pumps [3]. Acquired carbapenem resistance in *A. baumannii* can also be mediated by acquired carbapenemases of class A (KPC) [4], metallo- β -lactamases (MBLs) of IMP, VIM, SIM and NDM family [5-8] and carbapenem-hydrolyzing oxacillinases (CHDL) (OXA-23-like, OXA-40-like, OXA-58-like, OXA-143-like and OXA-235-like [9-12]. From 2008 carbapenem-resistant *A. baumannii* emerged with increasing frequency in most hospital centers in Croatia and became an important public health problem as shown by the multicenter study conducted in Northern Croatia and Istria demonstrating that OXA-40-like and OXA-58-like were the dominant types of acquired CHDL in Croatia. However, reduced susceptibility to carbapenems among Istrian isolates, belonging to ST1, was due to of the intrinsic OXA-51 β -lactamase upregulated by *ISAbal* [13]. Unlike other geographic regions, Istria county located in the southwest part of Croatia was spared of acquired carbapenemases until recently. During routine diagnostic laboratory work, the clinical microbiologist observed the emergence of carbapenem-resistant *A. baumannii* isolates, alongside with persistence of previous susceptible or intermediate susceptible ones and classified them into three categories; susceptible, intermediate and resistant. Isolates belonging to the same carbapenem susceptibility group exhibited identical susceptibility/resistance patterns to non- β -lactam antibiotics. The three groups circulated in different hospital wards during the study period (2013-2014). This prompted us to analyze the

underlying mechanisms of carbapenem-resistance and the molecular epidemiology of isolates with various antibiotic susceptibility profiles.

2. MATERIAL AND METHODS

2.1. *Material and methods*

A total of 59 consecutive *A. baumannii* isolates collected between May 2012 and August 2014 were analyzed. The isolates were tentatively identified to species level by VITEK2 automated system, and confirmed by *gyrB* multiplex PCR and the presence of the intrinsic *bla*_{OXA-51} gene [14].

2.2. *Antimicrobial susceptibility testing*

The antimicrobial susceptibility to a wide range of β -lactam antibiotics including ceftazidime (CAZ), cefotaxime (CTX), ceftriaxone (CRO), cefepime (FEP), imipenem (IMP), meropenem (MEM) and non- β -lactam antibiotics such as gentamicin (GEN), amikacin (AMK), ciprofloxacin (CIP), tigecycline (TGC) and colistin (COL) was determined by broth microdilution in Mueller-Hinton broth in 96-well microtiter plates according to CLSI guidelines [15]. *Pseudomonas aeruginosa* ATCC 27853 and *A. baumannii* ATCC 19606 were used as quality control strain. E-test MBL strips (AB Biodisk, Solna, Sweden) and combined disk test with EDTA were used for detection of metallo- β -lactamases (MBL) [16]. Extended-spectrum β -lactamases were detected by double disk synergy test (DDST) according to Jarlier and combined disk test with the addition of cloxacillin in the medium to inhibit chromosomal AmpC β -lactamase of *A. baumannii* [17]. *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive control strains, respectively.

2.3. *Molecular characterization of β -lactamases*

The presence of the genes encoding KPC, MBLs of IMP, VIM, SIM and NDM class [4, 5-8] and OXA β -lactamases (*bla*_{OXA-51-like}, *bla*_{OXA-23-like}, *bla*_{OXA-40-like}, *bla*_{OXA-58-like} and *bla*_{OXA-143-}

like) genes [18] was determined by PCR as previously described. The amplicons of the selected representative isolates were column-purified (QIAquick PCR purification kit, Inel Medicinska tehnika, Zagreb) and subjected to sequencing by the Macrogen sequencing service (South Korea) with the same primers used for PCR in order to determine the identity of the enzyme. Sequence alignment analysis was done online by utilizing the BLAST Program (www.ncbi.nlm.nih.gov). The genetic context of *bla*_{OXA-51} and *bla*_{OXA-23} genes was further investigated by PCR mapping with primers for *ISAbal* combined with forward and reverse primers for *bla*_{OXA-51} and *bla*_{OXA-23} [19]. Isolates phenotypically positive for extended-spectrum β -lactamases (ESBLs) in combined disk test were subjected to PCR with primers specific for SHV, TEM, PER-1, GES and CTX-M β -lactamases [20-24]. Reference strains positive for OXA-23, OXA-40, OXA-58, OXA-143, VIM-1, IMP-1, NDM-1, TEM-1, SHV-1, PER-1 and CTX-M-15 were used as positive controls for PCR.

2.4.Characterization of plasmids

Conjugation experiments were performed using *E. coli* J 53 resistant to sodium azide [25]. To determine if acquired oxacillinase genes were plasmid-encoded, plasmid DNA was extracted from representative isolates (ACB 22, ACB 23, ACB 24, ACB, 25 and ACB 26) and transferred to *Acinetobacter baumannii* ATCC 17978 by electroporation. Transformants were selected on LB medium containing 100 μ g/ml ticarcillin [26].

Plasmid incompatibility groups were determined by PCR-based replicon typing (PBRT) according to Bertini et al [27].

2.5.Molecular typing of isolates

Sequence groups (SGs 1-3) corresponding to international clonal lineages (IC1-3) determination was performed according to the procedure described by Turton et al. [28]. PFGE genotyping of *Xba*I-digested genomic DNA was performed with a CHEF-DRIII-system (Bio-Rad); the images were processed using Gel-Compar software, and a dendrogram

was computed after band intensity correlation using global alignment with 1.5% optimization and UPGMA (unweighted pair-group method using arithmetical averages) clustering [29]. The isolates were considered to be clonally related if they showed more than 87% similarity in their PFGE patterns. The Oxford MLST scheme was used to type a subset of six isolates, belonging to different clusters, using primers and conditions as described at PubMLST (<http://pubmlst.org/abaumannii/>).

RESULTS

3.1. Bacterial isolates

All isolates initially identified by VITEK 2 as *A. baumannii*, were confirmed by molecular methods as *A. baumannii*. Respiratory tract specimens (bronchoalveolar lavage fluid and tracheal aspirates) were the most important source of the tested isolates (35%), followed by wound swabs (25%), urine (23%), blood cultures (10%), cerebrospinal fluid and drains (3%), respectively (Figure 1). The isolates originated predominantly from intensive care unit (ICU) (40%), followed by surgery (32%), internal medicine (23%), neurology (3%) and otorhinolaryngology (1.7%).

3.2. Antimicrobial susceptibility

Results of antimicrobial testing are summarised in Table 1. High resistance rates were observed for CIP (93%), CAZ (87%), FEP (84%), GEN (66%) IMI(49%) and MEM (45%). TGC showed good activity with only 28% of resistant isolates (according to the resistance breakpoint for Enterobacteriaceae). There was no resistance observed for colistin as shown in Table 1.

Table 1.Antibiotic susceptibility of *Acinetobacter baumannii* isolates

Antibiotic (CLSI breakpoint) mg/L	MIC range	MIC ₅₀	MIC ₉₀	Resistant strains (%)
Ceftriaxone (≥ 64)	>256	256	>256	59/59 (100)
Cefotaxime (≥ 64)	>256	256	>256	59/59 (100)
Ceftazidime (≥ 32)	2 - >256	128	>256	51/59 (87,3)
Cefepime (≥ 32)	2 - 256	32	32	50/59 (84,7)
Imipenem (≥ 8)	0,25 - 32	8	32	27/59 (45,8)
Meropenem (≥ 8)	0,25 - 32	4	32	29/59 (49,2)
Ciprofloxacin (≥ 4)	0,25 - 256	128	>256	55/59 (93,2)
Amikacin (≥ 64)	2 - 256	4	>256	26/59 (44,0)
Gentamicin (≥ 16)	1 - 256	16	>256	39/24 (66,1)
Tigecycline (≥ 4)*	0.25 - 4	2	4	14/59 (28)
Colistin (≥ 4)	0.12 - 0,5	0.25	0.25	0/59 (0)

*Breakpoint for Enterobacteriaceae is applied

Isolates were grouped according to their imipenem-susceptibility according to CLSI criteria: group 1 were imipenem-susceptible (N=24), group 2 intermediate (N=8) and group 3 resistant (N=27).

The imipenem-susceptible group isolates showed resistance to CTX and CRO (intrinsic resistance in *A. baumannii*), and were all susceptible to IMI, COL and TGC (according to breakpoint for *Enterobacteriaceae*) and all but one were susceptible to MEM and AMK. They showed moderate resistance rates to ceftazidime and cefepime (67%) and high

resistance rate to ciprofloxacin (83%). COL was the most potent antibiotic with MIC₉₀ values of 0.12 mg/L.

The imipenem-intermediate group isolates were resistant to CAZ, CTX, CRO and CIP.

Eighty-seven percent of this group were resistant to FEP and 50% to GEN. The isolates were uniformly susceptible to TGC (according to CLSI breakpoints for Enterobacteriaceae), AMK and COL. Colistin was the most potent antibiotic with a MIC₉₀ of 0.12 mg/L. IMI and MEM displayed MIC₉₀ values of 4 mg/L that were slightly higher than those of TGC (2 mg/L).

The imipenem-resistant group isolates were multidrug-resistant according to Magiorakos et al [30] and were uniformly resistant to expanded-spectrum cephalosporins, CIP and GEN. They showed a moderate resistance rate to TGC of 52% (according to CLSI breakpoints for Enterobacteriaceae), but were uniformly susceptible to COL. The lowest MIC₉₀ was observed with COL (0.12 mg/L).

3.3.Characterization of β -lactamases

Imipenem susceptible isolates were uniformly negative in DDST for detection of ESBLs, but positive in CLSI combined disk test with clavulanate (augmentation of inhibition zone around cephalosporin disks ranging from 10 to 15 mm). They all tested negative in phenotypic test for MBLs. All 59 isolates were PCR positive for the intrinsic *bla*_{OXA-51-like} gene. Eight isolates had only *bla*_{OXA-51} gene without *ISAbal*, while in 16 isolates which displayed elevated MICs of meropenem (4-8 mg/L) *bla*_{OXA-51} gene was preceeded by *ISAbal*.

All imipenem-intermediate-susceptible isolates tested negative phenotypically for both ESBLs and MBLs. Imipenem-resistant isolates were negative for ESBLs, but the production of class B carbapenemases was suspected by positive inhibitor based test with EDTA and at least eight fold reduction of imipenem by EDTA MIC in E- test. However, they were PCR negative for the tested MBL genes. Out of 27 isolates, 20 harboured *bla*_{OXA-40-like}, and 7

*bla*_{OXA-23-like} genes. Sequencing of *bla*_{OXA} revealed the presence of *bla*_{OXA-23} and *bla*_{OXA-40} genes. *ISAbal* was found upstream of *bla*_{OXA-51} and *bla*_{OXA-23} genes.

3.4.Characterization of plasmids

ACB22 plasmid preparation transferred OXA-40 while ACB25 plasmid preparation transferred OXA-23 into *A. baumannii* ATCC 17978, leading to carbapenem resistance (data not shown). Other tested isolates did not transfer the CHDL to the recipient strain. The plasmids extracted from strains positive for OXA-23 belonged to Inc group 6 encoding *aci6*-replicase gene originally found on plasmid pACICU2 (amplicon size 662 bp).

3.5.Genotyping

The susceptible and intermediate isolates belonging to sequence groups (SGs) 1 and 2 were assigned to SG 2 corresponding to IC1, while isolates resistant to carbapenems (group 3) were allocated predominantly to SG 1 corresponding to IC2, apart from isolates 23, 24 and 30 (IC1, OXA-23 positive) and isolates 34, 37 and 38 (IC1, positive for OXA-40) (Figure 1). Three isolates were nontypeable using this method (AB 6, AB, 25 and AB 43). PFGE revealed the existence of three major clusters, summarised in Figure 1: the first dominant cluster with 32 isolates contained susceptible or intermediate susceptible obtained from different hospital units, positive only for *bla*_{OXA-51} with or without *ISAbal* and belonging to IC I, with one exception (isolate 26 with OXA-40-like, IC II), and the second and the third cluster included resistant isolates originating mostly from surgical or ICU wards, positive for *bla*_{OXA-23-like} or *bla*_{OXA-40-like}, and belonging to predominantly IC II, with one exception (isolate 55, carbapenem susceptible and negative for CHDL). Subclusters containing pairs, triplets or groups of identical isolates were identified within each clone. Interestingly, some of the OXA-23 positive isolates clustered together with OXA-40 positive isolates. Three isolates (AB 10, AB 25 and AB 32) were singletons (Figure 1). MLST of six representative isolates revealed four different STs: ST-195 (strain 27), ST-231 (strain 38), ST-775 (strains

10, 14 and 19), and ST-1095 (isolate 43). Analysis of STs using the PubMLST website revealed that ST-195 was associated with IC2, whereas ST-231 and -775 with IC1. None of the isolates belonged to ST1 previously reported among *A. baumannii* from Istria.

4.DISCUSSION

This study demonstrates the existence of at least three different clones of *A. baumannii* in GH Pula. A previous study conducted in 2009-2010 which included 23 isolates from Istria, showed that reduced susceptibility to carbapenems in GH Pula was only associated with upregulation of intrinsic OXA-51 β -lactamase by IS*Aba*1 located upstream of *bla*_{OXA-51} gene. In this study a shift to acquired CHDL was reported. The previous isolates from 2009-2010 belonged uniformly to SG 1 (IC II) and ST1. The fact that carbapenem-resistant isolates originated from patients without travel history or treatment history in other hospital centers in Croatia suggested that these isolates were not imported and arose independently.

In the present study OXA-23 and OXA-40 β -lactamases were found. OXA-23 was the first CHDL reported in 1986 in the UK as ARI-1 [31]. Later it was renamed to OXA-23. It has now spread all over the world with most reports originating from Europe and the Far-East [32]. OXA-23 is the dominant CHDL in Bulgaria [33], China [34], Germany [35], Italy [36], Poland [37], Spain [38], UK [39] and many other countries [32] and is often associated with nosocomial outbreaks [38]. OXA-23 was reported previously from two hospital centers in Zagreb [13] in the multicenter study conducted in 2009-2010 and it was also previously found in a nursing home in Zagreb [40].

OXA-40 group is the most prevalent CHDL among tested isolates, and this group was first described in the Iberian Peninsula [41], but was later identified in the Czech Republic [42], USA [43], and recently in Bulgaria [44] and in the neighbouring country of Serbia [45]. Similar to our isolates, the isolates from Spain and the Czech Republic belonged to IC II. Sequencing revealed the OXA-40 allelic variant in contrast with previous studies which

identified OXA-72 in other geographic regions of Croatia. The emergence of CHDL was associated with genetically related isolates, belonging into two PFGE clusters. *ISAbal*/OXA-51 positive isolates belonged to both groups: susceptible and intermediate susceptible, indicating that insertion sequence is not a good predictor of reduced susceptibility to carbapenems. However, even the susceptible isolates harbouring *ISAbal* upstream of the *bla*_{OXA-51} gene had carbapenem MICs above those in the wild type strains, similar as in German isolates [35] .

Interestingly, all imipenem-susceptible *A. baumannii* isolates and some of the OXA-23 and OXA-40 positive isolates belonged to IC I. In the previous studies in Croatia, IC I was associated with carbapenem-susceptible isolates whereas isolates positive for OXA-40 and OXA-58 belonged to IC II [13]. PFGE patterns were correlated with resistance phenotype with all resistant isolates in two clusters which contained subclusters. . The fact that isolates with different types of CHDL (OXA-23-like and OXA-40-like) clustered together indicates that the oxacillinase genes were acquired after clonal spread within the hospital. However, MLST revealed differences between isolates belonging to the same cluster. Population structure of the isolates was quite variable with five different STs reported. ST 195 was previously reported in China and associated with OXA-23 β -lactamase in contrast with our study in which this ST was found in OXA-40 positive isolates [46-47] . In both studies ST195 isolates belonged to IC II. The carbapenem-resistant isolates were mostly obtained from departments treating patients with severe infections. ST 231, 775, 1094 and ST 1095 are reported for the first time in this study according to the bibliographical data. The population structure of imipenem-susceptible isolates was highly diverse. Isolates from the previous study in 2009 belonged to ST1 [13] . In this study a shift to new STs was observed. All imipenem-resistant isolates tested positive in the inhibitor based test with EDTA, but PCR for common MBLs was negative. False positive results in inhibitor based tests can occur because

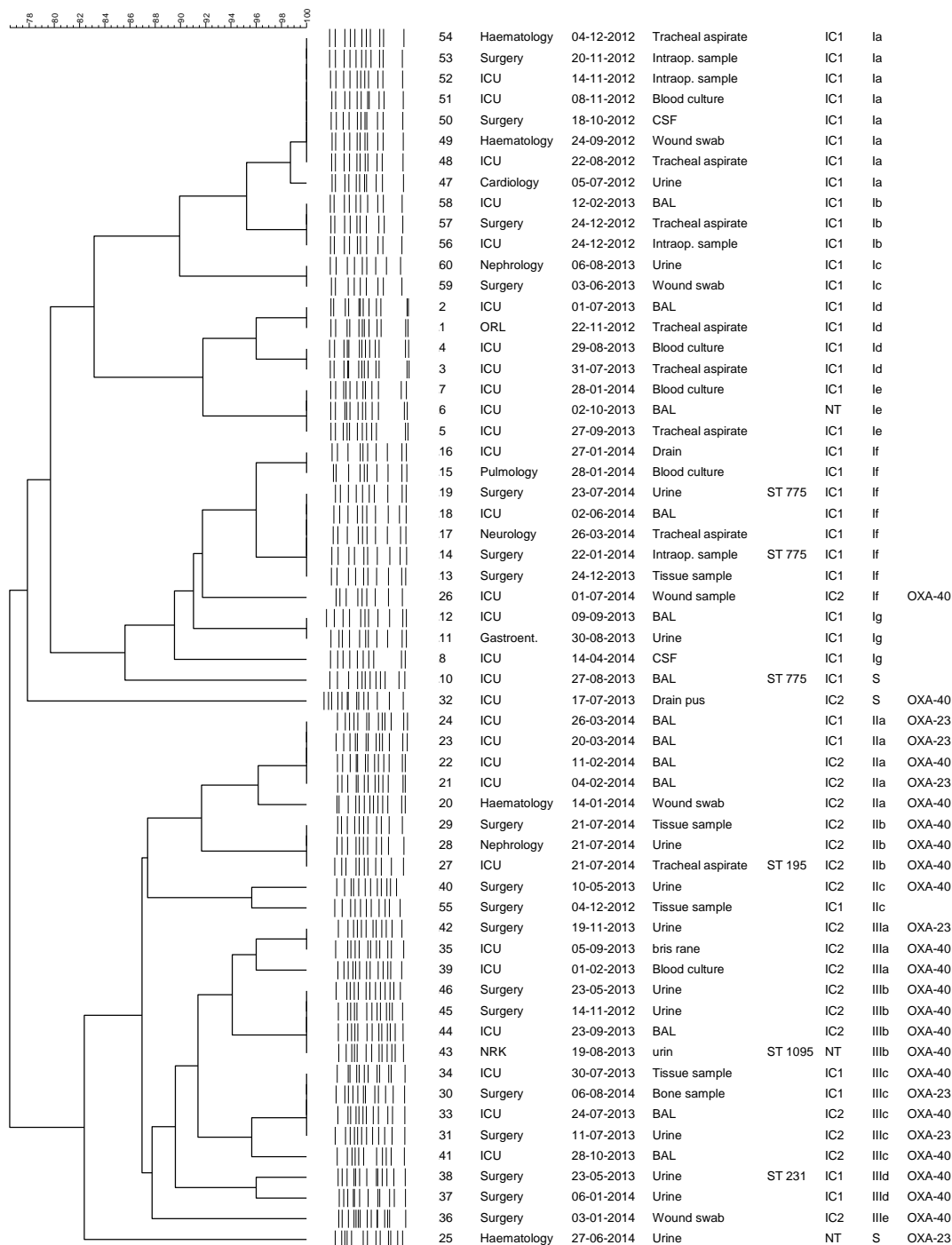
the chelating agent itself can increase the membrane permeability, thus increasing the chance for a bactericidal effect [48]. Moreover, false positive results were observed with combined disk test in detection of ESBLs which revealed augmentation of the inhibition zone above 5 mm in the presence of clavulanate among carbapenem- susceptible isolates. Classic double disk synergy test turned out negative and PCR revealed no *bla*_{ESBL} genes. Some of the isolates displayed significant difference in the MICs of imipenem and meropenem with MICs of meropenem being markedly higher. The possible explanation is that the isolates with elevated MICs of meropenem had upregulation of efflux pumps. Meropenem is a better substrate for efflux pumps, but the clarification of these mechanism was beyond this study. In conclusion, this study demonstrates the existence of several different clones of *A. baumannii* in GH Pula, which were mainly associated with IC1 and IC2 lineages. We also report that carbapenem-resistance was associated not only with overexpression of the intrinsic OXA-51 β -lactamase as was previously found in 2009-2010, but also with the acquisition of OXA-40 and OXA-23 which in some cases was shown to be plasmid encoded. The majority of resistant isolates were found to belong to the same clone. It is reasonable to suspect a vertical dissemination of CHDL positive isolates. Notwithstanding, the dissemination of successful clones may possibly contribute to the high rates and persistence of carbapenem-resistant *A. baumannii*.

These data demonstrate the importance of epidemiological studies to better understand the changing nature of carbapenem resistance. Prevention of outbreaks associated with such isolates should be focused on hospital hygiene measures, screening of the patients for carriage of MDR *A. baumannii* and restriction of carbapenem consumption.

FIGURE LEGEND: PFGE dendrogram of *A. baumannii* isolates. Cut off value of 87% was applied to define a clone. Hospital unit, date of isolation, specimen, sequence type, international clonal lineage, PFGE cluster and β -lactamase content are shown. Clusters are designated as I to III and subclusters as a to g. Abbreviations: BAL-bronchoalveolar lavage fluid; CSF-cerebrospinal fluid; ORL-otorhinolaryngology; ICU-intensive care unit; NRK-neurosurgery; IC-international clonal lineage; NT-non typeable; S- singleton

Dice (Opt:1.50%) (Tot 3.0%-3.0%) (F+0.0% S+0.0%) [0.0%-100.0%]
PFGE01

PFGE01



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