Emergence of different Acinetobacter baumannii clones in a Croatian hospital and correlation with antibiotic susceptibility

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

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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 $bla_{\text{OXA-40-like}}$ and 7 $bla_{\text{OXA-23-like}}$ genes. IS*Aba1* was found upstream of $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23}}$ 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 IS*Aba1* upstream of the $bla_{\text{OXA-51}}$ genes. Rarely, resistance can occurr 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 IS*Aba1* [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 susceptibile 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 *gyr*B multiplex PCR and the presence of the intrinsic $bla_{\text{OXA-51}}$ 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 antibiotis 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 ($bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-58-like}}$ and $bla_{\text{OXA-143-}}$

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*$_{\text{OXA-51}}$ and *bla*$_{\text{OXA-23}}$ genes was further investigated by PCR mapping with primers for IS*Aba1* combined with forward and reverse primers for *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{OXA-23}}$ [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 for 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 μg/ml ticarcillin [26] .

Plasmid incompatibility groups were determined by PCR-based replicon typing (PBRT) according to Bertini at 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 PubMSLT (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-susceptiblity 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 MIC_{90} 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 MIC_{90} of 0.12 mg/L. IMI and MEM displayed MIC_{90} 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 MIC_{90} 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 $bla_{\text{OXA-51-like}}$ gene. Eight isolates had only $bla_{\text{OXA-51}}$ gene without IS*AbaI*, while in 16 isolates which displayed elevated MICs of meropenem (4-8 mg/L)$bla_{\text{OXA-51}}$ gene was preceeded by IS*Aba*. 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 $bla_{\text{OXA-40-like}}$, and 7

bla$_{\text{OXA-23-like}}$ genes. Sequencing of *bla*$_{\text{OXA}}$ revealed the presence of *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-40}}$ genes. IS*Aba1* was found upstream of *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{OXA-23}}$ 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 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*$_{\text{OXA-51}}$ with or without IS*Aba1* 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*$_{\text{OXA-23-like}}$ or *bla*$_{\text{OXA-40-like}}$, 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 $bla_{\text{OXA-51}}$ 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 treament hystory 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 Penninsula [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

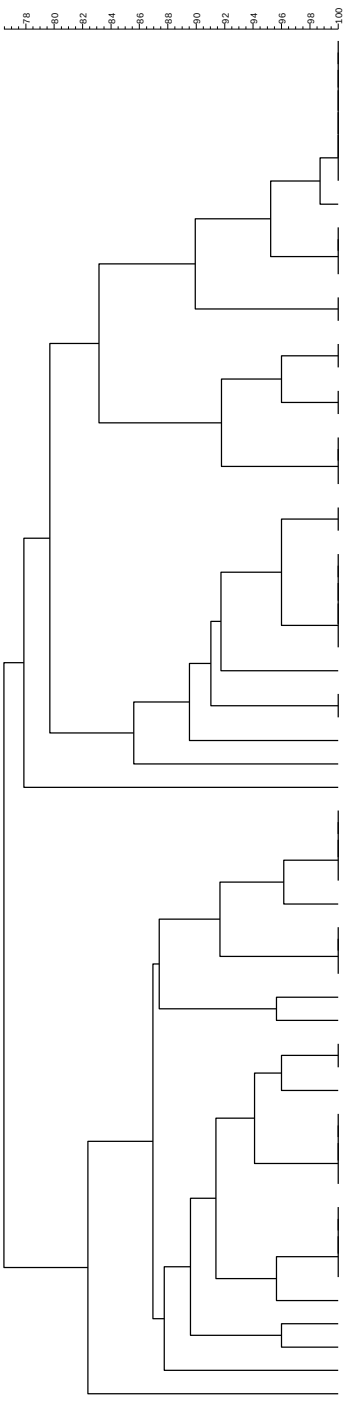
identified OXA-72 in other geographic regions of Croatia. The emergence of CHDL was associated with genetically related isolates, belonging into two PFGE clusters. IS*Aba1*/OXA-51 positive isolates belonged to both groups: susceptible and intermediate susceptible, indicating that insertion sequence is not a good predictor of reduced susceptibility to carbapenems. However, even the susceptible isolates harbouring IS*Aba1* upstream of the $bla_{\text{OXA-51}}$ 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 bactericial 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 carbapeneme- 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 persistance 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 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



54	Haematology	04-12-2012	Tracheal aspirate		IC1	Ia	
53	Surgery	20-11-2012	Intraop. sample		IC1	Ia	
52	ICU	14-11-2012	Intraop. sample		IC1	Ia	
51	ICU	08-11-2012	Blood culture		IC1	Ia	
50	Surgery	18-10-2012	CSF		IC1	Ia	
49	Haematology	24-09-2012	Wound swab		IC1	Ia	
48	ICU	22-08-2012	Tracheal aspirate		IC1	Ia	
47	Cardiology	05-07-2012	Urine		IC1	Ia	
58	ICU	12-02-2013	BAL		IC1	Ib	
57	Surgery	24-12-2012	Tracheal aspirate		IC1	Ib	
56	ICU	24-12-2012	Intraop. sample		IC1	Ib	
60	Nephrology	06-08-2013	Urine		IC1	Ic	
59	Surgery	03-06-2013	Wound swab		IC1	Ic	
2	ICU	01-07-2013	BAL		IC1	Id	
1	ORL	22-11-2012	Tracheal aspirate		IC1	Id	
4	ICU	29-08-2013	Blood culture		IC1	Id	
3	ICU	31-07-2013	Tracheal aspirate		IC1	Id	
7	ICU	28-01-2014	Blood culture		IC1	Ie	
6	ICU	02-10-2013	BAL		NT	Ie	
5	ICU	27-09-2013	Tracheal aspirate		IC1	Ie	
16	ICU	27-01-2014	Drain		IC1	If	
15	Pulmology	28-01-2014	Blood culture		IC1	If	
19	Surgery	23-07-2014	Urine	ST 775	IC1	If	
18	ICU	02-06-2014	BAL		IC1	If	
17	Neurology	26-03-2014	Tracheal aspirate		IC1	If	
14	Surgery	22-01-2014	Intraop. sample	ST 775	IC1	If	
13	Surgery	24-12-2013	Tissue sample		IC1	If	
26	ICU	01-07-2014	Wound sample		IC2	If	OXA-40
12	ICU	09-09-2013	BAL		IC1	Ig	
11	Gastroent.	30-08-2013	Urine		IC1	Ig	
8	ICU	14-04-2014	CSF		IC1	Ig	
10	ICU	27-08-2013	BAL	ST 775	IC1	S	
32	ICU	17-07-2013	Drain pus		IC2	S	OXA-40
24	ICU	26-03-2014	BAL		IC1	IIa	OXA-23
23	ICU	20-03-2014	BAL		IC1	IIa	OXA-23
22	ICU	11-02-2014	BAL		IC2	IIa	OXA-40
21	ICU	04-02-2014	BAL		IC2	IIa	OXA-23
20	Haematology	14-01-2014	Wound swab		IC2	IIa	OXA-40
29	Surgery	21-07-2014	Tissue sample		IC2	IIb	OXA-40
28	Nephrology	21-07-2014	Urine		IC2	IIb	OXA-40
27	ICU	21-07-2014	Tracheal aspirate	ST 195	IC2	IIb	OXA-40
40	Surgery	10-05-2013	Urine		IC2	IIc	OXA-40
55	Surgery	04-12-2012	Tissue sample		IC1	IIc	
42	Surgery	19-11-2013	Urine		IC2	IIIa	OXA-23
35	ICU	05-09-2013	bris rane		IC2	IIIa	OXA-40
39	ICU	01-02-2013	Blood culture		IC2	IIIa	OXA-40
46	Surgery	23-05-2013	Urine		IC2	IIIb	OXA-40
45	Surgery	14-11-2012	Urine		IC2	IIIb	OXA-40
44	ICU	23-09-2013	BAL		IC2	IIIb	OXA-40
43	NRK	19-08-2013	urin	ST 1095	NT	IIIb	OXA-40
34	ICU	30-07-2013	Tissue sample		IC1	IIIc	OXA-40
30	Surgery	06-08-2014	Bone sample		IC1	IIIc	OXA-23
33	ICU	24-07-2013	BAL		IC2	IIIc	OXA-40
31	Surgery	11-07-2013	Urine		IC2	IIIc	OXA-23
41	ICU	28-10-2013	BAL		IC2	IIIc	OXA-40
38	Surgery	23-05-2013	Urine	ST 231	IC1	IIId	OXA-40
37	Surgery	06-01-2014	Urine		IC1	IIId	OXA-40
36	Surgery	03-01-2014	Wound swab		IC2	IIIe	OXA-40
25	Haematology	27-06-2014	Urine		NT	S	OXA-23

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5.REFERENCES

1. Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: Emergence of a successful pathogen. *Clin Microbiol Rev* 2008; 21: 538-82.
2. Poirel L, Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clin Microb Infect* 2006; 12: 826-36.
3. Higgins PG, Dammhayn C, Hackel M, Seifert H. Global spread of carbapenem-resistant *Acinetobacter baumannii*. *J Antimicrob Chemother* 2010: 65(2):233-8.
4. Robledo I, Aquino EE, Sante MI, Santana JL, Oterko DM, Leon CF, Vazqueiz GJ. Detection of KPC in *Acinetobacter* spp in Puerto Rico. *Antimicrob. Agents. Chemother.* 2010; 43:1354-1357.
5. Cornaglia G, Riccio ML, Mazzariol A, Laureatti L, Fontana R, Rossolini GM. Appearance of IMP-1 metallo-β-lactamase in Europe. *Lancet* 1999; 353: 899.
6. Amudham MS, Sekar U, Kamalanathan A, Balaraman S. *Bla*$_{IMP}$ and *bla*$_{VIM}$ mediated carbapenem-resistance in *Pseudomonas* and *Acinetobacter* species in India. *J. Infect Ctries* 2012; 6: 757-762.
7. El-Ageery S, Al-Hazmi SS. Microbiology and molecular detection of VIM-1 metallo-β-lactamase-producing *Acinetobacter baumannii*. *Eur. Rev. Med. Pharmacol. Sci.* 2014; 18:965-970.
8. Hrabak J, Stolobova M, Studentova V, Fridrichova M, Chudackova E, Zemlickova H. NDM-1 producing *Acinetobacter baumannii* isolated from a patient repatriated to the Czech Republic from Egypt, July 2011. *Euro. Surveill.* 2012; 16:E pub.
9. Poirel L, Naas T, Nordmann P. Diversity, Epidemiology, and Genetics of Class D β-Lactamases. *Antimicrob Agents Chemother* 2010; 54: 24-38.

10. Brown S, Amyes S. OXA β-lactamase in *Acinetobacter*: the story so far. *J Antimicrob Chemother* 2006; 57:1-3.
11. Higgins P, Poirel L, Lehmann M, Nordmann P, Seifert H. OXA-143, a novel carbapenem-hydrolyzingclass D β-lactamase in *Acinetobacter baumannii*. Antimicrob.Agents Chemother. 2009. 53:5035–5038.
12. Higgins PG, Perez-Llarena FJ, Zander E, Fernandez A, Bou G, Seifert H. OXA-235, a novel Class D β-Lactamase Involved in Resistance to Carbapenems in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2013; 57: 2121-2126.
13. Vranić-Ladavac M, Bedenić B, Minandri F, Ištok M, Frančula-Zaninović S, Ladavac, et al.Carbapenem-resistance and acquired class D carbapenemases in *Acinetobacter baumannii* from Croatia 2009-2010. *Eur J Clin Microbiol Infect. Dis* 2014;33:471-8.
14. Higgins PG, Lehmann M, Wisplinghoff H, Seifert H gyrB multiplex PCR to differentiate between *Acinetobacter calcoaceticus* and *Acinetobacter* genomic species 3. 2010; J Clin Microbiol 48: 4592-4594.
15. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 24th informational supplement. M100-S24 Clinical and Laboratory Standards Institute, Supplement M100S Wayne, PA, 2014.
16. Walsh TR, Bolmstrom A, Gales A. Evaluation of new E test for detecting metallo-β-lactamases in routine clinical testing. *J Clin Microbiol* 2002; 40:2755-2759.
17. Jarlier V, Nicolas MH, Fournier G, Philippon A. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. Rev Infect Dis 1988; 10(4): 867-878.

18. Woodford N, Ellington MJ, Coelho J, Turton J, Ward ME, Brown S, Amyes SG, Livermore DM. Multiplex PCR for genes encoding prevalent OXA carbapenemases. *Int J Antimicrob Agents* 2006; 27: 351-353.

19. Turton JF, Ward ME, Woodford N, Kaufman ME, Pike R, Livermore DM, Pitt TL. The role of IS*Aba1* in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. *FEMS Microbiol. Lett.* 2006; 258: 72-7.

20. Arlet G., Brami G., Deere D., Flippo A., Gaillot O., Lagrange P.H., et al. Molecular characterization by PCR restriction fragment polymorphism of TEM β-lactamases. *FEMS Microbiol Lett* 1995; 134:203-208.

21. Nüesch-Inderbinen M.T., Hächler H., Kayser F.H. Detection of genes coding for extended-spectrum SHV β-lactamases in clinical isolates by a molecular genetic method, and comparison with the E test. *Eur J Clin Microbiol Infect Dis* 1996; 15:398-402.

22. Woodford N., Ward M.E., Kaufmann M.E., Turton J., Fagan E.J., James D., Johnson A.P.,et al. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum β-lactamases in the UK. *J Antimicrob Chemother* 2004; 54:735-743.

23. Pagani L., Mantengoli E., Migliavacca R., Nucleo E., Pollini S., Spalla M.,et al. Multifocal detection of multidrug-resistant *Pseudomonas aeruginosa* producing PER-1 extended-spectrum β-lactamase in Northern Italy. *J Clin Microbiol* 2004; 42:2523-2529.

24. Bonnin RA, Nordmann P, Potron A, Lecuyer H, Zahar JR, Poirel L Carbapenemhydrolyzing GES-type extended-spectrum beta-lactamase in *Acinetobacter baumannii*. Antimicrob Agents Chemother 2011; 55:349-354

25. Elwell LP, Falkow S. The characterization of R plasmids and the detection of plasmid-specified genes. In: Lorian V, ed. Antibiotics in Laboratory Medicine. 2nd edn. Baltimore MD: Williams and Wilkins, 1986: 683-721.

26. Choi KH, Kumar A, Schweizer HP A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. Journal of Microbiological Methods 2006: 64: 391-397 .

27. Bertini A, Poirel L, Mugnier P, Villa J, Nordman P, Caratoli A. Characterization and PCR-based replicon typing of resistance plasmids in *Acinetobacter baumannii*. *Antimicrobial. Agents. Chemother* 2010 54:4168-4177.

28. Turton JF, Gabriel SN, Valderrey C, Kaufmann ME, Pitt TL. Use of sequence based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*. *Clin Microbiol Infect 2007*; 13:807-815.

29. Kaufman ME. Pulsed-Field Gel Electrophoresis. *Molecular bacteriology. Protocols and clinical applications. 1st edn. New York: Humana Press Inc. Totowa* 1998;33-51.

30. Magiorakos AP, Srinivasan A, Carey R.B, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2002; **18**:268-281.

31. **P**aton R, Miles RS, Hood J, Amyes SG, Miles RS, Amyes SG. ARI 1: β-lactamase-mediated imipenem resistance in *Acinetobacter baumannii*. *Int J Antimicrob Agents.* 1993 Feb; **2(2)**:81-7.

32. Towner KJ, Levi K, Vlassiadi M**.** Genetic diversity of carbapenem-resistant isolates of *Acinetobacter baumannii* in Europe. *Clin Microbiol Infect* 2007;**14**:161-167.

33. Stoeva T, Higgins P, Bojkova K, Seifert H. Clonal spread of carbapenem-resistant OXA-23-positive *Acinetobacter baumannii* in a Bulgarian university hospital. Clin Microbiol Infect 2008;14(7):723-726.

34. Zong Z, Lu X, Valenzuela JK, Partridge SR, Iredell J. An outbreak of carbapenem-resistant *Acinetobacter baumannii* producing OXA-23 β-lactamase. Int J Antimicrob Agents 2008;31(1):50-54.

35. Schleicher X, Higgins PG, Wisplinghoff H, Korber-Irrgang B, Kresken M, Seifert H: Molecular epidemiology of *Acinetobacter baumannii* and *Acinetobacter nosomialis* in Germany over a 5-year period (2005-2009). Clin Microbiol Infect 2012;E pub

36. Minadri F, D'Arezzo S, Antuneus LC, Pourcel C, Principe L, Petrosillo N, et al. . Evidence of diversity among epidemiologically related carbapenemase producing *Acinetobacter baumannii* strains belonging to International Clonal Lineage II. J Clin Microbiol 2012;50(3)590-597.

37. Izdebski R, Fiett J, Hryniewicz W, Gniadkowski M. Molecular analysis of *Acinetobacter baumannii* isolates from invasive infections in 2009 in Poland. J Clin Microbiol. 2012 Nov;50(11):3813-5. doi: 10.1128/JCM.02271-12. Epub 2012 Sep 12

38. Merino M, Poza M, Roca I, Barba MJ, Sousa MD, Vila J, Bou G. Nosocomial outbreak of a multiresistant *Acinetobacter baumannii* expressing OXA-23 carbapenemase in Spain**.** Microb Drug Resist. 2014 20(4):259-63

39. Coelho JM Turton JF, Kaufmann ME, Glover J, Woodford N, Warner M, Palepou MF, Pike R, Pitt TL. Ocurrence of carbapenem-resistant *Acinetobacter baumannii* clones at multiple hospitals in London and Southeast England. J Clin Microbiol 2006;44(10):3623-3627.

40. Bedenić B, Beader N, Godič-Torkar K, Vranić-Ladavac M, Luxner J, Veir Z, et al. . Nursing home as reservoir of carbapenem-resistant *Acinetobacter baumannii*. Microb Drug Resist 2015; 21(3):270-278.

41. Bou G, Oliver A, Martinez-Beltran J. OXA-24, a novel class D β-lactamase with carbapenemase activity in an *Acinetobacter baumannii* clinical strain. *Antimicrob Agents Chemother* 2000;44:1556-1561.

42. Nemec A, Križova L, Maixnerova M, Diancourt L, Van der Reijden TJK, Brisse S, et al. Emergence of carbapenem resistance in *Acinetobacter baumannii* in the Czech Republic is associated with the spread of multidrug-resistant isolates of European clone II. *J Antimicrob Chemother* 2008;**62**: 484-489.

43. Lolans K, Rice TW, Munoz-Price S, Quinn JP. Multicity outbreaks of carbapenem-resistant *Acinetobacter baumannii* isolates producing the carbapenamase OXA-40. Antimicrob Agents Chemother 2006;50:2941-2045.

44. Todorova B, Velinov T, Ivanov I, Dobreva E, Kantardijev T. First detection of OXA-24 carbapenemase-producing *Acinetobacte baumannii* in Bulgaria. World J Microbiol Biotechnol. 2014:30(4):1427-1430.

45. Dortet, L, BonNin RA, Bernbeu S, Escaut L, Vittecoq D, Girlich D, et al. First occurrence of OXA-72-producing Acinetobacter baumannii in Serbia. Antimicrob Agents Chemother 2016;23:60(10):5724-30.

46. Ning NZ, Liu X, Bao cm, Chen SM, Cui EB, Zhang JL, et al. Molecular epidemiology of $bla_{\text{OXA-23}}$-producing carbapenem resistant Acinetobacter baumannii in a single instituion over a 65-month period in north China. BMC Infect Fis 2017;17(1):

47. Lean SS, Yeo Suhali Z, Thong KL. Comparative genomics of two ST 195 carbapenem-resistant *Acinetobacter baumannii* with different susceptibility to polymyxin revealed underlying resistance mechanisms. Front Microbiol 2016:&:

48. Ratkai C, Quinteira S, Grosso F, Monteiro N, Nagy E, Peixe L. Controlling for false positives: interpreting MBL E test and MBL combined disk test for the detection of metallo-β-lactamases. J Antimicrob Chemother 2009; 64(3): . 657-658.