Comparison of clinical and sewage isolates of Acinetobacter baumannii from two long-term care facilities in Zagreb; mechanisms and routes of spread

Bedenić, Branka; Siroglavić, Marko; Slade, Mia; Šijak, Dorotea; Dekić, Svjetlana; Šeruga Musić, Martina; Godan-Hauptman, Ana; Hrenović, Jasna

Source / Izvornik: **Archives of Microbiology, 2020, 202, 361 - 368**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.1007/s00203-019-01750-9

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:692719

Comparison of clinical and sewage isolates of *Acinetobacter baumannii* from two long-term care facilities in Zagreb; mechanisms and routes of spread

Branka Bedenić[1,2], Marko Siroglavić[2], Mia Slade[4], Dorotea Šijak[2], Svjetlana Dekić[5], Martina Šeruga Musić[5], Ana Godan-Hauptman[6], Jasna Hrenović[5]

[1]Department of Microbiology, School of Medicine, University of Zagreb, Šalata 2, Zagreb, Croatia

[2]Clinical Department of Clinical and Molecular Microbiology, University Hospital Centre Zagreb, Kišpatić Street 12, Zagreb, Croatia

[3]Clinical Department of Transfusion Medicine and Transplantation Biology, University Hospital Centre Zagreb, Kišpatić Street 12, Zagreb, Croatia

[4]Croatian Institute for Transfusion Medicine, Petrova 3, Zagreb, Croatia

[5]Faculty of Science, University of Zagreb, Bijenička 30, Zagreb, Croatia

[6]Clinic for Internal Medicine, University Hospital Centre Zagreb, Kišpatić Street 12, Zagreb, Croatia

*Corresponding author: B. Bedenić, Department of Microbiology, School of Medicine, University of Zagreb, Zagreb, Croatia, tel: +385 1 2367304, fax: +385 1 4590 130, e-mail: branka.bedenic@kbc-zagreb.hr; bbedenic@mef.hr

Abstract

In the previous studies OXA-23-like and OXA-24-like β-lactamase were reported among *Acinetobacter baumannii* in both hospitals and long-term care facilities (LTCF) in Croatia. The aim of this study was to analyze clinical and sewage *A. baumannii* isolates from two nursing homes in Zagreb, with regard to antibiotic susceptibility and resistance mechanisms, in order to determine the route of spread of carbapenem resistant isolates. Nine clinical isolates were collected from February to May 2017 whereas in April 2017, ten *A. baumannii* isolates were collected from sewage of two nursing homes in Zagreb. Antibiotics susceptibility was determined by broth microdilution method. The presence of carbapenemase and extended spectrum β-lactamases (ESBL) encoding genes was explored by PCR. Conjugation and transformation experiments were performed as previously described. Genotyping was performed by SG determination, PFGE and MLST. Seven clinical isolates were positive for $bla_{\text{OXA24-like}}$ whereas two clinical and environmental carbapenem-resistant isolates, respectively, were found to possess $bla_{\text{OXA-23-like}}$ genes. Attempts to transfer imipenem resistance were unsuccessful indicating chromosomal location of $bla_{\text{OXA-23}}$ gene. All carbapenem-resistant isolates belonged to SG- 1 (IC-2) whereas the rest of the isolates susceptible to carbapenems were allocated to SG- 2 (IC-1). PFGE analysis revealed low degree of genetic variability within both IC- I and IC- II. MLST corroborated that two enviromental OXA-23 isolates belong to the ST-195. This study showed dissemination of OXA-23 producing *A. baumannii* from the nursing home into the urban sewage. Disinfection of nursing home sewage should be recommended in order to prevent the spread of resistance genes into the community sewage.

Key words: carbapenem, OXA-23, resistance, long-term care facility, Croatia

1. INTRODUCTION

Acinetobacter baumannii is an important hospital pathogen causing severe infections affecting not only hospitalized patients, but also residents in the long-term care facilities (LTCF). Resistance to carbapenems is challenge for clinicians due to limited therapeutic options. It can be attributable to impaired permeability, upregulation of efflux pumps or alternation in penicillin-binding proteins (Poirel et al. 2006). However, the most common mechanism od resistance is the production of carbapenemases belonging to class D (OXA enzymes), known as CHDL (carbapenem-hydrolyzing class D oxacillinases) (Brown et al. 2006) which are classified into five phylogenetic groups (OXA-51-like enzyme and the acquired enzymes: OXA-23-like, OXA-24/40-like, OXA-58- like, OXA-143-like and OXA-235-like (Brown et al. 2006, Higgins et al. 2009), and rarely class A such as KPC (Robledo et al. 2010) or GES (Bonnin et al. 2011) and class B (metallo-β-lactamases of IMP (Cornaglia et al. 1999), VIM (Perilli et al. 2011), SIM (Lee et al. 2005) or NDM family (Hrabak et al. 2011).

Carbapenem resistance in *A. baumannii* in Croatia emerged in early 2000-ties (Goić-Barišić et al. 2007) and was found to be mediated by hyperproduction of OXA-51 due the IS*Aba1* location upstream of the genes (Goić-Barišić et al. 2009). Acquired carbapenem resistance in Croatia was attributed to the emergence of OXA-72 producing isolates in 2008-2009 in Split and Zagreb University Hospitals (Goić-Barišić et al. 2011, Franolić-Kukina et al. 2011). In 2008 and 2009 carbapenem resistant *A. baumannii* spread in most hospital centers in different geographic Croatia and became an important challenge for clinicians. These observations prompted us to carry out the multicenter study conducted in Northtern Croatia and Istria in 2009-2010. The study found CHDL belonging to OXA-24-like and OXA-58-like group to be the dominant mechanism of carbapenem resistance in Croatia (Vranić-Ladavac et al. 2014). The emergence of carbapenem resistant *A. baumannii* was observed in Godan nursing home

in Zagreb in 2013 (Bedenić et al. 2014). The isolates were positive for OXA-23 and OXA-24/40-like CHDL. At the same time dissemination of multiresistant *A. baumannii* was observed in the acid and petroleum-contaminated soil influenced by illegally disposed solid waste in Croatia (Hrenović et al, 2014) and in the urban wastewater treatment plant (Hrenović et al, 2017), showing the spread of carbapenem resistant isolates into the community. The first environmental isolates positive for CHDL (OXA-72) of probable clinical origin were found in the treated and untreated urban wastewater in Zagreb in 2016 (Goić-Barišić et al, 2014) indicating a significant source for horizontal gene transfer and implications for wider spread of antibiotic resistance genes. Further studies found close relatedness of clinical and environmental isolates which suggests the emission of extensively-drug-resistant (XDR) *A. baumannii* via the untreated hospital wastewater in the natural environment (Šeruga-Musić et al. 2017). *Polydesmus* sp. is a millipede belonging to the phyllum Arthropoda, subphyllum Myriapoda and class Diplopoda. They are mainly cylindrically shaped terrestrial animals up to 28 cm long that feed on decomposing plant material, mainly leaves, wood or both (Mrsić, 1997). Millipedes inhabit moist habitats and can feed on mammalian feces, fallen fruits and even dead invertebrates in search of more nutritious food (David, 2019). Urbanization causes alterations in the natural habitats, which are destroyed or fragmented. However, new habitats are created and new ecological niches opened forcing animals to adapt.

Although there were many studies on environmental pool of multidrug and extensively drug resistant *A. baumannii* in Croatia, there are no reports on the role of long-term care facilities as a source of environmental contamination with carbapenem resistant isolates and no reports on arthropods playing role in dissemination of multiresistant *A. baumannii*. The aim of our study was to analyze sewage water coming from two nursing homes in Croatia and to compare clinical and environmental isolates in order to determine the route of spread of carbapenem-resistant *A. baumannii.*

2. MATERIALS AND METHODS

2.1.Bacterial isolates

The consecutive non-duplicate (one per patient) clinical isolates were collected from 1t February to 30th May 2017 from two nursing homes in Zagreb: Godan and Lav Švarc (Kvatrić) located in different city regions in Zagreb. The nursing home sewage from these two institutions (Godan and Kvatrić nursing home, Zagreb, Croatia) was collected at the central manhole on two occasions in April at 9 a.m. The nursing home sewage is discharged into the urban sewage system without pretreatment. The sewage was aseptically taken in sterile 1L glass bottles and analysed within 1h. *A. baumannii* was isolated on CHROMagar Acinetobacter supplemented with CR102 after incubation at 42°C/48h. Ten *A. baumannii* isolates were collected from sewage of a nursing home. The isolates were identified by Gram-staining growth at 42 C, catalase and oxidase test and other conventional biochemical tests, and by MALDI-TOF and confirmed as *A. baumannii* by PCR for intrinsic, chromosomal $bla_{\text{OXA-51}}$ gene.

2.2.Antimicrobial susceptibility testing

The antimicrobial susceptibility to a wide range of antibiotics (piperacillin alone and combined with tazobactam, sulbactam/ampicillin, ceftazidime, cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, gentamicin, amikacin, ciprofloxacin and colistin) was determined by broth microdilution method in Mueller-Hinton broth in 96 well microtiter plates according to CLSI guidelines (CLSI, 2016). *Pseudomonas aeruginosa* ATCC 27853 was used as quality control strain. Minimum inhibitory concentrations (MICs) of imipenem and meropenem were determined also by agar dilution with addition of cloxacillin (200 mg/L)

and sodium chloride in order to tentatively determine the effect of chromosomal AmpC β-lactamase on the susceptibility to carbapenems and to detect OXA-58 group which is susceptible to inhibition with sodium chloride, respectively (Pournaras et al. 2006). The susceptibility to tigecycline was determined by E-test. Carbapenem resistant isolates were screened for the hyperexpression of efflux pumps. Efflux pump overexpression was initially screened for by testing susceptibility to imipenem and meropenem in the presence and absence of an efflux pump inhibitor, carbonylcyanide-m-chlorophenylhydrazone (CCCP). MICs of imipenem and meropenem were determined by agar dilution method with and without CCCP added in MH agar at concentrations of 12.5 mM. Modified Hodge test (MHT) and CIM were used to screen for the production of carbapenemases in carbapenem resistant strains (Lee et al. 2005, Van der Zwaluw et al. 2015).

The production of MBLs was detected in carbapenem-resistant isolates by combined disk test with EDTA (MBL) (Lee et al. 2005). Extended spectrum β-lactamases were detected in all isolates by combined disk test with cephalosporins and clavulanic acid according to CLSI with addition of cloxacillin in the medium (200 mg/L) (CLSI, 2016) to inhibit the chromosomal AmpC β-lactamase which can antagonize the synergistic effect with clavulanate. Isolates were defined as multidrug-resistant (MDR), extensively drug resistant (XDR) or pandrug resistant (PDR) according to Magiorakos et al. (Magiorakos et al. 2002).

2.3.Molecular characterization of β-lactamases

The presence of the genes encoding KPC (Robledo et al. 2010), MBLs of IMP, VIM, SIM and NDM series (Poirel, 2011) OXA β-lactamases (*bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-24/40-like}}$, *bla*$_{\text{OXA-58-like}}$ and *bla*$_{\text{OXA-143-like}}$) genes (Woodford, 2006) and extended-spectrum β-lactamases (ESBL) of TEM (Arlet et al. 1995), SHV (Neusch-Inderbinen et al. 1996), CTX-M

(Woodford et al. 2004), PER (Pagani et al. 2004), and GES family (Bonnin et al. 2011) was determined by PCR as previously described. The amplicons of the selected carbapenem resistant strains (strain 41378, Kvatrić 1 and Kvatrić 4) were column purified (QIAquick PCR purification kit, Inel Medicinska tehnika, Zagreb) and subjected to sequencing in 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. Reference strains producing OXA-23-like, OXA-24/40-like, OXA-58-like, and OXA-143-like β-lactamases were used as positive control strains for PCR. The strains were kindly provided by Dr. Paul Higgins (University of Cologne, Cologne, Germany). The reference strains positive for CTX-M-15, CTX-M-2 and CTX-M-9 were provided by Dr. Neil Woodford (Health Protection agency, London, UK) while reference strains positive for SHV-1 and TEM-1 were obtained from Prof. Adolf Bauernfeind (Max von Pettenkofer Institute, Munich, Germany). The genetic context of *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{OXA-23,}}$ genes was determined by PCR mapping with primers for IS*Aba1* combined with forward and reverse primers for *bla*$_{\text{OXA-51}}$ and *bla*$_{\text{OXA-23}}$ (Turton et al. 2006).

2.4.Characterization of plasmids

Conjugation experiments were performed using *E. coli* J 53 resistant to sodium azide (Elwel, Falkow. 1986). To determine if acquired oxacillinase genes were plasmid-encoded, plasmid DNA was extracted from carbapenem resistant strains and transferred to $CaCl_2$ treated *Acinetobacter baumannii* ATCC 17606 by transformation. Transformants were selected on MacConkey agar containing 10 μg/ml imipenem (Elwel, Falkow. 1986). Plasmids were extracted with Macherey Nagel kit and used as template for the PBRT determination.

Plasmid incompatibility groups were determined by PCR based replicon typing (PBRT) according to Bertini et al. (Bertini et al. 2010). Multiplex PCR was performed with primers specific for six incompatibility groups of plasmids found in *A. baumannii*.

2.5.Molecular typing of isolates

Sequence groups (SGs 1-3) corresponding to international clonal lineages (ICL I-III) determination was performed according to the procedure described by Turton et al. (Turton et al. 2007).

Pulsed field genotyping of *Apa*I digested genomic DNA was performed on all environmental and eight clinical isolates with the CHEF-DRIII system (Bio-Rad) (Kaufman. 1998); the images were processed using the Gel-Compar software, and a dendrogram was computed after band intensity correlation using global alignment with 1.5% optimization and tolerance by UPGMA (unweighted pair-group method using arithmetical averages) clustering. The strains were considered to be clonally related if they showed more than 80% similarity of their PFGE patterns (Tenover et al. 1995).

The Oxford MLST scheme encompassing seven housekeeping genes (*gltA, gyrB, gdhB, recA*, *cpn60, gpi* and *rpoD*) was used to type one clinical and two environmental carbapenem resistant isolates, using primers and conditions as described at PubMLST. Amplicons obtained by PCR (ProFlex™ 96-Well PCR System, Applied Biosystems) were sequenced on both strands (commercial service Macrogen Europe, the Netherlands). The assembly and manual editing of raw nucleotide sequences was performed using Geneious software. The sequence type (ST) were determined using allele sequences and profiles retrieved from the *A. baumannii* MLST website.

3. RESULTS

3.1.Isolates

Two nursing homes in Zagreb (Godan and Kvatrić) were enrolled in the study. In total, 9 clinical isolates were obtained from the Godan nursing home and 10 environmental isolates were collected from sewage water (three from Godan and seven from Kvatrić). The Kvatrić nursing home did not have any clinical isolates in the study period. All isolates were identified as *A. baumannii* by conventional biochemical testing and MALDI-TOF and confirmed by PCR for $bla_{\text{OXA-51}}$ genes.

3.2.Antimicrobial susceptibility testing and phenotypic tests for detection of β-lactamases

Clinical isolates

All clinical isolates were resistant to ceftazidime, cefotaxime, ceftriaxone, cefepime, imipenem, meropenem, gentamicin and ciprofloxacin and uniformly susceptible to colistin and tigecycline. One strain showed intermediate susceptibility to ampicillin/sulbactam whereas the rest were classified as susceptible (Table 1a).

Environmental isolates

Out of ten, eight isolates (80%) were resistant to gentamicin and ciprofloxacin, four (40%) to ceftazidime and cefepime and two (20%) to carbapenems as shown in Table 1b. There was uniform susceptiblity to colistin and tigecyline (Table 1b).

Eight environmental isolates were classified as MDR as they were resistant to at least one antibiotic in three classes. All clinical and two environmental isolates (Kvatrić 1 and Kvatrić

4) were assigned to XDR category since they were susceptibile only to colistin and tigecycline.

The addition of either sodium chloride or cloxacillin did not lower the MICs of carbapenems for more than one dilution indicating the lack of OXA-58 and hyperproduction of chromosomal AmpC β-lactamase. CCCP did not lower the carbapenem MICs either, excluding the effect of hyperexpression of efflux pumps on carbapenem MICs.

Carbapenem susceptible isolates, in contrast to carbapenem resistant isolates, yielded an increase of the inhibition zone around ceftazidime and cefepime disk of 10 to 15 mm with addition of clavulanic acid in combined disk test, indicating production of an ESBL. All carbapenem resistant strains were positive in Hodge and CIM test and demonstrated enlargement of the inhibition zone around imipenem disk supplemented with EDTA indicating the production of an MBL.

3.3.Molecular characterization of β-lactamases

All isolates were positive for chromosomal *bla*$_{\text{OXA-51}}$ gene. Seven carbapenem-resistant clinical isolates tested positive for *bla*$_{\text{OXA-24-like}}$ whereas two clinical and environmental isolates respectively (44087, 55754, Kvatrić 1 and Kvatrić 4) were found to possess *bla*$_{\text{OXA-23-like}}$ genes (Table 1a and b). Sequencing of representative *bla*$_{\text{OXA-24-like}}$, (strain 41378), *bla*$_{\text{OXA-23-like}}$ (Kvatrić 1 and Kvatrić 4) and *bla*$_{\text{OXA-51-like}}$ (Kvatrić 1 and Kvatrić 4) genes identified OXA-72, OXA-23 and OXA-66 allelic variants, respectively. IS*Aba1* was found upstream of *bla*$_{\text{OXA-51-like}}$ in all clinical and seven out of ten environmental isolates and upstream of the *bla*$_{\text{OXA-23-like}}$ in two environemntal strains. PCR for ESBLs and MBLs found in *A. baumannii* so far, was negative.

3.4.Characterization of plasmids

Attempts to transfer imipenem resistance by both conjugation and transformation were unsuccessful indicating chromosomal location of *bla*$_{OXA-23}$ and *bla*$_{OXA-24}$ genes. However, the plasmids extracted from four isolates positive for OXA-23 belonged to Inc group 6 encoding aci6-replicase gene.

3.5.Molecular typing of isolates

The carbapenem susceptible environmental isolates belonged to IC- I (SG-2), whereas carbapenem resistant clinical and environmental strains belonged to IC -II (SG- 1). Clinical isolates clustered together in one large clone with >80% similarity which contained four subtypes differing in one to two bands, as shown in Fig 1. There were three identical isolates (41834, 33979 and 41378). The clinical isolate 41378 was allocated to ST-2 by MLST.

PFGE performed on environmental isolates revealed all except one carbapenem-susceptible isolate to belong to one large cluster containing three subclusters differing in two to three bands and one containing two identical isolates (Kvatrić 6 and Kvatrić 7) with almost identical antibiotic susceptibility patterns (Fig. 1). Two carbapenem resistant isolates (Kvatrić 1 and Kvatrić 4) and one susceptible isolates (Godan 2) turned out to be singletons as shown in Fig. 2. Comparison of PFGE profiles of clinical and environmental isolates revealed two distinct clones: one containing clinical and the other environmental isolates. One environmental OXA-23 positive organism (K4) clustered together with the clinical isolates (Fig. 1). Additional MLST analysis corroborated that two OXA-23 environmental carbapenem-resistant isolates belong to the ST-195 clustering into the CC92 within the IC2, commonly reported worldwide.

4. DISCUSSION

The main finding of the study is the predominance of carbapenem susceptible *A. baumannii* isolates among those isolated from the nursing home sewage water in contrast to clinical isolates which were all carbapenem-resistant. The possible explanation is that environmental isolates originate from digestive tract or skin of the residents as the part of the microbiota. In contrast, clinical isolates obtained from the relevant clinical specimens such as urine or wound swabs have acquired resistance determinants under the selective pressure of antibiotics. OXA-23 and OXA-24 β-lactamases were previously reported in the clinical specimens from the same long-term care facility (Bedenić et al. 2014). OXA-23 was the first CHDL reported in 1986 in the UK as ARI-1 (Paton, 1993). Later it was renamed to OXA-23. OXA-24-like carbapenemases are the most prevalent in Croatia, with OXA-72 being the sole allelic variant reported sor far, in both clinical and environmental isolates [11-14,18-19]. All OXA-24-like and four OXA-23 positive isolates were endowed with high level of resistance to carbapenems and categorized as XDR, similarily as in the previous reports. Susceptibility to non-β-lactam antibiotics did not differ significantly between carbapenem resistant and carbapenem susceptible isolates. Although OXA-23 is typically plasmid mediated the conjugation and transformation experiments did not work out but we managed to identify the plasmid belonging to group 6 according to Bertini et al (Bertini et al. 2011). However, the presence of IS*Aba1-bla*$_{\text{OXA-23}}$ element in genetically unrelated isolates corroborated the transferable nature of this carbapenem-resistant determinant. Moreover, insertion elements functions as a promoter enhancing expression of OXA-23 encoding genes. All isolates positive for acquired CHDL were identified as carbapenemase producers by Hodge test and CIM test showing good correlation between phenotypic and molecular methods for detection of carbapenemases. However, inhibitor based tests with clavulanic acid and EDTA yielded

false positive results. Similar observation of false positive MBL test in strains positive for CHDL was previously reported by Vilalon et al (Vilalon et al. 2011). The possible explanation is that in the presence of EDTA, oxacillinase change to a less active state, leading to a drastic reduction in MIC or augmentation of inhibition zone. Imipenem susceptible isolates were positive in phenotypic test for ESBLs but PCR was negative for ESBLs reported in *A. baumannii* so far. The possible explanation is the production of the new type of ESBLs or false positive phenotyptic testing. PFGE revealed a low degree of genetic variability within both IC-1 and IC- II population, irrespective of the antibiotic susceptibility pattern. Most sewage isolates were susceptible and unrelated to the clinical isolates indicating that the clinical isolates probably arouse *de novo* and not by acquisition of the resistant determinants by susceptible isolates. Comparison of PFGE banding patterns of environmental and clinical isolates showed no genetic relatedness except for one OXA-23 organism from sewage which clustered together with the clinical isolates. OXA-23 positive isolates belonged to ST-195 which was previously reported from clinical isolates of *A. baumannii* from Pula in Croatia 2013 (Ladavac et al. 2018). OXA-23 positive clinical isolates *A. baumannii* from investigated nursing home were previously found to belong to ST-487, confirming the emergence of a new clone. ST-2 previously identified in one clinical isolate was previously the dominant ST in Croatia [Vranić-ladavac, 2014]. Two typing methods were performed because PFGE enables the outbreak analysis whereas MLST as portable methods allows international and interlaboratory comparison of isolates. Although OXA-24-like is the dominant type of CHDL among clinical isolates, the sewage water contained only OXA-23 positive organisms. Interestingly, the Kvatrić nursing home did not have any clinical carbapenem-resistant isolates in the study period but had two environmental OXA-23 positive strains. On the other hand, Godan nursing home had nine carbapenem-resistant clinical isolates but had only carbapenem susceptible strains in the sewage water. Carbapenem-resistant isolates of *A.*

baumannii were previously identified in nursing homes in China [Cheng et al. 2016) and Taiwan (Lee et al. 2017), but no molecular characterization of resistance mechanism was performed. Moreover, there are no reports on the *A. baumannii* from the nursing home sewage and the OXA-23 was documented for the first time in the sewage in this study. High resistance rates to gentamicin and ciprofloxacin were observed also in the strains without acquired β-lactam resistance determinants.

Interestingly, one strain was isolated from *Polydesmus* indicating a possibility of an arthropod mediated dissemination of *A. baumannii* which is reported for the first time in this study. OXA-23 confers high level of carbapenem-resistance and provides selective advantage in an antibiotic rich environment such as long-term care facilities. The limitation of the study is relatively small number of isolates and centers included in the study. However, the strength of the study is the detailed analysis of resistance phenotypes and molecular detection of resistance mechanisms and genotypes. Multicenter studies should be undertaken in the future to cover nursing homes in all geographic regions in Croatia. This study showed dissemination of OXA-23 producing *A. baumannii* from the nursing home into the urban sewage. Disinfection of nursing home sewage should be recommended in order to prevent the spread of resistance genes into the community sewage.

Funding

This work has been supported in a part by the Croatian Science Foundation (project no. IP-2014-09-5656).

Competing interests

None declared.

Ethical approval

Not necessary.

References

Arlet G, Brami G, Deere D, Flippo A, Gaillot O, Lagrange PH (1995) Molecular characterization by PCR restriction fragment polymorphism of TEM β-lactamases. FEMS Microbiol Lett 134: 203-208.

Bedenić B, Beader N, Godič-Torkar K, Vranić-Ladavac M, Luxner J, Veir Z, Zarfel G, Grisold A (2014) Nursing home as reservoir of carbapenem-resistant *Acinetobacter baumannii,* Microb Drug Resist 21: 270-278.

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

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

Brown S, Amyes S (2006) OXA β-lactamase in *Acinetobacter*: the story so far. J Antimicrob Chemother 57: 1-3

Cheng VC, Chen JH, Ng WC, Wong JY, Chow DM, Law TC. (2016) Emergence of Carbapenem-Resistant *Acinetobacter baumannii* in Nursing Homes With High Background Rates of MRSA Colonization. Infect Control Hosp Epidemiol 37: 983-6.

Clinical and Laboratory Standards Institute (2016) Performance Standards for Antimicrobial Susceptibility Testing; 23rd Informational SupplementWayne, PA: CLSI, M100-S23.

Cornaglia G, Riccio ML, Mazzariol A, Lauretti L, Fontana R, Rossolini GM (1999) Appearance of IMP-1 metallo-β-lactamase in Europe. Lancet 353: 899-900

David JF (2019) Ecology of millipedes (Diplopoda) in the context of global change. Soil organisms 81: 719-733.

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

Franolić-Kukina I, Bedenić B, Budimir A, Herljević Z, Vraneš J, Higgins P (2011) Clonal spread of carbapenem-resistant OXA-72 positive *Acinetobacter baumannii* in a Croatian University Hospital. Int J Infect Dis 15: 706-709.

Goić-Barišić I, Bedenić B, Tonkić M, Katić S, Kalenić S, Punda-Polić V (2007) Molecular characterisation of carbapenem resistant *Acinetobacter baumannii* in different Intensive Care Units in University Hospital Split, Croatia. J Chemother 2; 19: 462-464

Goić-Barišić I, Bedenić B, Tonkić M, Novak A, Katić S, Kalenić S, Punda Polić V (2009) Occurrence of OXA-107 and IS*Aba 1* in carbapenem-resistant isolates of *Acinetobacter baumannii* from Croatia. J Clin Microbiol 47: 3348-3349

Goić-Barišić I, Hrenović J, Kovačić A, Šeruga-Musić M (2016) Emergence of Oxacillinases in Environmental Carbapenem-Resistant *Acinetobacter baumannii* Associated with Clinical Isolates. Microb Drug Resist 22: 559-563

Goić-Barišić I, Towner KJ, Kovačić A, Šiško-Kraljević K, Tonkić M, Novak A, Towner K (2011) Outbreak in Croatia caused by a new carbapenem-resistant clone of *Acinetobacter baumannii* producing OXA-72 carbapenemase. J Hospit Infect 77: 68-369

Higgins P, Poirel L, Lehmann M, Nordmann P, Seifert H (2009) OXA-143, a novel carbapenem-hydrolyzing class D β-lactamase in *Acinetobacter baumannii.* Antimicrob Agents Chemother 53: 5035-5038

Hrabak J, Stolobova M, Studentova V, Fridrichova M, Chudackova E, Zemlickova H (2011) NDM-1 producing *Acinetobacter baumannii* isolated from a patient repatrieted to the Czech Republic from Egypt Eurosurveillance 16: 17 (7)

Hrenović J, Durn G, Goić-Barišić I, Kovačić A (2014) Occurrence of an environmental *Acinetobacter baumannii* strain similar to a clinical isolate in paleosol from Croatia. Applied and Environmental Microbiology 80: 2860-2866.

Hrenović J, Durn G, Šeruga-Musić M, Dekić S, Troskot-Čorbić T, Škorić D (2017) Extensively and multi drug-resistant *Acinetobacter baumannii* recovered from technosol at a dump site in Croatia. Science of the Total Environment 607-608 1049-1055.

Hrenović J, Goić-Barišić I, Kazazić S, Kovačić A, Ganjto M, Tonkić M (2016) Carbapenem-resistant isolates of *Acinetobacter baumannii* in a municipal wastewater treatment plant, Croatia, Euro Surveill 14;21(15). doi: 10.2807/1560-7917

hydrolyzing GES-type extended-spectrum β-lactamase in *Acinetobacter baumannii*.

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

Ladavac R, Bedenić B, Vranić-Ladavac M, Barišić N, Karčić N, Pompe K. (2017) Emergence of different *Acinetobacter baumannii* clones in a Croatian hospital and correlation with antibiotic susceptibility. J Glob Antimicrob Resist 10: 213-218.

Lee CM, Lai CC, Chiang HT, Lu MC, Wang LF, Tsai TLal. (2017) Presence of multidrug-resistant *A. baumannii* in the residents and environments of long-term care facilities in Taiwan. J Microbiol Immunol Infect 50: 133-144.

Lee K, Jum JH, Yong D, Lee HM, Kim HD, Docquier JD (2005) Novel acquired metallo-β-lactamase gene, $bla_{(SIM-1)}$ in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob Agents Chemother 49: 4485-91

Lee K, Lim YS, Yong D, Yum JH, Chong Y (2005) Evaluation of the Hodge test and the imipenem-EDTA-double-disk synergy test for differentiating metallo-β-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbiol 2005; 41: 4623-4629

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG (2002). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 18: 268-281.

Mrsić N (1997) Uvod v pedozoologijo-sistematika in ekologija s splošnim pregledom talnih živali. Tehnička založba Slovenije, Ljubljana.

Nüesch-Inderbinen MT, Hächler H, Kayser FH (1996) 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 15: 398-402.

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

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

Perilli M, Pelegrini C, Calenza G, Segatore B, Amicosante G (2011) First report from Italy of $bla_{\text{VIM-1}}$ and $bla_{\text{TEM-1}}$ genes in *Pseudomonas putida* and *Acinetobacter baumannii* isolated from wastewater. J Chemother 23: 181-2

Poirel L, Nordmann P (2006) Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. Clin Microb Infect 12: 826-36

Poirel L, Walsh TR, Cuveiller V, Nordman P (2011) Multiplex PCR for detection of acquired carbapenemases genes. Diagn Microbiol Infect Dis 70: 119-125.

Pournaras S, Markogiannakis A, Ikonomidis A, Kondyli L, Bethimouti K, Maniatis AN (2006) Outbreak of multiple clones of imipenem-resistant *Acinetobacter baumannii* isolates expressing OXA-58 carbapenemase in an intensive care unit. J Antimicrob Chemother 57: 557-561

Robledo IE, Aquino EE, Sante MI, Santana JL, Oterko DM, Leon CF (2010) Detection of KPC in *Acinetobacter* spp in Puerto Rico. Antimicrob Agents Chemother 2010; 43: 1354-1357

Šeruga-Musić M, Hrenović J, Goić-Barišić I, Hunjak B, Škorić D, Ivanković T (2017) Emission of extensively-drug-resistant *Acinetobacter baumannii* from hospital settings to the natural environment. J Hosp Infect 96: 323-327

Tenover F, Arbeit R, Goering RV, Mickelsen A, Murray BE, Persing DH et al. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33: 2233-9.

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

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

Van der Zwaluw K, de Haan A, Pluister GN, Bootsma HJ, de Neeling AJ, Schouls LM (2005) The carbapenem-inacitvation method (CIM) a simple and low-cost alternative for the Carba NP test o assess phenotypic carbapenemase activity in Gram-negative rods. Plos One 10: 3.

Vilalon P, Valdezete S, Medina-Pascual MJ, Curasco G, Vindel A, Saez-Nieto JA (2011) Epidemiology of the *Acinetobacter*-derived cephalosporinase, carbapenem-hydrolyzing

oxacillinase and metallo-β-lactamase genes, and of common insertion sequences, in epidemic clones of *Acinetobacter baumannii*. J Antimicrob Chemother 68: 550-553.

Vranić-Ladavac M, Bedenić B, Minandri F, Ištok IM, Bošnjak Z, Frančula-Zaninović S, Visca P (2014) Carbapenem resistance and acquired class D beta-lactamases in *Acinetobacter baumannii* from Croatia 2009-2010. Eur J Clin Microbiol Infect Dis 33: 471-8.

Woodford N, Ellington MJ, Coelho J, Turton J, Ward ME, Brown S (2006). Multiplex PCR for genes encoding prevalent OXA carbapenemases. Int J Antimicrob Agents 2006; 27: 351-353.

Woodford N, Ward ME, Kaufmann ME, Turton J, Fagan EJ, James D (2004). Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum β-lactamases in the UK. J Antimicrob Chemother 54: 735-743.

Table 1a Antibiotic susceptibility, β-lactamase production and genotypes of *A. baumannii* isolates from clinical isolates.

Abbreviations; TZP-piperacillin/tazobactam; SAM; sulbactam/ampicillin; CAZ-ceftazidime; CRO-ceftriaxone; FEP-cefepime;IMI-imipenem; MEM-meropenem; GM-gentamicin; CIP-ciprofloxacin; TGC-tigecycline; COL-colistin; ST-sequence type; IC-International clonal lineage; BL: beta-lactamase content

		Minimum inhibitory concentrations (MIC) mg/L														
Strain number	Date of isolation	CAZ	CTX	CRO	FEP	TZP	SAM	IMI	MEM	GM	CIP	TGC	COL	BL	IC	Specimen
(33979)	20.02.2017.	>128	>128	>128	32	>128	8	>128	>128	>128	>128	1	0.5	OXA-24-like	IC II	urin
(33980)	20.02.2017.	>128	>128	>128	64	>128	8	64	>128	>128	>128	1	1	OXA-24-like	IC II	Wound swab
(33981)	20.02.2017	>128	>128	>128	32	>128	16	>128	>128	>128	>128	1	1	OXA-24-like	IC II	Throat swab
(41378)	02.03.2017.	>128	>128	>128	>128	>128	8	64	>128	>128	>128	2	2	OXA-24-like	IC II (ST2)	urine
(41834)	03.03.2017.	>128	>128	>128	>128	>128	8	64	>128	>128	>128	2	2	OXA-24-like	IC I I	urine
(43581)	06.03. 2017.	>128	>128	>128	>128	>128	8	64	>128	>128	>128	2	2	OXA-24-like	IC II	Wound swab
(44087)	07.03.2017.	>128	>128	>128	>128	>128	8	32	>128	>128	>128	0.5	2	OXA-23-like	IC II	urine
(50072)	15. 03. 2017.	>128	>128	>128	>128	>128	8	32	>128	>128	>128	0.5	1	OXA-24-like	IC II	Wound swab
(55754)	24. 03. 2017.	>128	>128	>128	>128	>128	8	32	>128	>128	>128	1	0.5	OXA-23-like	IC II	Canulla swab

Table 1b Antibiotic susceptibility, β-lactamase production and genotypes of *A. baumannii* isolates from nursing home sewage water.

Abbreviations; TZP-piperacillin/tazobactam; SAM; sulbactam/ampicillin; CAZ-ceftazidime; CRO-ceftriaxone; FEP-cefepime;IMI-imipenem; MEM-meropenem; GM-gentamicin; CIP-ciprofloxacin; TGC-tigecycline; COL-colistin; ST-sequence type; IC-International clonal lineage; BL: beta-lactamase content

		Minimum inhibitory concentrations (MIC) mg/L														
Strain number	Date of isolation	CAZ	CTX	CRO	FEP	TZP	SAM	IMI	MEM	GM	CIP	TGC	COL	BL	IC	PFGE and ST
Poli 1 (P1)	24.4.2017	1	>128	>128	1	16	8	0.12	0.25	1	0.25	1	0.5	OXA-51-like	IC I	Ia
Godan 1 (G1)	24.4.2017	2	>128	>128	1	8	4	0.25	0.25	0.5	0.12	1	1	OXA-51-like	IC I	Ib
Godan 2 (G2)	24.4.2017	>128	>128	>128	>128	>128	16	0.5	2	>128	>128	2	1	OXA-51-like	IC I	S
Kvatrić 1 (K1)	2.5.2017	>128	>128	>128	>128	>128	16	32	>128	>128	>128	0.5	2	OXA-66, OXA-23	IC II	S, ST195
Kvatrić 2 (K2)	2.5.2017	16	>128	>128	16	64	4	0.5	1	>128	>128	0.5	1	OXA-51-like	IC I	Ic
Kvatrić 3 (K3)	2.5.2017	32	>128	>128	8	64	4	0.5	1	>128	>128	0.5	1	OXA-51-like	IC I	Ie
Kvatrić 4 (K4)	2.5.2017	>128	>128	>128	>128	>128	8	32	>128	>128	>128	0.5	2	OXA-66, OXA-23	IC II	S, ST195
Kvatrić 5 (K5)	2.5.2017	2	>128	>128	1	16	2	0.5	2	>128	>128	2	0.5	OXA-51-like	IC I	Id
Kvatrić 6 (K6)	2.5.2017	4	>128	>128	2	16	8	0.5	2	>128	>128	1	0.5	OXA-51-like	IC I	Ib
Kvatrić 7 (K7)	2.5.2017	4	>128	>128	1	32	8	0.25	1	>128	>128	2	0.5	OXA-51-like	IC I	Ib

Figure legend

Fig. 1 Comparison of environmental and clinical *A. baumannii* isolates. Date of isolation, source, specimen, protocol number, type of CHDL and international clonal lineage (IC) is shown. All clinical isolates belong to one large cluster. All except two environmental isolates clustered in one clone The environmental OXA-23 producing strain clusters together with the clinical isolates. Cut-off value of 80% is applied to determine the clone. P: Polydesmus; G: Godan nursing home; K: Kvatrić nursing home.

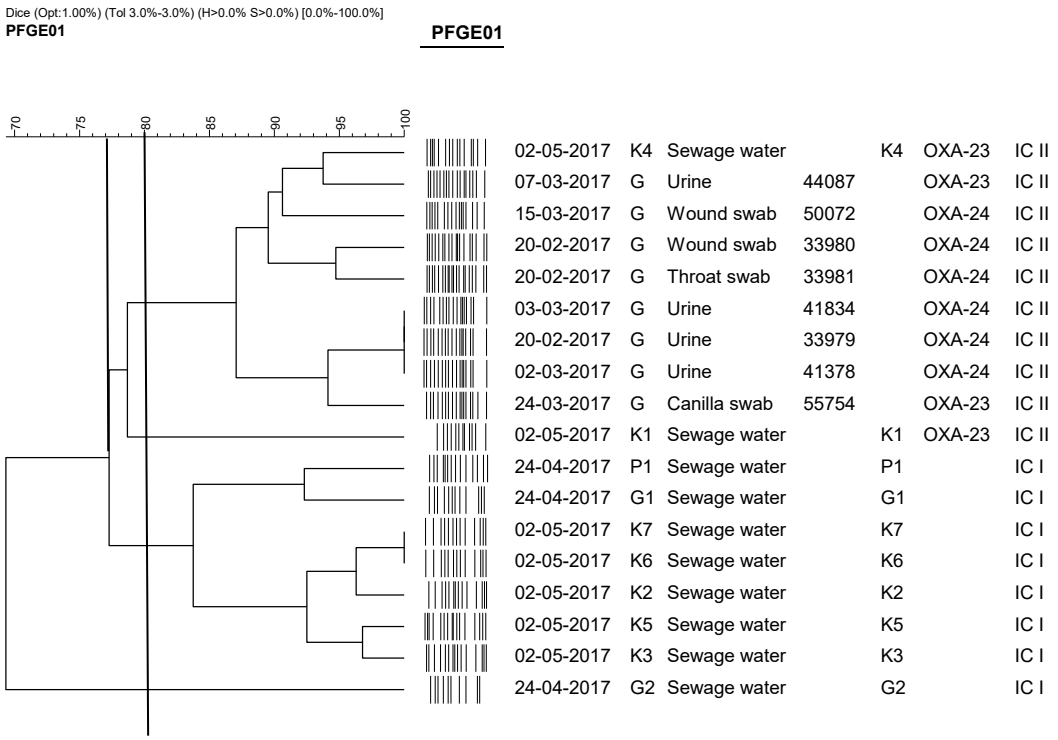
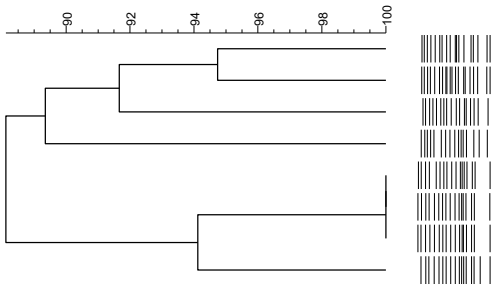


Fig. 1. Comparison of environmental and clinical *A. baumannii* isolates. Date of isolation, source, specimen, protocol number, type of CHDL and international clonal lineage (IC) is shown. All clinical isolates belong to one large cluster. All except two environmental isolates clustered in one clone The environmental OXA-23 producing strain clusters together with the clinical isolates The environmental OXA-23 producing strain clusters together with the clinical isolates. Cut-off value of 80% is applied to determine the clone. P: Polydesmus; G: Godan nursing home; K: Kvatrić nursing home.

Dice (Opt:1.00%) (Tol 3.0%-3.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE01

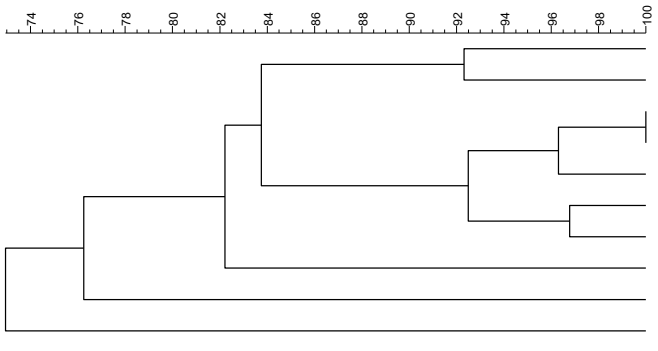


PFGE01

20-02-2017	G	Wound swab	33980	OXA-24	IC II
20-02-2017	G	Throat swab	33981	OXA-24	IC II
03-07-2017	G	Urine	44087	OXA-23	IC II
15-03-2017	G	Wound swab	50072	OXA-24	IC II
03-03-2017	G	Urine	41834	OXA-24	IC II
20-02-2017	G	Urine	33979	OXA-24	IC II
02-03-2017	G	Urine	41378	OXA-24	IC II
24-03-2017	G	Canilla swab	55754	OXA-23	IC II

Dice (Opt:1.00%) (Tol 3.0%-3.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
PFGE01

PFGE01



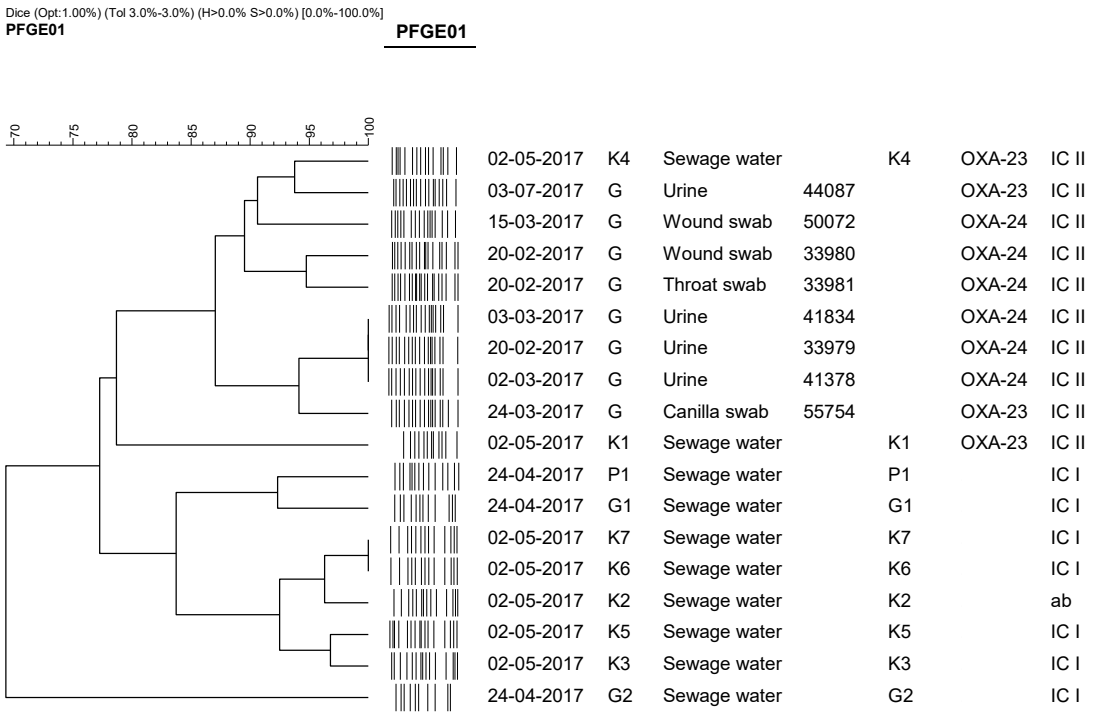


Fig.1. PFGE dendrogram of clinical *A. baumannii* isolates. Date of isolation, source, specimen, protocol number, type of CHDL and international clonal lineage (IC) is shown. All isolates belong to one large cluster. Cut-off value of 80% is applied to determine the clone. Cut-off value of 80% is applied to determine the clone.

Fg. 2. . PFGE dendrogram of environmental *A. baumannii* isolates. Date of isolation, source and international clonal lineage (IC) are shown. Cut-off value of 80% is applied to determine the clone.

Fig. 3 Comparison of environmental and clinical *A. baumannii* isolates. Date of isolation, source, specimen, protocol number, type of CHDL and international clonal lineage (IC) is shown. The environmental OXA-23 producing strain clusters together with the clinical isolates. Cut-off value of 80% is applied to determine the clone.