Survival of ESKAPE pathogen *Acinetobacter baumannii* in water of different temperatures and pH

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Abstract

Bacterium *Acinetobacter baumannii* is an emergent pathogen associated with nosocomial infections, which could be also found in natural waters. The impact of ecological factors on *A. baumannii* is insufficiently investigated. The aim was to examine the influence of temperatures (-20 to 80°C) and pH values (2 to 12) on the survival of environmental and clinical isolates of *A. baumannii* in nutrient-deprived spring water (SW) and nutrient-rich diluted Nutrient Broth (DNB) during 5 months. *A. baumannii* successfully survived at -20 to 44°C and neutral pH for 5 months, which is consistent to the persistence of this pathogen in the hospital environment. At temperatures 50 to 80°C the survival of *A. baumannii* ranged from 5 days to 5 min. The pH 2 was the most lethal with survival time up to 3 hours, suggesting that acidic conditions are promising for disinfection of water contaminated with *A. baumannii*. Although the type of media was not statistically significant for long-time survival, the extensively- or pandrug-resistant isolates survived better in SW than susceptible or multidrug-resistant isolates. Two distinct colony phenotypes were recorded at extreme temperatures and pH values. The results of this study provide insight into the behaviour of this emerging pathogen in the environment.

Keywords: *Acinetobacter baumannii*, survival, temperature, pH, water media

INTRODUCTION

Bacterium *Acinetobacter baumannii* is an emergent human pathogen of the 21st century. It belongs to the ESKAPE group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) These pathogens represent new paradigms in pathogenesis, transmission, resistance and possess the ability to "escape" the biocidal activity of antibiotics (Pendleton et al, 2013). In 2017, the World Health Organization has issued a list of the most dangerous pathogens with carbapenem-resistant *A. baumannii* strains at the top of that list (WHO, 2017). Carbapenems are broad-range antibiotics that have been successful so far in treating infections with multi-drug resistant *A. baumannii*. However, in Croatia carbapenem-resistance has grown drastically from 10% in 2008 to 86% in 2016 thus representing a major healthcare issue (CAMS, 2017). *A. baumannii* is an opportunistic pathogen that causes nosocomial as well as community-acquired infections (Dexter et al, 2005). In hospitals *A. baumannii* is extremely dangerous in the intensive care units and in operation rooms. In immunosuppressed patients, *A. baumannii* causes pneumonia, bacteraemia, meningitis, skin infections, urinary and bloodstream infections (McConnell et al, 2013). Outside the hospital environment clinically significant *A. baumannii* isolates have been reported in hospital wastewaters (Ferreira et al, 2011; Zhang et al, 2013) in different stages of the wastewater treatment process (Hrenovic et al 2016) and in the natural aquatic environment (Girlich et al, 2010; Seruga Music et al, 2017). *A. baumannii* forms biofilm on biotic and abiotic surfaces, resists desiccation and

expresses resistance to commercially available disinfectants and antibiotics (Espinal et al, 2012, Ivankovic et al, 2017). Various virulence factors that enable the persistence of *A. baumannii* in the environment such as outer membrane proteins, capsular polysaccharide and lipopolysaccharides, outer membrane vesicles, metal acquisition systems, protein secretion systems and β-lactamases have been identified (Lee et al, 2017). The influence of ecological factors such as temperature and pH on the growth and survival of *A. baumannii* has been scarcely investigated. There are several reports on *A. baumannii* survival but mostly concerning clinical isolates. Antunes et al (2011) have determined that clinical isolates of *A. baumannii* grow at temperatures ranging from 25 to 45°C with optimum growth at 37°C. Obeidat et al (2014) have monitored the survival time of clinical *A. baumannii* isolates up to 23 days in distilled, tap and saline water at 18 to 24°C and pH range of 4.5 to 8. Hrenovic et al (2014) have recovered one *A. baumannii* isolate from acid paleosol and demonstrated that it can survive pH 3.37, 50°C and resist desiccation. The aim of this study was to investigate the influence of temperature and pH on *A. baumannii* environmental isolates in order to predict the behaviour of this emergent pathogen in the environment.

MATERIAL AND METHODS

A. baumannii isolation and characterization

Four environmental *A. baumannii* isolates were recovered from the wastewater treatment plant in Zagreb (Croatia) and one clinical isolate from the Special Hospital for Pulmonary Diseases, Zagreb (Croatia) (Table 1). Isolation was performed according to Hrenovic et al (2016) on CHROMAgar Acinetobacter with the addition of 15 mg/L cefsulodin sodium salt hydrate both with or without CR102 supplement and incubation at 42°C/48h. Identification of the isolates was performed by Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) on cell extracts (Sousa et al, 2014). Antibiotic susceptibility to carbapenems (meropenem, imipenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (tobramycin, gentamicin, amikacin), tetracyclines (minocycline), penicillins/β-lactamase inhibitors (ampicillin-sulbactam, ticarcillin-clavulanic acid, piperacillin-tazobactam) and folate pathway inhibitors (trimethoprim-sulfamethoxazole) was determined by Vitek2 system (Biomerieux). Broth microdillution method was used for testing the susceptibility to polymyxins (colistin). Minimum inhibitory concentrations were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (2017) criteria for all antibiotics with defined breakpoints for *Acinetobacter* spp., while for penicillins/β-lactamase inhibitors and minocycline Clinical and Laboratory Standards Institute (2015) breakpoints were used. Isolates were grouped into categories according to (Magiorakos et al 2012): susceptible to all tested antibiotics (S), multidrug-resistant (MDR), extensively drug-resistant (XDR), pandrug-resistant (PDR) (Table 1).

Table 1. Origin and antibiotic susceptibility profile for selected isolates of *A. baumannii*

Isolate	Origin	Antibiotic susceptibility profile
OB4138	Patient with hospital-acquired pneumonia, bronchial aspirate, Special Hospital for Pulmonary Diseases, Zagreb	XDR (SXT, CST)[a]
IN39	Influent of wastewater treatment plant, Zagreb	MDR (MIN, CST, aminoglycosides)
EF7	Effluent of wastewater treatment Plant, Zagreb	PDR
EF8		XDR (SXT, CST)
EF11		S

[a]Antibiotics to which isolates remained susceptible are given in brackets; MIN: minocycline, SXT: trimethoprim/sulfamethoxazole, CST: colistin. Isolates from: influent (IN), effluent (EF), clinical isolate (OB). XDR: extensively drug-resistant, MDR: multidrug-resistant, PDR: pandrug-resistant, S: susceptible to all tested antibiotics. All isolates, except EF11, were resistant to carbapenems producing OXA-23 carbapenemase.

Experiment set up

Temperature

Commercially available spring water (SW) and Nutrient broth (Biolife) diluted with distilled water (DNB) to 1:100 were used in the experiments. The physiochemical properties of the tested media were measured according to the Standard Methods for Examination of Water and Wastewater (APHA et al., 2005) (Table 2). The choice of the water media simulated environmental conditions. SW represented nutrient deprived clean uncontaminated water, while DNB simulated water contaminated with nutrients such as wastewater. Overnight bacterial culture grown on Mueller-Hinton agar (Biolife) at 42°C/24h was suspended in SW and DNB. Bacterial suspensions were incubated at -20, 4, 22, 35 and 44°C during 5 months. Bacterial numbers were determined at the beginning of the experiment, after 1, 2 and further every 7 days on Mueller-Hinton agar) at 42°C/24h. At 50, 63, 72 and 80°C bacterial numbers were monitored until the death of all bacterial cells.

Table 2. Chemical analysis of commercially available spring water (SW) and nutrient broth diluted with distilled water 1:100 (DNB)

Chemical parametres	SW	DNB
pH	8.1	6.9
Dissolved Oxygen (mg/L O_2)	4.6	4.6
Chemical Oxygen Demand (mg/L COD)	3	99
Total Organic Carbon (mg/L C)	<1	44
Total Nitrogen (mg/L N)	0.7	13.2
Total Phosphorus (mg/L P)	0.1	1.1

pH

Prior to the beginning of the experiment, the pH of SW and DNB was set (WTW pH 330/SET-1) to 2, 5, 10, 12 with 1M NaOH and 1M HCl. In order to adjust pH to 2 and 12, concentrated HCl and NaOH granules were used. Prepared solutions were sterilized, inoculated with bacterial suspensions and incubated at room temperature (22 °C) during 5 months. Bacterial numbers were determined at the beginning, after 1, 2 and further every 7 days on Mueller-Hinton agar at 42°C/24h. At pH 2 and 12 measurements were made after 1, 3 and 5 hours of exposure.

Microscopic analysis

At the beginning and at the end of the experiment at 4, 22 and 44°C the samples were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS) and prepared for scanning electron microscopy (SEM) using standard techniques. Briefly, after removal of the fixative, the samples were rinsed in PBS, post-fixed in 1% osmium tetroxide in PBS, again rinsed with PBS and then dehydrated in a graded ethanol series up to absolute ethanol. Cells were dried with hexamethyldisilazane and carbon coated before examination at low voltage (0.5 kV) with a Zeiss Ultra PLUS FEG SEM.

Statistical analysis

Bacterial numbers were expressed as log CFU/mL (Colony Forming Unit). Statistical analysis was carried out using Statistica 13.3 (TIBCO Software, Inc.). Survival rate was calculated for graphical presentation as follows:

$$\boldsymbol{Survival}\ (\%) = \left(\frac{\log \boldsymbol{CFU/mL_{t(m)}}}{\log \boldsymbol{CFU/mL_{t(0)}}}\right) * \mathbf{100} \qquad (1)$$

where t(0) is the initial number and t(m) bacterial number at the time of measurement. For pairwise comparisons factorial ANOVA and Duncan post hoc test were conducted using values of bacterial reduction which was calculated as follows:

$$\boldsymbol{Reduction\ of\ bacteria = log\ CFU/mL_{t(0)} - log\ CFU/mL_{t(fin)}} \quad (2)$$

where t(fin) is the final bacterial number at the end of experiment. Decisions regarding statistical significance were made at $p<0.05$.

RESULTS

Temperature

The results are presented in Figures 1-5. *A. baumannii* successfully survived at -20, 4, 22, 35 and 44°C for 5 months. In SW there was no multiplication of bacteria regardless of temperature, whereas in DNB multiplication occurred only at 22, 35 and slightly at 44°C. At low temperatures isolates show little variation in behaviour. At -20°C decline in survival is slow and steady (Fig. 1). At 4°C survival of *A. baumannii* is constant with survival rates above 90% after 5 months for almost all isolates, except IN39 (51%) and EF11 (62%) in SW (Fig. 2).

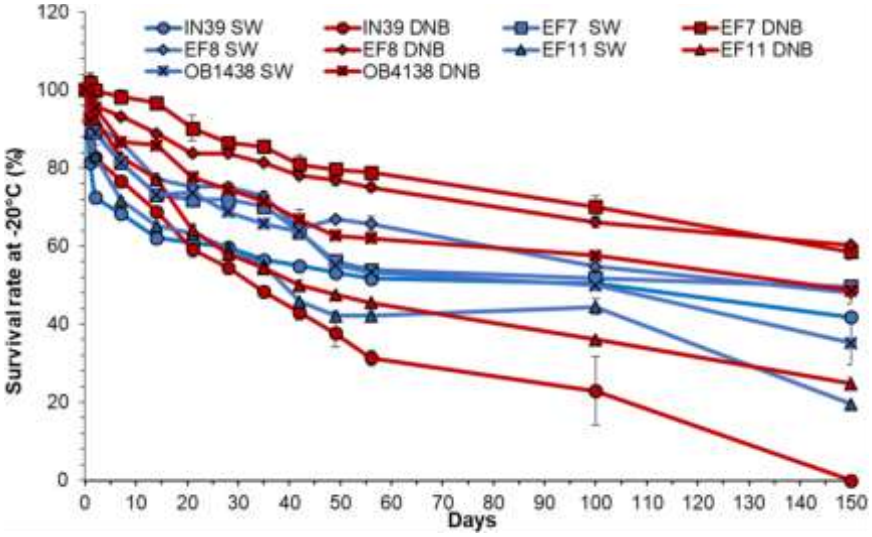

Figure 1. Survival of *A. baumannii* isolates at −20 °C during 5 months of monitoring. SW: commercially available spring water, DNB: nutrient broth diluted with distilled water 1:100.

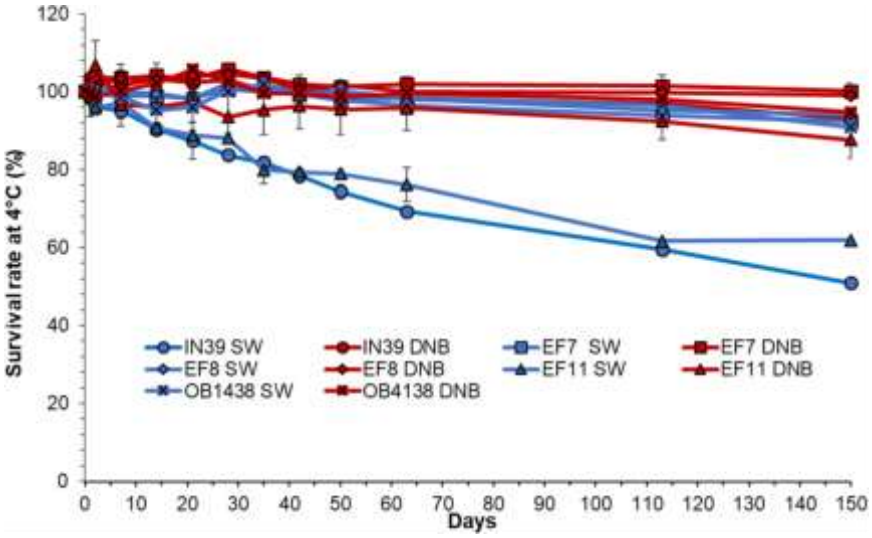

Figure 2. Survival of *A. baumannii* isolates at 4 °C during 5 months of monitoring. SW: commercially available spring water, DNB: nutrient broth diluted with distilled water 1:100.

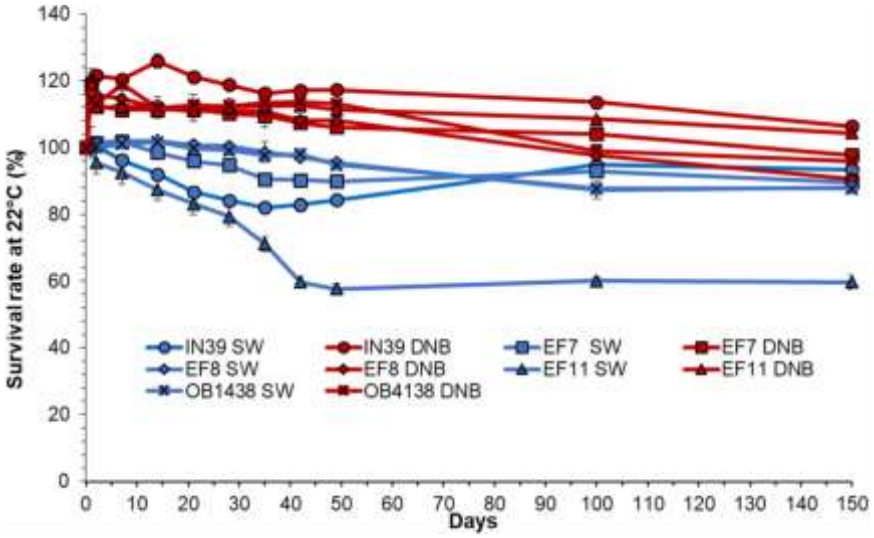

Figure 3. Survival of *A. baumannii* isolates at 22 °C during 5 months of monitoring. SW: commercially available spring water, DNB: nutrient broth diluted with distilled water 1:100.

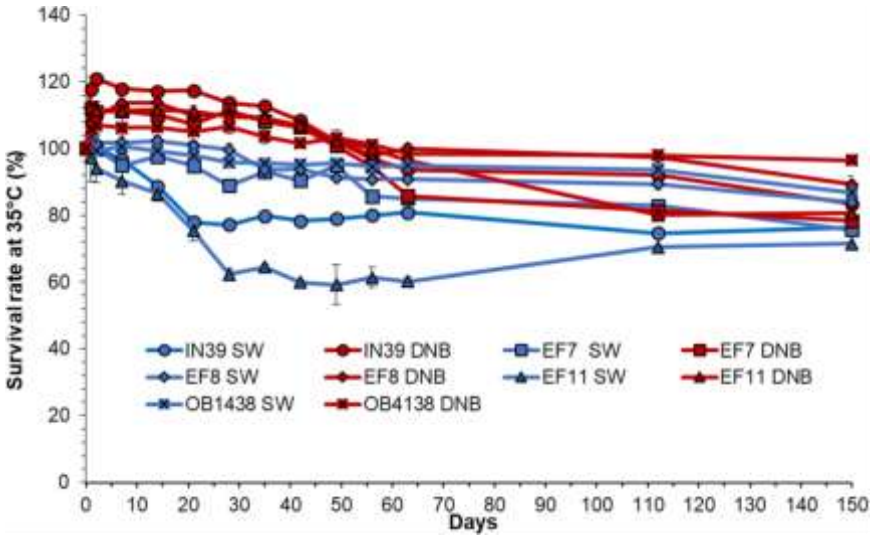

Figure 4. Survival of *A. baumannii* isolates at 35 °C during 5 months of monitoring. SW: commercially available spring water, DNB: nutrient broth diluted with distilled water 1:100.

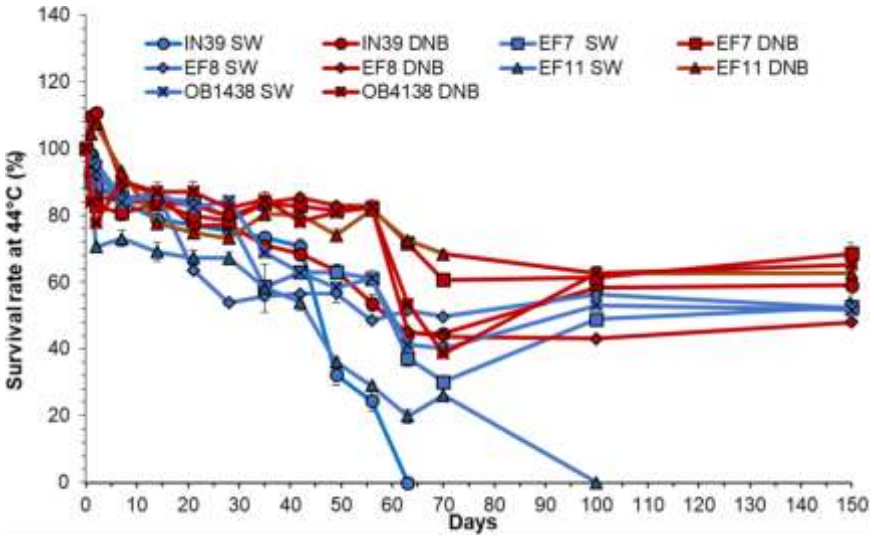

Figure 5. Survival of *A. baumannii* isolates at 44 °C during 5 months of monitoring. SW: commercially available spring water, DNB: nutrient broth diluted with distilled water 1:100.

The highest survival rates for almost all isolates were at 22°C, which were all above 90% in SW, except EF11 (60%) and up to 106% in DNB after 5 months (Fig. 3). At 35°C there was a significant rise in bacterial number at the beginning of the experiment in DNB up to day 50 when survival started to decline (Fig. 4). At the end of the experiment survival at 35°C in DNB was not statistically different from SW (p=0.065). At 44°C slight multiplication of bacteria was recorded after the first 2 days of incubation in DNB. Survival at 44°C was erratic with increases and decreases in bacterial numbers. Survival rates were 50-60% after 5 months. Isolates IN39 and EF11 in SW died after 63 and 100 days (Fig. 5).

A. baumannii at concentrations of 10^5 CFU/mL survived extreme temperatures at 50, 63, 72 and 80°C for up to 5 days, 2 hours, 10 and 5 minutes, respectively (data not shown). Survival of *A. baumannii* is slightly better in DNB in regard to SW at all tested temperatures after 5 months, but without statistical significance. However, better survival in DNB is statistically significant only at 22 (p=0.000) and 44°C (p=0.000).

A. baumannii had the highest survival rates at 4 and 22°C with no statistically significant difference between these two temperatures (p=0.336). There was a statistically significant difference at all tested temperatures in SW between EF11 and other isolates (EF7 p=0.018, EF8 p=0.014, OB4138 p=0.031) except IN39 (p=0.476) while in DNB isolates demonstrated no significant difference.

The results of the SEM analysis confirm that the bacterial cells have lost their osmotic stability after 5 months at 44°C and 4°C in both tested media (Fig. 6). After 5 months at 22°C bacterial cells were osmotically stable and resembled the cells from the beginning of the experiment in both media.

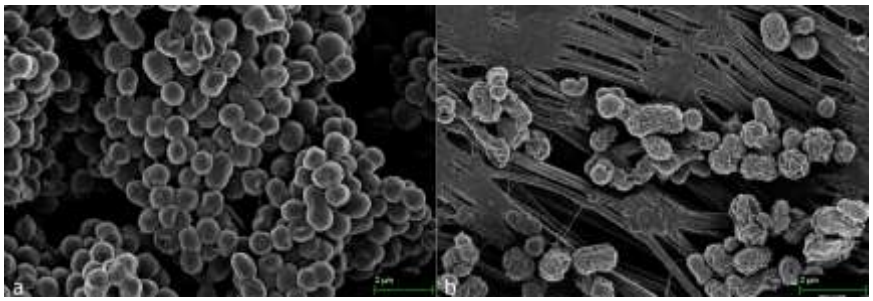

Figure 6. SEM analysis of *A. baumannii* samples (a) at the beginning of the experiment (b) after 5 months of exposure at 44 °C in diluted nutrient broth.

pH

A. baumannii successfully multiplied and survived in DNB of unmodified neutral pH 6.9 for 5 months, while in SW (pH 8.1) as mentioned above no multiplication occurred (data not shown). The pH of SW and DNB set to 5 and 10 changed after two days of exposure and, because of the buffer capacity of tested solutions, became neutral. The exposure of *A. baumannii* to pH 5 and 10 lasted only two days. However, in this short time no multiplication or decline in bacterial numbers was observed. At pH 12 the survival of *A. baumannii* was recorded up to 3 hours in SW and 5 hours in DNB. In addition, one isolate in DNB (EF11) was able to survive 24h at pH 12. pH 2 was the most lethal with survival time up to 3 hours in both media (data not shown). At extreme temperatures (-20, 4, 44, 50, 63, 72, 80°C) and pH values (2, 12) a part of *A. baumannii* population formed smaller translucent colonies.

DISCUSSION

According to literature, the optimal temperature for the growth of *A. baumannii* is 37°C with isolates entering stationary phase after 10-12 hours of incubation (Antunes et al, 2011) which is in accordance with the results of this study at 35°C. Bacterial metabolism is faster at 35°C, which means faster growth. However, the optimal temperature for long-term survival of *A. baumannii* is 4 and 22°C. Furthermore, at 22°C bacteria multiply in DNB in contrast to 4°C where no multiplication occurs in both tested media. These results suggest that *A. baumannii* has the best

chance of survival at room temperature and in refrigerators, which is extremely important in the hospital environment where occasional outbreaks occur. At almost all tested temperatures isolates demonstrate better survival rates in DNB, however with passing time the survival rates are not significantly different from SW suggesting that long-term persistence (5 months) is not largely dependent upon the type of media. DNB is richer with nutrients than SW, especially in phosphorus, which is a limiting factor in natural waters, therefore the bacteria were able to multiply at favourable temperatures (22 and 35°C). At low temperatures, bacteria had slower metabolism hence large variations in survival rates were not recorded, whereas at 44°C the survival curve is erratic with decreases and rises in survival rate. This behaviour is previously recorded as the "bust and boom" survival strategy where weak bacterial cells die in unfavourable conditions. The remaining "persistor" cells live at the expense of dead cells (Bravo et al, 2016). Another common survival strategy is entry into viable but non-culturable state (VBNC). In this strategy, bacteria cannot be cultivated on media normally used for cultivation. However, they retain metabolic activity and can possibly behave as pathogens. Bravo et al (2016) did not record the entry of *A. baumannii* ATCC 19606T into VBNC state under nutrient-deprived conditions. The results of this study indicate that *A. baumannii* has the ability to survive high temperatures. It can survive classic pasteurization procedure: 63°C/30 min; 72°C/15 sec (Pearce et al, 2001) and withstand exposure to 80°C for a short period. The results of high temperature tolerance of *A. baumannii* are unexpected since it is considered a mesophilic non-sporogenic bacterium. SEM analysis confirmed osmotically unstable cells at extreme temperatures. However, this osmotic instability had no apparent effect on long-term survival especially at low temperatures.

Isolate susceptible to all tested antibiotics (EF11) as well as MDR IN39 had lower survival rates in SW suggesting that isolates with resistance to a broader spectrum of antibiotics such as XDR (EF8, OB4138) and PDR (EF7) have the ability to survive longer in nutrient-deprived water media. According to Bazleyu and Kumar (2014) *A. baumannii* ATCC 19606T was more susceptible to imipenem, ciprofloxacin, gentamicin, and ceftriaxone at 30°C as a result of lower expression of efflux pumps. Resistance to tested antibiotics was greater at 37°C with no significant difference between 37 and 42°C.

Bacteria of the genus *Acinetobacter* have a slight acidic optimum for growth ranging from 5.5 to 6.5 (Garrity et al, 2005). However, the results of this study suggest that *A. baumannii* optimally grows at pH 6.9 in DNB. *A. baumannii* has the ability to persist in pH significantly above optimum (SW= 8.1). Due to considerably higher pH combined with a lack of nutrients in SW, no multiplication of the bacteria occurred. In addition, at the first two days of monitoring no change in *A. baumannii* numbers at pH 5 and 10 occurred, suggesting its possible persistence in the aqueous environment of a wide range of pH values. The most lethal was pH 2 with survival time up to 3 hours. At pH 12 some of the isolates were able to survive several hours and one isolate up to 24h. These results suggest that the disinfection of water contaminated with *A. baumannii* is more effective in acidic conditions. Similar research has been conducted by Obeidat et al (2014) who reported the survival of *A. baumannii* recovered from patients and the hospital environment for up to 23 days in distilled, tap and saline water (addition of 0.9% NaCl). Tested isolates were exposed to room temperature ranging from 18 to 24°C and pH range of 4.5 to 8. Furthermore, they reported the growth of *A. baumannii* at 37, 42 and 45°C, whereas no growth was observed at 4 and 48°C, which is in accordance with the results of this study. Furthermore, Hrenovic et al (2016) have reported the survival of *A. baumannii* environmental isolates in effluent water after 50 days. Additionally, Kovacic et al (2017) have reported the successful survival of two *A. baumannii* environmental and one clinical isolate in seawater after 50 days.

In conditions of extreme temperatures and pH *A. baumannii* formed smaller translucent colonies together with larger opaque colonies. Tipton et al (2015) have demonstrated that these smaller translucent colonies formed stronger biofilm and pellicle in comparison to normal opaque ones,

which expressed more virulence in *Galleria mellonella* model and better surface motility. Furthermore, translucent colonies have the ability to transition to opaque variants and vice versa (Tipton et al, 2015). The ability of *A. baumannii* to produce and quickly change different phenotypes could increase its adaptation to different environmental conditions within and outside the host.

CONCLUSION

A. baumannii is a versatile bacterium that can survive at different temperatures and pH values in the water media. Optimal conditions for the long-term survival of *A. baumannii* are temperature of 4, 22°C and neutral pH, which is a prerequisite for the existence of isolates in the hospital environment at room temperature and in refrigerators. *A. baumannii* prefers nutrient-rich DNB, however, long-term survival is not significantly dependent upon nutrient concentration in water media. Isolates with resistance to a broader spectrum of antibiotics (XDR, PDR) could have better survival rates in nutrient-deprived water media than susceptible and MDR isolates. In conditions of extreme temperatures and pH values, *A. baumannii* forms smaller translucent colonies, which could be a coping mechanism ensuring better survival. The results of this study provide insight into the behaviour of this emerging pathogen in the environment and suggest the need for additional control measures to prevent future outbreaks.

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