IMMOBILIZATION OF ACINETOBACTER BAUMANNII ONTO NATURAL ZEOLITE DEPENDENT ON THE NUTRIENT CONCENTRATION OF WATER MEDIA

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ABSTRACT

An emerging hospital pathogen, bacterium *Acinetobacter baumannii* occurs in the natural environment influenced by human liquid or solid waste. Bacterial biofilm formation on abiotic or biotic solid surfaces is considered as an important virulence factor of this pathogen. There are no data on the quantification of viable bacterial cells of *A. baumannii* (either of clinical or environmental origin) that are immobilized onto the solid surfaces. In this study, the environmental isolate of *A. baumannii* was immobilized onto the particles of natural zeolitizied tuff (NZ) at 20°C in the water media of different nutrient concentrations. Numbers of immobilized viable bacterial cells ranged from 6.7-9.1 log CFU/g. The intensity of bacterial immobilization onto the NZ was a function of total bacterial concentration which was statistically significantly positively correlated with the COD of surrounding water media. Immobilization of bacteria onto the NZ occurred via the bacterial extracellular substances that enable longer survival of bacteria in the environment. The presence of *A. baumannii* in the natural environment will possess a serious public health concern about the spread of this emerging human pathogen.

Keywords: bacteria; chemical oxygen demand; immobilization; zeolitized tuff.

INTRODUCTION

During two decades of successful pathogenesis, bacterium *A. baumannii* was considered as an exclusive hospital pathogen. From 2010 onwards, reports on the occurrence of *A. baumannii* outside the hospital environment can be found. Multi-drug resistant (MDR) *A. baumannii* were found in hospital[1] as well as in municipal[2] wastewaters and in acid paleosol influenced by illegally disposed solid waste[3].

Virulence factors that influence the success of *A. baumannii* as a pathogen are its immobilization and subsequent biofilm formation on abiotic or biotic solid surfaces. Biofilm is an assemblage of microbial cells enclosed in an extracellular matrix, which protects bacteria from harsh environmental conditions, and therefore bacteria in the form of biofilm survive longer in the environment[4]. Biofilm formation of clinical isolates of *A. baumannii* is routinely quantified by staining of the total biofilm formed on the polypropylene surface in nutrient rich media at 37°C[5].

There are no data on the quantification of viable bacterial cells of *A. baumannii* (either of clinical or environmental origin) that are immobilized onto the solid surfaces. The aim of this study was to quantify the numbers of environmental isolate of *A. baumannii* immobilized onto the particles of natural zeolitizied tuff (NZ) at 20°C in the water media of different nutrient concentrations.

EXPERIMENTAL

In this study bacterium *A. baumannii* isolated form acid paleosol influenced by illegally disposed solid waste of external origin was used[3]. This isolate is MDR to gentamicin, trimethoprim-sulfamethoxazole, ciprofloxacin and levofloxacin, and showed close relationship with the clinical isolate from adjacent hospital. For experiments NZ from quarry located at

Donje Jesenje, Croatia was used. The mineralogical analysis[6] revealed that NZ sample consisted mostly of clinoptilolite (50-55%) with major constituents being celadonite, plagioclase feldspars and opal-CT (10-15% each), while analcime (another zeolite group mineral) and quartz were present in traces. The NZ was crushed, sieved, and dry autoclaved size fraction of 0.122-0.263mm was used in experiments.

Overnight bacterial culture was suspended in 100mL of autoclaved spring water, nutrient broth diluted 100x with distilled water, and undiluted nutrient broth (composition in mg/L: beef extract 3, peptone 5). All water media were of neutral pH. The chemical oxygen demand (COD) of water media was measured spectrophotometrically (Hach reactor digestion method 8000). Into each bacterial suspension in water media, a 1.0g of NZ was added. Media were incubated during 3 days at 20°C with 170rpm.

Number of viable bacteria was determined as colony forming units (CFU), logarithmically transformed, and expressed as log CFU per 1mL of water or 1g of dry NZ. Samples were diluted in sterile saline solution, inoculated onto Nutrient agar plates, and bacterial colonies were counted after incubation at 42°C/24h. To confirm the immobilization of bacteria onto NZ, particles of NZ were taken at the end of experiments, stained with carbol fuxin and alcian blue dyes and examined under optical microscope. Statistical analyses were carried out using Statistica 12 software and decisions were made at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

The COD of water media was taken as a measure of nutrients available for bacterial multiplication. The COD values ranged from 3 to 930 and 93,000 mgO_2/L in spring water, diluted nutrient broth and undiluted nutrient broth, respectively (Table 1). *A. baumannii* multiplied in all three examined water media during 3 days of incubation (Table 1). Ratio of bacterial multiplication increased with the increase of COD of water media and statistically significantly positively correlated with the COD of water media ($r=0.843$; $p < 0.05$).

Table 1. Numbers of viable *A. baumannii* in water media of different nutrient concentrations. Ratio of immobilized:planktonic bacteria was calculated as (log $CFU/g_{immobilized}$: log $CFU/mL_{planktonic}$) and was used as a measure of intensity of bacterial immobilization. Ratio of bacterial multiplication was calculated as (log $CFU/mL_{total\ bacteria\ 3\ days}$: log CFU/mL_{start}).

Parameter	Spring water	Diluted nutrient broth	Undiluted nutrient broth
Start			
COD (mg O_2/L)	3	930	93,000
Total bacteria (log CFU/mL)	6.9±0.1	6.9±0.1	6.9±0.1
3 days			
Planktonic bacteria (log CFU/mL)	7.3±0.1	8.5±0.0	9.6±0.0
Immobilized bacteria (log CFU/g)	6.7±0.1	7.7±0.1	9.1±0.1
Total bacteria (log CFU/mL)	7.3±0.1	8.6±0.0	9.7±0.0
Ratio immobilized:planktonic	0.9±0.1	0.9±0.0	1.0±0.0
Ratio of multiplication	1.1±0.0	1.2±0.0	1.4±0.0

After 3 days of incubation, one part of total bacteria remained in water media as planktonic population, while the other part was immobilized onto particles of NZ. The intensity of bacterial immobilization indicated as a ratio of immobilized and planktonic bacteria (Table 1) around 1 suggested that the number of immobilized bacteria was dependent on the total bacterial count. The numbers of planktonic, immobilized and total bacteria, as well as the ratio of immobilized

and planktonic bacteria statistically significantly positively correlated with the COD of water media ($r=0.847, 0.912, 0.848, 0.936$; $p < 0.05$, respectively). Therefore, the intensity of bacterial immobilization onto the NZ was a function of total bacterial concentration which was determined by COD of surrounding water media.

The NZ examined for the immobilization of *A. baumannii* in this study was previously tested for the immobilization of other bacterial species in nutrient broth[6]. Another species of the genus *Acinetobacter*, phosphate-accumulating bacterium *A. junii*, was immobilized onto the same NZ in much higher numbers (10.1 log CFU/g) than *Escherichia coli* (8.7 log CFU/g) or *Enterococcus faecalis* (7.1 log CFU/g). Immobilization of *A. baumannii* (9.1 log CFU/g, Table 1) was something lower as compared to *A. junii*, but substantially higher than *E. coli* or *E. faecalis*. This confirms a good ability of *A. baumannii* to immobilize onto the particles of NZ.

Microscopical examination confirmed the immobilization of *A. baumannii* onto NZ particles by extracellular substances (Figure 1). Bacterial extracellular polymers are considered as an important virulence factor that protect bacteria in unsuitable environmental conditions[7]. Therefore, bacteria in the immobilized form survive longer in the environment[4]. *A. baumannii* can reach the natural environment via the human liquid[1,2] or solid waste[3]. Once in natural environment, bacteria could be immobilized onto the particles of natural minerals as shown for NZ even at environmental temperature of 20°C. Immobilized bacteria are protected by extracellular substances and can survive in the soil or sediment for prolonged period of time. The presence of MDR *A. baumannii* in the natural environment will possess a serious public health concern about the spread of this emerging human pathogen.

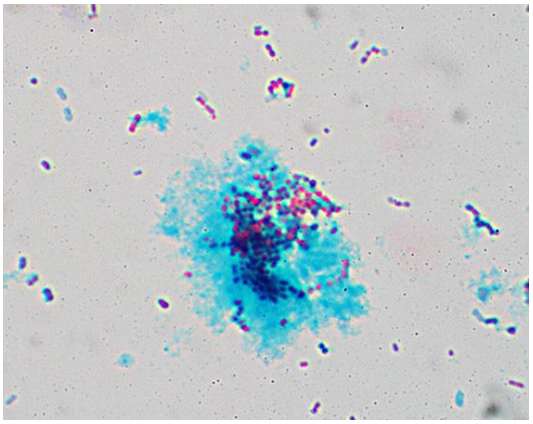

Figure 1. Thick layer of extracellular substances (blue) with embedded cells of *A. baumannii* (red).

CONCLUSION

The environmental isolate of *A. baumannii* was immobilized onto the particles of NZ at 20°C in the water media of different nutrient concentrations. Numbers of immobilized viable bacterial cells ranged from 6.7-9.1 log CFU/g. The intensity of bacterial immobilization onto the NZ was a function of total bacterial concentration which was statistically significantly positively correlated with the COD of surrounding water media. Immobilization of bacteria onto the NZ occurred via the bacterial extracellular substances.

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