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# Interaction of *Acinetobacter baumannii* 19606 and 1656-2 with *Acanthamoeba castellanii*

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***Acinetobacter baumannii* is virtually avirulent for healthy people but maintains a high virulence among critically ill patients or immuno-compromised individuals. The ability of *A. baumannii* to adhere to cells and persist on surfaces as biofilms could be central to its pathogenicity. In the present study, we compared the virulence of the *A. baumannii* 1656-2 clinical strain, which is able to form a thick biofilm, with the virulence of the *A. baumannii* type strain (ATCC 19606[T]). *Acanthamoeba castellanii*, a single-celled organism, was used as the host model system to study the virulence of *A. baumannii*. Compared to *A. baumannii* ATCC 19606[T], *A. baumannii* 1656-2 exhibited a higher ability to adhere and invade *A. castellanii* cells and had a higher killing rate of *A. castellanii* cells. Furthermore, co-incubation of the amoeba cells and the cell-free supernatant of *A. baumannii* resulted in the cell death of the amoebae. Heat inactivation or proteinase K treatment of the supernatant did not eliminate its cytotoxicity, suggesting heat stable non-protein factors are responsible for its cytotoxicity to *A. castellanii* cells. In conclusion, this study for the first time has revealed the capacity of the *A. baumannii* strain and/or its metabolic products to induce cytotoxicity in *A. castellanii* cells.**

***Keywords*:** invasion assay, cytotoxicity, protozoa-bacteria interaction, host model

*Acinetobacter* species are a genetically diverse group of aerobic, Gram-negative, non-fermenting bacteria that are distributed widely in the environment (Towner, 1995). Among them, *Acinetobacter* genomic species 2 (*Acinetobacter baumannii* or *A. baumannii*) is the most prevalent species among the clinical specimens (Bouvet and Grimont, 1987; Bergogne-Bérézin and Towner, 1996). Recently, *A. baumannii* has emerged as an important pathogen causing infections in severely ill patients in intensive care units or immuno-compromised individuals (Bergogne-Bérézin and Towner, 1996; Villegas and Hartstein, 2003) due to its remarkable ability to acquire resistance determinants to various kinds of antimicrobial agents (Perilli *et al.*, 1996). In addition to multi-drug resistance (MDR), it has been found that most of the MDR *A. baumannii* strains have the ability to form considerable amounts of biofilms (Lee *et al.*, 2008). The ability to form a biofilm is thought to be an important feature in the resistance to stresses in the bacterial colonization of abiotic surfaces and in persistence in diverse environmental niches, and could be central to its pathogenicity (Davey and O'Toole, 2000; Branda *et al.*, 2005). Furthermore, it has been reported that the ability of *A. baumannii* to form biofilms correlates to the resistance of complement mediated bacterial killing (King *et al.*, 2009). Thus, it is neccessanry to study the pathogenesis of *A. baumannii* which is proficient in forming biofilms.

*Acanthamoeba* is a free-living amoeba that is distributed in diverse environments worldwide. *Acanthamoeba* were first demonstrated to be lysed by bacteria in 1954 (Drozanski, 1956) and to harbor cytoplasmic bacterial endosymbionts in 1975 (Proca-Ciobanu *et al*., 1975). It is now well established that *Acanthamoeba* acts as a host for various bacterial pathogens, including *Legionella* species (Neumeister *et al.*, 1997), *Coxiella burnetii* (La Scola and Raoult, 2001), *Chlamydia pneumoniae* (Amann *et al.*, 1997), *Francisella tularensis* (Abd *et al.*, 2003), *Helicobacter pylori* (Winiecka-Krusnell, 2002), *Listeria monocytogenes* (Ly and Muller, 1990), *Mycobacterium avium* (Steinert *et al.*, 1998), and *Vibrio cholerae* (Abd *et al.*, 2005, 2007). Moreover, the host-pathogen interaction between *Acanthamoeba castellanii* and the various bacteria or even fungi have been extensively studied during the last two decades (Steinert *et al.*, 1998; La Scola and Raoult, 2001; Steenbergen *et al.*, 2001, 2004). However, to date, little is known about the interaction of *A. castellanii* with *A. baumannii* (Pagnier *et al.*, 2008; Thomas *et al.*, 2008).

In our previous study, the *A. baumannii* 1656-2 strain was found to produce the largest amount of biofilm among 23 clinical isolates of *A. baumannii* and formed a thick biofilm (Lee *et al.*, 2008). The full genome sequence of *A. baumannii* 1656-2 strain was analyzed and deposited in GenBank under the accession no. CP001921. To evaluate the virulence of *A. baumannii* 1656-2 strain, we compared its cellular adherence, invasiveness, and cytotoxicity with those of *A. baumannii* type strain ATCC 19606[T]. *Acanthamoeba castellanii* was used as a tool to study the virulence of the *A. baumannii* strains.

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## Materials and Methods

### Microorganisms and culture conditions

The *A. castellanii* strain Castellani (ATCC 30011; genotype T4) was maintained as monolayers in peptone-yeast extract-glucose (PYG) broth [ATCC medium 712; 2% proteose peptone, 0.1% yeast extract, 4 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $CaCl_2$, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2.5 mM $Na_2HPO_4 \cdot 7H_2O$, 2.5 mM $KH_2PO_4$, 0.1% sodium citrate dehydrate, and 0.1 M glucose, pH 6.5] in 75-$cm^2$ tissue-culture flasks (Corning, USA) at 25°C without shaking. For experimental use, *A. castellanii* was harvested from flasks, centrifuged, and suspended in fresh PYG medium or phosphate-buffered saline (PBS). *A. baumannii* 1656-2 strain that is able to form a thick biofilm (Lee *et al.*, 2008) and *A. baumannii* ATCC $19606^T$ strain were used in this study. Preparation of whole bacteria and cell-free supernatant.

*A. baumannii* strains were grown in Brain Heart Infusion (BHI) broth to exponential phase and harvested by centrifugation (10,000×g for 15 min at 4°C). Pellets were suspended in PBS and used in the subsequent assays. For each independent experiment, fresh whole bacteria were prepared. To prepare cell-free culture supernatants, *A. baumannii* strains were separately cultured overnight in BHI broth at 37°C in a shaking incubator and the cultures were adjusted to an optical density ($OD_{600}$) of 1. Then the cultures were diluted 50 times in BHI broth and cultured further for 24 h without shaking. The cultures were harvested by centrifugation and the supernatants were immediately filter sterilized by passage through 0.2-μm- pore-size syringe filters (Nalgene, USA). The cell-free culture supernatants were divided into aliquots and stored at -70°C for use in the subsequent assays. For the heat inactivation, the whole bacteria and culture supernatants were heated at 60°C for 1 h and 100°C for 15 min, respectively. They were then immediately placed on ice until they were applied to the monolayers of *Acanthamoeba* cells.

### Association assay

The amoebae cells ($5\times10^5$) were seeded in PYG medium in 24-well tissue-culture plates (Nalge Nunc international, Denmark) and incubated overnight at 25°C. The medium and unbound amoebae were aspirated and bound trophozoites were washed once with PBS. Next, PBS containing $5\times10^7$ CFU of *A. baumannii* (in PBS) were added per well. The plates were incubated for 1 h at room temperature and then rinsed with PBS twice to remove non-associated bacteria. Finally, the amoebae were harvested from each well and the number of amoebae for each well was counted using a hemocytometer. The number of bacteria was enumerated by a 10-fold serial dilution with spotting of each dilution onto dry LB plates. The bacteria associated with *A. castellanii* were calculated as follows: recovered *A. baumannii* (CFU)/ total *A. baumannii* (CFU) × 100 = % *A. baumannii* associated with *Acanthamoeba*. In addition, the ratio of bacteria to amoebae was calculated as follows: recovered *A. baumannii* (CFU) / number of *Acanthamoeba* × 100 = *A. baumannii* / *Acanthamoeba* ratio.

### Invasion assay

Amoebae cells, $5\times10^5$, were grown overnight at 25°C and washed once with PBS. *A. baumannii* at $5\times10^7$ CFU were added to the washed amoeba cells. After 1 h co-incubation with the *A. baumannii* strains, the amoebae monolayer was rinsed with PBS three times and treated with imipenem antibiotics (300 μg/ml) for 2 h at room temperature. Then, the amoebae monolayer was rinsed once with PBS to remove imipenem. Finally, the amoebae were harvested into microcentrifuge tubes from each well and the number of amoebae for each was well counted, and the intracellular bacteria were enumerated as described above. The percentage of the bacterial invasion/uptake was calculated as follows: recovered *A. baumannii* (CFU) / total *A. baumannii* (CFU) × 100 = % intracellular *A. baumannii*. In addition, the ratio of bacteria to amoebae was calculated as follows: recovered *A. baumannii* (CFU)/ number of *Acanthamoeba* × 100 = *A. baumannii* / *Acanthamoeba* ratio.

### Killing assay

*A. castellanii* cells ($5\times10^4$ cells) were incubated overnight at 25°C. The medium and unbound amoebae were aspirated and 100 μl of normal or heated whole bacteria in PBS containing $5\times10^7$ CFU of *A. baumannii* strains were added to each well at a 1:1,000 amoeba to bacteria ratio except in the control wells where sterile PBS was added. The co-culture plates were further incubated for 1, 5, and 24 h at 37°C. At each time interval, the number of viable *A. castellani* cells was determined by trypan blue exclusion assays using 0.4% trypan blue solution (Sigma-Aldrich, Inc, USA). The percentage of live amoebae was ascertained by counting the number of amoeba cells unable to exclude the dye per total amoebae counted. At each interval, three separate counts were done for each well and three wells per strain were counted. The experiments were repeated at least three times.

For the killing assay with *A. baumannii* cell-free supernatant, the amoebae cells ($5\times10^5$) were grown overnight at 25°C in 96-well tissue culture plates as described above. After removing the medium and

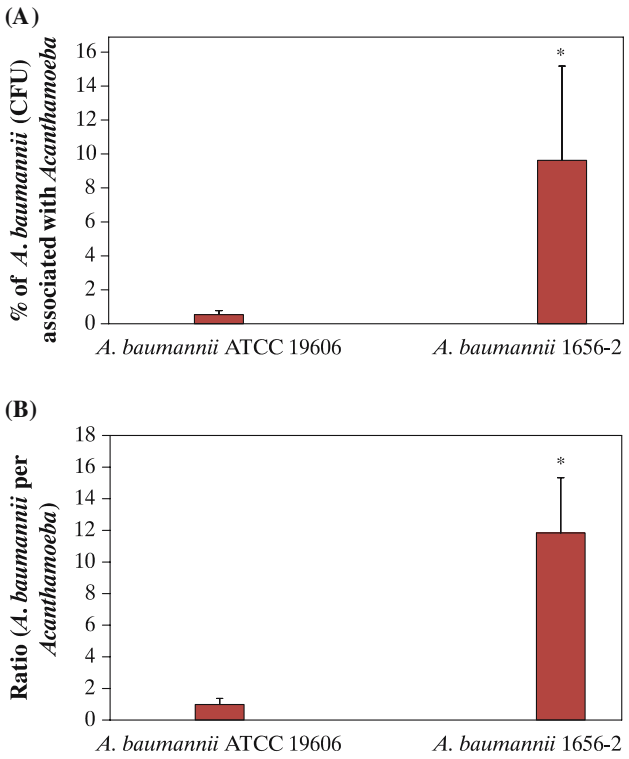


**Fig. 1.** Association of *A. baumannii* strains to *A. castellanii* cells. (A) The percentage of *A. baumannii* cells associated to *A. castellanii* cells and (B) the ratio of *A. baumannii* per amoeba. Results are the mean of three independent experiments done in triplicate. The error bars represent standard deviations from the mean. * $p<0.05$, *A. baumannii* ATCC $19606^T$ versus *A. baumannii* 1656-2.

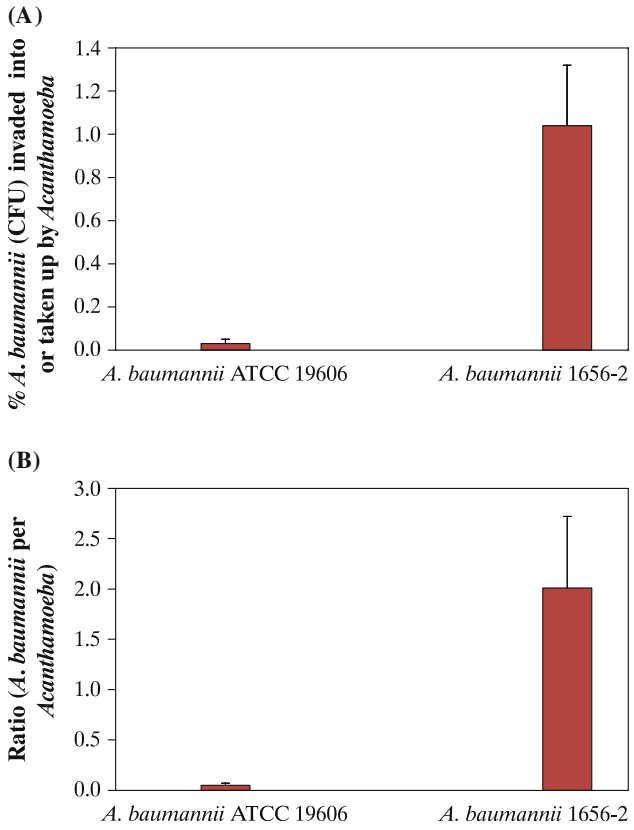


**Fig. 2.** Invasion of *A. baumannii* into *A. castellanii* cells. (A) The percentage of *A. baumannii* which invaded *A. castellanii* cells and (B) the ratio of *A. baumannii* per amoeba. Results are the mean of three independent experiments done in triplicate. The error bars represent standard deviations from the mean.

unbound amoebae, 100 µl of cell-free supernatant from an *A. baumannii* culture was added to each well and sterile BHI broth was added to the control well. The plates were further incubated for 24 h at 25°C. Cytopathic effects were observed visually using a Nikon Eclipse TE 2000-U inverted light microscope (Nikon Instruments Korea Co., Ltd, Korea) and the number of viable *A. castellani* cells was determined by Trypan blue exclusion assays using 0.4% trypan blue solution as described above. Amoebae viability was expressed as % live amoebae relative to that of total number of *Acanthamoeba* in the control wells (without culture supernatant), which were set at 100%. The sensitivity of the cell-free culture supernatant against heat or proteases were tested by pre-heating the cell-free culture supernatants at 100°C for 15 min or pre-incubating with 100 or 300 µg/ml of proteinase K for 1 h at 55°C, respectively. At least, three independent experiments were done in triplicate under the same conditions.

### Statistical analysis

Statistical analysis was done using GraphPad INSTAT (GraphPad Software, Inc, USA). Comparisons between groups were done using Fisher's exact test. Values of $P<0.05$ were considered significant.

## Results

### Adhesion and invasion of *A. baumannii* to *Acanthamoeba*

*A. baumannii* 1656-2 exhibited a higher association and adhesion ability with *A. castellanii* cells compared to *A. baumannii* ATCC 19606$^T$ (Fig. 1). Nearly 10% of the original inoculum of *A. baumannii* 1656-2 associated with *Acanthamoeba* compared to 0.54% of *A. baumannii* ATCC 19606$^T$ (Fig. 1A). Similarly, our results revealed that the ratio of *A. baumannii* 1656-2 associated to *Acanthamoeba* was higher compared to *A. baumannii* ATCC 19606$^T$ (11.83 vs 0.98) (Fig. 1B).

In conjunction with the results obtained from the associa-

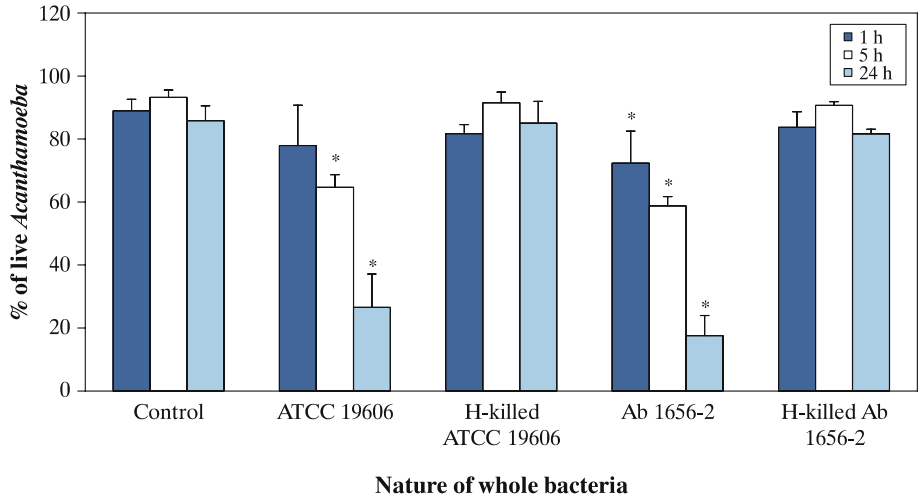


**Fig. 3.** Killing of *A. castellanii* cells by *A. baumannii* whole cells. Briefly, $5\times10^4$ amoebae were grown overnight in PYG medium in 96-well plates at 25°C. The cells were washed and $5\times10^7$ normal or heat-killed whole bacteria in PBS was added to each well at a 1:1,000 amoeba to bacteria ratio except in the control wells where sterile PBS was added. The co-culture plates were incubated for 1, 5, and 24 h at 37°C. For each time interval, the number of viable *A. castellani* cells was determined by trypan blue exclusion assay. Results are the mean of three independent experiments done in triplicate. The error bars represent standard deviations from the mean. Abbreviations: ATCC 19606, ATCC *A. baumannii* type strain 19606; H-killed, heat-killed; and Ab 1656-2, *A. baumannii* 1656-2 strain.

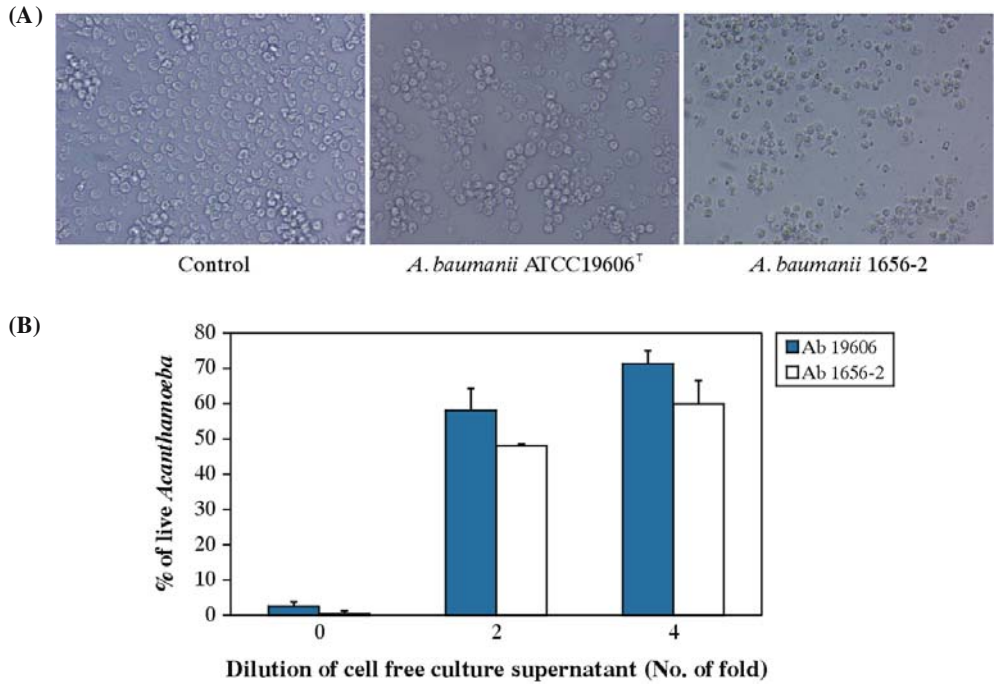


**Fig. 4.** Killing of *A. castellanii* by *A. baumannii* cell-free supernatant. (A) Amoeba monolayer damage by *A. baumannii* cell-free supernatant. Untreated *Acanthamoeba* monolayer control remained intact. *Acanthamoeba* cells treated with cell-free supernatant of *A. baumannii* 1656-2 caused complete lysis compared to that by *A. baumannii* ATCC 19606$^T$. All pictures were taken at 200× magnification. (B) Cytotoxicity of *A. baumannii* cell-free supernatant. Amoeba cell viability was determined by trypan blue exclusion assay. Results are the mean of three independent experiments done in triplicate. The error bars represent standard deviations from the mean. Abbreviations: Ab 19606, *A. baumannii* type strain 19606$^T$ and Ab 1656-2, *A. baumannii* 1656-2 strain.

tion assays, *A. baumannii* 1656-2 exhibited a higher ability to invade to *A. castellanii* compared to *A. baumannii* ATCC 19606$^T$ (Fig. 2). Around 1.0% of the original inoculum of *A. baumannii* 1656-2 invaded *Acanthamoeba* compared to 0.03% of *A. baumannii* ATCC 19606$^T$ (Fig. 2A). Similarly, the ratio of invaded *A. baumannii* 1656-2 to *Acanthamoeba* was higher compared to *A. baumannii* ATCC 19606$^T$ (2.02 versus 0.05) (Fig. 2B).

### Toxicity of *A. baumannii* to *Acanthamoeba*

The percentage of amoebae still alive after co-incubation with *A. baumannii* ATCC 19606$^T$ or *A. baumannii* 1656-2 whole bacteria was compared. As shown in Fig. 3, a significant proportion of the *Acanthamoeba* cells exposed to *A. baumannii* whole cells were killed and a maximum increase in amoeba cell death occurred after 24 h of co-incubation. *A. baumannii* 1656-2 demonstrated a higher rate of killing compared to that by *A. baumannii* ATCC 19606$^T$. Heat inactivated whole bacteria (60°C for 1 h) did not cause *Acanthamoeba* cells to die and the cytopathic effects were no different from those produced by the controls without bacteria (Fig. 3).

Co-incubation of amoebae and cell-free culture supernatant obtained from both *A. baumannii* ATCC 19606$^T$ or 1656-2 strains resulted in the cell death of the amoebae. In contrast, *A. castellanii* cells co-incubated with sterile BHI broth or PBS were not killed. The cytopathic effect was detected by the morphologically rounding of the cells and the eventual partial or complete lysis of the cells (Fig. 4A). The killing rate of

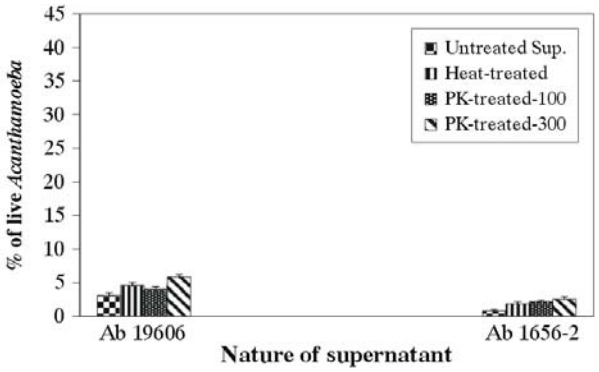


**Fig. 5.** Killing of *A. castellanii* by *A. baumannii* cell-free supernatant treated by heat or proteinase K. Aliquots of untreated, heat-treated, or Proteinase K treated cell-free culture supernatants prepared from *A. baumannii* strains were added to each well except in the control wells. The plates were further incubated for 24 h at 25°C. Amoeba cell viability was determined by trypan blue exclusion assay and expressed as % live amoebae relative to that of the total number of *Acanthamoeba* in the control wells. Results showed very negligible effects of the heat inactivation or proteinase K treatment for the supernatants derived from both of the *A. baumannii* strains. Results are the mean of three independent experiments done in triplicate. The error bars represent standard deviations from the mean. Abbreviations: Ab 19606, *A. baumannii* ATCC 19606$^T$; Ab 1656-2, *A. baumannii* 1656-2 strain; Sup., supernatant; PK-treated-100 and PK-treated-300, treated with 100 and 300 μg/ml of Proteinase K, respectively.

the cell-free culture supernatant obtained from the *A. baumannii* strains supported the data obtained with the whole bacterial cells showing a relatively higher killing rate for *A. baumannii* 1656-2 strain compared to *A. baumannii* ATCC 19606$^T$ (Fig. 4B).

We also tested whether the culture supernatant is sensitive to heat or proteinase K treatment. Heat inactivation (100°C for 15 min) of undiluted *A. baumannii* supernatant retained almost all of its toxicity for the *Acanthamoeba* cells and treatment with protease K did not eliminate the cytotoxicity either (Fig. 5).

## Discussion

In this work, we compared the virulence of *A. baumannii* ATCC 19606$^T$ and a clinical strain of *A. baumannii* 1656-2, which was previously reported to have a very high ability to produce biofilms, using a free living unicellular organism, *Acanthamoeba*. The higher capacity for adherence and invasion to *A. castellanii* cells by *A. baumannii* 1656-2 is probably due to its higher ability to form biofilms. Our observation is in agreement with a previous study (Lee *et al.*, 2008) in which higher adherence to respiratory epithelial cells by MDR clinical isolates of *A. baumannii* was attributed to their ability to form large amounts of biofilms.

Our results revealed an antagonistic interaction between *A. baumannii* and *A. castellanii*. Indeed, this is the first report describing the cytotoxicity of *A. baumannii* cells to *A. castellanii* cells. Thomas *et al.* (2008) reported the recovery of *Acinetobacter* species including *A. baumannii* by co-cultivating water and biofilm samples from a drinking water plant with *A. castellanii* ATCC 30010. Furthermore, Pagnier *et al.* (2008) also described the isolation of *A. baumannii* from environmental water samples using a co-culture with *A. polyphaga*, suggesting that *A. baumannii* could be associated with the amoebae in the environment. More importantly, Pagnier *et al.* (2008) reported that *A. baumannii* failed to cause the lysis of *A. polyphaga* during co-cultivation. However, only environmental strains of *A. baumannii* were studied and they did not test the ability to cause lysis in other species of amoebae. Thus, the inconsistency in the outcome of the interaction is probably due to the differences between bacterial strains or *Acanthamoeba* strains.

The cytotoxic effect of *A. baumannii* on *A. castellanii* apparently did not require amoeba-*A. baumannii* cell contact since the incubation of cell free *A. baumannii* culture supernatant with *A. castellanii* cells resulted in increased amoeba cell death. The fact that the responses obtained with the culture supernatants was higher than those detected with whole bacterial cells indicate that contact between the bacteria and the *A. castellanii* cells is less important. In addition, the cell-free supernatants of *A. baumannii* strains cultured in the absence of *A. castellanii* cells have a cytotoxic activity, which indicates that *A. baumannii* secretes cytotoxic factors independent of the presence of *A. castellanii* cells. Thus, cell contact independent factors of *A. baumannii* might play major role in the death of *A. castellanii* cells.

The important finding of our study is the cytotoxicity of the cell-free supernatant obtained from *A. baumannii* cultures to *A. castellanii* cells. Heat inactivation or proteinase K treatment of undiluted *A. baumannii* supernatant retained almost all of its toxicity, suggesting heat stable non-protein factors are responsible for the *A. baumannii* supernatant cytotoxicity. It was reported that secretion of rhamnolipids (non-protein factors) by *P. aeruginosa* was responsible for the fast lysis of *Dictyostelium discoideum* (Cosson *et al.*, 2002). Since *A. baumannii* is virtually avirulent for healthy people and has not been demonstrated to carry specific toxins to induce cytotoxicity, further studies should be done in order to identify and characterize the specific cytotoxic factors present in the supernatant.

The study of pathogenesis in mammalian models is complicated by several factors such as high cost, difficulties in handling, long reproductive cycles, small brood sizes, regulatory requirements, and ethical considerations. Thus, there is a continuing need for the development of simple model systems for the study of host-pathogen interactions. Non-mammalian model organisms such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and *D. discoideum* have been used with success in the past (Mylonakis *et al.*, 2007). It has been demonstrated that *C. elegans* is an appropriate model host for the study of virulence mechanisms deployed by *P. aeruginosa* (Tan *et al.*, 1999). The use of *C. elegans* for such studies has also been applied to model mammalian bacterial pathogenesis for a range of Gram-positive and Gram-negative bacteria including *Salmonella* Typhimurium (Labrousse *et al.*, 2000), *Staphylococcus aureus* (Sifri *et al.*, 2003), and *L. monocytogenes* (Thomsen *et al.*, 2006). In recent years, one species, *A. castellanii,* has been increasingly used as model to study the virulence of human pathogenic fungi (Steenbergen *et al.*, 2004; Mylonakis *et al.*, 2007). In this study, we used *A. castellanii* as a model of eukaryote-prokaryote interaction to test the virulence of *A. baumannii* 1656-2 and 19606$^T$ strains. The use of the *Acanthamoeba* species as a model organism to test the virulence of bacterial isolates was reported previously for *P. aeruginosa* (Fenner *et al.*, 2006) and *M. kansasii* (Goy *et al.*, 2007). Similarly, *D. discoideum* was successfully used as a simple nonmammalian host system to assess pathogenicity of *P. aeruginosa* strains (Cosson *et al.*, 2002). Overall, our findings suggest that the unicellular organism *A. castellanii* could be a host for studying the pathogenesis of *A. baumannii* as an alternative to or as a complement to a mammalian host system.

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