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Investigation of Relevant Genes and Pathogenicity Factors in Multidrug-Resistant Acinetobacter Baumannii Strains Isolated from Clinical Specimens

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Abstract

Acinetobacter baumannii has become a clinical and epidemiologically relevant microorganism word wide. Several studies reveal aspects important to this pathogen to cause infectious disease, such as membrane proteins apoptosis-inducing, biofilm formation, resistance to oxidative stress, besides of their potential to acquire antimicrobial resistance genes. However, the mechanisms underlying the success of this pathogen remain of great interest. This study evaluated some pathogenicity factors presented by strains of Multidrug-Resistant (MDR) A. baumannii isolated from different clinical specimens of patients in Belo Horizonte city, Brazil. Genes associated to the capsule, biofilm, apoptosis-inducing and quorum sensing were researched by Polymerase Chain Reaction. The hemolytic activity of the strains in six blood types from three different mammalian species (sheep, horse and human blood type A, B, O, AB, all Rh positive) and the sensitivity to oxidative stress by hydrogen peroxide occurred by disc diffusion test. In vitro apoptosis was evaluated in bone marrow macrophages obtained from BALB/c mice. Overall, patients had mean age of 60.6 years (± 17.6) and most of them (95%) were using some invasive device. All strains (29) were positive for gaiU and bap, 76% to wzc, 86% to luxI and 79% luxR, 45% to omp33 and 31% to ompA genes. In the oxidative stress test, 49% were more resistant (p<0.05) than the reference strains. In relation to in vitro tests, eight strains led to a significant reduction in the number of macrophages as compared to controls (p <0.05), among them 63% was positive to omp33 and ompA genes. The results show significant pathogenicity factors that contribute to the colonization and to the success of an infectious disease in hospitalized patients. Therefore, these data contributed to confirm the potential for aggression by these microorganisms, in addition to alerting to the need for constant improvements in nosocomial infection control protocols.

Keywords: Acinetobacter baumannii; Pathogenicity; Oxidative stress; Biofilm; Cell death; Quorum sensing
Introduction

Healthcare-Associated Infections (HAIs) are recognized as having a substantial impact on the lethality and morbidity of hospitalized patients, as well as on the social and economic costs to health systems [1]. In this context, *Acinetobacter baumannii* has been highlighted as an important opportunistic pathogen, responsible for a large percentage of HAIs and also for hospital outbreaks [2].

Its ability to form biofilm on biotic surfaces plays an important role in nosocomial infections caused by this microorganism, which usually follows colonization of hospital equipment and medical devices [3]. In addition, other pathogenicity factors exhibited by *A. baumannii* representing a bacterial adaptation to or escape from challenges posed by the environment or by immune system of the host. Bacterial capsule are the most relevant of these factors [4]. It is believed that it provides a coating that protects cells from the external environment and also from phagocytosis [5].

Another mechanism of protection observed in *Acinetobacter* is the quorum sensing. This regulatory mechanism impacts the expression of a range of pathways that increase the persistence of this microorganism under hostile environmental conditions, such as dissection, nutrient restriction and antimicrobial treatments [4].

Hydrogen peroxide is a potent disinfectant with bactericidal activity and has been used to control Multidrug-Resistant *A. baumannii* (MDR) outbreaks in health care units. In addition, it plays a key role in containing bacterial infections by the immune system through phagocytes. This process is often referred to as a "respiratory burst", and is critical to contain and eliminate infection by the innate immune system. *A. baumannii* strains contain several genes encoding catalases, as well as Superoxide Dismutase (SOD), which leads to a different phenotype in relation to resistance to oxidative stress [10].

Despite its clinical importance, relatively little is known about the innate host defense mechanisms against respiratory *A. baumannii* infection. Recent studies have shown that the macrophage is an important phagocyte that is involved in host defense, and are crucial in the control of local bacterial. In this regard, play a critical role in host resistance against both intracellular and extracellular bacterial pathogens [5]. Several pathogens have been reported as inducers of cell death due to different mechanisms [6]. It’s known that the exposure to purified OmpA protein leads to apoptosis of eukaryotic cells [7-9]. Omp33 protein also is capable of inducing apoptosis in eukaryotic cells, in addition to modulating autophagy, with the consequent accumulation of autophagosomes to avoid bacterial degradation [10].

In this study, we investigated some pathogenicity aspects presented by MDR *A. baumannii* strains, isolated from different clinical specimens of patients in Belo Horizonte city, Brazil. It was evaluated the presence of genes associated to virulence, tolerance to hydrogen peroxide, the hemolytic activity, as well as the induction of death of bone marrow derived macrophages.

Materials and methods

Study design and characterization of patients

The *A. baumannii* strains were isolated from specimens obtained from critically ill infected patients, admitted to the University Hospital of Universidade Federal de Minas Gerais state, from August 2012 to May 2013. This study was approved by the Research Ethics Committee (RCP Certificate of Presentation for Ethical Appreciation - CAAE - 01402312.60000.5149). The authorization for inclusion was obtained by signing the patients or legal guardians of the Informed Consent Term (ICT). Adult patients (age ≥18 years) were included if they presented an acute infectious process related to *A. baumannii*, according to the treating physician judgment. The variables collected upon inclusion were as following: invasive procedures performed during hospitalization; use of invasive medical devices during hospitalization; site of infection, presence of blood stream infection, presence of sepsis, admission to the Intensive Care Unit (ICU) and hospital mortality.

**Acinetobacter baumannii** strains characterization

All 29 *A. baumannii* strains were identified by the Vitek II system® and through the presence of the chromosomal gene *bla*<sub>GAAS</sub>. Strains showing to be resistance to at least one antimicrobial agent of three different classes were included in this study [11,12]. The reference strain, *A. baumannii* ATCC 19606, was used as control for all experiments.

All strains were submitted to antimicrobial susceptibility to Ampicillin-sulbactam (AB), Ceftazidime (TZ), Gentamicin (GM), Meropenem (MP), Polymyxin B (PO) and Tigecycline (TGC) by E-Test®. The PCR reaction was performed for detection of the following genes: *bla*<sub>GAAS</sub>, *bla*<sub>GAAS</sub>, *bla*<sub>GAAS</sub>, *bla*<sub>GAAS</sub>, *bla*<sub>GAAS</sub>, *bla*<sub>VIM1</sub>, *csuE*, *ompA* and insertion element ISAba1. The genetic diversity among the strains was evaluated by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR, and phenotyping assays of biofilm formation on polystyrene microplate. All these tests were realized in previous study of the same research group [13].

**Detection of genes associated with pathogenicity**

The following genes related to induction to apoptosis in host cells, biofilm formation, quorum sensing and capsule presence were selected and screened using PCR: *omp33, ompA, bap, luxL, luxR, wzc and gaiU*. The primers used are demonstrated in Table 1.

### Table 1: Sequence of bases, amplicons and PCR reaction conditions used in the research of genetic determinants of pathogenicity factors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Pathogenicity factor</th>
<th>Primers sequence (5’- 3’)</th>
<th>Amplification conditions</th>
<th>Amplicon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bap</td>
<td>Biofilm formation</td>
<td>PF 5’-TCGGACATGCCGAGCTAGAACCACA-3’ PR 5’-TCAACTGGTAGGAAATGCGCA-3’</td>
<td>Initial denaturation 95°C, 2 min; 30x: 95°C, 45s; 61°C, 45s; Final extension: 72°C, 1min.</td>
<td>184pb</td>
<td>[22]</td>
</tr>
<tr>
<td>ompA</td>
<td>Induction of apoptosis in host cells</td>
<td>PF 5’-GATGCGCTGAAATGCGAGCTAGAACCACA-3’ PR 5’-CAACTGGTAGGAAATGCGCA-3’</td>
<td>Initial denaturation 94°C, 3 min; 30x: 94°C, 45s; 57°C, 45s; 72°C, 1min, Final extension: 72°C,5 min.</td>
<td>355pb</td>
<td>[33]</td>
</tr>
<tr>
<td>omp33</td>
<td>Induction of apoptosis in host cells</td>
<td>PF 5’-CAAGTGTCGTCAACCATCTTGCTGCA-3’ PR 5’-GTTTCTCTGAGCCAGCTGCAC-3’</td>
<td>Initial denaturation 94°C, 3 min; 30x: 94°C, 45s; 65°C, 45s; Final extension: 72°C, 1min.</td>
<td>194pb</td>
<td>[7]</td>
</tr>
</tbody>
</table>
**Quorum sensing**

PF 5’-TCGGATTTGATTATTGCG CTTATG-3’

- 170pb

4

183pb

PF 5’-GGTTGGGAG TTGAACTGTCC-3’

[5]

**Capsule**

- 370pb

2

PF 5’-CTCCACCAGTGCTTGCAGTA-3’

[5]

**Research Trends of Microbiology**

**in the Multiplicity of Infection Index (MOI): 100.**

The experiment was performed using a 24 h incubation period, the coverslips were stained with panoptic and fixed in microscopy slides for evaluation under capture microscopy.

**Cell count**

Macrophages were photographed under a microscope (15 fields per coverslip), using the program Q Capture Pro 7 and counted through Image J software by densitometry.

**Evaluation of hydrogen peroxide tolerance**

The stress oxidative caused by Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was evaluated on the 29 A. baumannii strains according to Heindorf et al, by disk diffusion [15]. Incremental concentrations of hydrogen peroxide used were: 1%, 5%, 10% and 20%, and a disc with sterile milli-Q water to negative control. After 20h at 37°C, the inhibition halos formed around the paper discs were measured in millimeters (mm). Three independent experiments were performed and the mean halos obtained (in mm) were used for the statistic analysis.

**Evaluation of A. baumannii hemolytic activity**

The evaluation of hemolytic activity of the A baumannii tested strains was performed according to Tayabali et al [16]. In this test, six different kinds of blood were used: Sheep, horse and human. In the latter, four different blood types were tested (O, A, B, AB, all of them being Rh+). Streptococcus pyogenes ATCC 19615 and Klebsiella pneumoniae ATCC 13882 strains were used as positive and negative controls respectively.

**Statistic analysis**

The Stata version 12.0 (STATA Corp. TX, USA) was used to perform the statistical analyses. Linear regression analysis was chosen to compare the variables.

**Results**

Clinical and demographic profile of the study patients

Overall, 29 MDR A. baumannii strains from 20 infected adult patients, 50% of them were female, and the mean age was 60.6 years (± 17.6). The mean hospital length of stay was 61.5 days (± 41.4). All patients had at least one comorbidity, with heart failure (35%) being the most prevalent (Table 2).
Table 2: Frequency of comorbidities and patient intervention included in the study.

<table>
<thead>
<tr>
<th>Comorbidity (n=20)</th>
<th>Frequency (n)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart failure</td>
<td>7</td>
<td>35%</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>6</td>
<td>30%</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>5</td>
<td>25%</td>
</tr>
<tr>
<td>Chronic Renal Failure</td>
<td>5</td>
<td>25%</td>
</tr>
<tr>
<td>Hematologic neoplasia</td>
<td>5</td>
<td>25%</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>2</td>
<td>10%</td>
</tr>
<tr>
<td>Dialysis</td>
<td>2</td>
<td>10%</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>Muscle- degenerative</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>Solid organ neoplasm</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>Pressure ulcer</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>Bedridden</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>Patient intervention</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Admission in ICU</td>
<td>16</td>
<td>80%</td>
</tr>
<tr>
<td>Invasive devices</td>
<td>19</td>
<td>95%</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>15</td>
<td>75%</td>
</tr>
<tr>
<td>Tracheotomy</td>
<td>7</td>
<td>35%</td>
</tr>
<tr>
<td>Hemodialysis</td>
<td>7</td>
<td>35%</td>
</tr>
</tbody>
</table>

Most included patients (95%) received some invasive device during hospitalization and 75% of them underwent mechanical ventilation. All patients received one or more courses of antibiotic therapy during hospitalization, with a mean duration of 31.6 days (±25.0). Finally, five (20%) out of the 20 patients died, three of them due septic shock.

Detection of genes and association to the pathogenicity

Figure 1 shows the frequency of the studied genes in A. baumannii strains. It is known that bap, as well as genes related to capsule and quorum sensing, were associated with biofilm formation. All strains had the bap and gaiU gene, wzc was present in 76% of them. In 72% of the strains both of the quorum sensing genes was observed. In a previous study, using the same strains, the quick biofilm production were evaluated in 4h at 37°C, besides in 24h/25°C, 24h/37°C and 48h/25°C [13]. In this present study we also try to find an association between biofilm productions found before with the presence of the genes researched now. It was observed that all strains were able to form biofilm in at least some conditions, presenting better results at 48h/25°C, in which 97% of the strains formed biofilm, and the non-formed strain contained neither wzc nor the quorum sensing genes. In this condition, among of the strongly adherent strains, 97% present at least one quorum sensing gene. At 37°C, 97% of the strains were able to form biofilm in only 4h and, in this condition; all the four strongly adherent samples contained the wzc and quorum sensing genes. These results reveal the ability of this species to form biofilm and remain for long periods in the environment and, despite no tests related with expression genic had been done, all of them genes seems be importance in this process.

Some studies have shown that OMP33, as well as OMPA, are proteins that cause cytotoxicity in host cells, and since pneumonia associated with mechanical ventilation is the comorbidity caused by A. baumannii that affects the most of the patients, it is interesting to study the presence and the pathogenicity of these proteins in cells of the innate immune. This study also detected the presence of this genes and show that omp33 and ompA genes was present in 45% and 31% of the strains, respectively, that reveals their high prevalence in clinical strains.

Figure 1: Frequency of genes related to pathogenicity of Acinetobacter baumannii strains (n = 29).

Phenotypic detection of pathogenicity factors

Evaluation of A. baumannii tolerance to hydrogen peroxide

Most of the tested strains (69%) showed higher tolerance to H₂O₂ when compared to the reference strain. Further analysis presented significant difference in six strains (21%), showing to be more susceptible to the H₂O₂ in comparison to the reference one (Figure 2). On the other hand, 14 (49%) strains were more resistant to the H₂O₂ than the reference strain (p<0.05) (Figure 2). The overall results of this experiment strongly reveals the pathogenic potential of these strains, on this analysed parameter, since the oxidative stress response is critical to survive to this conditions that the bacterial pathogens may encounter due to the host immune response.

Figure 2: Mean size of the inhibition halos for the thirty Acinetobacter baumannii strains tested.
Research Trends of Microbiology

Evaluation of hemolytic activity

None of the strains of A. baumannii tested in the present study demonstrated hemolytic capacity to the erythrocytes of any of the blood types evaluated.

Evaluation of cell death induced by A. baumannii strains

Figure 3 describes the results of the macrophage culture in vitro assays. Different intensities of decrease in the cell density of macrophages were observed for all A. baumannii strains evaluated, including reference one, and this reduction compared to control (without bacterial inoculum) was statistically significant in 8 (43%) clinical strains tested (p<0.05).

Discussion

The knowledge of the mechanisms involved in the A. baumannii pathogenesis may be useful to better understand the interaction between this microorganism and the affected patients as well as potential target for therapy [2]. Rocha et al [17] evaluated the predictors of an unfavorable outcome in patients with A. baumannii infections at a university hospital in Brazil. The factors associated with mortality in patients with A. baumannii MDR, that was 39.7%, were: age> 60 years, presence of chronic diseases and use of invasive procedures. In our study, all patients presented one or more comorbidity, and the majority underwent some invasive procedure, that was associated to mortality rate elevated (25%).

The investigation of quorum sensing has allowed a better understanding of the host bacteria interaction [18]. The quorum sensing system effectors of Acinetobacter spp. are homologues of the LuxR (receptor) and LuxI (synthase) proteins. Previous studies have shown that surface motility mediated by this quorum sensing regulatory network plays a crucial role in the biofilm development of A. baumannii, by the production of N-(3-hydroxydodecanoyl)-L-HSL (3-hydroxy-C12-HSL) [20, 21]. Accordingly, the disruption of LuxI, which produces the A-HSL molecule results in a 30–40% reduction in the biofilm production compared to that of the isogenic parental strain [31]. In our study, the quorum sensing genes was present in 72% of the strains, and 97% of the positive strains for both genes was strongly adherent at least one condition tested. At room temperature, 97% of the strains formed biofilm. Only one, that did not promote biofilm, also was negative to the quorum sensing genes. Our results reveals that these strains have high potential to form biofilm, especially in room temperature, and the regulation of biofilm formation may be linked to quorum sensing system.

Brossard et al [22] also demonstrated that the presence of biofilm-associated protein (Bap) is necessary for the formation of mature biofilm in medically relevant devices. In our study, we observed the presence of this gene in the all tested strains. Similar result was found in a study realized by Goh et al. (2013) in which 22 of the 24 strains of multi-resistant A. Baumannii to carbapenems carried this gene [23]. Together, this data’s of the high prevalence show the important role that bap and quorum sensing genes plays in biofilm formation, although are needed further studies related to expression genic to clarify the process.

In relation to the polysaccharide capsule, mutants for the ptk (wzc) gene were completely cleared after 24 hours of infection in a model of soft tissue infection [24]. In the present study, 76% of the strains had the wzc gene and all strains had the gaiU gene. The latter is responsible for the synthesis of a UDP-glucose and is part of the central metabolism in E. coli [25]. Recently one study suggested that gaiU gene may be involved in more than one function on the cell [5]. Otherwise, genes such as wzc have been shown to be essential for the expression of the capsule on the cell surface in A. baumannii [24]. Although there are no studies relating these genes to the formation of biofilms, it is known that the presence of capsule is an essential factor in this process, so the high prevalence of biofilm-forming strains in this study may be due to the presence of these genes as well, in addiction to quorum sensing genes and bap, but further studies are needed to confirm this hypothesis.

Capsular polysaccharide could act as a barrier to penetration of the Reactive Oxygen Species (ROS) extracellular into the interior of the microorganism, lead to cell death [32]. In addition, previous studies have shown that mice deficient in ROS-producing enzymes are highly susceptible to A. baumannii infection [30]. Sun et al. (2016) have shown that differences in the production and expression of anti-ROS enzymes, such as the different types of catalases in Acinetobacter spp. resulted in different virulence phenotypes [10]. In a general context, in our study the tested A. baumannii strains presented differences in the sensitivity profile to hydrogen peroxide stress. Approximately half of the samples proved to be significantly more resistant to oxidative stress than the reference strain (p<0.05), and interestingly they all presented the wzc and gaiU genes. These data strongly suggest that the presence of capsule could also be involved in this process, in addition to the formation of biofilm. However, further investigations are required to confirm this hypothesis.

In A. baumannii study has been reported that the presence of OmpA protein relates to induction of death in respiratory tract cells, besides antimicrobial resistance [26]. On the same way, Omp33 is a porine involved in resistance to carbapenems, induction of apoptosis and modulation of autophagy in host cells, besides biofilm formation [7, 27]. The study by Kryzminska et al. evidenced that A. baumannii induces apoptosis in epithelial cells [29]. In our study, 45% of the strains presented omp33, and 31% the ompA. Between the 19 clinical strains tested, eight led to a significant reduction in the macrophage count in vitro (p<0.05), among them, 63% had both ompA and omp33 genes. Thus, these data suggest that these porins may indeed be involved in this process, but as there were different pathogenicity.

Footnote: MOI - Multiplicity of Infection.

Figure 3. Cellular density after infection of macrophages with A. baumannii strains, representing the samples in which a significant statistical difference (p<0.05) was observed in relation to the control (without bacterial inoculum).
profiles, it also shows that may be associated to a difference of expression of these porins [28], or even other factors inducing apoptosis not evaluated in our study.

Regarding to the hemolytic activity of A. baumannii, none of the strains evaluated induced hemolysis in the blood sample tested. Antunes et al [31] evaluated the hemolytic activity of A. baumannii strains. Sheep and horse blood agar were tested, and only in the latter all strains showed some type of hemolysis. In addition, it was noticed that the hemolytic activity was higher in liquid medium than in solid. This interesting result could explain why this species is still classified as non-hemolytic.

This study has some limitations. The small patient population and the retrospective nature of the study did not allow us to make a direct and significant association between the presence of the pathogenicity genes and the clinical outcome. Studies with a larger number of patients are required, as well as a more detailed follow-up of these.

**Conclusion**

In conclusion, all strains showed at least two of the seven genes associated with pathogenicity in this species. The high prevalence of biofilm-forming strains and the concomitant presence of bap, genes related to capsule and quorum sensing, suggests a strong correlation between these factors. The resistance to oxidative stress of the clinicals trains in relation to the reference one was also high (p <0.05). None of the strains were able to hemolyze any of the blood types tested, but most of them induced macrophage death (p <0.05) in vitro, and the death may have occurred due to the presence of ompA and omp33 genes. These interesting data suggested that virulent A. baumannii strains could trigger severe diseases. However, further studies on A. baumannii pathogenicity factors are needed to find novel therapeutic agents or vaccines against this nosocomial pathogen.

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