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Original Article

Evaluation of biofilm production, gelatinase activity, and mannose-resistant hemagglutination in *Acinetobacter baumannii* strains

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Background and Purpose: *Acinetobacter baumannii* is an important nosocomial pathogen, but its pathogenic characteristics are not well defined. The purpose of this study was to evaluate biofilm production, mannose-resistant hemagglutination, and gelatinase production in *A. baumannii* strains isolated from various clinical specimens.
Methods: Eighty six strains of *A. baumannii* isolated from 86 hospital inpatients were studied for biofilm formation, gelatinase activity, and mannose-resistant hemagglutination. The isolates were identified using conventional techniques and/or the API 2ONE system. Comparisons of biofilm production, gelatinase activity, and mannose-resistant hemagglutination were made by chi-squared analysis.
Results: Twenty two and 61 of the isolates agglutinated human group O and AB erythrocytes in the presence of mannose, respectively. Gelatinase activity was detected in 12 isolates (14%), while 64 isolates formed biofilms.
Conclusions: Several parameters may play important roles in causing infection in colonized patients. Identifying the factors that influence virulence may help to separate the colonizing strains into those with high or low potential virulence.

Key words: *Acinetobacter baumannii*; Biofilms; Gelatinases; Hemagglutination

Introduction

Acinetobacter baumannii is a glucose non-fermentative, Gram-negative coccobacillus that is widely distributed in the hospital environment. It is an important opportunistic pathogen that is responsible for a variety of nosocomial infections, including bacteremia, urinary tract infections, secondary meningitis, surgical-site infections, and nosocomial or ventilator-associated pneumonia, especially in patients in the intensive care unit [1,2].

In the past, *Acinetobacter* was considered to be a low-virulence organism. However, the occurrence of fulminant community-acquired *Acinetobacter* pneumonia indicates that these organisms may sometimes be of high pathogenicity and cause invasive disease [3]. Knowledge of *Acinetobacter* virulence factors is still at an elementary stage. Non-specific adherence factors, such as fimbriae, have been associated with *Acinetobacter* and, in the presence of iron-deficient conditions, bacterial growth may be accompanied by the production of receptors and iron-regulated catechol siderophores, which favor bacterial growth and the expression of virulence factors [3].

The purpose of this study was to evaluate the capacity of *A. baumannii* strains isolated from various clinical specimens for biofilm formation and gelatinase production, along with the presence of mannose-resistant hemagglutinin.

Methods

Bacterial strains

Eighty six clinical isolates of *A. baumannii* were collected from 86 inpatients at the Pamukkale University

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School of Medicine Research Hospital, Denizli, Turkey, between January 2006 and December 2007. The isolates were identified using conventional techniques [4] and/or the API 20NE system (bioMérieux, Marcy l'Étoile, France) used according to the manufacturer's instructions.

Gelatinase assay

Gelatinase production was assessed using Luria-Bertani (LB) agar containing gelatin (30 g/L). All isolates were grown overnight on Brain Heart Infusion (Manufacturer, Town, Country) agar plates at 37°C. One loop of each of the colonies was inoculated onto LB agar containing gelatin. After inoculation, the plates were incubated overnight at 37°C and then cooled for 5 h at 4°C. Those colonies with the appearance of a turbid halo were considered to be positive for gelatinase production [5,6].

Biofilm assay

The ability of the *Acinetobacter* strains to form a biofilm on an abiotic surface was quantified as described by Toledo-Arana et al [7]. *A. baumannii* strains were grown overnight in trypticase soy broth (TSB) with 0.25% glucose at 37°C. The next day, each culture was diluted at a ratio of 1:40 in TSB-0.25% glucose, and 200 µL of the cell suspension was used to inoculate sterile 96-well polystyrene microtiter plates. After 24 h at 37°C, the wells were gently washed 3 times with 200 µL of phosphate-buffered saline (PBS), dried in an inverted position, and stained with 1% crystal violet for 15 min. The wells were then rinsed again and the crystal violet was solubilized in 200 µL of ethanol-acetone (80:20, v/v). The optical density (OD) at 595 nm (OD_{595}) was determined using a microplate reader. Each assay was performed in triplicate and repeated 3 times. The following values were assigned for biofilm determination: $OD_{595}<1$, non-biofilm forming; $1<OD_{595}<2$, weak; $2<OD_{595}<3$, medium; and $3<OD_{595}$, strong.

Hemagglutination assay

Hemagglutination was determined via a micro-hemagglutination test using 96-well round-bottom plates and fresh human group O and AB Rhesus-positive erythrocytes. Bacteria were grown on colonization factor antigen (CFA) agar plates at 37°C for 18 h, then suspended and serially diluted in PBS to yield a starting concentration of 10^9 bacteria/mL. 100 µL of the bacterial suspension was then added to each well, followed by an equal volume of a 1% suspension of erythrocytes in PBS. The microplate was then incubated at 4°C for 1 h. The presence of a small pellet of erythrocytes after incubation indicated a negative result, while an even sheet of erythrocytes was considered positive [8,9]. CFA agar was chosen because the hemagglutinating activity of the bacteria was expressed more clearly on that medium [8,10].

Hemagglutination inhibition testing with mannose

Bacteria were grown on CFA agar plates according to the method for the hemagglutination assay. A 100-µL aliquot of each suspension was added to the wells and mixed with an equal volume of a suspension of 1% erythrocytes and 1% D-mannose. Wells containing only the erythrocyte suspension were used as a negative control. A small pellet of erythrocytes at the bottom of the well after 1 h of incubation at 4°C was considered negative, while an even sheet of erythrocytes was considered positive [8,9].

Statistical analysis

Comparisons of biofilm formation, gelatinase activity, and mannose-resistant hemagglutination with the isolation source were made by chi-squared analysis. A p value of <0.05 was considered significant.

Results

Fifty one *A. baumannii* strains (59.3%) were isolated from tracheal aspirates. The source of each isolate is shown in Table 1.

The presence of fimbrial structures in the isolates correlated with the capacity to agglutinate human group O and AB erythrocytes in the presence of mannose (mannose-resistant hemagglutinating activity; Fig. 1). Of 86 isolates of *A. baumannii*, 22 agglutinated human group O erythrocytes and 61 agglutinated human group AB erythrocytes in the presence of mannose (Table 2).

Table 1. Sources of *Acinetobacter baumannii* isolates

Culture	No. (%)
Tracheal aspirate	51 (59.3)
Wound swab	10 (11.6)
Blood	8 (9.3)
Cerebrospinal fluid	5 (5.8)
Urine	4 (4.7)
Sputum	4 (4.7)
Pleural fluid	4 (4.7)
Total	86 (100.0)

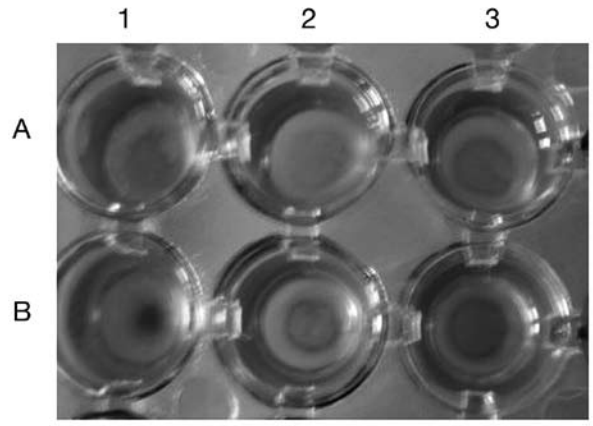

Fig. 1. Hemagglutinating activity in *Acinetobacter baumannii* strains. A, wells without mannose; B, wells with mannose; 1, strains without mannose-sensitive hemagglutinating activity; 2 and 3, strains with mannose-resistant hemagglutinating activity.

Gelatinase activity was detected in 12 isolates (14%) [Table 3 and Fig. 2].

Sixty four isolates were able to form a biofilm (Fig. 3), but the isolates varied in their biofilm-forming capacity. Ten isolates formed a strong biofilm, 27 formed medium-strength biofilms, and 27 formed weak biofilms (Table 4).

No relationship was detected between biofilm formation, gelatinase activity, or mannose-resistant hemagglutination and the isolation source (p>0.05).

Discussion

Acinetobacter, once considered to be a low-grade pathogen, is currently a major problem in intensive care units, particularly in patients receiving artificial ventilation. Antibiotic resistance and virulence are amongst the most important pathogen-related outcome-determining variables in nosocomial infections. Although the significance of multiple antibiotic resistance associated with *Acinetobacter* has been described, the sources of its virulence have not been fully elucidated [3,5]. This study assessed the prevalence of 3 virulence factors: gelatinase production, biofilm production, and the presence of mannose-resistant hemagglutinin.

Table 2. Mannose-resistant hemagglutinating activity in *Acinetobacter baumannii* strains

Culture	Mannose-resistant hemagglutinating activity	
	O erythrocytes	AB erythrocytes
Tracheal aspirate	11	35
Wound swab	2	7
Blood	1	6
Cerebrospinal fluid	3	4
Urine	1	3
Sputum	2	3
Pleural fluid	2	3
Total	22	61

Table 3. Gelatinase activity in *Acinetobacter baumannii* strains

Culture	Gelatinase activity		Total
	Positive	Negative	
Tracheal aspirate	7	44	51
Wound swab	1	9	10
Blood	2	6	8
Cerebrospinal fluid	-	5	5
Urine	-	4	4
Sputum	1	3	4
Pleural fluid	1	3	4
Total	12	74	86

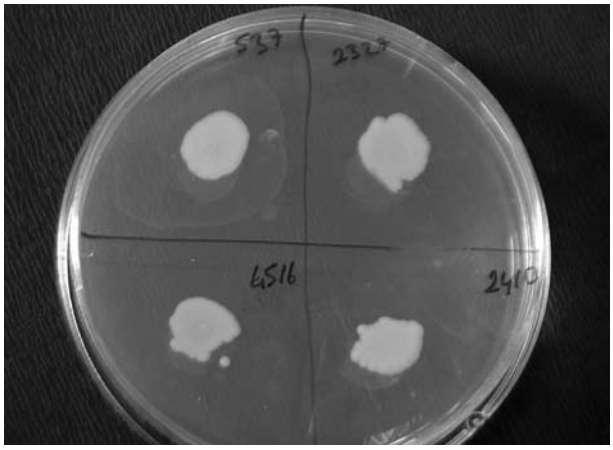

Fig. 2. Gelatinase activity. Strain 537 is positive, while the other strains are negative.

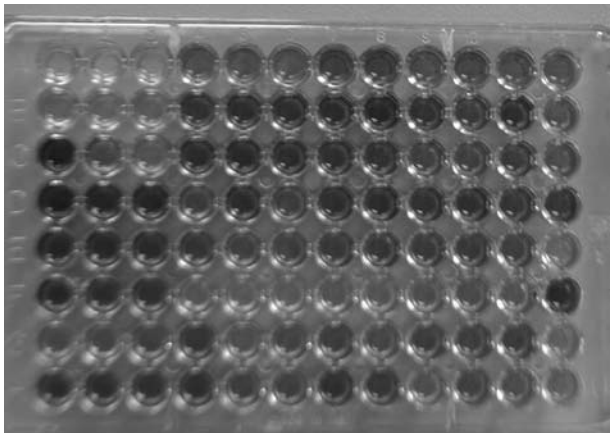

Fig. 3. Microplate-based assay for biofilm activity.

Table 4. Biofilm formation by *Acinetobacter baumannii* strains

Culture	Biofilm formation				Total
	Weak	Medium	Strong	Non-biofilm forming	
Tracheal aspirate	16	18	5	12	51
Wound swab	4	3	1	2	10
Blood	3	2	1	2	8
Cerebrospinal fluid	2	-	2	1	5
Urine	-	3	-	1	4
Sputum	2	-	-	2	4
Pleural fluid	-	1	1	2	4
Total	27	27	10	22	86

The role of *Acinetobacter* as a nosocomial opportunistic pathogen is well documented, but information regarding the factors determining its epidemicity and virulence is limited. Nosocomial *Acinetobacter* strains are striking in their ability to colonize the skin and mucosal surfaces of patients. These organisms may also colonize medical devices, and biofilm formation may play a role in the process of colonization [11]. The potential for biofilm formation of *A. baumannii* may explain its exceptional antibiotic resistance and survival properties. This potential has been demonstrated in a limited number of studies showing that a clinical isolate of the bacterium can attach to and form biofilms on glass surfaces [2,12]. Bacterial biofilms are arrangements in which the cells are morphologically, metabolically, and physiologically different from their planctonic counterparts. Biofilms have been found on the surfaces of medical devices, which are targets for colonization by complex microbial communities [2].

Vidal et al showed that an *A. baumannii* biotype 9 strain isolated from a patient with a respiratory tract infection formed biofilms on glass cover slips using an amorphous material similar to the exopolysaccharide that covers the sessile cells [12]. Sechi et al demonstrated that 16 of 20 *Acinetobacter* strains were able to form a biofilm [5]. In this study, 64 of 86 *A. baumannii* isolates were positive for biofilm formation, with 10 isolates forming strong biofilms, 27 forming medium-strength biofilms, and 27 forming weak biofilms.

Adherence is an important property in bacterial pathogenicity. Pathogenic bacteria usually develop surface structures whose primary function is to interact with receptors on the membranes of target cells, but these structures may also cause erythrocyte agglutination in some animal species. This interaction with erythrocytes reflects the presence of surface structures with a role in adherence to epithelial cells that may be essential for the infection process [8].

Little is known about the surface structures formed by *Acinetobacter* strains that play a role in adherence. Lipopolysaccharide O-antigens within the *Acinetobacter* genus are hydrophobic due to deoxy and amino sugars in their repeating units and the structural branching of most of these polymers. This hydrophobicity may be a factor in non-specific adherence [11].

Pili (fimbriae) or associated polypeptides appear to mediate the adherence of *Escherichia coli* to uroepithelial cells and erythrocytes. Mannose-resistant adhesins such as type P pili, defined by receptor specificity, recognize a glycolipid receptor on human epithelial cells and erythrocytes [13]. Type P fimbriae are the prototypal adhesins responsible for mannose-resistant hemagglutination, and their expression and high hydrophobicity are the major characteristics of pyelonephritogenic strains of *E. coli* [14].

Many enteropathogenic bacteria have well-characterized hemagglutinating properties that are indicative of an ability to adhere to intestinal mucosal surfaces. A correlation among hemagglutination, adhesion, and bacterial pathogenicity has been demonstrated [8]. This correlation suggests that cellular adhesins other than those of the MR type must be present on the cell surface. Additional adhesins have been found in strains isolated from respiratory and urinary tract infection samples, but not in isolates from blood cultures [15].

The fimbriae of *A. baumannii* strains that mediate adherence may be mannose-resistant, although the mechanism of adherence to human epithelial cells remains to be elucidated [16]. Gospodarek tested 309 strains of *Acinetobacter calcoaceticus* for hemagglutinating activity and obtained a positive result for 75% to 85% of the strains [17]. The data obtained from electron microscopic and hemagglutination studies suggest that thin and thick fimbriae of *A. baumannii* participate in the adhesion of these bacteria to red

blood cells [18]. Ruiz et al showed that *A. baumannii* strains also have D-mannose- and D-galactose-resistant hemagglutinating ability [16]. Braun and Vidotto demonstrated that 9 of 13 isolates agglutinated human group AB erythrocytes in the presence of mannose, but none agglutinated group O erythrocytes [9]. In this study, 22 of 86 *A. baumannii* isolates agglutinated human group O erythrocytes and 61 agglutinated human group AB erythrocytes in the presence of mannose. A previous study indicated the presence of thin pili in *Acinetobacter*; the data showed that the thin pili were not essential for motility on solid surfaces, but for adhesion to hydrophobic surfaces and erythrocytes [19].

Gelatinase is a protease that is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other bioactive peptides. Proteinases are associated with inflammation, and this has been shown to contribute to virulence in human and animal models [6]. Gelatinase-producing strains of *Enterococcus faecalis* have been shown to contribute to the virulence of endocarditis in an animal model [20]. It may be advantageous for enterococci to hydrolyze collagen in subcutaneous tissues during wound infection [6]. Sechi et al did not detect gelatinase activity in *A. baumannii* strains [5]. In contrast, gelatinase activity occurred in 12 isolates (14%) in this study.

Several parameters, including host factors, the bacterial burden, and the virulence of the individual strain, may play important roles in causing infection in colonized patients. Considering that *Acinetobacter* is often resistant to multiple antibiotics, the identification of the factors that influence its virulence could help separate colonizing strains into those with high and low potential virulence. Thus, antibiotic therapy could be avoided for strains with low potential virulence, whereas patients with highly virulent colonizing strains in the respiratory tract could be selected for preventive cross-infection measures and early antibiotic treatment [3].

In this study, the prevalence of 3 virulence factors was assessed: gelatinase production, biofilm production, and the presence of mannose-resistant hemagglutinin. These virulence factors may play important roles in *A. baumannii* infections. Many factors may contribute to infection among colonized patients. Understanding the factors that influence virulence in this organism will aid in the identification of those colonizing strains with high or low potential virulence.

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