Evaluation of Adherence, Hemagglutination, and Presence of Genes Codifying for Virulence Factors of *Acinetobacter baumannii* Causing Urinary Tract Infection

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Acinetobacter baumannii *is a strictly aerobic bacterium which causes severe infections, however its pathogenic characteristics are not well defined. Thirteen* A. baumannii *strains isolated from urine of hospitalized and nonhospitalized patients with different ages were investigated for the presence of virulence factors. The isolates belonged to biotypes 2, 6, and 9 and were sensitive to imipenem. The majority of them showed resistance to amikacin, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, norfloxacin, and trimethoprim-sulfamethoxazole. None of* A. baumannii *strains presented genes codifying for 17 different virulence factors previously described in uropathogenic* Escherichia coli*, when tested by polymerase chain reaction (PCR). Nine isolates agglutinated human group AB erythrocytes, in presence of mannose, but none of them agglutinated group O erythrocytes. Adherence to polystyrene was observed in 7 isolates, and this result did not correlate with that obtained in hemagglutination assay. All the isolates were able to grow in iron-limiting conditions, showing that* A. baumannii *produces some type of siderophore. However, the genes* iutA *and* fyuA*, from iron uptake system of* E. coli *and* Yersinia *sp., respectively, were not present in the isolates, suggesting the presence of a different type of siderophore. The fimbriae of* A. baumannii *strains that mediates the adherence are possibly mannose-resistant, eventhough the mechanism of adherence to human epithelial cells still remains to be elucidated.*

Key words: *Acinetobacter baumannii* - adherence - hemagglutination - siderophore - virulence factors

Acinetobacter baumannii is a gram-negative coccobacillus, strictly aerobic, nonmotile, and usually commensal. During the last few decades it emerged as an important opportunistic pathogen due to characteristics that favor its persistence at the hospital environment. *A. baumannii* is resistant to the action of many antimicrobial drugs, spreads easily from patient to patient and survives desiccation, persisting in the environment for many days causing extended epidemic outbreaks (Bergogne-Bérézin & Towner 1996). Nineteen biotypes have been described within the species (Bouvet & Grimont 1987, Bouvet et al. 1990), but only some of them are prevalent in certain geographical areas.

The more common sites of infection by *A. baumannii* are respiratory and urinary tracts, and wounds, and septicemia is a frequent event (Schreckenberger & Graevenitz 1999). The potential risk factors for these infections are advanced age, immunodepression, surgery, presence of invasive devices, use of antimicrobial agents, and the increased length of stay in hospital and intensive care units (ICU) (Bergogne-Bérézin & Towner 1996).

Although *A. baumannii* is considered to be relatively low-grade pathogen, some characteristics of this bacterium may enhance the virulence of the strains involved in infections. Among the virulence factors (VF) it is included: the adhesion to human epithelial cells in the presence of fimbriae and/or capsular polysaccharide (Rosenberg et al. 1981, 1983); the polysaccharide capsule, that probably renders the surface of strains more hydrophilic (Kaplan et al. 1985); the high surface hydrophobicity of certain strains, that facilitates the adherence to prostheses and catheters (Boujaafar et al. 1990); ability to grow in iron-chelated medium by secreting iron-regulated siderophores (Actis et al. 1993); and adhesion to rat bladder tissue what may be a factor contributing to the pathogenicity in the urinary tract or in other tissues (Sepulveda et al. 1998).

During the past decades, new VF have been described in *Escherichia coli*. Pathogenicity-associated islands (PAI) are blocks of VF genes that provide a mechanism to coordinate horizontal transfer of VF genes between lineages, and even between species, have emerged as characteristic of diverse pathogenic bacteria, including uropathogenic *E. coli* strains (Johnson & Stell 2000). Recognized VF in uropathogenic *E. coli* include diverse adhesins, as P fimbriae (*pap* genes), S and F1C (*sfa*), Dr-antigen family (*afa/dra*), type 1 fimbriae (*fimH*) (Le Bouguenec et al. 1992, Johnson & Stell 2000) and curli fibers (*csg*) (Gophna et al. 2001); fibronectin receptor (*fbn*) (Sarén et al. 1999); toxins, as cytotoxic necrotizing factor (*cnf*) (Blanco et al. 1990); siderophores, as yersiniabactin (*fyuA*) and aerobactin (*iutA*); invasins as *IbeA*; polysaccharide coatings as group II and III capsules (*kps*MT); serum resistance (*traT*) (Montenegro et al. 1985) and colicin V production (*cvaC*).

The purposes of this work were to evaluate the ability of *A. baumannii* strains isolated from urine to grow in

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iron-chelated medium; to detect the adherence to polystyrene, the presence of mannose-resistant hemaglutinins and genes codifying for VF previously described in uropathogenic *E. coli*.

MATERIALS AND METHODS

Bacterial isolates - Thirteen *A. baumannii* strains were isolated from urine of hospitalized and nonhospitalized patients in different wards of a university hospital in the city of Londrina, Paraná, Brazil, between January and April 2001. The strains were stored in glycerol tryptcasein soy broth (TSB) at –20ºC.

A. baumannii ATCC 19606 was utilized as control. The strains *E. coli* FV35 (*afa/dra*), *E. coli* FVL2 (*cnf*1, *papC* and *sfa/foc*DE), *E. coli* S5 (*cnf*2), *E. coli* C600 (*csgA*), *E. coli* LG1315 (*cvaC*), *E. coli* V27 (*fim*H, *fyu*A, *iut*A, *kps*MT, *pap*G II,III, and PAI), *E. coli* RS218 (*ibe*A, *sfaS* and *tra*T), *Staphylococcus aureus* ATCC 25923 (*fbn*) were utilized as positive controls for VF.

Biotyping and susceptibility to antimicrobial drugs - Bacteria were biotyped according to Bouvet and Grimont (1987), using M70 defined broth with the specific C sources: levulinate, citraconate, L-phenylalanine, phenylacetate, 4-hydroxybenzoate, and L-tartrate (Sigma Chemical Co, US). Filter-sterilized carbon sources were added at a final concentration of 0.1% (wt/vol). Growth of bacteria was recorded after 2 and 6 days at 37°C.

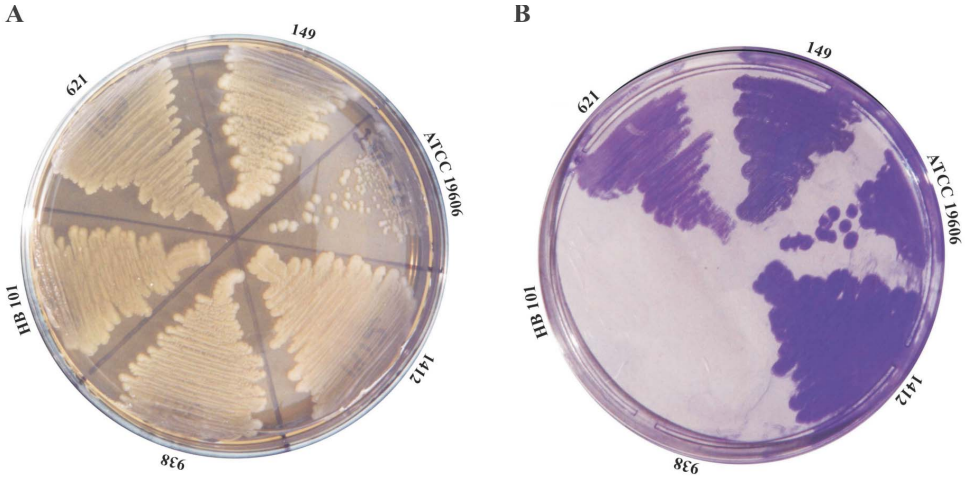
Susceptibility to antimicrobial drugs was assessed by MicroScan Walk-Away (Dade Behring, West Sacramento, CA), utilizing panel Combo 1 to amikacin, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, norfloxacin, tobramycin, and trimethoprim-sulfamethox-azole, according to the manufacturer's recommendations. Isolates showing an intermediate level of susceptibility were classified as resistant.

Growth under iron-limiting conditions - The isolates were grown on M9 minimal medium containing the iron chelator 2,2-dipyridyl (DIP) (Sigma) in concentrations of 50 µM and 200 µM and 2% agar (Echenique et al. 1992). The plates were incubated at 37ºC for 18 h. DIP was prepared as a 20 mM stock solution in distilled water and kept at –20ºC.

Hemagglutination assay - Hemagglutinating activity was determined by micro-hemagglutination test using 96-well round-bottom plates and fresh human group O and AB, Rh positive erythrocytes. Bacteria were grown on Luria Bertani plates at 37°C for 24 h, suspended and serially diluted in PBS, and the starting concentration was 10^{10} bacteria/ml. A suspension of 1% erythrocytes containing 1% D-mannose (Sigma) was added to each well and mixed. Wells containing only the suspension of erythrocytes were utilized as negative control. A small pellet of erythrocytes at the bottom after 1 h incubation at 4°C were considered negative, and those containing an even sheet of erythrocytes across the well were considered positive (Sepulveda et al. 1998).

Adherence to polystyrene - A simple replica method was used to study adherence to polystyrene (Rosenberg 1981). A polystyrene disk is pressed onto an agar plate containing bacterial isolates growth after incubation at 37°C for 24 h. The replica formed is then extensively washed under tap running water and stained with gentian violet. Colonies of adherent bacteria remain bound to the polystyrene surface, whereas, the nonadherent ones are removed during the washing. *E. coli* strain HB101 was used as negative control.

PCR - Bacterial DNA to be amplified was released from whole microorganisms by boiling (Le Bouguenec et al. 1992); the oligonucleotides used are shown in Table I. PCR was carried out in a total volume of 25 µl containing 5 µl of template DNA, 20 pmol of each of the primers, the four deoxynucleoside triphosphates (each at 200 µM), PCR buffer with 2.5 mM of $MgCl_2$, and 1.5 U of Taq DNA polymerase (Gibco). Amplifications were carried out as follows: a cycle of 95ºC for 12 min, 25 cycles of 94°C for 30 s, annealing at 63°C for 30 s, 68°C for 3 min, and a final extension at 72°C for 10 min in a Thermal Cycler (Gene Amp PCR System 9700/Perkin Elmer). Different temperatures of annealing of 60ºC, 50ºC, and 40ºC were only utilized with the primers Fbn, CsgA and Cnf2, respectively.



Adherence to polystyrene. A: nutrient agar plate containing *Acinetobacter baumannii* isolates; B: polystyrene replica stained with gentian violet. 621, 149, and 1412: adherent isolates; 938: nonadherent isolate; *Eschirechia coli* strain HB101: negative control; *A. baumannii* ATCC 19606: positive control.

The amplified DNA was visualized in 1.5% agarose gels stained with ethidium bromide. The 100-bp ladder (Gibco) was used as standard for determining molecular mass of PCR products.

RESULTS AND DISCUSSION

Numerous outbreaks of nosocomial infections caused by multiresistant *A. baumannii* have been reported in various countries, including Brazil (Oliveira et al. 1993, Bergogne-Bérézin & Towner 1996, Levin et al. 1996, Vaz et al. 1996). Important risk factors are involved in the urinary tract infections, which occur mostly in elderly debilitated patients confined to ICU, and with permanent urinary catheters. In this work the isolates were obtained from patients aging 18 to 88 years, and the 18 year-old patient was in use of permanent urinary catheter (Table II). Six strains were isolated from inpatients, being 2 from ICU and 4 from infirmaries; and 7 strains were isolated from outpatients being 3 from emergency and 4 from ambulatory.

The isolates belonged to biotypes 2 (6/13), 6 (6/13), and 9 (1/13); they did not present association with the origin of isolation, and were uniform in their sensitivity to imipenem. Three isolates from nonhospitalized patients were sensitive to the majority of the tested drugs. The others, showed resistance to amikacin, ceftriaxone,

TABLE I

Primers to virulence factors utilized in polymerase chain reaction

Gene	Virulence factor	Name of primer	Sequence (5'-3')	Amplified DNA (bp)	Reference
afa/draBC	Dr fimbriae	afa1 afa2	GCTGGGCAGCAAACTGATAACTCTC CATCAAGCTGTTTGTTCGTCCGCCG	750	Le Bouguenec et al. (1992)
cnf1	cytotoxic necrotizing factor	cnf1 cnf2	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498	Yamamoto et al. (1995)
cnf2	cytotoxic necrotizing factor	cnf2a cnf2b	AATCTAATTAAAGAGAAC CATGCTTTGTATATCTA	543	Blanco et al. (1996)
csgA	curli fiber	M464 M465 R	ACTCTGACTTGACTATTACC AGATGCAGTCTGGTCAAC	200	
cvaC	colicin V	ColV-Cf ColV-Cr	CACACACAAACGGGAGCTGTT CTTCCCGCAGCATAGTTCCAT	680	Johnson & Stell (2000)
fbn	fibronectin receptor	Fbn F1 Fbn R1	GGTAATCAGTCATTCGAG TGGCACACTGTCGAAGTC	207 93	
fimH	type 1 fimbriae	FimH f FimH r	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508	Johnson & Stell (2000)
fyuA	yersiniabactin	FyuA f FyuA r	TGATTAACCCCGCGACGGGAA CGCAGTAGGCACGATGTTGTA	880	Johnson & Stell (2000), Schubert et al. (1998)
ibeA	invasin	ibe10 fibe10 r	AGGCAGGTGTGCGCCGCGTAC TGGTGCTCCGGCAAACCATGC	170	Huang et al. (1995), Johnson & Stell (2000)
iutA	aerobactin	AerJ f AerJ r	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300	Johnson & Brown (1998)
kpsMT II	capsule	kpsII f kpsII r	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	272	Johnson & Stell (2000)
PAI	pathogenicity -associated island	RPAi f RPAi r	GGACATCCTGTTACAGCGCGCA TCGCCACCAATCACAGCCGAAC	930	Johnson & Stell (2000)
papC	P fimbriae	pap1 pap2	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328	Le Bouguenec et al. (1992)
PapG II, III	P fimbriae	pGf pGr	CTGTAATTACGGAAGTGATTTCTG ACTATCCGGCTCCGGATAAACCAT	1070	Marklund et al. (1992)
sfa/focDE	S fimbriae	sfa1 sfa2	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410	Le Bouguenec et al. (1992)
sfaS	S fimbriae	SfaS f SfaS r	GTGGATACGACGATTACTGTG CCGCCAGCATTCCCTGTATTC	240	Johnson & Stell (2000)
traT	serum resistance	TraT f TraT r	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	290	Johnson & Stell (2000)

ciprofloxacin, trimethoprim-sulfamethoxazole; the resistance to other antimicrobials varied among the isolates (Table II). Hospitalized patients, from ICU and infirmaries, presented resistance to most antimicrobial agents tested, while nonhospitalized patients, from ambulatory and emergency, showed variable drug resistance patterns (Table II). Since these patients had been previously hospitalized the high resistance rates observed in these isolates could be due to the selection of the strains in the hospital environment.

Little is known about the mechanisms of pathogenicity of *A. baumannii*. In this work, we tested *A. baumannii* strains, isolated from urine, for the presence of genes that codify for various VF by PCR, as described for uropathogenic *E. coli*. All the isolates were negative to the VF analyzed.

Adherence is related to adhesins filamentous (fimbrial structures) or non-filamentous (Hornick et al. 1991). Fimbrial structures and their participation in adhesive properties involving fibronectin were investigated by means of agglutination assays (Köljelg et al. 1996). Sepulveda et al. (1998) verified that adherence to bladder tissue is a natural attribute of strains belonging to different *A. baumannii* biotypes, a comparable trait found in a uropathogenic *E. coli* strain. Additionally, the presence of fimbrial structures in *A. baumannii* isolates was correlated with the capacity to agglutinate human group O erythrocytes, in presence of mannose or galactose.

Hemagglutination tests have been used for the in vitro assay of the expression of many mannose-resistant hemaglutinins, designed fimbriae type P, S, Dr, and F, frequently found in extra-intestinal *E. coli*. In this work, 9 of 13 *A. baumannii* isolates agglutinated human group AB erythrocytes, in presence of mannose, but none of the isolates agglutinated human group O erythrocytes. In contrast, Sepulveda et al. (1998) described that all *A. baumannii* strains agglutinated human group O erythrocytes in presence of mannose. Although the isolates here studied present mannose-resistant hemaglutinins, the genes *pap*, *afa/dra* and *sfa* codifying for the fimbriae P, Dr, and S, respectively, were not detected by means of PCR with specific primers.

Rosenberg (1981) described a high correlation between affinity of bacteria to polystyrene and surface hydrophobicity. The existence of thin fimbriae in *A. baumannii* which are a major factor in adherence to polystyrene was de-

TABLE II

Characteristics of patients and *Acinetobacter baumannii* strains isolated from urine

Strain	Date of isolation (mo/day/yr)	Age (yr)	Ward	Resistance profile (MIC in µg/ml)	Biotype B & G	Polystyrene adherence	Hemagglutination O^+	Hemagglutination AB^+
135	01/15/01	25	ICU	AM (>32), CZ (>16), CX (>32), CP (>2), ST (>2/38)	2	-	-	+
149	01/03/01	25	Amb	-	6	+	-	-
621	01/10/01	44	Amb	AM (>32), CZ (>16), CX (>32), CP (>2), GN (>8), NF (>8), ST (>2/38)	2	+	-	-
922	04/09/01	32	ICU	AM(32), CZ (16), CX (>32), CP (>2), GN (>8), TO (>8), ST (>2/38)	2	-	-	+
931	01/10/01	41	Inf	AM (>32), CZ (>16), CX (>32), CP (>2), GN (8), NF (>8), ST (>2/38)	6	+	-	+
938	01/15/01	88	Eme	AM (>32), CZ (>16), CX (>32), CP (>2), GN (>8), NF (>8), ST (>2/38)	2	-	-	+
1185	01/18/01	44	Inf	AM (>32), CX (16), CP (>2), GN (>8), NF (>8), ST (>2/38)	6	+	-	+
1412	01/22/01	41	Eme	-	6	+	-	+
1711	01/26/01	81	Eme	AM (>32), CZ (>16), CX (>32), CP (>2), GN (8), NF (>8), ST (>2/38)	2	-	-	+
6001	03/29/01	75	Inf	AM (>32), CZ (>16), CX (>32), CP (>2), GN (8), NF (>8), ST (>2/38)	2	-	-	-
6526	04/06/01	83	Amb	NF (8)	6	+	-	-
6531	04/06/01	18	Amb	AM (>32), CZ (16), CX (>32), CP (>2), GN (>8), NF (>8), TO (>8), ST (>2/38)	9	+	-	+
6808	04/11/01	42	Inf	AM (>32), CX (>32), CP (>2), GN (>8), NF (>8), TO (>8), ST (>2/38)	6	-	-	+

ICU: intensive care unit; Amb: ambulatory; Inf: infirmary; Eme: emergency; MIC: minimal inhibitory concentration; AM: amikacin; CZ: ceftazidime; CX: ceftriaxone; CP: ciprofloxacin; GN: gentamicin; NF: norfloxacin; ST: trimethoprim/sulfamethoxazole; TO: tobramycin; B & G: Bouvet & Grimont (1987); –: negative; +: positive

scribed by Rosenberg et al. (1982). The surface hydrophobicity of microorganisms appears to play an important role in their attachment to various polymers (Magnusson 1982), and is related to adherence of microorganism to the plastic surface, as catheters and prostheses. In our work, 7 of 13 isolates from urine adhered to polystyrene (Figure), but no association was found with hemagglutination assay.

Another VF described in *A. baumannii* is the production of siderophores by isolates growing under iron-limiting conditions. These bacteria are capable to express high-affinity iron uptake systems composed by siderophores, components capable to convert polymeric ferric oxy-hydroxides into soluble iron chelates, and iron-repressible outer membrane proteins (IROMPS) (Neilands 1995). Smith et al. (1990) detected the presence of iron chelator 2,3-dihidroxi-benzoic acid (DHBA) and IROMPS in the culture supernatant of *Acinetobacter* sp. Besides DHBA, *A. baumannii* strains present other siderophores type catechol (Echenique et al. 1992, Actis et al. 1993). The Fur protein of *A. baumannii*, which regulates the genes involved in iron uptake, was sequenced and indicated that it is 63% identical to that of *E. coli* (Daniel et al. 1999).

Considering that, in our work, all *A. baumannii* strains were able to grow in M9 medium plates containing 50 µM and 200 µM DIP, we conclude that this bacterium produces siderophores. However, the genes *iut*A and *fyuA* from iron uptake system of *E. coli* and *Yersinia* sp., respectively, were not present in the studied isolates, suggesting the presence of a different type of siderophore.

In conclusion, *A. baumannii* strains here studied do not possess DNA sequences similar to the codifying for VF present in uropathogenic *E. coli*; and possibly the fimbriae of *A. baumannii* mediating the adherence are mannose-resistant, eventhough the mechanism of adherence of *A. baumannii* still remains to be elucidated.

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