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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Multidrug-resistant *Acinetobacter baumannii*: differential adherence to HEp-2 and A-549 cells

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Abstract

Acinetobacter baumannii has been associated with antimicrobial resistance and ability to form biofilms. Furthermore, its adherence to host cells is an important factor to the colonization process. Therefore, this study intended to identify some virulence factors that can explain the success of *A. baumannii* in causing nosocomial infections. We studied 92 *A. baumannii* isolates collected from hospitals in Rio de Janeiro, Brazil. Isolates were identified and the susceptibility to antimicrobials was determined. Oxacilinase type β-lactamase encoding genes were amplified by polymerase chain reaction, and genetic diversity was investigated by pulsed-field gel electrophoresis (PFGE). In addition, biofilm formation on polystyrene plates using crystal violet staining was quantified, and adherence to human cell lines was evaluated. Eighty-six isolates were multidrug-resistant, of which 93% were carbapenem-resistant. All isolates had the $bla_{\text{OXA-51}}$ gene and 94% had the $bla_{\text{OXA-23}}$ gene, other searched bla_{OXA} genes were not detected. PFGE typing showed two predominant clones, and biofilm production was observed in 79% of isolates. *A. baumannii* isolates adhered better to HEp-2 cell compared with A-549 cell. Clones A, B, E, and F showed a significantly increased adherence to HEp-2 compared with adherence to A-549 cell. Our findings revealed that *A. baumannii* isolates had high frequencies of resistance to antimicrobial agents, ability to form biofilm, and capacity to adhere to HEp-2 cells.

Keywords *Acinetobacter baumannii* · Antimicrobial resistance · PCR · PFGE · Biofilm · Adherence to cell

Introduction

Acinetobacter baumannii is an aerobic, non-fermentative Gram-negative coccobacillus that can survive for long periods in the environment [1]. *A. baumannii* infections are a major *cause* of hospital-acquired infections worldwide, mainly due to *A. baumannii*'s ability to live under critical situations in this environment [2, 3]. Resistance to desiccation and antibiotics [4, 5], high production of biofilm on abiotic surfaces, and the adherence to human cells [6–9] might justify the prevalence of this microorganism in hospitals in outbreaks and endemic situations.

Extensive use of antibiotics in hospitals has contributed to the rapid appearance of resistant *A. baumannii* isolates [10]. Carbapenems are widely used to treat infections caused by MDR *A. baumannii*, but increasing resistance to carbapenems in *A. baumannii* infections in the past decade has been creating therapeutic challenges [11]. Carbapenem resistance in *A. baumannii* is generally associated to the oxacillinase (OXA) enzyme group. Among the phylogenetic subgroups of OXA already identified, six were described in *A. baumannii*: OXA-23-like, OXA-24-like, OXA-51-like, OXA-58-like, OXA-143-like, and OXA-235-like [12–14].

In Brazil, the first report of carbapenem-resistant *A. baumannii* isolates was in 2003 with the description of OXA-23–producing *A. baumannii* belonging to a single clone [15]. In 2009, Carvalho et al. (2009) described the spread of two predominant clones of MDR OXA-23–producing *A. baumannii* in the city of Rio de Janeiro [16]. However,

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another study done in the same city demonstrated no predominant clones and distribution of the bla_{OXA-23} gene in several clones analyzed [17].

There are few papers on the virulence-associated factors of *A. baumannii* [18]. Biofilm formation plays a significant role in the pathogenicity of this microorganism and is associated with increased bacterial survival [19]. Bacteria in biofilms are more resistant to antimicrobial agents when compared with planktonic cells [20]. Moreover, biofilm allows bacteria to persist on biotic and abiotic surfaces and helps them to evade the host response. The ability of bacteria to adhere to eukaryotic cells is critical for pathogenesis and generally considered an essential early step in the colonization process, and the biofilm formation and its adherence to cells are common features in clinical *A. baumannii* isolates [21, 22].

The features that make *A. baumannii* able to induce outbreaks and human diseases are not totally understood. Therefore, this study intended to identify some virulence factors present in *A. baumannii* isolates obtained from patients with nosocomial infections. To this purpose, we characterized the antimicrobial resistance, clonal relationships, biofilm formation, adherence, and invasion to eukaryotic cells ability of 92 *Acinetobacter baumannii* isolates from two public hospitals in Rio de Janeiro, Brazil.

Material and methods

Bacterial isolates

Ninety-two clinical *Acinetobacter baumannii* isolates collected from two public hospitals in the city of Rio de Janeiro, between 2010 and 2011, were included in this study. *A. baumannii* isolates were identified by automated VITEK 2 System (bioMérieux Vitek Systems Inc., Hazelwood, MO, CA) and by partial *rpoB* sequencing [23]. The sources of isolates collected included urine, blood, catheter, tracheal secretion, and wound and bronchoalveolar lavage. In an earlier study, we described molecular identification, sources, and antimicrobial susceptibility for polymyxin B by minimum inhibition concentration (MIC) of these isolates [24].

Antimicrobial susceptibility testing

To evaluate the antimicrobial susceptibility of the isolates, the automated VITEK 2 System (bioMérieux Vitek Systems, Inc., Hazelwood, MO) was used with a Gram-negative identification card (NG-105) following the manufacturer's instructions. The following antibiotics were tested: amikacin, gentamicin, ampicillin-sulbactam, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, ciprofloxacin, imipenem, and meropenem. Values were interpreted according to the guidelines of CLSI [25], and isolates were classified as non-multidrug-resistant (n-MDR) and multidrug-resistant (MDR) according to the criteria of Magiorakos et al. (2012) [26].

Oxacillinases detection

The genomic DNA was extracted using DNeasy Tissue Kit (QIAGEN, Valencia, CA), according to manufacturer's instructions. Multiplex-PCR reactions using specific primers for amplifying bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51},bla_{OXA-58}, and $bla_{OXA-143}$ genes were done as described previously by Woodford et al. (2006) and Higgins et al.(2010) [27, 28]. For the bla_{OXA-23} and bla_{OXA-51} positive isolates, new PCR reactions were performed [29, 30]. The PCR products were purified using PureLink PCR purification Kit (Invitrogen) and sequenced using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequences were analyzed using DNASTAR software, and subsequently, the obtained sequences were submitted to BLAST (*Basic Local Alignment Search Tool*) [2].

Pulsed-field gel electrophoresis (PFGE)

Isolates were typed by PFGE [31] following digestion of genomic DNA with *Apa* I (Invitrogen). DNA fragments were separated on 1% (w/v) agarose gels in 0.5% TBE [Tris–borate–ethylene diamine tetra-acetic acid (EDTA)] buffer using a CHEF-DR III apparatus (Bio-Rad, Hercules, CA) with 6 V/cm, pulsed from 5 to 15 s, for 18 h at 14 °C. Gels were stained with ethidium bromide and photographed under ultraviolet light. The *Apa* I restriction profiles were initially compared by visual inspection according to the criteria of Tenover et al. (1995) [32]. Computer-assisted analysis was also performed using BioNumerics v.4.0 (Applied Maths, Sint-Martens-Latem, Belgium). Comparison of the banding patterns was accomplished by the unweighted pair group method with arithmetic averages (UPGMA) using the Dice similarity coefficient.

Quantification of biofilm formation

The biofilm formation was performed in 96-well microplates of polystyrene assay (Nunclon ™ – Nalgene Nunc International, Rochester, NY Catalog No. 269 787), using an overnight culture, according to Moskowitz et al. (2004) [33]. Each strain was tested in triplicate. We used the *A. baumannii* ATCC 19606 type strain as positive control. Wells with culture medium not inoculated were considered as negative control and *Escherichia coli* K-12 isolate weakly biofilm producing. All tests were performed in triplicate and the results were averaged. The biofilm formation was graded as described by Stepanovic et al. (2000) [34].

Eukaryotic cell culture

Human alveolar epithelial tumor cells A-549 and human epithelial laryngeal carcinoma cells (HEp-2) were obtained from the American Type Culture Collection. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 1% of penicillin, 1% of streptomycin, and 10% fetal bovine serum at 37 °C in 5% CO_2. Confluent growth was obtained in 100 mm diameter in 24-well plates (Nunclon™ – Nalgene Nunc International, Rochester, NY).

Bacterial adherence assays

The adherence assays of *A. baumannii* isolates to eukaryotic cells were determined as described previously with some modifications [7]. Strains of *A. baumannii* were cultured in 10 mL of nutrient agar at 37 °C for 24 h. Bacterial suspensions were washed and adjusted to match the turbidity standard of 2 McFarland units (approximately 6.0×10^8 CFU/mL) in DMEM. The adherence assays were conducted by exposing eukaryotic cells at a multiplicity of infection (MOI, bacterium: eukaryotic cell ration) ~ 50:4 of *A. baumannii* isolates. The infected plates were centrifuged for 10 min at 700 ×g prior to the incubation to promote adherence of bacteria to cells and to synchronize infections. Each strain was tested in triplicate and experiments were performed in duplicate. After 2 h of incubation at 37 °C, the coverslips were washed, and the cells fixed and stained with Giemsa solution for 30 min. The coverslips were placed on glass slides. Bacterial adherence to the cells was determined by optical microscopy. In each field displayed on the optical microscope, bacteria that have adhered to human epithelial cells were counted. For each coverslip, 300 ± 45 cells were inspected to determine the number of adhered bacteria [9, 35]. Two independent experiments were performed with two coverslips each. *A. baumannii* ATCC 19606 was included in each test series as a reference strain.

Bacterial invasion assay

For quantification of adherent bacteria, the cells were plated in 24-well plates (Nunclon™ – Nalgene Nunc International, Rochester, NY) without glass coverslips. The cells grown in plates were infected with *A. baumannii* isolates under the same conditions as those described for bacterial adherence assays. After the period of 2 h incubation, we added 250 μg/ml of polymyxin B to the wells to kill any extracellular bacteria. After this time, the wells were washed with PBS, and the cells were lysed with Triton X-100 in 0.1X PBS 1X for 20 min at 37 °C in 5% CO_2. The CFU was determined by successive dilution in 0.85% saline and plated on MacConkey agar. The *Yersinia enterocolitica* O:3 isolate was used as positive control for bacterial invasion [8]. Two independent experiments were performed.

Cell viability by the tetrazolium reduction assay

In addition to the bacterial invasion assay, we evaluated the cell viability of HEp-2 cell following exposure to *A. baumannii* isolates by the tetrazolium reduction assay [36], which was performed in a *96-well* microtiter plate (Corning Costar® Sigma-Aldrich, catalog No. 32190102). After incubation of the confluent monolayers of cells HEp-2 previously infected with 10 μL (~ 10^6 CFU) of *A. baumannii* isolates for 2 h, 20 μl of the complete DMEM medium were removed and 20 μl of MTT (0.2 mg/ml) were added to each well. Then the cells were incubated for 4 h at 37 °C in 5% CO_2, and after which, 20 μl of sodium dodecyl sulfate 10% (SDS, GE Healthcare, Uppsala, Sweden) was added. The absorbance was read in spectrophotometer at 590 nm [37]. Cell viability in infected cultures was calculated considering the absorbance values of negative control as 100%. Two independent experiments were carried out in duplicate.

Statistical analysis

Data obtained in quantification of biofilm formation and cell viability by MTT were analyzed for statistical significance using Grubb's test. The p value < 0.05 was considered significant.

Student's t test was used to determine the differences in the percentage of cell viability infected with *A. baumannii* ATCC 19606 type strain and the selected isolates. Mann–Whitney test was used to determine the differences in the number of bacteria that adhered to A-549 or HEp-2 cells.

Results

Distribution of isolates and antimicrobial resistance

A total of 92 isolates of *A. baumannii* were collected from two hospitals in Rio de Janeiro (Brazil). Resistance and intermediate resistance to cefotaxime was the most common (87, 94.6%), followed by piperacillin/tazobactam (85, 92.4%), cefepime and ciprofloxacin (83, 90.2%), carbapenems (80, 87%), ceftazidime (77, 83.7%), ampicillin/sulbactam (76, 82.6%), and gentamicin (53, 57.6%). High percentage susceptibility was observed for amikacin (19, 20.7%). Thirteen isolates were resistant to all of the 10 antibiotics. Only 6 isolates were classified as n-MDR, and all of them were susceptible to carbapenems. Most of the isolates that showed resistance to carbapenems were collected from tracheal aspirates (23%, $n = 18$).

Oxacillinases encoding genes

Using multiplex-PCR, all isolates were positive for the bla_{OXA-51} gene, and 85 isolates (92%) were positive for bla_{OXA-23}.The sequences were submitted to BLAST and similarity values > 99% were found among all. No isolate was positive for bla_{OXA-24}, bla_{OXA-58}, or $bla_{OXA-143}$ genes.

PFGE typing

According to Tenover's criteria [32], the PFGE analysis revealed 22 clones among the 92 isolates, with a similarity > 85% [38]. Clone A (30% of isolates; $n = 28$) was the predominant, followed by clone B (19% of isolates; $n = 17$). Forty-seven of the total 92 *A. baumannii* isolates were classified in 20 sporadic clones named from C to U, which included at least one isolate of each profile (Fig. 1). Clones A, B, and C were present in both hospitals, whereas others were present in only one of them. The isolates belonged to the predominant clones A and B were mainly collected from tracheal aspirates (24% and 41%, respectively). The majority of n-MDR isolates was grouped only in sporadic clones (S, Q, R, F, V), only one n-MDR *A. baumannii* isolate belonged to the predominant clone A.

Biofilm formation

The quantification of biofilm formation assay showed that 39 isolates were considered strongly adherent (42%), 26 isolates were moderately adherent (28%), and 8 isolates were weakly adherent (9%). Nineteen isolates were considered non-biofilm producers. Most isolates that were considered strongly adherent were collected from wound (58%, $n = 7$), tracheal aspirates (58%, $n = 11$), and catheter (53%, $n = 15$).

Cell adherence assay

Two distinct eukaryotic cells were used to examine adherence of six *A. baumannii* isolates (Figs. 2 and 3). All isolates were selected as MDR, carbapenem resistant and biofilm producers. Four isolates were considered strongly adherent, and the other two were considered moderately adherent (30,654, clone F) and weakly adherent (33,677, clone A). Verification of the ability of differing clones to adhere to the eukaryotic cells revealed no clonal specific trends. Clones A, B, E, and F showed a *significantly* increased ($p > 0.05$) adherence to HEp-2 when compared with A-549 cells. For the other isolates (clones C and D), there was no statistical significance.

All *A. baumannii* isolates studied and the *A. baumannii* ATCC 19606 type strain were not able to invade HEp-2 cells. *Y. enterocolitica* O:3, used as positive control for bacterial invasion, was able to grow after plating in MacConkey agar (0.5×10^3 CFU/mL).

HEp-2 cells were examined by MTT-assay to determine the viability after infection with *A. baumannii* isolates. Cells infected by the *A. baumannii* ATCC 19606 type strain showed 45% cell viability and by clones A, B, C, D, E, and F exhibited 22%, 19%, 38%, 80%, 36%, and 20% cell viability, respectively. After infection, all isolates, except clone C and D, induced a significant decrease in HEp-2 cell viability ($p < 0.05$) compared with the HEp-2 cell viability after infection by the type strain.

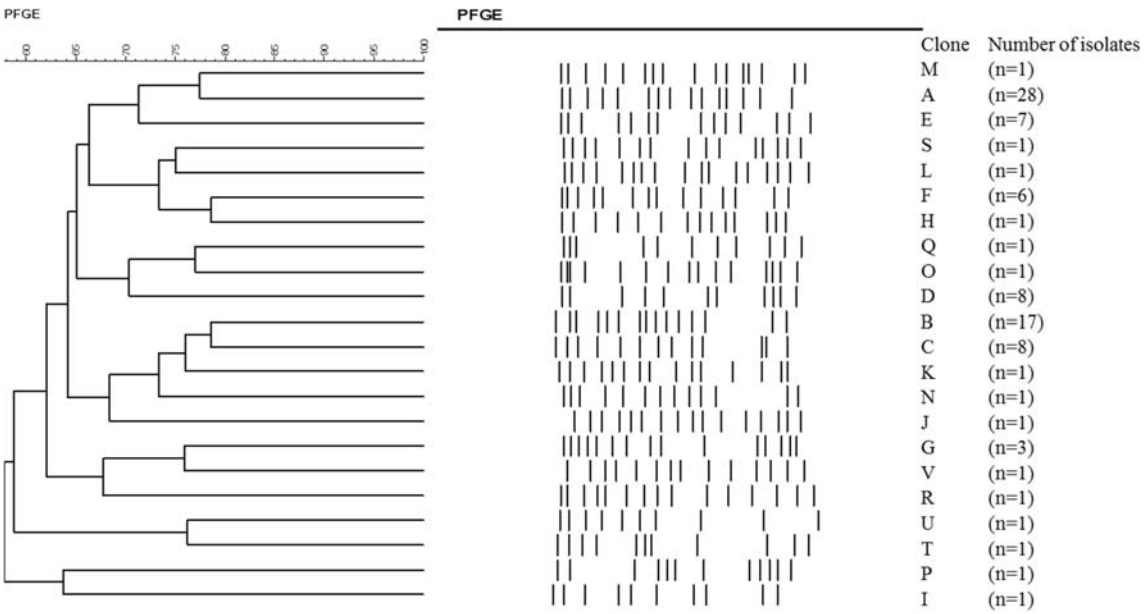

Fig. 1 Dendrogram of *A. baumannii* isolates, built with GelCompar II version 4.0 (Applied Maths). Pulsed-field gel electrophoresis (PFGE) detected the patterns of bands after digestion of chromosomal DNA with restriction endonuclease *Apa* I

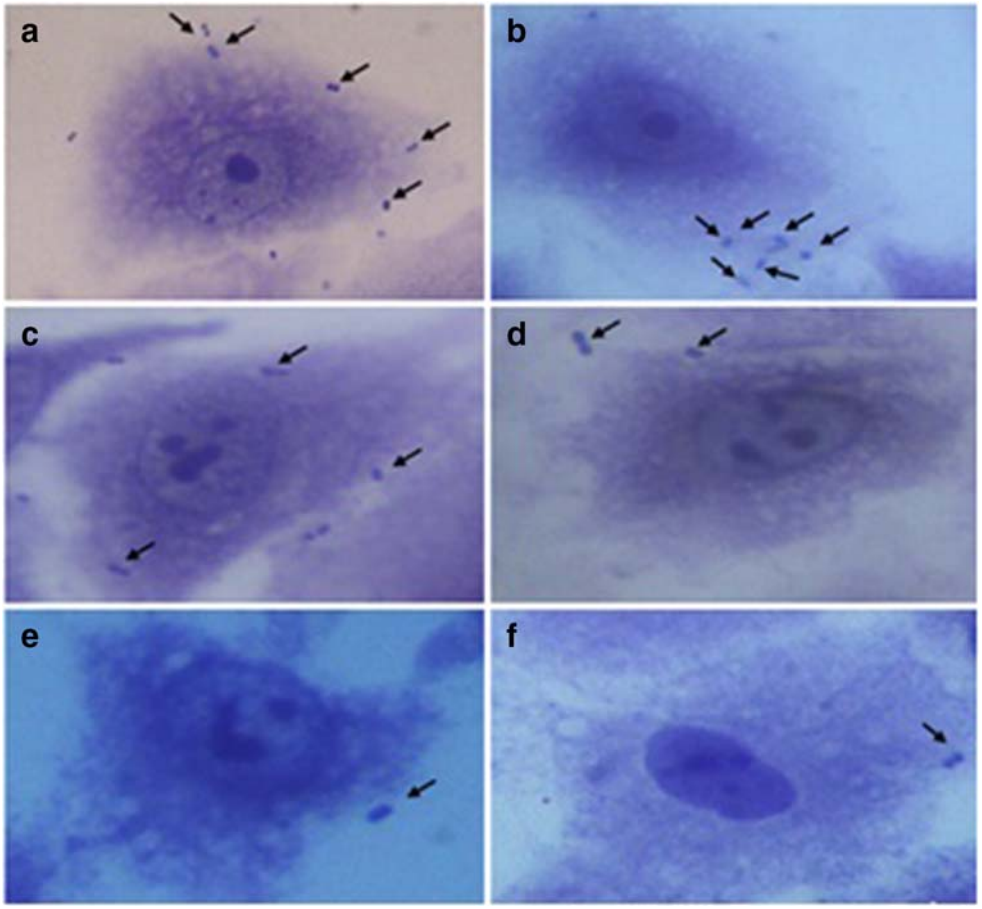

Fig. 2 A-549 cells infected for 2 h with *A. baumannii* stained and photographed in an optical microscope at a 40x magnification. **a** Clone A. **b** Clone B. **c** Clone C. **d** Clone D. **e** Clone E. **f** Clone F

Discussion

Antibiotic resistance and persistence in hospital environments are the two factors that most contribute to the success of *A. baumannii* as an opportunistic pathogen. However, it is possible that the emergence of isolates over others is the result of their different individual characteristics that allow colonization and disease. Biofilm formation and its adherence to eukaryotic cells were studied as potential virulence-related characteristics among *A. baumannii* isolates.

Our data show high predominance of MDR *A. baumannii* and high susceptibility to amikacin, as observed previously [37]. Carbapenems were used to be one of the last resorts when treating resistant *Acinetobacter* infections, but today high resistance to them is reported. While in 2001 isolates collected in Brazil exhibited only 10% of resistance [39], recently this resistance has increased dramatically [8, 10, 40]. In general, the *A. baumannii* carbapenem-resistant isolates evaluated here were also resistant to other antimicrobial agents, except to polymyxin B. However, the intensive use of polymyxins has selected resistant isolates, and this has already been shown in Spain, Korea, Iran, USA, and Brazil [24, 40–44].

Different mechanisms are involved in the *A. baumannii* resistance to carbapenems, but the β-lactamase production is the most important factor [29]. Our study found that the $bla_{\text{OXA-51}}$ gene was the sole carbapenemase gene detected in all the isolates, as expected [45]. The $bla_{\text{OXA-23}}$ gene appeared in most *A. baumannii* isolates. These data may indicate that carbapenem resistance of isolates most often mediate by oxacillinases like $bla_{\text{OXA-23}}$ gene [46]. Moreover, we also observed the $bla_{\text{OXA-23}}$ gene in carbapenem-susceptible isolates. These results suggest the importance of the occurrence of the silent carriage $bla_{\text{OXA-23}}$ gene in hospital environments and the detection of isolates as reservoir oxacillinase genes [46]. Previously, Carvalho et al. (2011) described that this susceptibility occurs due to different associations between the $bla_{\text{OXA-23}}$ and ISAba1 genes [46]. With regard to $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, and $bla_{\text{OXA-143}}$ genes, in this study, no *Acinetobacter* isolate was positive, although international epidemiologic studies have indicated that these oxacillinases are spread among *A. baumannii* clinical isolates [47].

Various genotyping methods have been used to facilitate the understanding of the epidemiology of an *A. baumannii* infection [26]. PFGE typing showed two main clones, indicating clonal dissemination. A high prevalence of clones A and B in the studied hospitals showed that health professionals should be very careful to prevent the spread of these clones. Isolates positive for the $bla_{\text{OXA-23}}$ gene were associated with predominant and

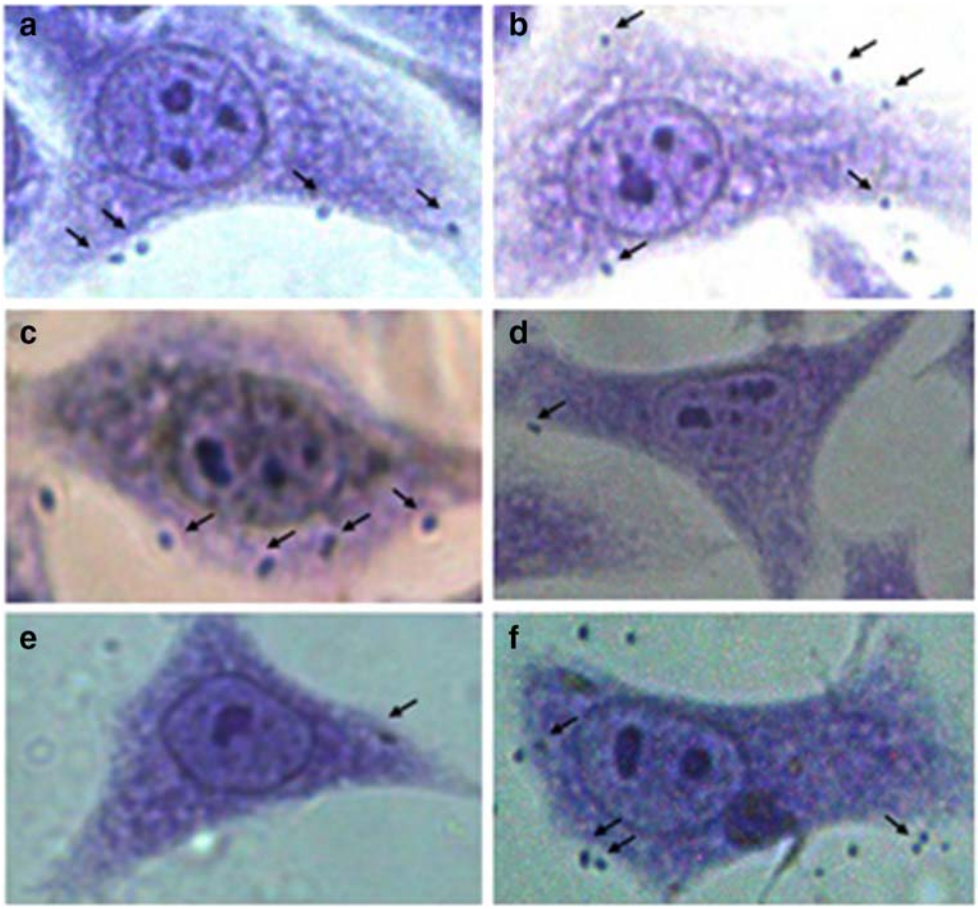

Fig. 3 HEp-2 cells infected for 2 h with *A. baumannii* stained and photographed in an optical microscope at a × 40 magnification. **a** Clone A. **b** Clone B. **c** Clone C. **d** Clone D. **e** Clone E. **f** Clone F

sporadic clones, unlike a previous study that found the presence of this gene only in few distinct clones [17, 48]. These findings show the diversity of clinical *A. baumannii* isolates circulating in Rio de Janeiro.

The ability of *A. baumannii* to persist in hospital environments could be attributed to its ability to form biofilm on abiotic surfaces. Our data show that *A. baumannii* isolates have different abilities to attach to polystyrene microplates. Previously, Rodríguez-Baño et al. (2008) showed that the ability to form biofilms on abiotic surfaces is common in *A. baumannii* isolates and that one of the most frequent sites of collection of these isolates is related to the catheter [22]. But in this same study, a higher biofilm formation was also observed in isolates collected from bloodstream infection; these data differ from our study [22].We observed that the MDR isolates were not significantly strong biofilm-forming, since carbapenem-susceptible isolates produced more biofilm than resistant isolates, as observed before [19, 20, 47]. No difference has been verified regarding biofilm-forming capacity between predominant and sporadic clones. Previous reports also did not describe a significant relationship between biofilm formation and PFGE clones [48–50]. Multiple factors contribute to *A. baumannii* virulence and pathogenicity. The diversity of virulence characteristics among *A. baumannii* isolates has been previously shown [49, 50], but little information exists about *A. baumannii* adhesion to eukaryotic cells. The bacterial adhesion assay demonstrated that *A. baumannii* clones differ in their abilities to adhere to A-549 and HEp-2 cells [7, 9, 51]. Moreover, the isolate belonging to clone F was not able to form strong biofilm in polystyrene microplates, but was able to adhere better to the HEp-2 cells. The non-correlation of biofilm formation on abiotic and biotic surfaces indicates that different mechanisms are involved in these colonization processes, such as the participation of structures such as pili, associated only with biofilm formation on abiotic surfaces [52]. In addition, the isolates belonging to the major PFGE clones (A and B) did not adhere to eukaryotic cells more than others [7]. The optical microscopy analyses also suggest that bacteria can adhere but not invade eukaryotic cells, and this was consistent with the invasiveness experiment performed. Reports indicate that *A. baumannii* infection leads to apoptotic death without invasion of the pathogen [8, 53]. Cell viability by the MTT reduction assay showed consistent data with that observed in optical microscopy. However, the isolates belonging to A and B clones induced significant decrease in the viability of HEp-2 cells, which was not observed in optical microscopy.

In summary, the results presented in this study showed the ability of the *A. baumannii* clinical isolates to form biofilm and attach to eukaryotic cells. Furthermore, we indicated

individual differences among the isolates, irrespective of their belonging to the predominant or sporadic clones, mainly in relation to biofilm formation and adherence to epithelial cells.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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