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Multidrug Resistance Related to Biofilm Formation in *Acinetobacter baumannii* and *Klebsiella pneumoniae* Clinical Strains from Different Pulsotypes

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Abstract The emergence of *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains in the hospital environment has been associated with the presence of multiple genetic elements, virulence factors and the ability to form biofilms. This study evaluated the biofilm formation ability of clinical and environmental *A. baumannii* and *K. pneumoniae* strains, isolated from various sources and presenting different molecular characteristics, resistance profiles and pulsed-field gel electrophoresis patterns. Fifty-three isolates were recovered from 2009 to 2014 in a Brazilian university hospital. Investigation of biofilm formation was performed for 10 strains of each species assessed by an initial adhesion assay, biofilm cell concentration and biofilm biomass, evaluated by quantitative assays in replicates, in three independent experiments. All strains of *A. baumannii* were able to attach to polystyrene plates, although two strains adhered to a lesser degree than the control. *K. pneumoniae* strains showed opposite behaviour, where only three strains adhered significantly when compared to the control. Quantitative evaluation revealed that in five *A. baumannii* and four *K. pneumoniae* isolates the biomass production could be characterised as moderate. None of the isolates were strong biofilm producers. Our results demonstrate: (1) biofilm formation is a heterogeneous property amongst *A. baumannii* and *K. pneumoniae* clinical strains and it was not associated with certain clonal types; (2) no relationship between multidrug resistance and biofilm production was observed; (3) more virulent *K. pneumoniae* strains tended to present higher production of biofilm.

Introduction

In recent years the emergence and spread of resistance to the β-lactam class of antibiotics in members of the Enterobacteriaceae family and gram-negative non-fermenting bacilli such as *Acinetobacter baumannii* has become a significant problem. In hospitals this is mainly due to the large increase in resistance to extended spectrum (3rd and 4th generation) cephalosporins (ESBL) and carbapenems [34]. *A. baumannii* and *Klebsiella pneumoniae* together are known to be responsible for a significant proportion of nosocomial infections, such as ventilator-associated pneumonia, bacteraemia and urinary tract infections; pneumonia and bacteraemia are associated with substantial morbidity and mortality rates and high-cost health care [21].

Currently, the increased incidence of *A. baumannii* and *K. pneumoniae* multidrug-resistant strains is a global problem [18], a consequence of the ability of these microorganisms to acquire resistance genes to almost all antibiotics available for the treatment of severe infections [13]. In addition to antimicrobial resistance, numerous studies have described the spread of predominant clones in Brazil [3, 26]. Another important aspect that complicates the treatment of serious infections with these phenotypes is the ability to form biofilms [15]. Biofilms are bacterial communities where the cells adhere to each other and to an inert or living surface, in which the aggregated microorganisms produce large

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quantities of extracellular polymeric substances [8]. Biofilms improve the persistence of bacterial growth in harsh environments, serve as a reservoir for the dissemination of pathogens under favourable conditions and act as a nidus for the exchange of antimicrobial resistance genes [12].

Complex interactions between pathogenicity, epidemicity and antibiotic resistance have resulted in the successful global spread of gram-negative bacilli. In this study we evaluated the ability to form biofilms of clinical and environmental *A. baumannii* and *K. pneumoniae* strains recovered in a university hospital. Additionally, the biofilm formation phenotype was evaluated in the context of virulence, resistance genes and genetic patterns.

Materials and Methods

Bacterial Strains, Media and Growth Conditions

The origin and epidemiological characteristics of the strains used in this study are described in Table 1. The clinical strains were recovered from hospitalised patients and the environmental strains were collected in the bedroom of the patients at the Clinical Hospital of the Federal University of Uberlândia, a 533-bed public tertiary care teaching hospital in the southeast of Brazil, obtained from 2009 to 2014. The identification of the clinical strains and antimicrobial susceptibility testing was done using Vitek II (bioMérieux, Craponne, France) and environmental strains were identified using gram, fermentation of carbohydrates, citrate utilisation test, motility test, oxidase and indole test. Confirmation of species identification of *A. baumannii* was done by detecting the bla_{OXA-51} gene by PCR. For *A. baumannii* strains resistance to tigecycline and imipenem was also determined by the E-test® method according to the manufacturer's guidelines (AB Biodisk, Solna, Sweden). All the resistance tests were done in accordance with the Clinical and Laboratory Standards Institute recommended practices [6]. Since there were no breakpoints available for tigecycline for *Acinetobacter* spp., US Food and Drug Administration (FDA) tigecycline breakpoints listed for Enterobacteriaceae (≤ 2, 4 and ≥ 8 μg/ml for susceptible, intermediate and resistant strains, respectively) were applied to *Acinetobacter* spp. in this study. Strains were stored at -80 °C and routinely cultured on Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) plates (Becton, Dickinson and Company—BD, USA) for 24 h at 37 °C unless stated otherwise. The characteristics of the strains used for adhesion and biofilm assays are specified in Table 2.

Genetic Techniques

PCR Amplification

For *A. baumannii* isolates the following resistance genes were investigated: bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58}, $bla_{OXA-143}$, IS*Aba1* (insertion sequence), IS*Aba1*/$bla_{OXA-23-like}$, IS*Aba1*/$bla_{OXA-51-like}$, *carO* (29 kDa) and outer membrane protein (OMP) of 33–36 kDa. For *K. pneumoniae* strains the resistance genes were investigated:

Table 1 Characteristics of *Acinetobacter baumannii* and *Klebsiella pneumoniae* isolates

Bacterial species	Site of isolation	Pulsed-field type (PFTs)	Resistance profile[a]	Phenotype
Acinetobacter baumannii (n = 23)	Endotracheal aspirate (n = 17)	A (n = 9)	Cephalosporins of 4th generation (n = 23)	MDR (n = 22)[b]
		G (n = 3)	Carbapenem (n = 19)	
		B–F (n = 5)	Fluoroquinolones (n = 15)	
	Environment (n = 6)	E (n = 1)	Aminoglycosides (n = 15)	
		G (n = 1)	Tigecycline (n = 18)	
		H (n = 4)		
Klebsiella pneumoniae (n = 30)	Blood (n = 14)	A (n = 2)	Cephalosporins of 3rd and 4th generation (n = 30)	MDR (n = 20)
	Urine (n = 11)	B (n = 3)	Fluoroquinolones (n = 19)	
	Endotracheal aspirate (n = 3)	C–X (n = 21)	Aminoglycosides (n = 17)	
	Swab rectal (n = 2)	Non-typable (n = 4)	Carbapenems (n = 6)	

MDR multidrug-resistant organism

[a] For *A. baumannii* strains: cephalosporins of 4th generation, cefepime; carbapenem, imipenem; fluoroquinolones, ciprofloxacin; aminoglycosides, amikacin and/or gentamicin. For *K. pneumoniae* strains: cephalosporins of 3rd and 4th generation, ceftriaxone and cefepime; fluoroquinolones, ciprofloxacin and/or levofloxacin; aminoglycosides, gentamicin—only one strain was resistant to amikacin; carbapenems, ertapenem, imipenem and meropenem

[b] *A. baumannii* strains of endotracheal aspirate and environment

Table 2 Characterisation of *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains used in the study of adhesion and biofilm formation

Strains	Date of isolation	Source	Resistance profile[a]	Resistance genes	Porines genes[b]
Acinetobacter baumannii					
Ab 05	10.14.2011	Endotracheal aspirate	AMK; SAM; FEP; CIP; GEN; IPM; TGC	bla_{OXA-51}; bla_{OXA-23}; *ISAba1*/OXA-23; *ISAba1*/OXA-51	*carO*; 33–36 kDa
Ab 09	03.17.2012	Endotracheal aspirate	SAM; FEP; CIP; TGC	bla_{OXA-51}; bla_{OXA-23}; *ISAba1*; *ISAba1*/OXA-51	*carO*; 33–36 kDa
Ab 10	05.03.2012	Endotracheal aspirate	SAM; FEP; CIP; IPM	bla_{OXA-51}; bla_{OXA-23}; *ISAba1*; *ISAba1*/OXA-51	33–36 kDa
Ab 12	06.25.2012	Endotracheal aspirate	AMK; SAM; FEP; CIP; IPM	bla_{OXA-51}; bla_{OXA-23}; *ISAba1*; *ISAba1*/OXA-51	33–36 kDa
Ab 13	02.02.2012	Endotracheal aspirate	SAM; FEP; GEN; IPM; TGC	bla_{OXA-51}; bla_{OXA-23}; *ISAba1*; *ISAba1*/OXA-23; *ISAba1*/OXA-51	*carO*; 33–36 kDa
Ab 15	10.18.2011	Environment	SAM; FEP; GEN; IPM; TGC	bla_{OXA-51}; bla_{OXA-23}; *ISAba1*; *ISAba1*/OXA-23; *ISAba1*/OXA-51	*carO*; 33–36 kDa
Ab 18	02.07.2012	Environment	SAM; FEP; GEN; IPM	bla_{OXA-51}; bla_{OXA-23}; *ISAba1*; *ISAba1*/OXA-23; *ISAba1*/OXA-51	*carO*; 33–36 kDa
Ab 20	05.11.2012	Environment	SAM; FEP; IPM	bla_{OXA-51}; bla_{OXA-23}; *ISAba1*; *ISAba1*/OXA-23; *ISAba1*/OXA-51	*carO*; 33–36 kDa
Ab 21	02.22.2012	Endotracheal aspirate	FEP; CIP; TGC	bla_{OXA-51}; *ISAba1*; *ISAba1*/OXA-51	*carO*; 33–36 kDa
Ab 22	01.10.2012	Endotracheal aspirate	FEP; CIP; TGC	bla_{OXA-51}; *ISAba1*	*carO*; 33–36 kDa

Strains	Date of isolation	Source	Resistance profile	Beta-lactamase genes	Virulence-associated genes[c]
Klebsiella pneumoniae					
Kp 11	07.09.2009	Urine	CET, CRO, FEP, AMP, AMC	–	*fimH*, *wabG*, *ecpA*
Kp 30	08.15.2009	Tracheal aspirate	CET, CRO, FEP, AMP, AMC, LVX, GEN	bla_{TEM}	*fimH*, *fimA*, *wabG*, *ecpA*
Kp 37	08.18.2009	Blood	CET, CRO, FEP, AMP, AMC, LVX, GEN	bla_{SHV}, bla_{CTX-M}, bla_{TEM}	*fimH*, *fimA*, *wabG*, *ecpA*, *khe*
Kp 60	09.14.2009	Urine	CET, CRO, FEP, AMP, AMC	–	*fimH*, *fimA*, *wabG*, *ecpA*
Kp 109	11.12.2009	Blood	CET, CRO, FEP, AMP, AMC, LVX, GEN	bla_{SHV}, bla_{CTX-M}, bla_{TEM}	*fimH*, *fimA*, *wabG*, *iucC*, *khe*
Kp 129	01.01.2010	Blood	CET, CRO, FEP, AMP, AMC, GEN	bla_{SHV}, bla_{CTX-M}, bla_{ampC}	*ecpA*
KPC 02	05.22.2013	Blood	CET, FOX, CRO, FEP, AMP, AMC, ETP, IPM, MEM, LVX, CIP, GEN	bla_{KPC}, bla_{SHV}, bla_{CTX-M}, bla_{TEM}	*fimH*, *fimA*, *wabG*
KPC 03	05.25.2013	Urine	CET, FOX, CRO, FEP, AMP, AMC, ETP, IPM, MEM, CIP	bla_{KPC}, bla_{SHV}, bla_{CTX-M}, bla_{TEM}	*fimH*, *fimA*, *wabG*, *iucC*, *khe*
KPC 04	07.07.2014	Rectal swab	CET, FOX, CRO, FEP, AMP, AMC, ETP, IPM, MEM, CIP	bla_{KPC}, bla_{TEM}	*wabG*, *ecpA*
KPC 05	07.07.2014	Rectal swab	CET, FOX, CRO, FEP, AMP, AMC, ETP, IPM, MEM, CIP	bla_{KPC}, bla_{SHV}, bla_{CTX-M}, bla_{TEM}	*fimH*, *fimA*, *wabG*, *iucC*, *ecpA*, *khe*

PFGE pulsed-field gel electrophoresis

[a] Antibiotics evaluated: *AMP* ampicillin; *AMC* amoxicillin/clavulanic acid; *AMK* amikacin; *CET* cephalothin; *CRO* ceftriaxone; *CIP* ciprofloxacin; *CRO* ceftriaxone; *ETP* ertapenem; *FEP* cefepime; *FOX* cefoxitin; *GEN* gentamicin; *IPM* imipenem; *LVX* levofloxacin; *MEM* meropenem; *SAM* ampicillin/sulbactam; *TGC* tigecycline

[b] Virulence factors: *fimH*/*fimA*/*mrkD*, encode type 1 and the type 3 pili; *ecpA*, adhesins; *khe*, haemolysin; *iucC*, iron acquisition system; *wabG* and *rmp*, capsular polysaccharide

[c] Porines genes: *carO*, 29 kDa protein

bla_{KPC}, bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{ampC}; and the virulence genes *khe*, *fimH*, *iucC*, *mrkD*, *rmp*, *wabG*, *ecpA* and *fimA*. All the conditions for the amplification of *A. baumannii* and *K. pneumoniae* genes, as well as the primers used in the PCR reactions are further described in supplementary Table 1.

Pulsed-Field Gel Electrophoresis (PFGE)

Of the total of 23 *A. baumannii* and 30 *K. pneumoniae*, 23 *A. baumannii* and 26 *K. pneumoniae* strains were typed by PFGE (Table 1). For *A. baumannii* isolates intact genomic DNA was digested with *ApaI* (Invitrogen) according to the protocols described by Romão et al. [27] and for *K. pneumoniae* the chromosomal DNA was digested with *XbaI* as described by Gruteke et al. [11]. The similarity of the PFGE fingerprints was determined by computer comparison and interpreted using the BioNumerics software (Applied Maths, Belgium). A dendrogram was generated using the Dice method and by clustering by the unweighted-pair group method using average links with a 1 % band and position tolerance. A similarity coefficient of 85 % was chosen for cluster definition.

Investigation of Biofilm Formation

The isolates were grown for 18 ± 2 h, at 37 °C with shaking at 120 rpm in 20 ml of TSB using bacteria grown on TSA plates not older than 2 days as inoculum. After cells were harvested by centrifugation (5000 rpm, 10 min, 4 °C), they were washed twice and resuspended in saline (0.9 % NaCl prepared in distilled water) at a concentration of approximately 1×10^9 cells/ml, prior to use in biofilm and adhesion assays. *A. baumannii* ATCC 19606 and *K. pneumoniae* NTUH-K2044 were used as controls.

Initial Adhesion Assay

In order to evaluate the initial adhesion, 200 µl of a cell suspension containing 1×10^7 cells/ml prepared in TSB was added to 96-well polystyrene plates. Initial adhesion was allowed to occur for 2 h at 37 °C with rotation at 120 rpm. Bacteria adhered to 96-well polystyrene plates were washed twice with a 0.9 % NaCl solution and harvested by scraping of wells for 90 s. The cell suspension obtained was plated on TSA for colony-forming unit (CFU) enumeration. All experiments were done in quadruplicate, in three independent experiments.

Biofilm Formation Assay

Biofilms were formed as described previously [4], with some modifications. Briefly, 200 µl of a cell suspension containing 1×10^7 cells/ml prepared in TSB was added to 96-well polystyrene plates. Biofilm formation was allowed to occur for 24 h at 37 °C with rotation at 120 rpm. Bacteria grown in 96-well polystyrene plates were washed twice with a 0.9 % NaCl solution and allowed to dry in an inverted position. The total biomass was measured by methanol (Merck) fixation, crystal violet (Merck) staining and acetic acid (Merck) elution as previously described. The eluted dye was removed from each well and placed in a new 96-well microtitre plate and its absorbance read on an ELISA plate reader (Polaris® Celer, Brazil) at 570 nm. The experiments were done with eight replicates for each strain, in six independent experiments. TSB without bacteria was used as a negative control. Biofilm production was interpreted according to the criteria of Saxena et al. [30]. The optical density cut-off value (ODc) was established as three standard deviations (SD) above the mean of the optical density (OD) of the negative control: ODc = average OD of negative control + 3 × SD of negative control. For easier interpretation of the results, the strains were divided into the following categories according to their optical densities (ODi): ODi ≤ ODc = non-biofilm producer; ODc < ODi ≤ 2 × ODc = weak biofilm producer; 2 × ODc < ODi < 4 × ODc = moderate biofilm producer; 4 × ODc < ODi = strong biofilm producer.

Biofilm Cell Concentration

The biofilm cell concentration was determined by CFU enumeration. After biofilm formation as described, the biofilms were washed twice with a 0.9 % NaCl solution and harvested after scraping the wells for 90 s. The cell suspension obtained was plated onto TSA plates. All experiments were done in quadruplicate, on three independent occasions.

Definition

Multidrug-resistance (MDR) was defined as resistance to at least one agent in three or more antimicrobial categories [20].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software, San Diego, CA). Quantitative assays were compared using the Kruskal–Wallis test, applying Dunn's multiple comparison test. All tests were performed with a confidence level of 95 % and statistical significance was defined as $P < 0.05$.

Results

Overall, from 53 isolates previously described, among 30 and 23 non-repetitive isolates of *K. pneumoniae* and *A. baumannii*, respectively, that were recovered from clinical specimens and the environment from 2009 to 2014 in a Brazilian university hospital, the strains were resistant to various antibiotics, including cephalosporins (53/53; 100 %) and carbapenems (25/53; 47.2 %). Among *A. baumannii* isolates, 82.6 % (19/23) showed resistance to imipenem and 78.3 % (18/23) to tigecycline. In *K. pneumoniae* strains, the resistance to third and fourth generation cephalosporins was highlighted and the resistance to fluoroquinolones was accentuated. Multidrug-resistant isolates were frequent, comprising 66.7 % of *K. pneumoniae* and 95.7 % of *A. baumannii* isolates. A polyclonal profile was observed for both microorganisms, however in *A. baumannii* the genotypes A (n = 9, 52.9 % of clinical isolates) and H (n = 4, 66.6 of environmental isolates) were predominant. In *K. pneumoniae* the presence of sporadic clones was more frequent (Table 1).

In *A. baumannii* strains the resistance rates to ampicillin/sulbactam, cefepime and imipenem were similar in predominant genotypes and sporadic clones, in contrast to resistance rates observed for amikacin, gentamicin and tigecycline that were higher in predominant genotypes than in sporadic ones. The resistance rates to all antimicrobials tested were lower for the strains that presented the pulsotype H (Fig. 1a). In *K. pneumoniae* the resistance rates to all antibiotics evaluated were higher in sporadic genotypes than in predominant A and B clones (Fig. 1b).

Of the 23 *A. baumannii* isolates analysed, 43.5 % (10/23) harboured all of the resistance genes associated or not with the element IS*Aba1* as well as the genes evaluated for porins. Among the 30 isolates of *K. pneumoniae*, the most prevalent ESBL types were bla_{SHV} (n = 22), bla_{TEM} (n = 22) and bla_{CTX-M} (n = 21). Four of the six bla_{KPC} + isolates carried bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes concomitantly, and one of these isolates was also positive for almost all virulence genes evaluated, the *wabG* and *fimH* genes being prevalent in these strains (data not shown). Among the *A. baumannii* isolates selected for the evaluation of biofilm production, the

Fig. 1 **a** Antimicrobial resistance patterns of *A. baumannii* and **b** *K. pneumoniae* isolates clustered according to predominant and sporadic genotypes. AMP: ampicillin; AMC: amoxicillin/clavulanic acid; AMK: amikacin; CIP: ciprofloxacin; CRO: ceftriaxone; ETP: ertapenem; FEP: cefepime; GEN: gentamicin; IPM: imipenem; LVX: levofloxacin; MEM: meropenem; SAM: ampicillin/sulbactam; TGC, tigecycline.
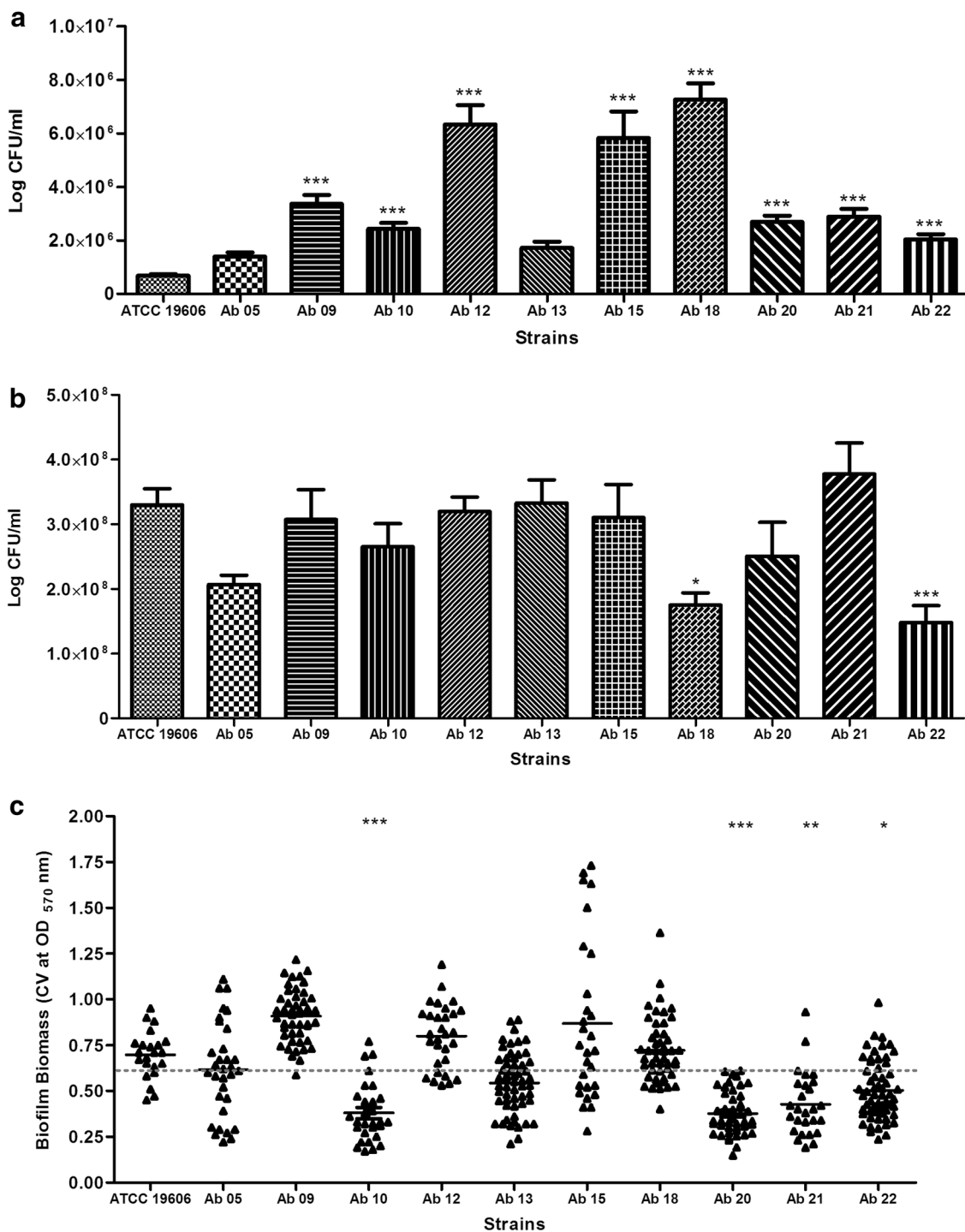185 mm × 236 mm
(300 × 300 DPI)
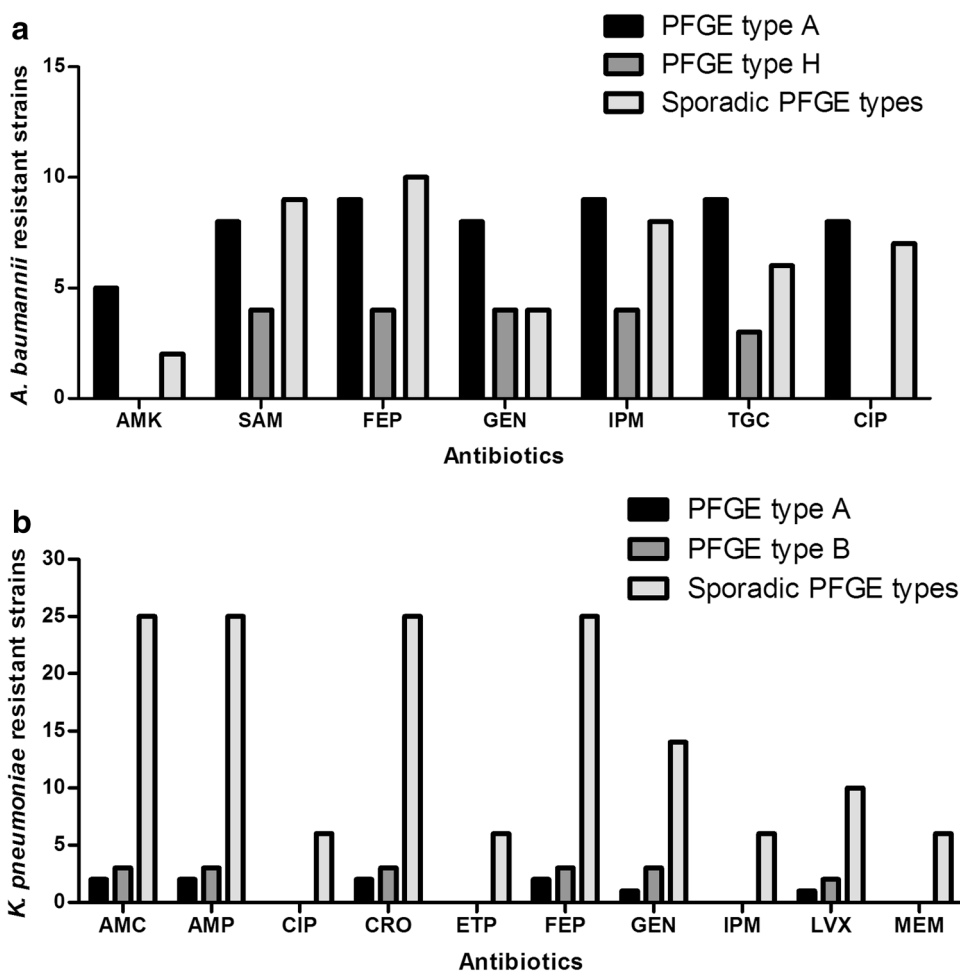
Fig. 2 Evaluating the number of adhered cells, biomass and biofilm formation in strains of *A. baumannii*. **a** Number of adhered cells onto polystyrene plates after 2 h period of contact, expressed by Log CFU/ml; **b** biofilm cellular concentration expressed by Log CFU/ml; **c** biofilm biomass expressed as crystal violet optical density ($OD_{570\ nm}$). Results represent means plus standard deviations (error bars) of three independent experiments. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$, using Kruskal–Wallis, Dunn's multiple comparison test. 199 mm × 276 mm (300 × 300 DPI)

strains that were included in the study possessed different resistance and porin genes and belonged to different pulsotypes. For *K. pneumoniae*, besides consideration of genetic typing, the strains were selected according to the presence or absence of resistance genes and showed different frequencies of the presence of the virulence genes (Table 2).

All *A. baumannii* strains assessed were able to adhere to an unmodified polystyrene surface (Fig. 2a). However,
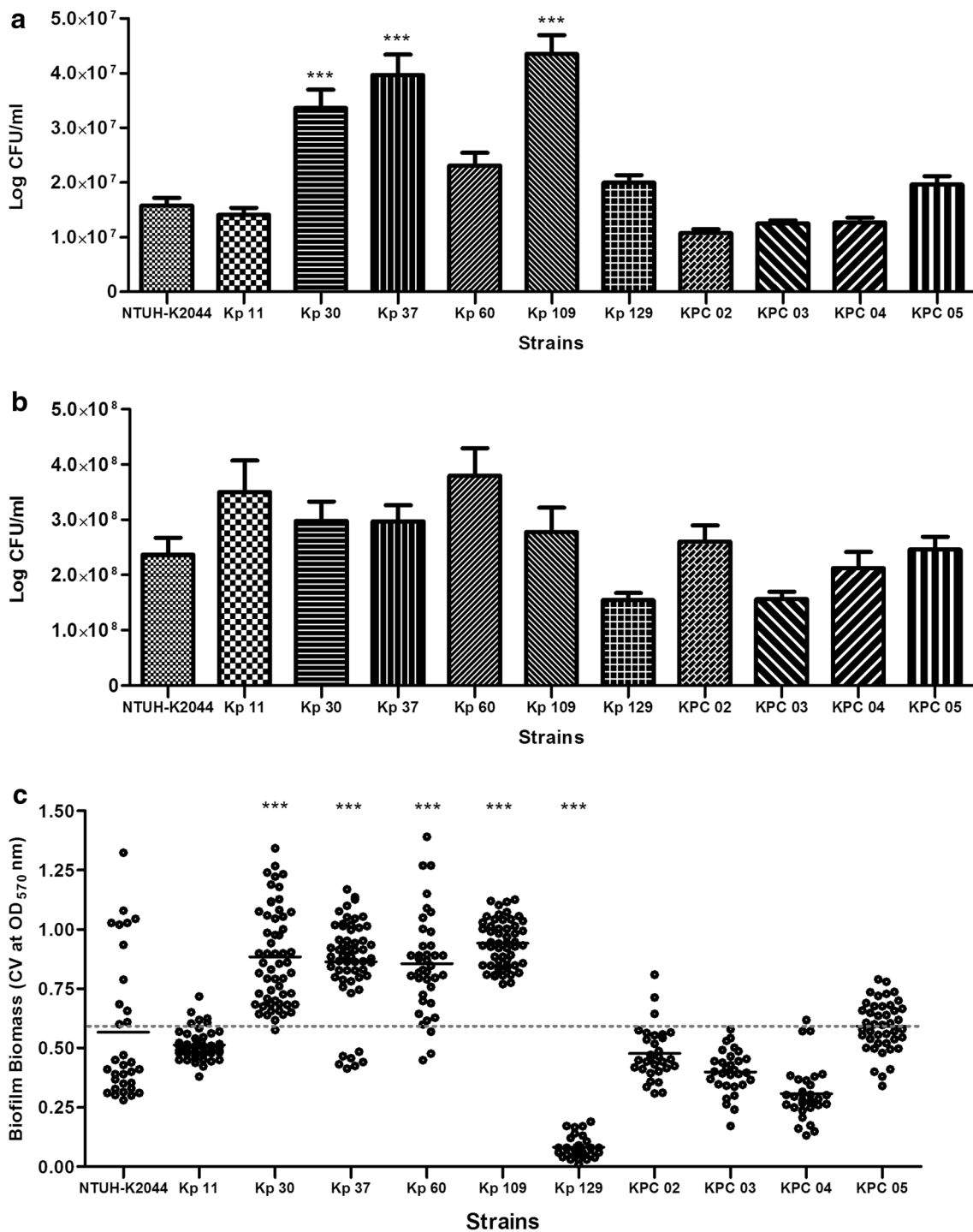
Fig. 3 Evaluating the number of adhered cells, biomass and biofilm formation in clinical strains of *K. pneumoniae*. **a** Number of adhered cells onto polystyrene plates after 2 h period of contact, expressed by Log CFU/ml; **b** biofilm cellular concentration expressed by Log CFU/ml; **c** biofilm biomass expressed as crystal violet optical density ($OD_{570 \text{ nm}}$). Results represent means plus standard deviations (error bars) of three independent experiments. $*P < 0.01$, $**P < 0.001$, $***P < 0.0001$, using Kruskal–Wallis, Dunn's multiple comparison test. 193 mm × 284 mm (300 × 300 DPI)

statistical analysis showed that only the Ab 05 and Ab 13 strains were not different in adhesion compared to the positive control ($P < 0.001$). The *K. pneumoniae* strains showed a different behaviour, wherein only the Kp 30, Kp 37 and Kp 109 strains differed significantly in adhesion compared to the control ($P < 0.001$) (Fig. 3a).

Regarding the number of cells present on each biofilm, the Ab 18 and Ab 22 strains had a lower number of cells in

Table 3 Classification of bacterial biofilm formation by microtiter plate method

Strains	Mean OD values	Biofilm formation	MDR	PFGE type
Acinetobacter baumannii				
ATCC 19606 (control strain)	0.7	Moderate	Non-MDR	–
Ab 05	0.62	Moderate	MDR	D
Ab 09	0.91	Moderate	MDR	C
Ab 10	0.38	Weak	MDR	B
Ab 12	0.8	Moderate	MDR	C
Ab 13	0.54	Weak	MDR	A
Ab 15[a]	0.87	Moderate	MDR	H
Ab 18[a]	0.72	Moderate	MDR	G
Ab 20[a]	0.38	Weak	Non-MDR	E
Ab 21	0.43	Weak	MDR	G
Ab 22	0.5	Weak	MDR	F
Klebsiella pneumoniae				
NTUH-K2044 (control strain)	0.57	Weak	MDR	–
Kp 11	0.51	Weak	non-MDR	G
Kp 30	0.88	Moderate	MDR	J
Kp 37	0.86	Moderate	MDR	B
Kp 60	0.86	Moderate	non-MDR	C
Kp 109	0.94	Moderate	MDR	U
Kp 129	0.08	Non	non-MDR	M
KPC 02	0.48	Weak	MDR	V
KPC 03	0.4	Weak	MDR	N
KPC 04	0.31	Weak	MDR	H
KPC 05	0.59	Weak	MDR	P

Optical density cut-off value (ODc) = average OD of negative control + 3 × standard deviation (SD) of negative control

MDR multidrug-resistant organism

[a] Environmental strains

the *A. baumannii* biofilm, and there was no significant difference in biofilm formation between patients and environmental isolates. Nevertheless, two isolates belonging to clone G, one clinical (Ab 21) and another environmental (Ab 18), showed different characteristics (Fig. 2b). For *K. pneumoniae*, all strains produced biofilms similar to the control (Fig. 3b).

Biofilm development determined by biofilm biomass was measured by optical absorbance (570 nm) for 20 isolates using crystal violet. The strains Ab 10, Ab 20, Ab 21 and Ab 22 produced less biomass than the cut-off established by Saxena et al. [30] (Fig. 2c). The mean optical density of the *A. baumannii* isolates was 0.622 ($\pm$0.191), and for *K. pneumoniae* it was 0.589 ($\pm$0.273). Also, four *K. pneumoniae* isolates produced more biofilm mass than the control ($P < 0.001$) whereas only the Kp 129 strain was characterised as non-biofilm producer (0.081 ± 0.0476), as shown in Fig. 3c.

Biofilm production as assessed by the quantitative method revealed five (50 %) moderate biofilm producers and another five (50 %) weak producers for *A. baumannii*. For *K. pneumoniae*, four isolates (40 %) showed moderate biofilm production, six (60 %) weak production and one strain was a non-biofilm producer (Table 3). None of the isolates were strong biofilm producers. Those *K. pneumoniae* strains that moderately produced biofilm possessed at least four virulence genes. In *A. baumannii* isolates, 80 % (4/5) presented both *carO* and 33–36 kDa porin genes as well as all resistance genes assessed. This study demonstrated high incidence rates of MDR in *A. baumannii* and *K. pneumoniae* (90 and 70 %, respectively) among isolates from both clinical and environmental sources as shown in Table 3. Independent of the microorganism, no relationship between MDR and biofilm production was observed.

The genetic relationship between the isolates and the possible association with the ability to form biofilm was investigated by PFGE. Different pulsotypes were selected to evaluate the biofilm production and in both cases there was no relationship between the moderate biofilm production and the pulsotype (Table 3).

Discussion

Currently, in developing countries like Brazil, the gram-negative bacilli, including *K. pneumoniae*, *A. baumannii* and *Pseudomonas aeruginosa* has emerged as the main agents of infections related to health assistance [3, 10, 13]. Despite the literature which describes different epidemiological models for the study of biofilms in clinical strains, in our study *K. pneumoniae* and *A. baumannii* were selected due to the significant increase in resistance to carbapenems rates observed in clinical strains evaluated in the hospital.

Recent research has shown that gram-negative strains specially *A. baumannii* and *K. pneumoniae*, particularly those of nosocomial origin, have multiple genetic elements, mobile or not, that confer resistance to several classes of antimicrobials [13, 18, 34]. Allied to these elements, the presence of virulence factors and the ability to form biofilms may contribute to the emergence and spread of these organisms in the hospital environment [7, 17]. Among these factors, biofilm formation is crucial, not only for environmental survival but also for successful infection caused by various pathogenic bacteria. Despite this, not much attention has been given to the significant role of biofilm formation in these pathogens [37].

Our data indicate that individual *A. baumannii* and *K. pneumoniae* strains had different abilities to attach to polystyrene plates. In *K. pneumoniae* we further investigated the potent influence of virulence on biofilm formation in 10 different strains. More virulent strains showed higher biofilm formation than less virulent ones. Exceptions were observed in some KPC-producer strains, however there are few studies that relate to the association between the virulence of these strains and biofilm formation ability, as in the study of Naparstek et al. [24], wherein *K. pneumoniae* bla_{KPC} + -ST258 strains do not form massive biofilms. Moreover, no relationship was observed between biofilm production and presence of porins in *A. baumannii*. However there are no studies described in the literature that have detected this relationship. In *A. baumannii* this work demonstrated a wide variation in biofilm formation among 10 well-described isolates. Although differences between the production of biofilms in dominant and sporadic clones have not been observed, the literature shows that there is a significant relationship between biofilm formation and pulsed-field type for *A. baumannii* isolates [29].

Together these findings demonstrate that biofilm formation is a prevalent feature among clinical strains associated with numerous infectious syndromes [7, 15, 29]. This is clear in the literature, which shows that isolates from non-fluid culture sites (deep tissue, bone, respiratory tract) have increased biofilm formation ability as determined by evaluation of crystal violet, compared with isolates recovered from blood and urine, for example [29]. In a previous study by our team with clinical isolates of methicillin-resistant *Staphylococcus aureus* (Batistão et al. unpublished) strong biofilm production in isolates recovered from the surgical site was reported, which was less frequently observed in isolates associated with other infections. Although these relationships were not assessed in the present study, a high frequency of biofilm-producing strains was found in all clinical isolates regardless of the site of infection.

To date, it has been demonstrated that some correlations exist between antibiotic resistance and biofilm-forming ability of *K. pneumoniae* and *A. baumannii* strains [2, 29, 37]. For instance, the study of Yang and Zhang [39] demonstrated that the ability of 150 *K. pneumoniae* strains to form biofilm evaluated with crystal violet exhibited a significant association with their ESBL production. Among the strains producing biofilm, 43.5 % produced ESBLs. In this work, of the *K. pneumoniae* strains evaluated for biofilm production, six presented the bla_{CTX-M} gene, which was found in two moderate producers of biofilm.

In *A. baumannii*, strains capable of forming biofilms are more often observed to be resistant to various antimicrobials including aminoglycosides, carbapenems and cephalosporins [17, 29]. In our study, the strains that produced biofilms mainly presented higher resistance to beta-lactam and tigecycline and others when compared to strains characterised as weak biofilm producers. Most environmental isolates were MDR and two isolates of the same clone, one clinical and one from the environment, showed different abilities of biofilm formation. However, our study demonstrated that there was no significant difference in biofilm formation on 96-well plates in both groups (clinical and environmental isolates), as observed in the Obeidat et al. [25] study. Additionally, in general, when it comes to clinical strains, previous studies have shown that biofilm formation is higher in MDR strains [17, 24, 29]. Regarding this aspect, similar to the study of Batistão et al. (unpublished) and Bocanegra-Ibarias et al. [2], regardless of whether the isolated microorganism was *A. baumannii* or *K. pneumoniae*, no relationship between biofilm formation and MDR was found in the clinical strains evaluated in this study.

Another important aspect to be evaluated in the biofilm formation process is the initial adhesion of the microorganism on the surface. This step is considered to be essential for the biofilm formation. Unfortunately, the majority of published studies evaluate biofilm production only by crystal violet and/or Congo red agar experiments without assessing the biofilm cell concentration or adherence. As in the paper of Kaliterna et al. [16], they found a certain relationship between the production of biofilm and

antimicrobial resistance in *A. baumannii* strains, however this study evaluated only the biofilm production by crystal violet assay. In our work, this relationship was not observed. Despite the small number of strains used in this study (*K. pneumoniae*, n = 10; *A. baumannii*, n = 10), the power of the statistic was corrected by the amount of replicas performed for each strain. For CFU analysis and adhesion, the experiments were performed in quadruplicate, in three independent experiments, totalling 120 values for CFU and adhesion for *K. pneumoniae* and 120 for *A. baumannii*. Regarding the analysis of crystal violet, i.e. the quantification of the biofilm itself, eight replicates per strain were performed in six independent experiments in 20 different strains, totaling 480 CV values for *K. pneumoniae* and 480 for *A. baumannii*. Therefore, the statistical analysis was based on repetitions of the experiments. To the best of our knowledge, this work represents one of the few studies in the world that uses a laborious methodology in the study of biofilm, to statistically evaluate the bacterial adhesion, the amount of cells in the biofilm and the biofilm formation in clinical strains from different sources, by *A. baumannii* and *K. pneumoniae*.

Collectively, our results confirm previous findings as regards the biofilm formation capacity in clinical and environmental strains of *A. baumannii* and *K. pneumoniae*, independent of the date of the strain isolation. However, in experimental design performed in this study, we found no relationship between genetic type and multidrug resistance and the ability of the strains to form biofilm. Furthermore, additional investigations are required to enhance understanding of the relationship between virulence and ability to form biofilm as well as the presence of resistance genes.

Compliance with Ethical Standards

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