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Relationship Between Biofilm Formation and Antimicrobial Resistance in Gram-Negative Bacteria

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Gram-negative microorganisms are a significant cause of infection in both community and nosocomial settings. The increase, emergence, and spread of antimicrobial resistance among bacteria are the most important health problems worldwide. One of the mechanisms of resistance used by bacteria is biofilm formation, which is also a mechanism of virulence. This study analyzed the possible relationship between antimicrobial resistance and biofilm formation among isolates of three Gram-negative bacteria species. Several relationships were found between the ability to form biofilm and antimicrobial resistance, being different for each species. Indeed, gentamicin and ceftazidime resistance was related to biofilm formation in *Escherichia coli*, piperacillin/tazobactam, and colistin in *Klebsiella pneumoniae*, and ciprofloxacin in *Pseudomonas aeruginosa*. However, no relationship was observed between global resistance or multidrug-resistance and biofilm formation. In addition, compared with other reported data, the isolates in the present study showed higher rates of antimicrobial resistance. In conclusion, the acquisition of specific antimicrobial resistance can compromise or enhance biofilm formation in several species of Gram-negative bacteria. However, multidrug-resistant isolates do not show a trend to being greater biofilm producers than non-multiresistant isolates.

Keywords: biofilm, gram-negative, infections, antimicrobial resistance

Introduction

The rise in the emergence and spread of antimicrobial resistance among the different microorganisms (bacteria, fungi, virus, and parasites) is one of the most important health problems worldwide today. Resistance to antibiotics is increasing at both community and hospital levels, being especially relevant in hospital settings, in which strong selective pressure favors the selection, persistence, and maintenance of resistant, multidrug-resistant (MDR) and even pan-resistant strains (resistant to all the current groups of antibiotics for therapeutic use) causing antibiotic treatment failure, increased mortality, and morbidity, and having a significant impact on the cost of medical treatment and prevention of bacterial infectious diseases.[1,2] It has been estimated that the annual cost due to antimicrobial-resistant *Staphylococcus aureus* infections is about $4.6 billion only in the United States of America.[3]

Bacterial resistance to antibiotics is primarily the consequence of a variety of phenomena such as alteration of the target of the drug, impermeability of the bacteria to the antibiotic, and genetically associated changes (mutational events, genetic transfer of resistance genes through plasmids, and mutations of target genes).[4] However, this is not the only reason for antimicrobial treatment failure. In fact, the ability to form communities called biofilms embedded in an exopolysaccharide matrix is one of the mechanisms of resistance used by bacteria to survive in the presence of an antibiotic.[5] In this state, bacteria can be up to 1,000-fold more resistant to antibiotics than those in a planktonic state.[6–8] Several studies recommend combined antibiotic therapy as the treatment of choice in biofilm-associated infections caused by Gram-negative bacteria, with macrolides (erythromycin, clarithromycin, and azithromycin) being the main antibiotics chosen due to their high antibiofilm activity *in vitro* and *in vivo*.[9] However, antibiotic treatment of biofilm-associated infections requires further study, since the selection of a specific treatment is difficult because of the wide variability of the microorganisms involved.

Several studies have demonstrated that low doses of certain antibiotics can induce biofilm formation indicating that

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biofilm regulation includes the presence of antibiotics. However, the correlation between biofilm formation and antibiotic resistance is currently unclear and remains under investigation.[10,11]

Previous studies carried out in our laboratory showed a relationship between the acquisition of resistance (specifically resistance to quinolones) and the ability to form biofilm[12] among uropathogenic *Escherichia coli* (UPEC). It was found that a decrease in biofilm formation was mainly due to a decrease of type 1 fimbriae expression.[13] However, more studies are needed to elucidate this relationship in other bacteria.

Thus, the aim of this study was to analyze the possible relationship between the ability to form biofilm and antimicrobial resistance among susceptible, resistant, and MDR Gram-negative clinical isolates from different hospitals in Catalonia.

Materials and Methods

Bacteria

Four hundred eight bacterial isolates were collected from four Catalan hospitals (Hospital Clinic of Barcelona, Hospital Universitario de Bellvitge, Hospital del Mar, and Hospital Universitario Mutua de Terrassa) over a 6-month period from 2016 to 2017. Among these, 142 were *E. coli*, 117 *Klebsiella pneumoniae*, and 149 were *Pseudomonas aeruginosa*. The bacteria were isolated from blood, urine, and respiratory (including, sputum, and tracheal aspirate) samples and processed at the corresponding Microbiology Laboratory. All the isolates were confirmed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF) and were stored in skim milk (BD) at −80°C. The samples used in our study were sourced through institutional tissue repositories.

Analysis of antimicrobial resistance

Resistance profiles were determined using the standard Kirby-Bauer disk diffusion method following the Clinical and Laboratory Standards Institute (CSLI) guidelines.[14] *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 strains were used as controls. The antimicrobial agents tested were: amikacin (30 μg), amoxicillin/clavulanic acid (30 μg), ceftazidime (30 μg), cefepime (30 μg), imipenem (10 μg), meropenem (10 μg), trimethoprim–sulfamethoxazole (30 μg), gentamicin (10 μg), tobramycin (10 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), aztreonam (15 μg), piperacillin/tazobactam (100/10 μg), fosfomycin (200 μg), tigecycline (15 μg) and colistin (10 μg).

Biofilm formation

Biofilm formation was analyzed using a modified protocol previously described by O'Toole *et al.*[15] Briefly, all isolates were cultured in aerobic conditions in Luria Bertani (LB) agar (Condalab) for 24 hr at 37°C to obtain single colonies. These colonies were established by the direct colony suspension method in LB broth for 24 hr at 37°C with shaking at 180 rpm.

The biofilm formation assay was tested in 96-well microtiter plates using an appropriate medium, M63 medium in *E. coli* strains, and LB for *P. aeruginosa* and *K. pneumoniae,* both mediums supplemented with 0.25% glucose. The plates were inoculated with the overnight culture diluted 1:100 in fresh medium and incubated for 24 hr at 37°C or 24 hr at 30°C in case of *E. coli* strains, both in static conditions. The final volume of liquid in each well was 200 μL. All plates include a sterility control (culture medium without inoculum) and a growth control (control medium with inoculum). To avoid evaporation, all plates were covered with adhesive foil lids.

The biofilm formation assay for *P. aeruginosa* was performed using the Calgary protocol as described previously.[16] The bacterial biofilm was formed by immersing the pegs of a modified polystyrene microtiter lid into a 96-well microtiter plate containing 200 μL of the overnight culture diluted 1:100 in fresh LB medium (catalog no. 445497; Nunc TSP system, Nunc, Roskilde, Denmark).

Biofilm quantification

After incubation, liquid culture was carefully removed and washed once with 210 μL of PBS and dried at 65°C until complete desiccation. Biofilms were stained with 200 μL of 1% (v/v) solution of Crystal Violet (CV) stain and incubated 10 min at room temperature. Afterward, CV stain was completely removed, washing once with 210 μL of PBS, and heat fixed at 65°C for 60 min.

The CV was eluted by the addition of 200 μL of 33% glacial acetic acid. The optical density (OD) was measured at 580 nm using a Microplate reader (EPOCH 2 microplate reader; BioTek, VT).

Biofilm classification

In this study, the heterogeneity in the biomass of the samples requires definition of a cutoff value that would divide the samples in non-adherent, weakly, moderately, and strongly adherent. For this reason, all samples were tested in triplicate and calculated the OD average using negative controls (medium without inoculum). The cutoff value was defined for each species. For easier interpretation of the results, strains were classified into the following categories using an adaptation of a previous study.[17]

The isolates were categorized in quartiles according to OD value using GraphPad Prism 5. The quartile below 25% percentile was classed as non-adherent (OD580 = 0.0640, 0.1605, and 0.3145 for *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, respectively). If their biomass absorbances were compressed between 25% percentile and median (0.1920, 0.2560, and 0.5560 for *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, respectively), they were classed as weakly adherent. Value between the median and 75% percentile (0.4165, 0.3765, and 0.8080 for *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, respectively) were classified as moderately adherent, and the isolates with OD over 75% percentile were deemed as strong biofilm producer. According to OD, value of positive control of each microorganism was categorized as strong biofilm.

Statistical analysis

Chi-square test and Spearman rank correlation test performed by SPSS 24.0 (for Windows) were used to study the association and correlation between biofilm formation and

antimicrobial susceptibility categories and the respective origin of microorganisms.

Results

Approximately 40% of all the isolates studied were resistant to ciprofloxacin. In addition, 50% of the *E. coli* isolates were resistant to cotrimoxazole, 36% of *K. pneumoniae* were resistant to ceftazidime, and about 30% of the *P. aeruginosa* isolates were resistant to imipenem, meropenem, aztreonam, and fosfomycin (Fig. 1). According to the number of antibiotic families to which the isolates were resistant, they were classified into susceptible (S; not resistant to any family), resistant (R; resistant to 1–2 categories), MDR (resistant to three or more antibiotic families), and extensively drug resistant (XDR; non-susceptible to at least one agent in all but two or fewer antimicrobial categories [*i.e.*, bacterial isolates remained susceptible to only one or two categories]). Thus, 35% of all the isolates were S, 35% were R, and 30% were MDR (data not shown). Among the *E. coli* isolates, 29% were S, 41% R, and 30% MDR. In the case of *K. pneumoniae*, 29%, 33%, and 38% were S, R, and MDR, respectively. Finally, 41%, 31%, 19%, and 9% of *P. aeruginosa* isolates were S, R, MDR, and XDR (Fig. 1).

On analysis of the antimicrobial resistance of each species according to the type of sample (blood, respiratory, and urine), several differences were found. *K. pneumoniae* isolates collected from blood were less resistant to fosfomycin than

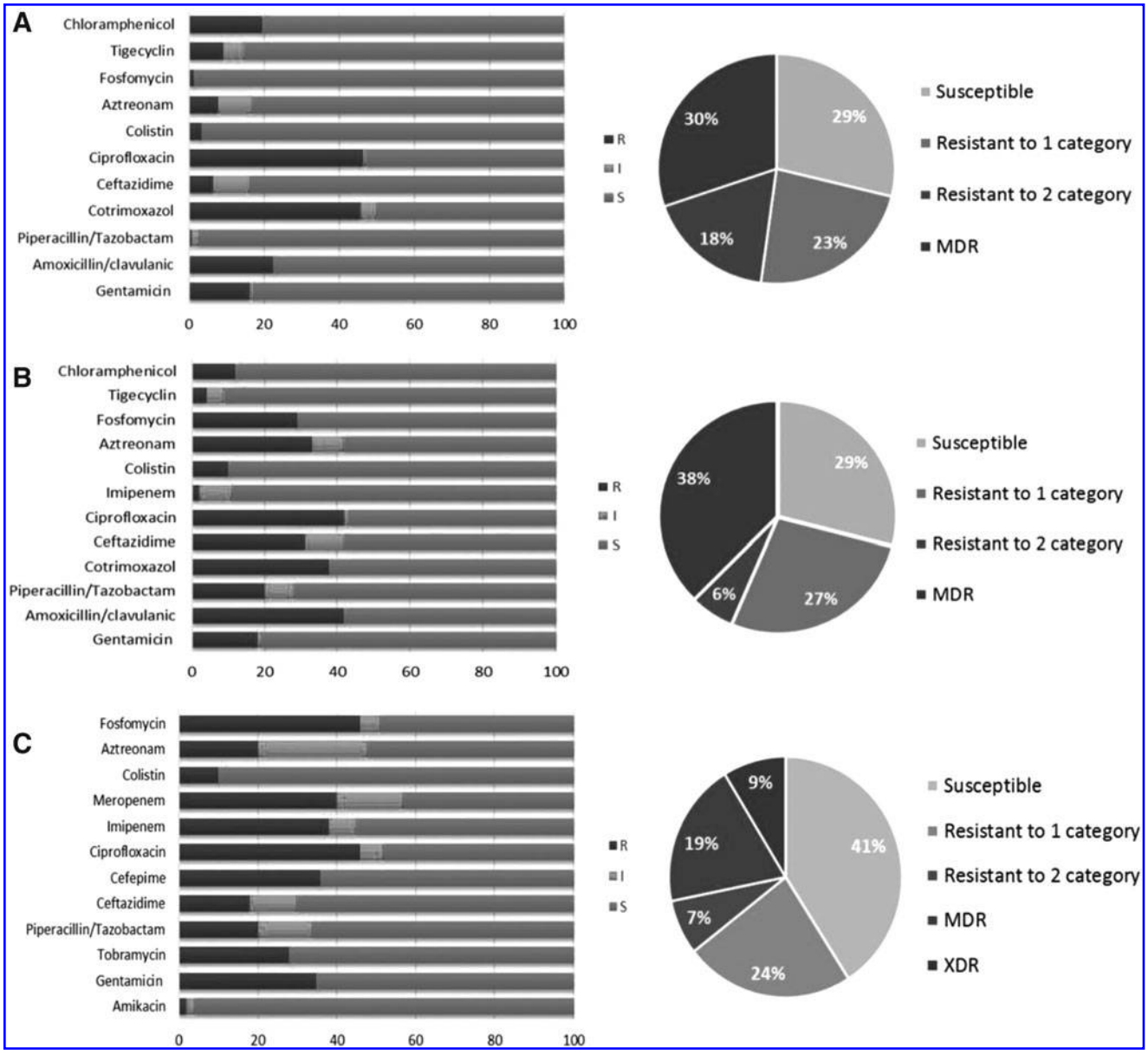

FIG. 1. Percentages of isolates resistant to the different antibiotics used in the treatment of each microorganism (**A**: *Escherichia coli*, **B**: *Klebsiella pneumoniae*, and **C**: *Pseudomonas aeruginosa*). MDR, multidrug-resistant and XDR, extensively drug resistant.

Table 1. Percentage of Susceptibility of Microorganism Isolates from Blood, Respiratory, and Urine Against Different Antimicrobials Commonly Used in This Study

		Escherichia coli				Klebsiella pneumoniae				Pseudomonas aeruginosa			
		S%	*R%*	p	*ρ*	*S%*	*R%*	p	*ρ*	*S%*	*R%*	p	*ρ*
Ceftazidime	Blood	26.8	4.2			16.2	9.4			13.4	2.7		
	Respiratory	35.2	1.4			20.5	7.7			59.1	3.4		
	Urine	31.7	0.7	0.054	–0.186	36.8	9.4	0.267	–0.148	15.4	6	**0.002***	0.142
Imipenem	Blood	100	0			25.6	0			10.1	6		
	Respiratory	100	0			16.5	1.7			51	11.4		
	Urine	100	0	a	a	46.2	0	0.075	–0.050	13.4	8.1	**0.033***	0.03
Gentamicin	Blood	28.2	2.8			22.2	3.4			10.7	5.4		
	Respiratory	28.2	8.5			25.6	2.6			51	11.4		
	Urine	27.5	4.9	0.175	0.063	36.8	9.4	0.344	0.103	14.8	6.7	0.152	0.007
Ciprofloxacin	Blood	16.9	14.1			16.2	9.4			10.7	5.4		
	Respiratory	16.2	20.4			18.8	9.4			45.6	16.8		
	Urine	20.4	12	0.174	–0.071	29.1	17.1	0.936	0.011	12.8	8.7	0.335	0.063
Aztreonam	Blood	26.8	4.2			17.1	8.5			13.5	2.7		
	Respiratory	35.5	2.1			20.5	7.7			55.4	6.8		
	Urine	31	1.4	0.206	–0.137	34.2	12	0.762	–0.062	17.6	4.1	0.471	0.032
Piperacillin/ tazobactam	Blood	31	0			20.5	5.1			14.1	2		
	Respiratory	35.9	0.7			18.8	11			55	7.4		
	Urine	32.4	0	0.418	–0.002	43.6	2.6	**0.003***	–0.216	17.4	4	0.606	0.063
Fosfomycin	Blood	31	0			23.9	1.7			8.1	8.1		
	Respiratory	36.6	0			18.8	9.4			49	13.4		
	Urine	31	1.4	0.120	0.148	32.5	13.7	**0.027***	0.180	12.1	9.4	**0.005***	–0.004
Colistin	Blood	28.9	2.1			23.1	2.6			14.8	1.3		
	Respiratory	35.9	0.7			27.4	0.9			59.7	2.7		
	Urine	31.7	0.7	0.360	–0.099	41	5.1	0.403	0.045	18.8	2.7	0.262	0.067

*Bold numbers are statistically significant ($p < 0.05$).
a, no statistics have been calculated; R, resistant; S, susceptible; ρ, Spearman rank correlation coefficient.

those collected from sputum and urine (1.7% vs. 9.4% and 13.7%, respectively). *P. aeruginosa* isolates collected from respiratory were, in general, more resistant to all the antimicrobial agents studied in common in the three species than their counterparts isolated from blood and urine (Table 1).

We studied the ability of all the isolates collected to form biofilm *in vitro* and found that 49.3% were able to do so, 30.3% of the *E. coli*, 37.6% of *K. pneumoniae*, and 76.5% of *P. aeruginosa* isolates, respectively. No significant differences were found in the frequency of biofilm-forming isolates in relation to each type of sample (blood, sputum, and urine). However, some trends were observed. For example, in the case of *E. coli*, the isolates collected from respiratory were less biofilm forming than those collected from blood or urine On the other hand, the *P. aeruginosa* isolates collected from respiratory were more biofilm forming than those from the other types of samples (Fig. 2).

Relationships between the ability to form biofilm and antimicrobial resistance were scarce and differed for each species. In the case of *K. pneumoniae*, the isolates resistant to colistin showed a strong capacity to form biofilm than the susceptible isolates ($p = 0.026$) and the biofilm formation was strong in *P. aeruginosa* isolates susceptible to ciprofloxacin than in their resistant counterparts ($p = 0.041$) (Table 2).

Finally, there was no significant relationship between global resistance or multidrug resistance and biofilm formation. However, the *P. aeruginosa* isolates susceptible to

Table 2. Relationship Between Biofilm Formation and Antimicrobial Resistance

	p *(>0.05)*		
Antimicrobials	E. coli	K. pneumoniae	P. aeruginosa
Amikacin	ND	ND	0.561
Gentamicin	0.133	0.826	0.254
Tobramycin	ND	ND	0.607
Amoxicillin/ clavulanic	0.351	0.713	ND
Piperacillin/ tazobactam	0.397	0.118	0.128
Ceftazidime	0.109	0.396	0.580
Cefepime	ND	ND	0.161
Imipenem	1	0.572	0.861
Meropenem	ND	ND	0.775
Ciprofloxacin	0.06	0.898	**0.041**[a]
Fosfomycin	0.113	0.148	0.935
Aztreonam	0.780	0.310	0.428
Colistin	0.639	**0.026**[a]	0.128
Chloramphenicol	0.448	0.3	ND
Tigecycline	0.669	0.098	ND
Cotrimoxazole	0.783	0.667	ND

[a]Bold numbers are statistically significant ($p < 0.05$).
ND, not determined.

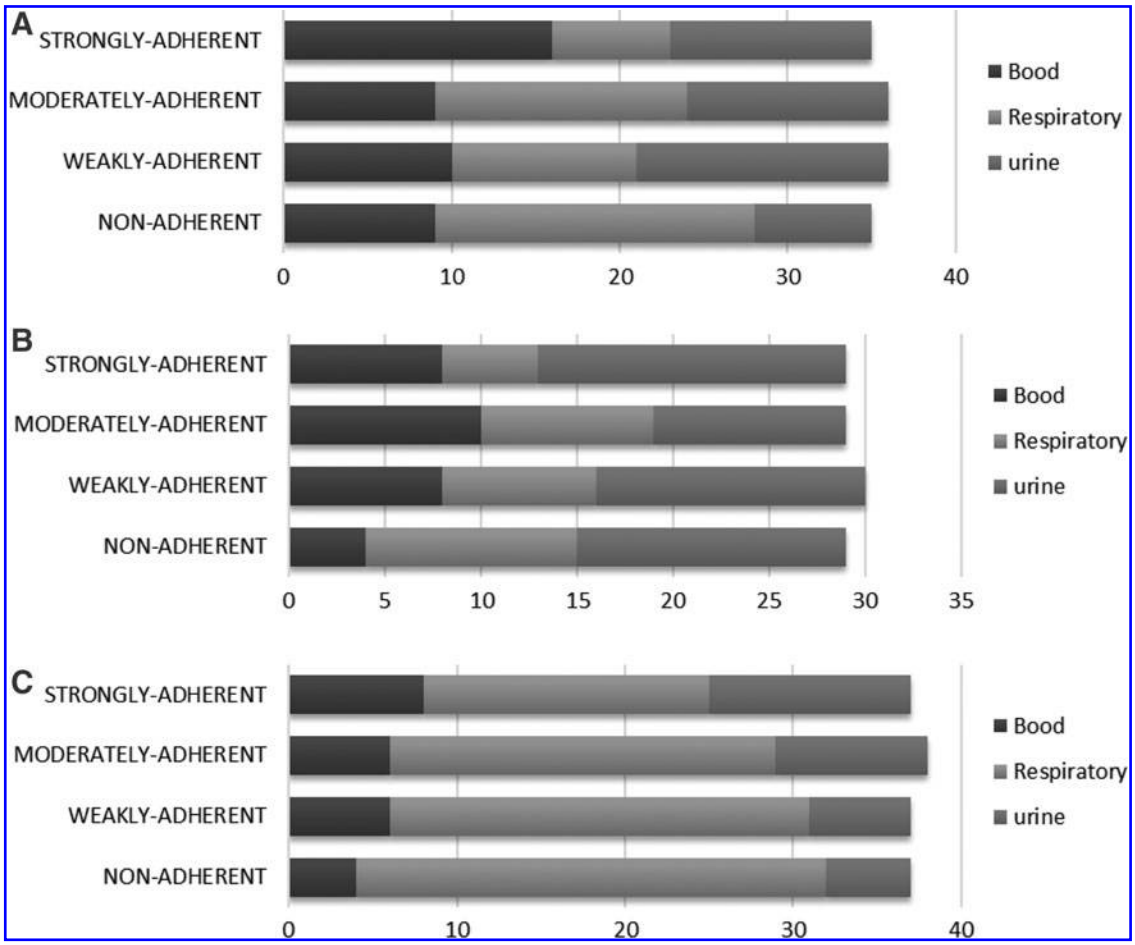

FIG. 2. Relationship between origin of microorganism and biofilm-forming capacities. **(A)** *E. coli*, **(B)** *K. pneumoniae*, and **(C)** *P. aeruginosa*.

all the antibiotics studied or resistant to only one antimicrobial category tended to be more biofilm forming than the MDR and XDR (Fig. 3).

Discussion

Gram-negative microorganisms are a significant cause of infection in both community and nosocomial settings.[18] The emergence of microorganisms resistant to multiple antibiotics used in the treatment of infections has become an important health problem worldwide. The present study analyzed three species of microorganisms included among the ESKAPE pathogens: *K. pneumoniae* and *P. aeruginosa*, as well as *E. coli* isolates.

The percentage of isolates resistant to the different antibiotics studied was higher in comparison with other studies (Table 3).

It was of note that the hospitals participating in this study showed higher rates of ciprofloxacin resistance ranging from 37% to 45% compared with other studies reporting a rate of resistance of less than 29%. The high percentage of resistance found among the isolates collected from blood in the hospitals participating in the study could be due to the fact that patients had received antimicrobial treatment before the sample was obtained. It is also well known that the misuse of antibiotics leads to selective pressure that favors the acquisition of resistance. We evaluated the possible relationship between antimicrobial resistance and the ability to form biofilm among the collected isolates. No relationship was found between multidrug resistance and biofilm formation, but similar to other studies[19] we found a comparable level of biofilm production in both multidrug- and nonmultidrug-resistant isolates with no significant differences between the two groups. High rates of biofilm-producing *K. pneumoniae* have been reported in MDR strains, mainly Extended Spectrum Betalactamases producers harboring $bla_{\text{CTX-M}}$ genes.[20]

However, there are reports regarding relationships between biofilm formation and resistance to specific antibiotics. Thus, the acquisition of quinolone resistance has been related to a decrease in biofilm production in both UPEC and *Salmonella typhimurium*.[12,21] In the present study, we also found this relationship between quinolone resistance and biofilm formation in *P. aeruginosa*, with the susceptible isolates showing a greater capacity to form biofilm than the resistant isolates. However, there are discrepancies among the different studies in the literature. One example of this is the study of the effect of meropenem resistance on biofilm formation. Several studies found that the strains resistant to meropenem showed Gram-negative bacteria to have a greater capacity to form biofilm[22] in contrast to other studies that found an inverse

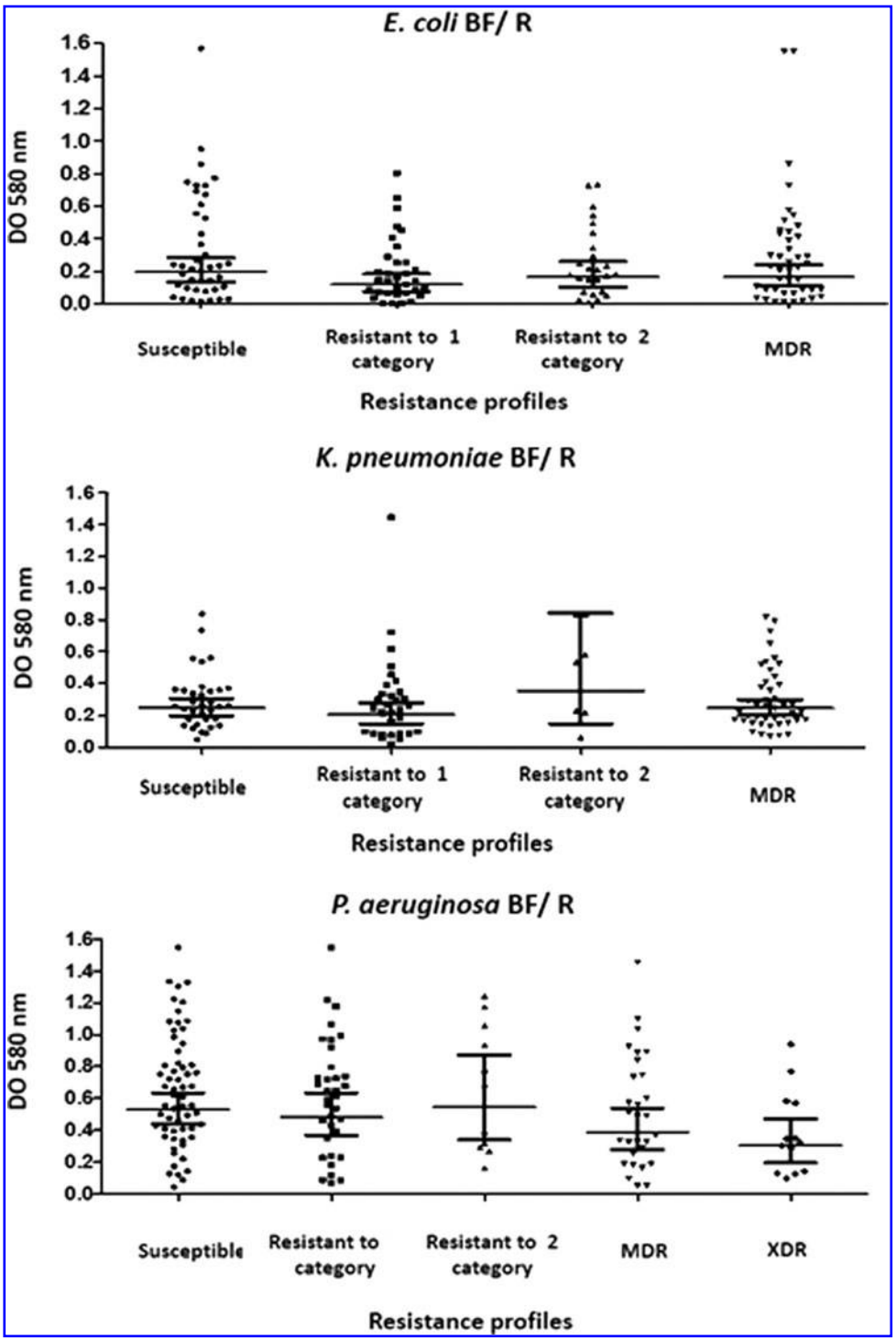

FIG. 3. Distribution of biofilm formation of isolate with different resistance phenotype. The distribution was separate in quartiles according to OD580 value. The OD range of positive control biofilm is between 0.81. OD, optical density.

relationship between meropenem resistance and biofilm formation among other Gram-negative bacteria, such as *Acinetobacter baumannii*.[23] Resistance to imipenem has been associated with less biofilm production in *P. aeruginosa* isolates,[24] although we did not observe this association. This is the first time that a relationship between gentamicin resistance and biofilm formation has been reported in *E. coli*.

In conclusion, the acquisition of specific antimicrobial resistance can compromise or enhance biofilm formation in several species of Gram-negative bacteria. However, MDR strains did not tend to have greater biofilm production than non-multiresistant isolates. Further studies are needed to determine how the acquisition of gentamicin resistance affects biofilm formation.

Table 3. Percentage of Resistance in Blood Isolates Reported in Different Studies

E. coli	*Yang (2017) [25]*	*Guy (2016) [26]*	*Bell (2016) [27]*	*Wong (2014) [28]*	*Present study*
Gentamicin	—	9.6	7.5	—	34.8
Amikacin	7.12	—	—	—	—
Piperacillin/tazobactam	7.49	11	3.2	3.9	0.2
Cotrimoxazole	—	—	29.2	34.30	15.9
Ceftazidime	—	11.1	4.4	24	2.2
Ciprofloxacin	—	18.7	10.4	28.8	16.2
Imipenem	1.28	0.1	0.1	—	0
K. pneumonia					
Gentamicin	—	7.5	5.5	—	4.4
Amikacin	12.1	—	—	—	—
Piperacillin/tazobactam	24.2	16.9	4.8	8.30	4.9
Cotrimoxazole	—	—	15.5	12.5	9.3
Ceftazidime	—	12.1	6.1	13.3	7.6
Ciprofloxacin	—	10.9	5	16.7	10.3
Imipenem	7.26	1.5	1.1	—	0.5
P. aeruginosa					
Gentamicin	—	—	—	—	8.6
Amikacin	25.25	—	—	—	0.5
Piperacillin/tazobactam	25.59	—	—	8	4.9
Cotrimoxazole	—	—	—	—	—
Ceftazidime	—	7.4	—	12.70	4.4
Ciprofloxacin	—	—	—	21.10	11.3
Imipenem	15.82	11.5	—	4.20	9.3

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Disclosure Statement

No competing financial interests exist.

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