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Abstract:	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 is one of 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 to 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.

Relationship between biofilm formation and antimicrobial resistance in Gram-negative bacteria

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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, multi-drug-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 USA.[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 via 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 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 multidrug-resistant Gram-negative clinical isolates from different hospitals in Catalonia.

Cepas

MATERIAL 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 2016-2017. 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 -80ºC. 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 & 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 (30µg), gentamicin (10µg), 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 h at 37ºC to obtain single

colonies. These colonies were established by the direct colony suspension method in LB broth for 24 h at 37ºC 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 h at 37ºC or 24 h at 30ºC 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 ON 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 65ºC 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. Afterwards, CV stain was completely removed, washing once with 210 µL of PBS and heat-fixed at 65ºC 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, USA).

Biofilm classification

In this study, the heterogeneity in the biomass of the samples requires definition of a cut-off 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 cut-off 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 categorised in quartiles according OD value using Graphpad Prism 5. The quartile below 25% percentile were 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, 0.5560 for *E. coli*, *K. pneumoniae* and *P. aeruginosa*, respectively) as weakly adherent. Value between the median and 75% Percentile (0.4165, 0.3765, 0.8080 for *E. coli*, *K. pneumoniae* and *P. aeruginosa*, respectively) were classified as moderate adherent and the isolate with OD over 75 % percentile were deemed as strong biofilm producer. According to OD value of positive control of each microorganism were categorized as strong biofilm.

Statistical analysis. Chi-square test and Spearman's rank correlation test was performed by SPSS 24.0 for Windows) were used for study de association and correlation between biofilm formation among 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 cotrimoxazol, 36% of *K. pneumoniae* were resistant to ceftazidime, and about 30% of the *P. aeruginosa* isolates were resistant to imipenem, meropenem, azthreonam and fosfomycin (Figure 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), multidrug-resistant (MDR - resistant to 3 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 (Figure 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 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 (Figure 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 1).

Finally, there was no significant relationship between global resistance or multidrug-resistance and biofilm formation. However, the *P. aeruginosa* isolates susceptible to all the antibiotics studied or resistant to only 1 antimicrobial category tended to be more biofilm forming than the MDR and XDR (Figure 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 to 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 non-multidrug resistant isolates with no significant differences between the two groups. High rates of biofilm producing *K. pneumoniae* have been reported in multidrug-resistant strains, mainly ESBL 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 uropathogenic *E. coli* 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

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, multidrug-resistant 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.

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AUTHOR DISCLOSURE STATEMENT

No commercial associations that might create a conflict of interest in connection with the submitted manuscript exists.

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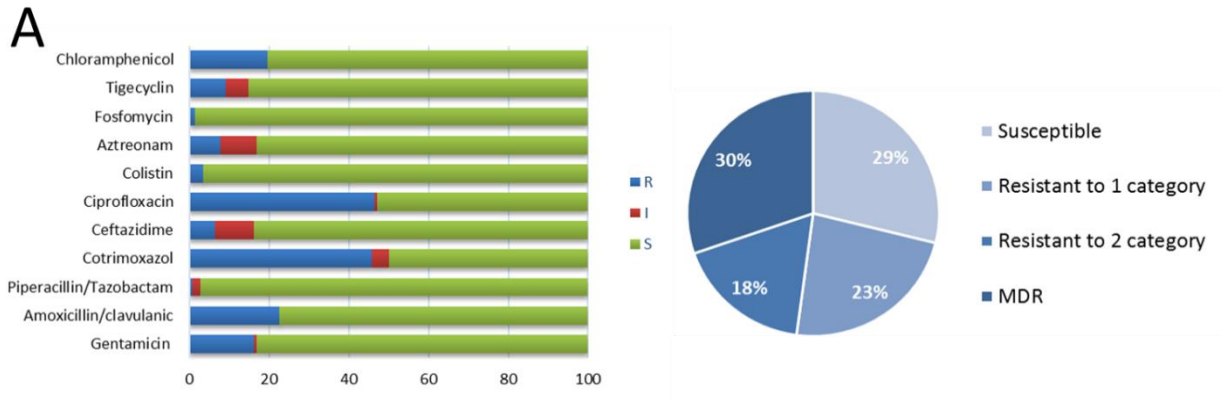

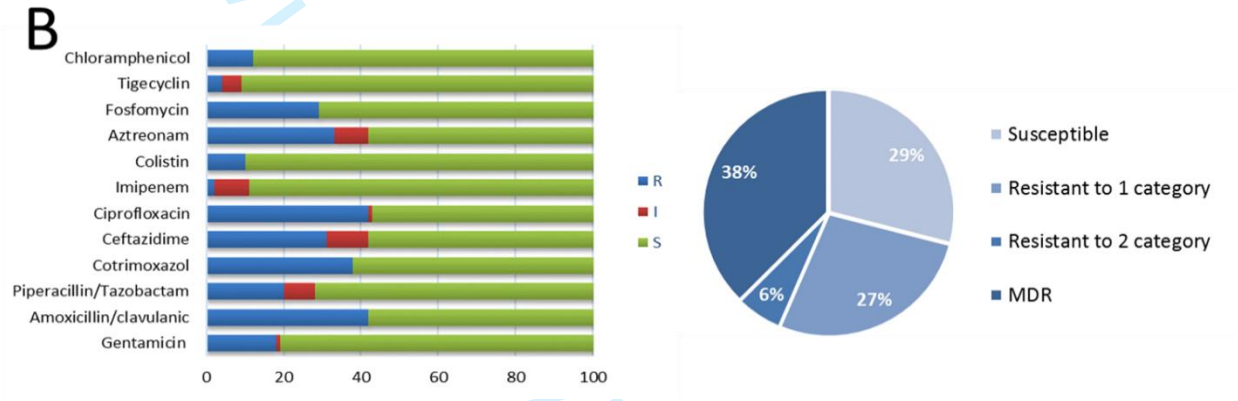

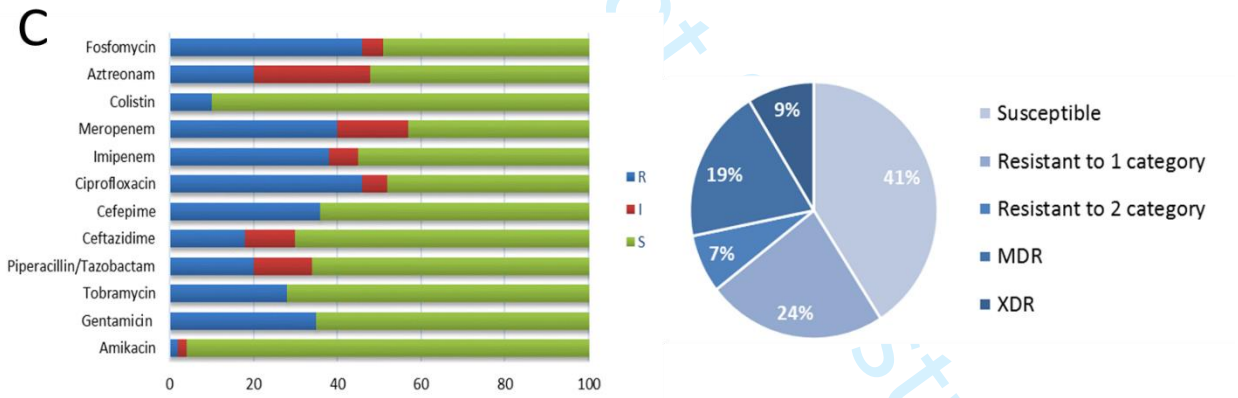

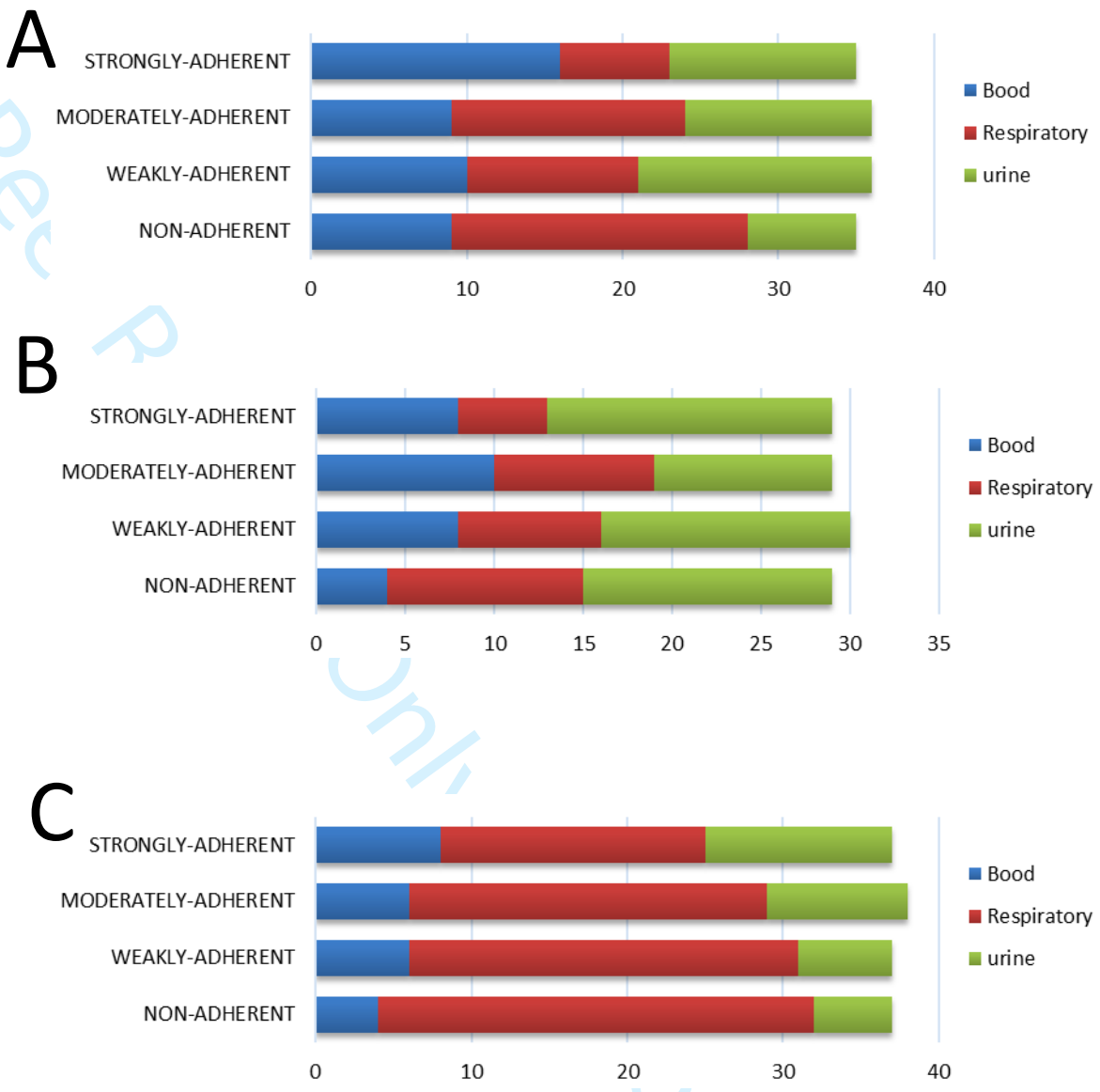

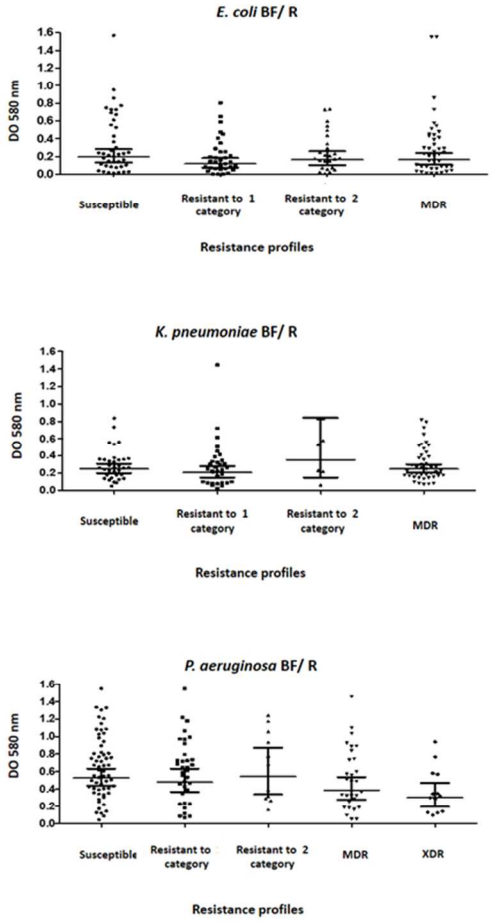

Figure 3

190x275mm (96 x 96 DPI)

FIGURE LEGENDS

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

Figure 2. Relationship between origin of microorganism and biofilm forming capacities

Figure 3. Distribution of biofilm formation of isolate with different resistance phenotype.

MDR: multidrug-resistant; XDR: extensively drug-resistant.

The distribution was separate in quartile according OD580 value. The OD range of positive control biofilm is between 0.8-1.

Table 1. Percentage of susceptibly of microorganisms isolate from blood, respiratory and urine against different antimicrobials in common used in this study.

		E. coli				*K. pneumoniae*				*P. aeruginosa*			
		S %	R%	p-value	ρ	S%	R%	p-value	ρ	S%	R%	p-value	P
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

Susceptible (S), Resistant (R), Spearman´s rank correlation coefficient (ρ), No statistics have been calculated (a).

* Statically significant ($p < 0.05$).

Table 2. Relationship between biofilm formation and antimicrobial resistance.

Antimicrobials	p-value (> 0.05)		
	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	0351	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***
Fosfomycin	0.113	0.148	0.935
Aztreonam	0.780	0.310	0.428
Colistin	0.639	**0.026***	0.128
Chloramphenicol	0.448	0.3	ND
Tigecyclin	0.669	0.098	ND
Cotrimoxazol	0.783	0.667	ND

ND, Not determined

* Statically significant ($p < 0.05$).

Table 3. Percentage of resistance in blood isolates reported in different studies.

	Yang Q (2017) [17]	Guy R (2016) [18]	Bell JM (2016) [19]	Wong PH (2014) [20]	Present study
E. coli					
Gentamicin	-	9.6	7.5	-	34.8
Amikacin	7.12	-	-	-	-
Piperacillin/Tazobactam	7.49	11	3.2	3.9	0.2
Cotrimoxazol	-	-	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. pneumoniae					
Gentamicin	-	7.5	5.5	-	4.4
Amikacin	12.1	-	-	-	-
Piperacillin/Tazobactam	24.2	16.9	4.8	8.30	4.9
Cotrimoxazol	-	-	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
Cotrimoxazol	-	-	-	-	-
Ceftazidime	-	7.4	-	12.70	4.4
Ciprofloxacin	-	-	-	21.10	11.3
Imipenem	15.82	11.5	-	4.20	9.3