Pontificia Universidad JAVERIANA Bogotá

Univ. Sci. 25 (3): 545-571, 2020.
doi: 10.11144/Javeriana.SC25-3.dpbo

ORIGINAL ARTICLE

Biofilm and persister cell fo mation variability in clinical isolates of *Klebsiella pneumoniae* in Colombia

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Received: 12-09-2019
Accepted: 07-09-2020
Published on line: 31-12-2020

Citation: Posada L, Acosta IC, Zárate L, Rodríguez P, Huertas MG, Zambrano MM. Bio lm and persister cell formation variability in clinical isolates of *Klebsiella pneumoniae* in Colombia, *Universitas Scientiarum,* 25 (3): 545-571, 2020. doi: 10.11144/Javeriana.SC25-3.bapc

Funding:
COLCIENCIAS
Project no. 657054531675

Electronic supplementary material:
N.A.

OPEN ACCESS

Abstract

Klebsiella pneumoniae is an opportunistic pathogen associated with nosocomial infections. Persister cells are a fraction of a bacterial population that can escape antibiotic treatment and are associated with antibiotic therapy failure. In this work, we analyzed persistent cells in planktonic cultures and biofilms using 10 *K. pneumoniae* clinical isolates and four different antibiotic types. The isolates had different antibiotic susceptibility profiles that did not correlate with their capacity to form biofilms. Persister cells were found under all conditions tested, although their population numbers varied depending on the antibiotic used. A larger number of persister cells were found in biofilms than in planktonic cultures. Antibiotic treatment with trimethoprim-sulfamethoxazole resulted in the largest persister cell sub-population compared with other antibiotics tested, while ciprofloxacin was the antibiotic that produced fewer persister cells. These results indicate that *K. pneumoniae* clinical isolates vary not only in their susceptibility to antibiotics but also in properties relevant to diseases, such as biofilm formation and persister cell populations.

Keywords: Persistence; biofilm; antibiotic resistance; *Klebsiella pneumoniae*

Introduction

The Gram-negative opportunistic pathogen *Klebsiella pneumoniae* is a significant cause of community-acquired and nosocomial infections. *K. pneumoniae* may colonize the gastrointestinal tract or the nasopharynx leading to diseases such as necrotizing pneumonia and pyogenic liver abscesses [1, 2]. In hospital settings this bacterium may lead to urinary tract and bloodstream infections, pneumonia, and infections in newborns and vulnerable individuals within intensive care units [1, 2]. *K. pneumoniae* is also a member of the "ESKAPE" group of pathogens (*Enterococcus faecium*,

Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and *Enterobacter* spp.) that are among the most common causes of life-threatening nosocomial infections [3–5]. Nosocomial infections are also relevant in the current Covid-19 pandemic. Recent reports indicate that nosocomial infections are frequent among Covid-19 patients and higher among those who died as opposed to those who were discharged [6, 7]. Given the symptoms of SARS-CoV-2 infections, it is likely that diseases like necrotizing pneumonia in hospitalized patients may be under-registered or remain unnoticed as possible comorbidities.

K. pneumoniae is also capable of causing severe clinical outbreaks and is recognized for its capacity to acquire resistance determinants via horizontal gene transfer [3, 8]. The global emergence and recent spread of antibiotic-resistant and multidrug-resistant (MDR) pathogens entails a threat to human health that has triggered a worldwide call to action [8, 9]. In the case of *K. pneumoniae*, the reported increase in resistance to first-line drugs and to carbapenems, which are often the last line of therapy effective against MDR strains, makes the control of this pathogen very challenging [8].

Bacteria, including *K. pneumoniae*, also can form biofilms, complex multicellular structures that are more tolerant of environmental insults, such as free radicals from the immune system and antibiotic treatments. Biofilms have been associated with chronic infections or persistence in clinical settings [10, 11]. Antibiotic tolerance has also been implicated in treatment failure and relapses [12, 13] and is defined as the capacity of bacteria to withstand exposure to a lethal antibiotic concentration. This phenomenon was described many years ago and involves a subpopulation of persister cells that survives by adopting a transient phenotypic state [14–16]. In contrast to resistant mutants, persister cells are phenotypic variants that can resume growth only when the antibiotic has been removed, giving rise to a population that is genetically susceptible and indistinguishable from the initial population.

Persisters arise stochastically [17] but are also affected by environmental conditions such as growth phase or exposure to various stresses, including antibiotics [18–20]. Persisters are considered to play an important role in chronic infections, especially when bacteria grow as biofilms where cells are more recalcitrant to the antimicrobial treatment and can serve as a continual source of infectious agents [12, 13]. The existence of persister phenotypic variants indicates heterogeneity within microbial populations and functional diversity that can prove beneficial to a population under fluctuating environmental conditions [21]. In microbial pathogens,

persister phenotypic variants may constitute a survival strategy for microbial populations exposed to adverse conditions such as lethal concentrations of antibiotics.

Persister cells have been studied in *K. pneumoniae* and other ESKAPE pathogens [18, 19]; periodic, high-dose antibiotic administration led to an increase in persistence and provided cross-tolerance to other antibiotics without the emergence of resistance [12]. In addition to persister cell formation, *K. pneumoniae* isolates are also known to have variability in genome composition, biofilm-forming ability, and antibiotic resistance [10, 22–24]. Given the clinical relevance of *K. pneumoniae* and its previously reported genetic variability [21], in this work we analyzed phenotypic traits in a group of *K. pneumoniae* clinical strains isolated from hospitals in Colombia. These sort of *Klebsiella* spp isolates are reservoirs of drug resistance determinants [25] and are among the most common pathogens isolated within intensive care units [26]. This work aimed at assesing if clinical strains with different antibiotic susceptibility patterns varied in their capacity to form biofilms and to develop persister cell populations. The results indicate variability among clinical isolates that should be considered as an additional confounding factor when undergoing treatment of infections by this pathogen.

Materials and Methods

Strains and growth conditions

A total of ten *K. pneumoniae* clinical isolates were obtained from the strain collection at the Bacterial Molecular Genetics research group, Universidad El Bosque, Bogotá, Colombia. Control strains consisting of previously characterized *K. pneumoniae* isolates, the LM21 *gfp* strain [27], and mutants lacking genes relevant to biofilm formation [28] were also included. Unless otherwise indicated, all strains were grown at 37 °C in bacterial culture media Luria Bertani (LB; Oxoid) or Mueller-Hinton (MH; Oxoid), with agitation at 180 rpm, overnight for 16 hours. Media were supplemented with antibiotics (Sigma- Aldrich) when necessary: Meropenem (MER), amikacin (AMK), ciprofloxacin (CIP), trimethoprim-sulfamethoxazole (TS); the latter prepared at a 1:19 combination of two antibiotics that inhibit two steps in the synthesis of tetrahydrofolate. LB agar, supplemented with Congo red (Sigma-Aldrich), Coomassie blue (Sigma-Aldrich), and calcofluor (fluorescent brightener 28; Sigma-Aldrich), was used to assess colony morphology [29, 30].

Antibiotic susceptibility determination

Overnight cultures grown in MH medium were diluted 1/30 in the same medium, incubated for 45 min, and diluted again to obtain 10^6 CFU/mL. The minimal inhibitory concentration (MIC) was determined following standard protocols by growing 150 uL of each freshly diluted culture in 96-well plates (COSTAR 3595) in the presence of different antibiotic concentrations. Resistance was assesed following CLSI [31]. The MIC was determined as the concentration in which no growth was observed after overnight incubation at 37 °C. The minimum biofilm eradication concentration (MBEC) was determined following Harrison JJ *et al*. [32], by placing 150 uL of each freshly diluted culture in wells of a 96-well plate with a lid containing pegs (Calgary device, NUNC) where biofilms are formed. After incubation for 24 h at 37 °C the lids with biofilms were washed twice with PBS, placed on a new plate with fresh MH medium supplemented with antibiotics and incubated overnight. The lid with biofilms exposed to different antibiotic concentrations was washed twice in PBS, placed on a new 96-well plate with MH free of antibiotics, and incubated overnight. The MBEC was defined as the concentration where no growth was observed after this last incubation in fresh MH medium [32, 33].

Determination of persister cells

Cells were grown overnight in MH medium, after a 1/30 dilution in the same medium, cells were incubated for 45 min and further diluted 1/100 to obtain 10^6 CFU/mL. To determine the number of persister cells in planktonic cultures, 75 μL of the freshly diluted culture at 10^6 CFU/ml were placed in wells of a 96-well plate containing 75 μL of MH medium supplemented with antibiotic at concentrations above the MIC (2X, 4X, and 8X). After 4h incubation at 37 °C, cells were transferred to 1.5 ml centrifuge tubes and recovered by centrifugation at 5 000 rpm for 5 min. CFUs were determined using the microdilution method [34].

To determine the number of persister cells in biofilms, cells were grown in MH medium overnight at 37 °C in a 96-well Calgary device (NUNC) to form biofilms on lid pegs. Lids were then washed twice in PBS and transferred to a plate with fresh medium supplemented with antibiotic concentrations below the MBEC (1/2X 1/4X and 1/8X). Concentrations below the MBEC were used since, by definition, the MBEC is the amount of antibiotic that eradicates all cells. After overnight incubation, pegs were broken off the lids and placed in 1.5 mL tubes containing 200 μL PBS and 2 % Tween 80. Biofilms were then removed by sonicating for 10 min at 60 Mhz followed by vortexing for 2 min. CFUs were determined using the microdilution method [34].

Killing curves

Cells were grown overnight in MH medium, then diluted 1/30 into the same medium, and incubated for 45 min and diluted again 1/100 in MH medium to obtain 10^6 CFU/mL. A total of 1 mL of each culture was transferred to a 24-well plate (COSTAR) and challenged with four times the MIC of each antibiotic. Plates were incubated at 37 °C and cultures were sampled at seven different time points. Cells were recovered by centrifugation at 5 000 rpm for 5 min, resuspended in the same volume of PBS and CFUs were determined using the microdilution method [34]. Sample volumes at each time point varied in order to get better sensitivity.

Biofilm formation assays and cluster analysis

Biofilms were formed on 96-well plates and quantified using crystal violet (CV), as previously reported [35]. Briefly, 1 μL cells were inoculated into 150 μL LB mdium in 96-well polystyrene plates (Microtest 96; BD Falcon), and allowed to grow overnight. Planktonic cells were removed, wells washed with dH_2O and allowed to dry for 15 min prior to fixing biofilm cells with methanol (99.9 %) for 15 min and staining with 180 μL 1 % CV for 15 min. After removing CV and washing plates with dH_2O, attached cells were resuspended in 10 % SDS and their optical density (OD) was determined at 595 nm in a plate reader (Tecan, GENIOS MOD). At least three independent experiments were carried out, each with four technical replicates. The biofilm formation data (OD values) were used to make a dendrogram based on Euclidian distances using PAST 3 software (https://folk.uio.no/ohammer/past/).

Antibiotic penetration assays

Cells were grown overnight in MH and 10 μL were spotted onto 0.2 μm cellulose acetate filters (1.5 cm in diameter, Millipore) and allowed to grow for 48 hours on MH agar to form a colony biofilm, as previously reported [36, 37]. These preformed biofilms were then placed on sensidics (Whatman) on a freshly made lawn of the sensitive strain *Escherichia coli* J53 [38]. This lawn was done by spreading 50 μl of a culture at OD_{595nm} of 0.3 onto MH agar plates. 5 μl of each antibiotic (0.5 X MBEC) were then spotted on the biofilms, plates were incubated overnight and halos of growth inhibition were measured and compared with controls of filters without biofilm and with the antibiotic.

Results

Biofilm formation does not correlate with a resistance profile

A total of 56 *K. pneumoniae* strains isolated from clinical infections and with previously determined antibiotic susceptibility profiles were analyzed for their capacity to form biofilms after overnight growth. We used as controls the laboratory wild type (WT) strain LM21*gfp* and the mutant strain LM21*gfp* Δ*yfiR* [27, 28], previously shown to vary in their ability to form biofilms. Consistent with previous observations, the wild type LM21*gfp* strain performed poorly in this assay, whereas the *yfiR* mutant strain formed a more robust biofilm [28]. All clinical strains grew well, as assessed by their measured OD600nm (data not shown), and their capacity to form biofilms varied widely.

Our cluster analysis based on biofilm-forming ability (**Fig. 1**) showed no correlation between biofilm formation and site of infection, place of

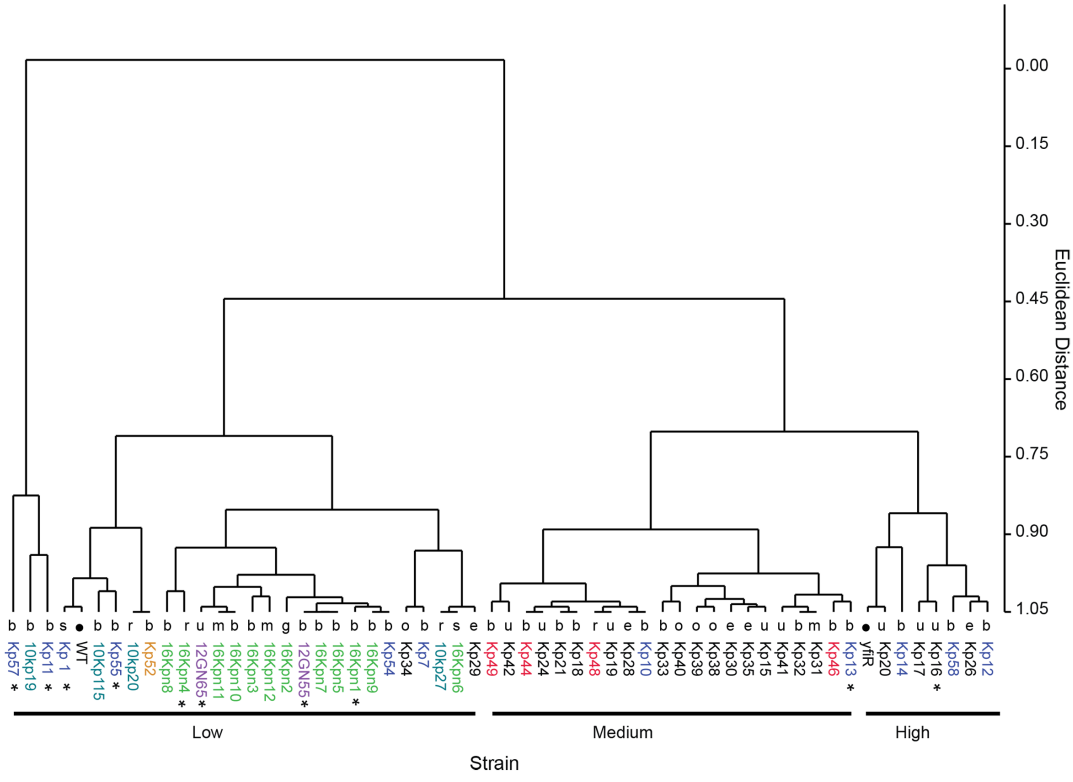

Figure 1. Biofilm formation of clinical *Klebsiella pneumoniae isolates*. Clinical isolates and lab strain controls (WT LM21*gfp* and Δ*yfiR*, black circles) were assayed for their capacity to form biofilms and separated based on arbitrarily defined CV staining parameters as low, middle or high biofilm formers. Strains selected for additional analyses are indicated with an asterisk. Colors indicate different hospitals, letters indicate collection site (b, bloodstream; g; gastrointestinal tract; e, environmental; m, medical device; o, other; r, respiratory tract; s, skin; and u, urinary tract). The dendrogram was made using PAST 3 software (https://folk.uio.no/ohammer/past/).

isolation, or antibiotic resistance profiles (Fig. 1). For example, strains Kp7, Kp13, and Kp14 had identical antibiotic resistance profiles yet differed in terms of biofilm forming performance (low, middle, and high, respectively). The few identified high performance biofilm-forming strains originated from more than one hospital and corresponded to different sample types (bloodstream and urinary tract infections). However, strains with a reduced biofilm-forming ability also shared these characteristics. An analysis of colony morphology, done by growing strains in LB medium containing Congo red + Coomassie blue, showed differences in colony phenotypes (**Fig. 2**), suggesting variation in cell wall properties. These morphological differences serve as indicators of changes in the composition of the cell envelope of Enterobacteriaceae that can be critical for cell adhesion [26, 27]. Some strains also resulted in fluorescent colonies when grown on LB + calcofluor medium, a phenotype indicative of extracellular matrix cellulose production that has been previously associated with increased biofilm formation in *Klebsiella* sp. and other bacteria [28–30]. Again, no correlation was seen between biofilm-forming ability and colony morphology upon incubation on these media.

Persister cell identification

We selected ten *K. pneumoniae* isolates based on their biofilm-forming ability (Fig. 1) and their antibiotic susceptibility profiles, obtained by determining the MICs for the antibiotics MER, AMK, CIP, and TS, which are used for the treatment of *K. pneumoniae* infections and have different mechanisms of action. The strains displayed different susceptibilities to these four antibiotics (**Table 1**). Five strains were sensitive to all tested antibiotics, and two (12GN55, 16Kp1) were sensitive to two of the four drugs tested. Six strains (KpI, Kp11, Kp13, Kp55, 12GN55, and 12GN65), which were sensitive to at least two of the antibiotics tested, were therefore studied for their capacity to resist antibiotics in biofilms (MBEC). Biofilm formation was consistent with our intial observations (Fig. 1) and was comparable to the wild type laboratory strain LM21, except for strain Kp11 that formed a very poor biofilm (**Fig. 3**). The obtained MBECs indicated that all strains showed greater resistance to antibiotics when grown as biofilms (Table 1), as has been already reported [33, 39]. Even strain Kp11, which formed a very poor biofilm, revealed increased resistance (>1000 X) to AMK and TS in biofilms (MBEC) when compared to planktonic cells (MIC) (Table 1).

These six strains were then assayed for the presence of persister cells in planktonic cultures and in biofilms by incubating cells in the presence of antibiotics. In general, there were more persister cells in biofilms than in planktonic cultures, as has been previously reported [16, 40]. In many cases,

Table 1. *Klebsiella penumoniae* strains analyzed in this study. Shown for each strain are sample type and hospital of isolation, as well as the minimal inhibitory concentration (MIC) and the minimal biofilm eradication concentration (MBEC) for the following four antibiotics (μg/ml): AMK, amikacin; CIP, ciproflaxicin; TS, trimethoprim-sulfamethoxazole; and MER, meropenem. Resistance was established following previously defined criteria [31]. Underlined names indicate the six strains selected for further analyses; numbers in bold indicate resistance; > indicates no growth inhibition at the maximum concentration used; ND, not determined; asterisks indicate a >1000X increase (MBEC vs MIC). SC, ET, UC correspond to hospital names, provided as acronyms.

Strain	Sample	Hospital	AMK		CIP		TS		MER	
			MIC	MBEC	MIC	MBEC	MIC	MBEC	MIC	MBEC
Kp1	Brain Abscess	SC	4	32	0.125	0.5	1.25/23.75	80/1520	0.125	16
Kp11	Blood	SC	0.5	512*	0.06	16	0.31/5.94	500/9500*	0.125	128
Kp13	Blood	SC	4	64	0.015	64	1.25/23.75	>500/9500	0.25	256*
Kp16	Urine	ET	>128	ND	>64	ND	>40/760	ND	64	ND
Kp55	Blood	SC	8	32	0.03	32	1.25/23.75	500/9500	0.5	128
Kp57	Blood	SC	4	ND	32	ND	>40/760	ND	128	ND
16Kp4	Respiratory tract	ET	128	ND	>64	ND	>40/760	ND	>256	ND
16Kp1	Blood	ET	128	ND	0.015	ND	2.5/47.5	ND	128	ND
12GN55	Blood	UC	16	512	8	>16	>40/760	>500/9500	0.125	4
12GN65	Urine	UC	4	32	0.015	1	0.63/11.88	80/1520	0.25	128

fewer cells were recovered when exposed to AMK and CIP in comparison with incubation in MER and TS (**Fig. 4**). This is seen for example, in the case of planktonic cultures of Kp1, Kp13, and 12GN65 (Fig. 4A) and in biofilms of Kp11 and Kp13 (Fig. 4B). However, the amount of persisters cells recovered after treatment with amikacin was higher in Kp11 cultures (Fig. 4A) and in Kp55 biofilms (Fig. 4B).

Antibiotic penetration in biofilms

To determine whether the observed differences in persister cell recovery were due to differences in the ability of antibiotics to reach cells in biofilms, we assayed antibiotic for their capacity to penetrate cells grown as colonies

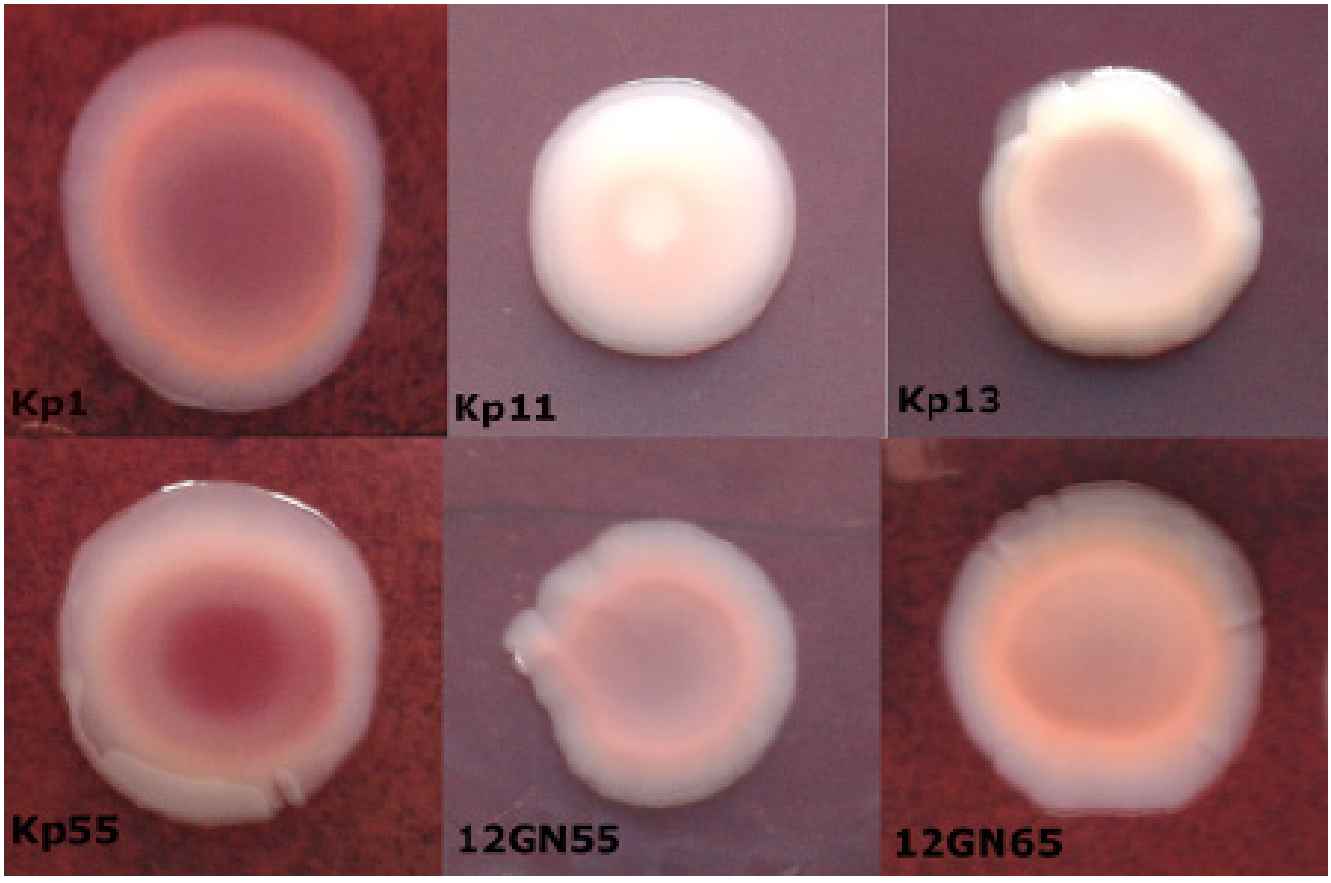

Figure 2. Colony morphology of six *Klebisella pneumoniae* strains grown on LB agar supplemented with Congo red and coomassie blue

[41]. Pre-grown colonies were transferred to fresh solid medium spread with *E. coli* J53 [38], which is sensitive to antibiotics, and spotted with antibiotics. Penetration was assayed based on the ability to inhibit the growth of the lawn of *E. coli* J53 tester cells. As depicted in **Fig. 5**, the antibiotics used differed in their capacity to penetrate these colony biofilms and penetration by a particular antibiotic also varied depending on the clinical isolate tested. The antibiotic AMK, for example, was unable to penetrate any of the preformed biofilms (not shown), while the growth of the *E. coli* tester strain was affected only by TS in two cases and by CIP in three cases.

Killing curve assays

We assessed the clinical isolates by performing killing curves, revealing the presence of the persister subpopulation over time. In this case, we used the antibiotics TS and MER because they gave rise to a larger number of

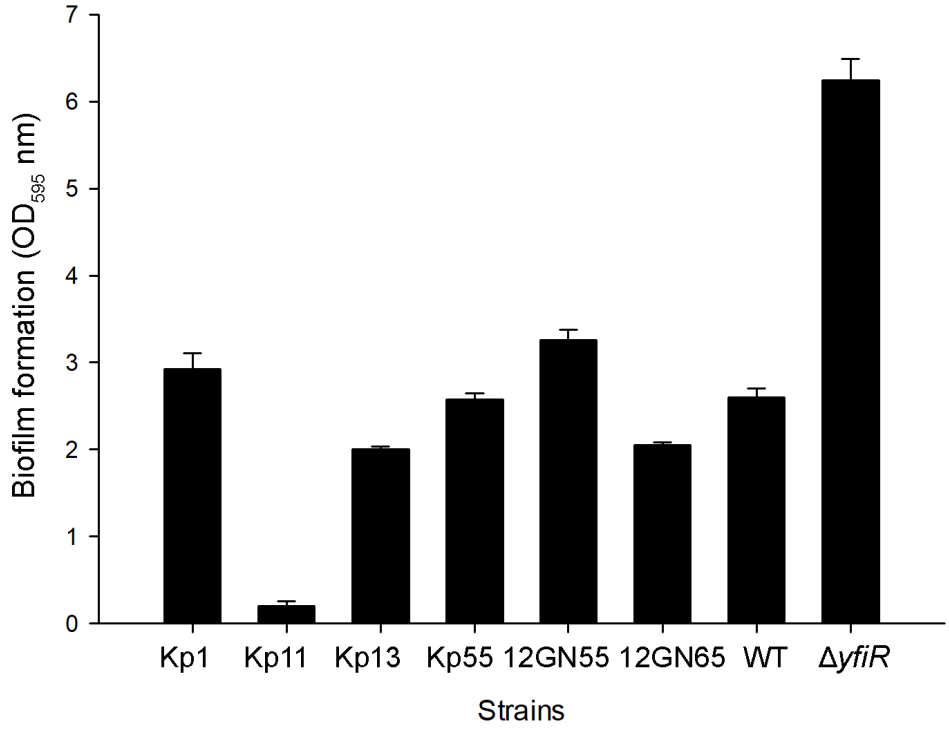

Figure 3. Biofilm formation of selected Klebisella pneumoniae strains. Biofilms were determined by staining with CV for the six selected strains and two laboratory strains (LM21 WT and *δyfiR*). Error bars represent the standard deviation for four technical replicates and 3 biological replicates.

persister cells. Cultures of the five strains that were sensitive to these two antibiotics (Kp1, Kp11, Kp13, Kp55, and 12GN65) were exposed up to 48 hours to 4X the MIC of each antibiotic to avoid the appearance of resistant mutants. Surviving cells were determined by plating for CFU to assess the kinetics of death under incubation with an antibiotic. All strains showed the typical biphasic curve with an initial rapid killing of the sensitive population, followed by a slower phase that represents persisters [42]. Strain Kp1 was the most susceptible since no viable cells were recovered after eight hours of incubation in either of the antibiotics tested (**Fig. 6**). Cultures of the other strains still had viable cells in the presence of TS at 24 hours (12GN65) or 48 hours (Kp11, Kp13 and Kp55). Cell viability upon incubation with MER was evident up until 24 hours for three strains (12GN65, Kp13, and Kp55) and at 48 hours for only one strain (Kp11). The laboratory strain LM21 also showed a similar killing curve and no recovery of viable cells after 48 hours

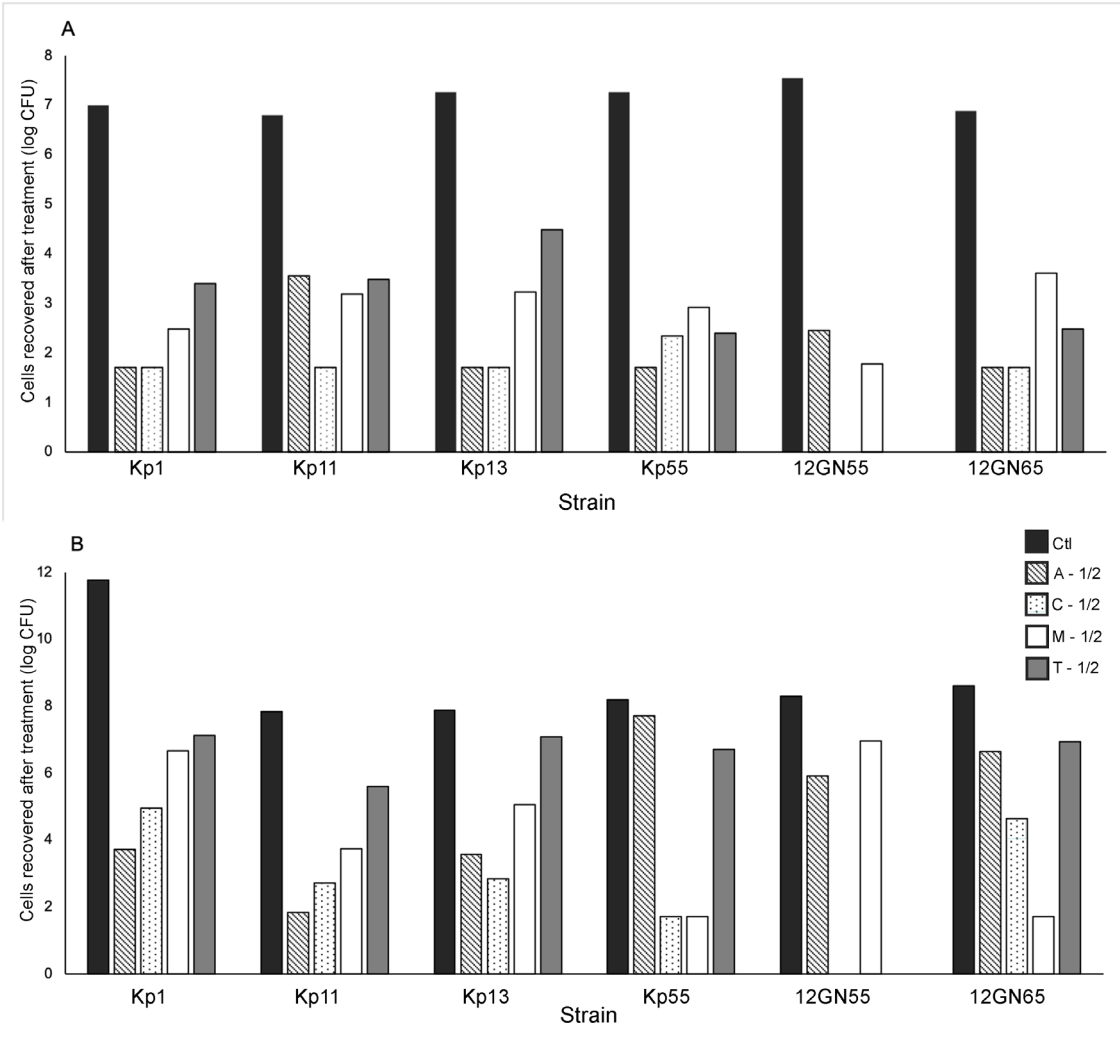

Figure 4. Persister cells in clinical *Klebisella pneumoniae* isolates. Persister populations were determined by treating A) planktonic cultures using 2X the MIC and B) biofilms using ½ the MBEC and then plating survivor cells on media without antibiotics to determine CFU. Cultures were both untreated (controls, Ctl, black bars) and treated with AMK (A, striped bars), CIP (C, dotted bars), MER (M, white bars) or TS (T, grey bars). The limit of detection was 50 CFU.

of incubation. In some cases, a slight increase was observed after the initial killing phase, but no growth was observed thereafter, and thus these were not presumed to be resistant mutants, as has been observed upon incubation with low antibiotic concentrations [19].

Discussion

The success of *K. pneumoniae* as a pathogen relies not only on its wide repertoire of virulence factors but also on its capacity to thrive

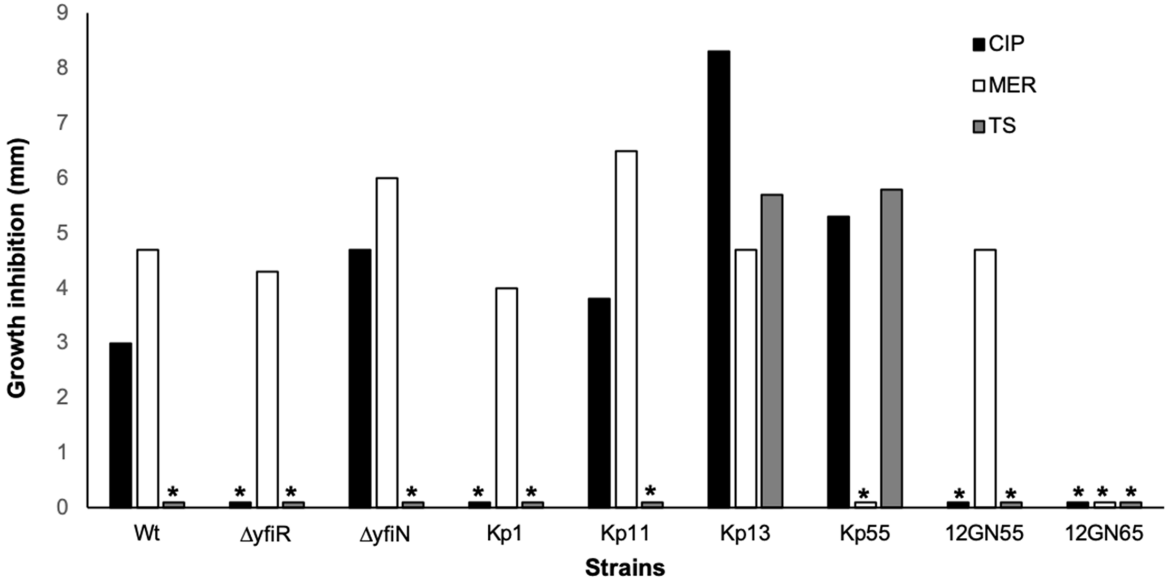

Figure 5. Antibiotic penetration of preformed *Klebsiella penumoniae* colony biofilms. Laboratory strains (LM21 WT, Δ *yfiR*), and Δ *yfiN*) and clinical isolates were grown as colonies, placed over a lawn of the tester strain *E. coli* J53 and overlayed with antibiotics. Penetration is indicated by growth inhibition of the sensitive tester strain. CIP, black bars; MER, white bars; TS, grey bars. Asterisks indicate no growth inhibition.

under many antibiotics. This Gram-negative pathogen mainly acquires antibiotic-resistance genes via horizontal gene transfer, playing a role as a resistome reservoir [43]. In addition to resistance due to particular genetic determinants, *K. pneumoniae* can also withstand antibiotic treatment through a non-inherited mechanism known as persistence. Persistence is used to indicate the capacity of a clonal subpopulation of cells to survive on a high concentration of antibiotic while the rest of the population is killed [14][44]. Even though persistence has gained attention lately, the precise nature of the signals that activate this dormant state has yet to be fully defined. Our data provide insight into how some antibiotics and biofilm formation are crucial players in the formation of persister bacterial cells. These characteristics are commonly found in clinical settings where the overuse of antimicrobials primarily selects pathogens like *K. pneumoniae*, which have a remarkable ability to form biofilms on abiotic or biotic surfaces [27, 28].

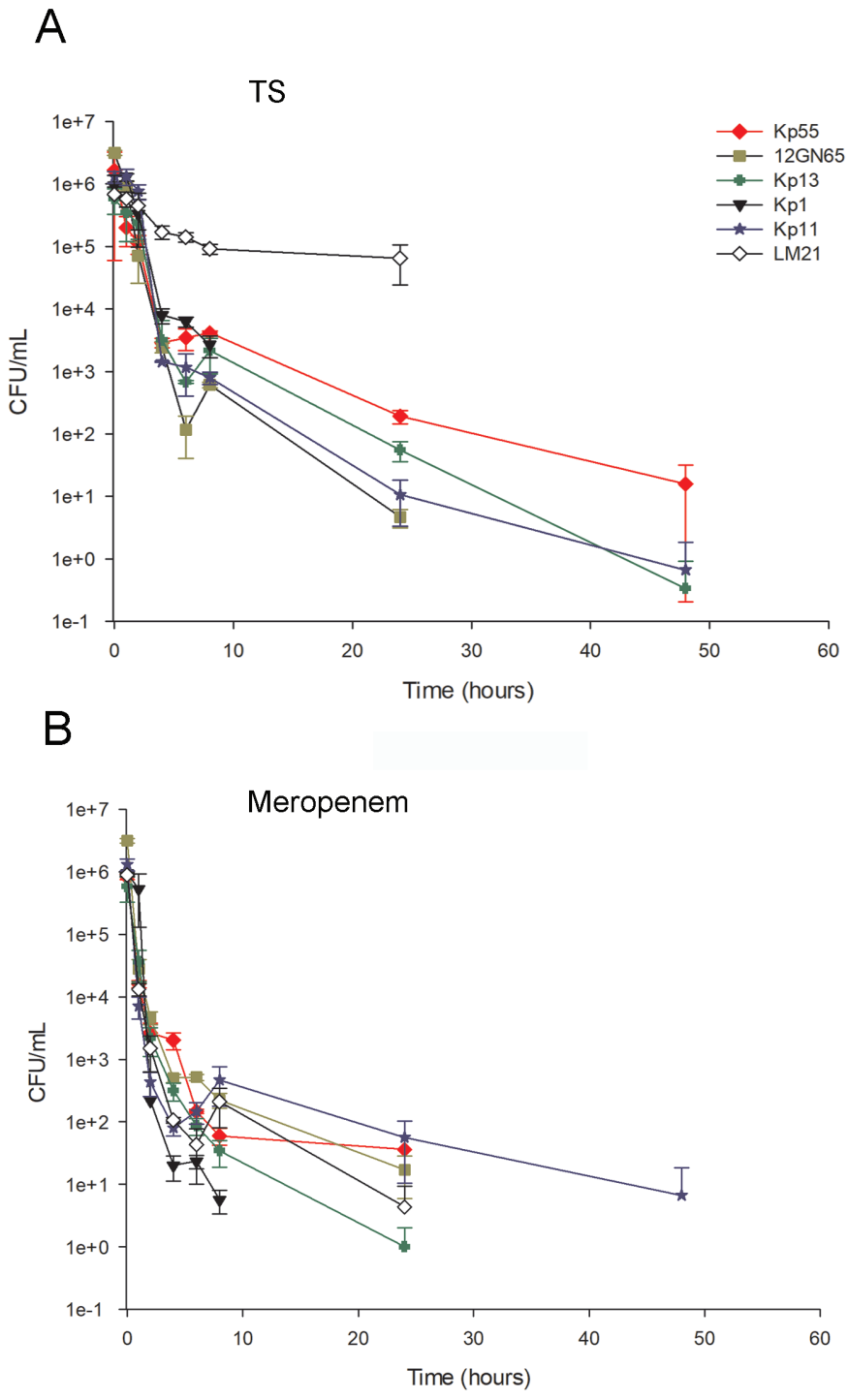

Figure 6. Killing curves for *Klebsiella penumoniae* cultures incubated with antibiotics at 4x their MIC. Cells were incubated in the presence of TS (**A**) or MER (**B**). Only time points in which CFUs were recovered for the various strains are shown. Error bars represent the standard deviation for three replicate cultures.

Biofilm and antibiotic resistance

The analysis presented here indicated that there was no correlation between biofilm-forming ability and antibiotic resistance profiles (Fig. 1) among our clinical *K. pneumoniae* isolates. These isolates differed, nonetheless, in phenotypes associated with multicellular behavior, such as biofilm formation and production of extracellular matrix components. These results are consistent with work showing that clinical strains of *K. pneumoniae* and other species display a broad range of biofilm-forming phenotypes [10], but differed from a study where *K. pneumoniae* strains producing extended-spectrum β-lactamases revealed a higher probability of forming more biofilm [24]. Our results and the discrepancies with previous studies could be due to differences in the conditions used for these assays and to genotypic variability among isolates [28], which was not examined in this study. It is also important to note that one limitation of using *in-vitro* systems for biofilm evaluation is the inability to imitate the natural host environment. Therefore, further studies would be required to address how host components such as tissue composition influence bacterial adhesion and antibiotic resistance gene expression during infections.

Our MBEC data indicates that all strains show higher resistance to antibiotics when grown as biofilms (Table 1), as has been shown previously [33, 39]. Even the strains that formed a very poor biofilm were more resistant than their planktonic state counterpart. Many properties of these multicellular biofilm structures have been linked with increased capacity to survive upon antibiotic exposure. For instance, low antibiotic permeability and reduced metabolic activity increase the persister cell formation and the capacity to survive antimicrobials, a process which is unrelated to the acquisition of resistance genes [42]. Together, our results suggest that biofilm conditions enable bacteria to survive in the presence of antibiotics.

Persister cells vary among isolates

Our experiments indicate that the number of persister cells varies among *K. pneumoniae* isolates when exposed to the same antibiotic. They also suggest that the persister population depends on the antibiotic tested, consistent with previous findings in other bacterial species [18, 45]. These differences could be due to viarable antibiotic modes of action or to the presence of strain-specific persister populations. Similar results have been observed for *E. coli*, where it was postulated that different types of persister cells occur within populations due to treatment with various antibiotics and even when treated with antibiotics that have similar modes of action [46].

A low antibiotic penetration might result in a dramatic increase in the number of persister cells in biofilms. However, penetration did not necessarily correlate with the observed number of persister cells detected with the antibiotics AMK and TS. AMK did not penetrate (data not shown), being presumably less able to reach cells inside the biofilm and resulting in a low number of persister cells (Fig. 4). In contrast, TS failed to penetrate in several biofilms, yet it resulted in an overall more significant number of persister cells, even in strains where antibiotic penetration was observed (Fig. 4). Therefore, it seems that the number of persister cells recovered is due not only to the ability of the drug to penetrate biofilm structures, as assayed here, but also to the particular drug in which cells are incubated.

The previous observation was validated by conducting killing curves, which showed that the number of persister cells was higher under treatment with TS than MER (Fig. 4A). This result indicates that the antibiotic-mode of action might dictate the activation of a particular response. Antibiotics TS and MER trigger different cell responses, as previously reported [47]. MER does not readily diffuse through the cell wall and acts on penicillin-binding proteins that catalyze peptidoglycan biosynthesis [48]. On the other hand, TS blocks the synthesis of tetrahydrofolate and interferes with nucleic acid and protein metabolism [47]. Previous evidence demonstrated that each antibiotic type gives rise to the persister state in *K. pneumoniae* independently, indicating the activation of separate cellular pathways [49]. Consistent with this idea, different persistent profiles were observed for two different clinical pathogens, *K. pneumoniae* and *Proteus mirabilis* [50]. Even though the role of antibiotics in persister formation is clear, the precise mechanisms involved are still unknown. A recent study suggested, for example, that persisters can arise due to fluctuations in expression of genes for energy-generating components, such as TCA enzymes, that result in reduced cellular ATP levels and a low energy state in which cells are more tolerant to antibiotics [51]. In our case, the analysis of gene expression could, for example, help to identify responses upon exposure to various antibiotics or to the same antibiotic among strains with different genetic backgrounds [52].

In hospitals, one consequence of the misuse of antibiotics is the failure in treating infections. The exposure of bacteria to antimicrobials promotes the selection of MDR strains and the emergence of persister cells due to their capacity to survive when exposed to stress conditions [53]. A recent study found, however, that persister formation rates in *K. pneumoniae* were not necessarily associated with clinical outcome or underlying diseases, at least when MER is used [49]. Again, it is essential to keep in mind that pure in-vitro culture analysis may not accurately reflect the *in vivo* conditions,

mostly when little is known regarding persisters in the host environment [54]. The phenotypic variability among the *K. pneumonia* strains in our study reveals the complexity underlying persister formation and opens the possibility of using this group of clinical strains to further study possible response and mechanistic differences in persister formation. Recognizing that clinical strains vary in phenotypes can also help to anticipate possible complications during an antibiotic treatment.

Conclusions

This study shows that *K. pneumoniae* clinical strains, which are important in terms of nosocomial infections, have variable pathogenicity-relevant phenotypes. These strains had different drug susceptibilities and differed in their capacity to form biofilms. Their biofilm-forming capacity did not correlate with antibiotic resistance profiles or with the site of isolation.

Exposure of a strain to different groups of antibiotics resulted in variable persister cell populations. Exposure to the antibiotic TS, which inhibits the tetrahydrofolate route, led to a higher number of persister cells than exposure to any of the other antibiotics tested. AMK followed in terms of producing more persistent cells in biofilm and planktonic cultures, while CIP, a fluoroquinolone antibiotic, generated the smallest persister cell populations in planktonic cultures and in biofilms.

Killing curves were biphasic and consistent with the presence of persister cells, with a rapid killing phase until hour 6 followed by slow killing up to 48h [17, 28]. The phenotypes of these clinical isolates differed in antibiotic susceptibility, biofilm formation and persister cell presence, indicating strain variability and population heterogeneity that can impact treatment and control of this pathogen in hospitals.

Acknowledgments

We would like to thank Dr. Javier Escobar (Universidad El Bosque, Bogota, Colombia) for facilitating most of the *K. pnuemoniae* strains employed in this study. Likewise, we acknowledge Dr. Damien Balestrino and Dr. Christine Forestier (University of Auvergne, Clermont-Ferrand, France) for sharing the *K. pneumoniae* LM21*gfp* strain. We thank Gabriela Portilla for her help with killing curve experiments.

This work was supported by Colciencias (project no. 657054531675)

Conflict of interest

The authors certify that they have no conflict of interest, such as affiliations with or involvement in any organization or entity with any financial interest or related to the subject matter or materials discussed in this manuscript.

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Variabilidad en la formación de biopelículas y de células persistentes en aislados clínicos de *Klebsiella pneumoniae* en Colombia

Resumen: *Klebsiella pneumoniae* es un patógeno oportunista asociado a infecciones nosocomiales. Las células persistentes son una fracción de la población bacteriana que escapa al tratamiento con antibióticos y está asociada con el fracaso de la terapia antibiótica. En este trabajo se analizaron células persistentes en cultivos planctónicos y biopelículas, usando 10 aislados clínicos *K. pneumoniae* y cuatro tipos de antibióticos diferentes. Los aislados tuvieron diferentes perfiles de susceptibilidad al antibiótico, que no se correlacionaron con la capacidad de formar biopelículas. Se encontraron células persistentes bajo todas las condiciones evaluadas, aunque sus números poblacionales variaron dependiendo del antibiótico usado. Se encontró un mayor número de células persistentes en biopelículas que en cultivos planctónicos. El tratamiento antibiótico con trimetoprim-sulfametoxazol dio como resultado la mayor sub-población de células persistentes, en comparación con otros antibióticos evaluados, mientras que ciprofloxacin fue el antibiótico que produjo menos células persistentes. Estos resultados indican que los aislados clínicos de *K. pneumoniae* varían no solamente en su susceptibilidad a antibióticos, sino también en propiedades relevantes en las enfermedades, como la formación de biopelículas y poblaciones de células persistentes.

Palabras clave: Persistencia; biopelícula; resistencia a antibióticos; *K. p umoniae*.

Variabilidade na formação de biofilme e de células persistentes em isolamentos clínicos de *Klebsiella pneumoniae* em Colômbia

Resumo: *Klebsiella pneumoniae* é um patógeno oportunista associado a infecções nosocomial. As células persistentes são uma fração da população bacteriana que escapa ao tratamento com antibióticos e está associada ao fracasso da terapia antibiótica. Neste trabalho foram analisadas células persistentes em culturas planctônicas e biofilmes, usando 10 isolamentos clínicos de *K. pneumoniae* e quatro tipos de antibióticos diferentes. Os isolamentos tiveram diferentes perfis de susceptibilidade ao antibiótico, que não se correlacionaram com a capacidade de formar biofilme. Se encontraram células persistentes sob todas as condições avaliadas, entretanto, seus números populacionais variaram dependendo do antibiótico usado. Encontrou-se um maior número de células persistentes em biofilmes que em cultivos planctônicos. O tratamento antibiótico com trimetoprima-sulfametoxazol obteve como resultado a maior subpopulação de células persistentes, em comparação com outros antibióticos avaliados, enquanto que ciprofloxacino foi o antibiótico que produziu menos células persistentes. Estes resultados indicam que os isolamentos clínicos de *K. pneumoniae* variam não só quanto a sua susceptibilidade a antibióticos, como também em propriedades relevantes nas doenças, como a formação de biofilmes e populações de células persistentes.

Palavras-chave: persistência; biofilme; resistência a antibióticos; *K. p umoniae*.

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