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RESEARCH ARTICLE

Heteroresistance to colistin in *Klebsiella pneumoniae* is triggered by small colony variants sub-populations within biofilms

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One sentence summary: This work represents the first evidence that biofilm formation can trigger the emergence of heteroresistance to colistin from an apparently susceptible strain.

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ABSTRACT

The emergence of *Klebsiella pneumoniae* multidrug-resistant strains paves the way to the re-introduction of colistin as a salvage therapy. However, recent planktonic studies have reported several cases of heteroresistance to this antimicrobial agent. The aim of this present work was to gain better understanding about the response of *K. pneumoniae* biofilms to colistin antibiotherapy and inspect the occurrence of heteroresistance in biofilm-derived cells. Biofilm formation and its susceptibility to colistin were evaluated through the determination of biofilm-cells viability. The profiling of planktonic and biofilm cell populations was conducted to assess the occurrence of heteroresistance. Colony morphology was further characterized in order to inspect the potential role of colistin in *K. pneumoniae* phenotypic differentiation. Results show that *K. pneumoniae* was susceptible to colistin in its planktonic form, but biofilms presented enhanced resistance. Population analysis profiles pointed out that *K. pneumoniae* manifest heteroresistance to colistin only when grown in biofilm arrangements, and it was possible to identify a resistant sub-population presenting a small colony morphology (diameter around 5 mm). To the best of our knowledge, this is the first report linking heteroresistance to biofilm formation and a morphological distinctive sub-population. Moreover, this is the first evidence that biofilm formation can trigger the emergence of heteroresistance in an apparently susceptible strain.

Keywords: *Klebsiella pneumoniae*; colistin; heteroresistance; biofilm; small colony variants

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative pathogen that frequently infects the tissues and organs of immunocompromised patients and can easily develop antibiotic resistance (Nordmann, Naas and Poirel 2011; Queenan and Bush 2007). It can cause serious infections in the urinary tract and wounds as well as intraabdominal infections, pneumonia and bacteremia (Tzouvelekis *et al.* 2012). This opportunistic behaviour makes *K. pneumoniae* responsible for many nosocomial infections Usually, treatment involves the administration of penicillin-based

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antibiotics, but the emergence of strains producing extended-spectrum β-lactamases (ESBL) has rendered this antibiotic class ineffective (Pitout and Laupland 2008). Carbapenems were introduced for treating ESBL-producing strains, but eventually, this led to the appearance and dissemination of carbapenem-resistant *Klebsiella* pneumonia (CRKP) strains (van Duin *et al.* 2013).

Since 1959, polymyxin E, also known as colistin, has been used to treat Gram-negative bacterial infections (Reed *et al.* 2001). In the early 1970's, colistin was largely abandoned due to its toxicity and the advent of safer treatment alternatives, such as those based on aminoglycosides (Li *et al.* 2005). However, the emergence of multidrug-resistant (MDR) microorganisms and the lack of effective antimicrobials have promoted the re-introduction of colistin as a salvage therapy (Falagas and Kasiakou 2005). Currently, colistin is one of the last resort options that remains effective against MDR strains (Ah, Kim and Lee 2014).

It is now well established that most bacterial infections are associated to biofilms, and most notably biofilm formation has been identified as one of the major mechanisms of virulence in *Klebsiella* pneumonia infections in humans (Murphy and Clegg 2012). Biofilms are communities of microorganisms that live on a reversible sessile state, attached to a surface or to each other and surrounded by a self-produced polymeric matrix (Moscoso, García and López 2009). Biofilm-associated cells differ phenotypically from cells in planktonic state, showing altered gene transcription and growth rate, and displaying enhanced resistance against antibiotherapy (Costerton 1999; Balcázar, Subirats and Borrego 2015). Therefore, studies using biofilms models to more comprehensively understand and fight bacterial resistance towards antimicrobials are in demanding.

Antibiotic resistance is routinely screened in the clinical practice and used as treatment guider. However, routine antibiograms fail to detect specific resistance phenomena, such as heteroresistance, which is generally defined as the presence, within a larger population of fully antimicrobial-susceptible microorganisms, of resistant sub-populations (Rinder 2001; Wang *et al.* 2014). Heteroresistant strains are usually classified as susceptible in routine antibiograms, which can be easily explained by the extremely low fraction of the resistant sub-population that usually goes unnoticed (Falagas *et al.* 2008). Nevertheless, this small sub-population has an important clinical significance, seeing as, in the presence of the antimicrobial, and it can be selected and dominate the infection. It is therefore easy to conclude that the failure of antimicrobial-based treatments may be closely linked to the heteroresistance. This phenomenon should not be confused with tolerance, which corresponds to the appearance of sub-populations capable of surviving antimicrobial pressure, but whose minimal inhibitory concentration is similar to that of raw population (Morosini and Cantón 2010).

Although several cases of heteroresistance to colistin among *K. pneumoniae* strains have been reported, a few works study biofilms consortia (Poudyal *et al.* 2008; Meletis *et al.* 2011; Ah, Kim and Lee 2014). The understanding of heteroresistance phenomena and its role in the failure of in-use antimicrobial strategies is attracting considerable research interest. Therefore, the aim of this present work was to study the response of *K. pneumoniae* biofilms to colistin antibiotherapy and examine the putative development of associated resistance phenomena.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The experimental work was conducted, using a strain of *K. pneumoniae* isolated from a urinary sample and gently provided by Braga Hospital, Portugal. This strain belongs to the Centre of Biological Engineering collection of University of Minho and was stored at –70°C in broth medium supplemented with 20 % (v/v) of glycerol. Bacteria were first streaked on a Tryptic Soy Agar (TSA, Merck, Portugal) plate, grown for 24 h at 37°C and were subsequently stored at –4°C for no longer than one week. Prior to each experiment, some colonies were collected from the TSA plates and grown overnight in medium broth at 37°C and 120 rpm (OS-20). Subsequently, bacteria were harvested by centrifugation at 9000 *g* for 5 min at room temperature, washed and suspended in a fresh medium for further analysis. Cell concentration was adjusted by measuring optical density (OD) at 640 nm, using a previously prepared calibration curve [OD versus Colony Forming Units (CFU)]: CFU $mL^{-1} = 1 \times 10^9 \times OD - 2 \times 10^7$

Biofilm formation

Biofilm formation was performed according to the microtiter plate test developed by Stepanović *et al.* (2000), using 96-well flat bottom microtiter plates (Orange Scientific) and Tryptic Soy Broth (TSB, Merck, Portugal) as culture medium. Bacterial suspensions were adjusted to a final concentration of 1×10^6 CFU mL^{-1}, and 200 μL were transferred to each well. Microtiter plates were incubated at 37°C in a horizontal shaker (120 rpm) for 24 h. After incubation, the content of the plates was discarded and the wells washed 2× with sterile deionized water to remove non-adherent cells. A characterization of biofilms was then performed as follows

Biofilm analysis

Biofilm viability

Biofilm viability was assessed by the CFU method. Briefly, 200 μL of sterile deionized water were introduced in each well, and biofilms were detached by sonication using an ultrasonic cleaning bath (Sonicor). The content of five wells was recovered to a single Eppendorf, homogenized and serial diluted. Posteriorly, 10 μL of each dilution was spread onto TSA plates. After 13 h of incubation at 37°C, the CFU were counted.

Antimicrobial assays

The antimicrobial peptide utilized throughout the work was colistin sulphate salt ≥15000 U mL^{-1} (Sigma). Aliquots of 1 mg mL^{-1} were prepared in sterile ultrapure water and stored at –20°C until being used.

Planktonic susceptibility

Determination of minimal inhibitory (MIC) and minimum bactericidal concentration (MBC) of colistin was performed by the microdilution method according to Clinical and Laboratory Standards Institute (CLSI) (NCCLS 2003), using 96-well round bottom microtiter plates (Orange Scientific) and bacterial suspensions prepared in Muller Hinton Broth (MHB, Merck, Portugal) adjusted to a final 1×10^6 CFU mL^{-1}. In each well, 100 μL of fresh medium with increasing colistin concentrations were introduced and 100 μL of bacterial suspension were added,

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reaching a final concentration of 5×10^5 CFU mL^{-1}. A range of 0.25–64 μg mL^{-1} of colistin was tested. Microtiter plates were incubated at 37°C in a horizontal shaker (120 rpm) for 24 h, and the OD was measured at 640 nm in a microtiter plate reader (Sunrise, Tecan). MIC was recorded as the lowest concentration, where no growth was detected. Determination of MBC was conducted by plating 10 μL from the wells with no visible growth in Muller Hinton Agar (MHA Merck, Portugal) plates. Following 24 h of incubation at 37°C, MBC was recorded as the lowest colistin concentration that yielded no colony growth.

Biofilm susceptibility

For determination of biofilm-derived cells susceptibility to colistin, a similar procedure to that, described for biofilm formation, was followed. In each well, 100 μL of a bacterial suspension adjusted to 2×10^6 CFU mL^{-1} was added to 100 μL of fresh medium supplemented with increasing colistin concentrations, testing a range of 0.25–64 μg mL^{-1} of colistin. Biofilms were posteriorly characterized as previously described.

Population analysis profile

The population analysis profile (PAP) is a widely used method for detection of heteroresistance among diverse microorganisms as it allows the detection and quantification of resistant subpopulations in a single isolate (Morand and Mühlemann 2007; Meletis *et al.* 2011; Hung *et al.* 2012).

Planktonic cells

A *K. pneumoniae* culture, grown for 24 h in MHB, was adjusted to 1×10^8 CFU mL^{-1}. Serial dilutions were prepared in a saline solution 0,9 % (w/v) NaCl and 50 μL of each were spread on MHA plates containing 0, 1, 2, 4 and 8 μg mL^{-1} of colistin. CFU were counted after 24 h of incubation at 37°C. In order to assess the impact of the culture media in PAP analysis, some previous experiments were carried out with TSB instead of MHB and TSA replacing MHA. As no significant differences were detected (data not shown), it was decided to hold the remaining tests with MHB and MHA to strengthen the comparison with MIC and MBC values previously established.

Biofilm cells

A similar procedure was implemented to evaluate the presence of resistant sub-populations in *Klebsiella* pneumonia biofilms. Biofilms were formed in 24-wells microtiter plates (Orange Scientific), using TSB according to the biofilm formation procedure previously described. However, in these assays, 1.5 mL of bacterial suspension was introduced in each well, and after the biofilms were formed, the wells were scraped instead of sonicated in order to collect an adequate amount of biomass. Biofilm cells were resuspended in saline solution and gently vortexed for 1 min to disrupt possible cell aggregates (these parameters were previously optimized in order to accomplish the complete resuspension of all the biofilm-attached cells without lysis). Afterwards the solution was adjusted to 1×10^8 CFU mL^{-1}. Serial dilutions were prepared in saline solution, and 50 μL of each were spread onto TSA plates containing 0, 1, 2, 4 and 8 μg mL^{-1} of colistin. CFU were counted after 24 h of incubation at 37°C.

In order to quantify the resistant sub-populations, both in planktonic and biofilm assays, the number of CFU counted in each colistin concentration was divided by the number of CFU counted in plates without colistin (positive control).

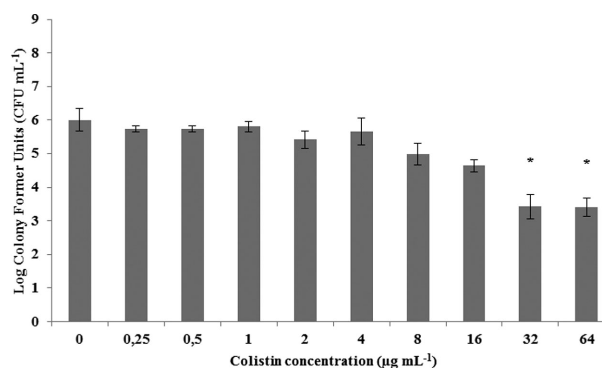

Figure 1. Number of cultivable cells obtained for biofilms formed by *Klebsiella* pneumonia developed in TSB supplemented with increasing concentrations of colistin; mean (±SD), $n = 6$. *indicates significant differences ($P < 0,05$) between means.

Observation of Colony Morphology

To evaluate the impact of the bacterial mode of growth on *K. pneumoniae* phenotypic diversification, the morphology of the colonies formed by bacteria derived from planktonic and biofilm cultures was characterized. Serial bacterial dilutions were prepared in saline solution and plated in TSA plates at 37°C. Colonies were observed, photographed and classified after 45 h of growth. In order to do so, a magnifying glass (Olympus SZ-CTV) was used and the images recorded with a CCD camera (AVC, D5CE, Sony, Tokyo, Japan).

Statistical analysis

A statistical analysis was conducted in order to assess the individual influence of the various parameters studied. All the experiments were accomplished by at least three independent assays. PAPs were analysed by a model of Mann–Whitney test and biofilm formation and susceptibility was analysed by a model of Kruskal–wallis of *GraphPad Prism 6* software. Differences between means were considered statistically significant for P-values ≤ 0.05.

RESULTS

Even though colistin is still showing effectiveness against *K. pneumoniae* infections, the formation of biofilm consortia makes it more difficult to handle antibiotherapy phenomenon. Therefore, the characterization of biofilm responses to colistin has become a pressing analysis need within the scope of clinical practice. Here, a preliminary analysis of MIC and MBC values was performed in order to be able to compare the responses of *K. pneumoniae* planktonic and biofilm populations. Considering EUCAST breakpoints (EUCAST 2015), *K. pneumoniae* is deemed susceptible to colistin presenting an MIC = 2 μg mL^{-1} and an MBC = 2 μg mL^{-1}. Regarding the biofilm mode of life, the susceptibility profile of the biofilm-associated cells to colistin is presented in Fig. 1. Biofilms presented a dose-dependent response to the antimicrobial, i.e. the increment in colistin concentration promoted a continuously reduction of viability, although only observed at concentrations above 4 μg mL^{-1}. The MIC value previously determined in the planktonic assays (2 μg mL^{-1}) had no effect when used against biofilms. Furthermore, in the range of concentrations tested, no concentration was able to totally inhibit biofilm formation and only the two higher concentrations (32 and 64 μg mL^{-1}) resulted in a considerable reduction of

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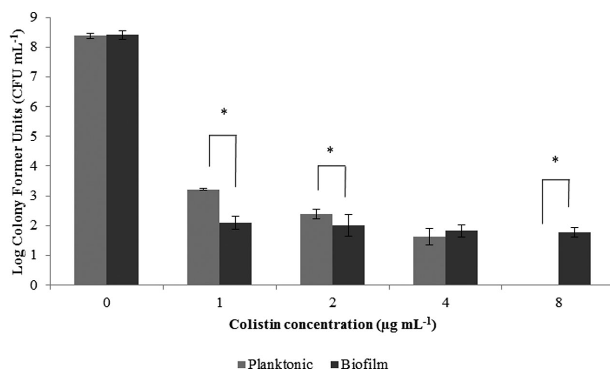

Figure 2. PAP of K. pneumoniae: (**A**) planktonic cultures and (**B**) biofilm cultures; mean (±SD), $n = 6$. *indicates significant differences ($P < 0,05$) between means.

biofilm-cells viability ($P < 0.05$). Nonetheless, from the biological point of view, the log reduction achieved with these two colistin doses fall short of the desired for a successful therapy.

In the course of biofilm susceptibility assays, it was observed a heterogeneous behaviour when in the presence of high doses of colistin. In fact, the response of the strain was quite different between replicates, i.e. in some replicates, it was verified a complete inhibition of growth, while in other replicates of the same colistin concentration the growth was quite noticeable. This atypical behaviour led to the suspicion of heteroresistance to colistin. In order to investigate this suspicion, PAP was conducted for planktonic cultures of *K. pneumoniae* as well as for bacteria derived from pre-formed biofilms (Fig. 2). Results showed that the population able to grow on the MHA plates changed according to the colistin concentrations applied (Table 1).

Both in planktonic and biofilm cultures the proportion of the population able to grow in presence of colistin was very small, e.g. for the concentration of 1 μg mL^{-1}, the resistant fraction of the population was 6.7E^{-06} ± 4.7E^{-07} and 4.2E^{-07} ± 2.8E^{-07} for planktonic and biofilm bacteria, respectively. These values are in accordance with other studies where *K. pneumoniae* isolates also presented extremely low fractions of population able to survive in the presence of colistin (Poudyal *et al.* 2008; Meletis *et al.* 2011). Planktonic cultures revealed bacteria able to grow up to 4 μg mL^{-1} of colistin, as it was never detected growth in plates containing 8 μg mL^{-1}. The increase in colistin concentration decreased the fraction of planktonic bacteria capable of growing, whereas biofilms seemed to be less sensible to the increase of antimicrobial concentration. Interestingly, biofilms revealed an extremely low fraction of the population able to grow in the presence of 8 μg mL^{-1}. It should be stressed that in planktonic cultures it was never detected any bacteria capable of surviving at this concentration. As previously referred, PAP is carried out by the CFU method, which allowed an easy examination of the colony morphologies. In particular, the observation of the biofilm sub-population resistant to 8 μg mL^{-1} of colistin sparked a further interest as it exhibited a smaller morphology (small morphotype, diameter of 4.8 ± 1 mm) compared to what is normally observed in this strain [wild-type morphotype (WTM), diameter of 9.8 ± 1 mm] (Fig. 3). The small morphotype (SM) was only observed in plates containing 8 μg mL^{-1} of colistin and this morphology was never detected in experiments using any other concentration.

These data gave an important contribution to heteroresistance investigation. Moreover, the observation of a new morphotype capable to survive in a concentration of colistin superior to the MIC led to the formulation of a new hypothesis: SM could represent a sub-population responsible for heteroresistance to colistin. As a first step to verify this hypothesis, the MIC and MBC of colistin against the SM variants were assessed. Results revealed that SM was resistant to colistin (Table 2). However, it is known that resistances and morphotypes may not be stable and may even reverse their behaviour (Meletis *et al.* 2011). Therefore, SM was tested for stability throughout nine daily sub-cultures on antibiotic free solid medium and also with an overnight growth in liquid medium, with subsequent determination of MIC and MBC values. Results confirmed the stability of the resistance and the non-affectation of MIC and MBC values (Table 2). Colony morphology remained unchanged both for the cells coming from the 24-h-old planktonic culture and from the nine daily sub-cultures on antibiotic free solid medium.

As a complement to the planktonic assays, a subsequent analysis of biofilm formation and susceptibility to colistin was performed for the SM variants, resorting to the analysis of its viability (Fig. 4).

SM biofilms presented a completely different response to colistin compared to that observed in the wild-type morphotype. Colistin was ineffective in this variant, i.e. it never achieved any reduction in viability. Biofilms were more resistant than planktonic cultures considering that the MIC (64 μg mL^{-1}) caused no effect in the communities. Although the colony morphology was carefully observed in order to detect possible (even minor) changes related to biofilm formation, the morphology remained unaltered (Fig. 5).

DISCUSSION

The mechanism of action of colistin is based on the electrostatic interaction between the cationic polymyxin and the anionic outer membrane of Gram-negative bacteria. Divalent cations (calcium and magnesium), responsible for stabilizing lipopolysaccharide (LPS), are competitively displaced by colistin, decreasing the membrane stability, which leads to its permeabilization and consequently cell death (Newton 1956; Davis, Iannetta and Wedgwood 1971; Storm, Rosenthal and Swanson 1977). Mechanisms of resistance to colistin are usually related to structural remodellings in LPS composition mainly by diminution of the membrane overall negative charge,

Table 1. Resistant fractions of the population calculated for each concentration of colistin based on the PAP; mean (±SD), $n = 6$.

		Resistant fractions of the population	
		Planktonic cultures	Biofilm cultures
Colistin concentration (μg mL^{-1})	0	1 ± 2.2E-01	1 ± 3.0E-01
	1	6.7E-06 ± 4.7E-07	4.2E-07 ± 2.8E-07
	2	1.1E-06 ± 4.6E-07	4.7E-07 ± 2.4E-07
	4	2.1E-07 ± 1.6E-07	2.7E-07 ± 1.2E-07
	8	0	2.4E-07 ± 8.7E-08

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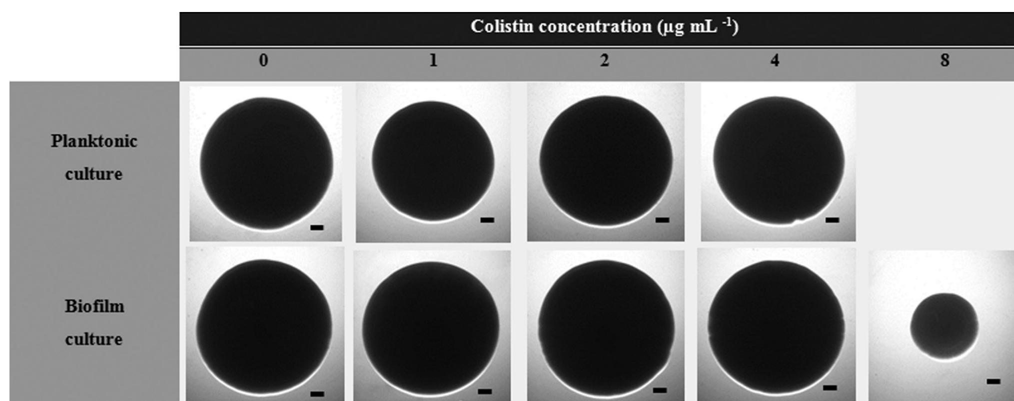

Figure 3. Colony morphology observed in the PAP of planktonic cultures and biofilms; No differences were observed between independent assays. Each black bar represents 1 mm.

Table 2. MIC and MBC of colistin for the *K. pneumonie* wild-type morphotype (WTM), the SM, and for the SM after growth in antibiotic free media: nine generations in solid media (SM 9th G) and 24 h culture in liquid media (SM 24 h); $n = 3$.

	MIC (μg mL^{-1})	MBC (μg mL^{-1})
WTM	2	2
SM	64	>64
SM (9th G)	64	>64
SM (24 h)	64	>64

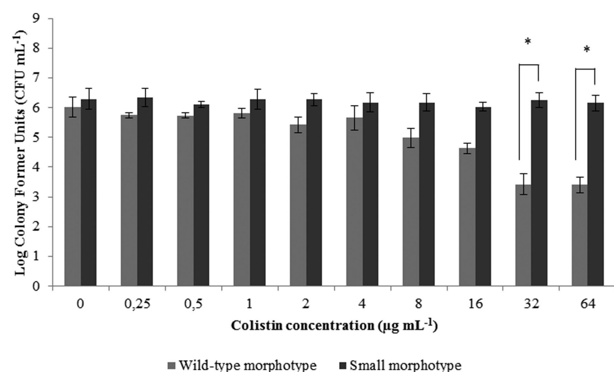

Figure 4. Number of cultivable cells derived from biofilms formed by *K. pneumoniae* wild-type morphotype and by SM developed in TSB supplemented with increasing concentrations of colistin; mean (±SD), $n = 6$. *indicates significant differences ($P < 0{,}05$) between means.

modification of the outer membrane porines (Kim *et al.* 2014) and presence of efflux pumps (Pamp *et al.* 2008). In *K. pneumoniae*, alterations in the mgrB gene like insertions, point and also large deletions leading to its inactivation were already identified as a common mechanism of acquired resistance. The mgrB is responsible for encoding a negative feedback for the phoP/phoQ system, and consequently, its inactivation frequently leads to an upregulation of this system. As an upregulation of the phoQ/phoP leads to an overexpression of the Pmr LPS modification system, the LPS target changes and the affinity to colistin binding decreases (Cannatelli *et al.* 2014; Poirel *et al.* 2014). Notwithstanding, a recent study, addressing the resistance mechanisms to colistin in *K. pneumoniae*, also identified an insertion in mgrB, however, pointing out that this insertion is capable to induce a capsule modification that confers resistance to the bacteria (Formosa *et al.* 2015). The authors showed that, whereas the susceptible strain had the capsule removed by the presence of colistin, the resistant strain presented a multilayered capsule that colistin was unable to remove. In effect, it is obvious that for an encapsulated bacterium colistin has to first overlap the capsule and only then can reach LPS. Thus, it can be concluded that the first colistin mechanism of action is focused in the penetration of capsule and only afterwards it may bind to LPS. Further, studies about the role of the capsule in *K. pneumoniae* tolerance to colistin are already being conducted by our group. The response to antibiotherapy is usually very different between planktonic bacteria and biofilms (Aslam 2008; Ramage *et al.* 2009), and it has already been demonstrated that *K. pneumoniae* is more resistant to various classes of antimicrobials in its biofilm form (Černohorská and Votava 2004; Naparstek *et al.* 2014). Several mechanisms contribute to seriously increase resistance in biofilms, i.e. the transference of large number of genes that encoded for resistance or the extremely difficult diffusion of antibiotics throughout the matrix (Borriello *et al.* 2004; Francolini and Donelli 2010).

However, studies regarding *K. pneumoniae* biofilms already demonstrated that the increase in resistance is not correlated with the amount of biofilm formed, indicating that slow diffusion of antibiotics into the biofilm is not the key factor or at least a major cause (Naparstek *et al.* 2014). Probably, the mechanisms of resistance are in the bacteria itself and not in its arrangement. However, the identity and origin of these mechanisms remain unknown. According to Pamp and colleagues (2008), activity of colistin in *P. aeruginosa* biofilms is linked to a spatial organisation within biofilms where metabolic active cells develop tolerance while the cells with low metabolic activity are killed by colistin. Despite the fact that this is a tolerance and not a resistance mechanism and also another species, this study points that activity of colistin is related to heterogeneity within biofilms which is in accordance to our results. As already stated, bacteria living in biofilms usually exhibit different phenotypes, which is a result of the intense phenotypic switching characteristic of biofilm formation. According to Sousa, Machado and Pereira (2011), it is possible to detect phenotypic switching by observing colony morphology and also to establish a relationship between colony morphotypes and antimicrobial resistance. This behaviour is in accordance with this study, where colonies with SM were found to be strictly linked to a resistant behaviour. Of particular interest are the small colony variants (SCV), a form of slow-growing bacteria that present small colony morphology and different biochemical properties. SCV are closely linked to

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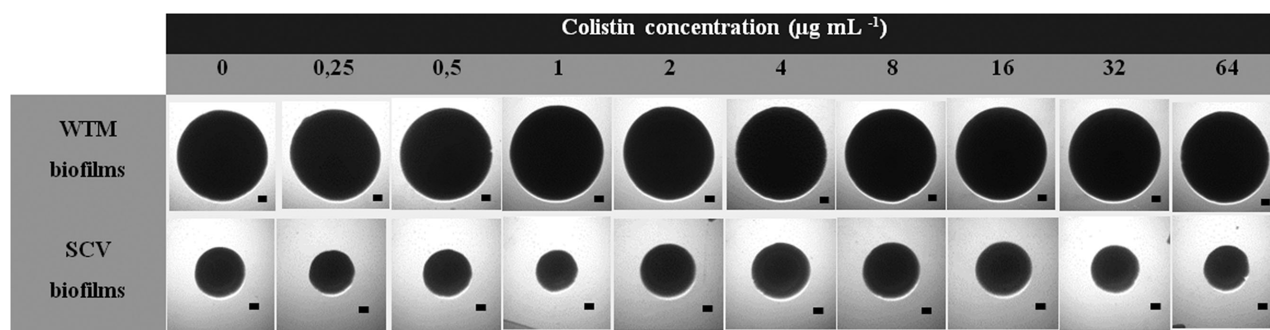

Figure 5. Colony morphology observed for biofilms of *K. pneumoniae* wild-type and SM formed in TSB supplemented with increasing colistin concentrations. No differences were observed between independent assays. Each black bar represents 1 mm.

Wild-type Morphotype	Small Colony Variants
Diameter: 9,8 ± 1 mm Texture: mucoid Margin: entire Elevation: flat Colour: white **MIC: 2 μg mL $^{-1}$** **Origin: planktonic and biofilm**	**Diameter: 4,8 ± 1 mm** Texture: mucoid Margin: entire Elevation: flat Colour: white **MIC: 64 μg mL $^{-1}$** **Origin: biofilm**

Figure 6. Characterization of wild-type morphotype versus SCV. Each black bar represents 1 mm.

biofilm formation and responsible for persistent infections in various species (Häussler *et al.* 2003; Proctor *et al.* 2006). To the extent of our knowledge, this is the first report describing slow-growing variants in *K. pneumoniae* and characterizing its morphology. For this reason and as its resistance was stable, the classification of these colonies as SCV of *K. pneumoniae* (Fig. 6) is here proposed.

It should be highlighted that this is the first evidence of a relation between these variants and a heteroresistance phenomenon. Heteroresistance can be considered a very heterogeneous behaviour, so it is pivotal trying to understand what lies behind this heterogeneity. Many authors have tried to identify mechanisms involved in heteroresistance, mostly looking for an explanation in genes codifying for resistance or structural abnormalities (Markova *et al.* 2008; Pournaras *et al.* 2010; Lee *et al.* 2011; Campanile *et al.* 2012; Deresinski 2013; Engel *et al.* 2014). A recent study already revealed that mutations in PhoP are responsible for modulating *K. pneumoniae* response to colistin however only to explain how a resistant strain can present some susceptible sub-populations (Jayol *et al.* 2015). The mechanism underlying the triggering of resistant sub-populations from an apparently susceptible strain is still a mystery and represents an actual clinical concern. Nowadays, research is becoming mainly focused on a genetic basis, where every phenomena must be attributed or related to a gene or to a mutation and less importance is given to the factors that can trigger certain processes or activate those genes. The fact is that heteroresistance appears through a single clone, which alone might indicate that all sub-populations are genetically identical, thus try to find explanation in DNA mutations might be a dead end. On the other hand, it is well known that heterogeneity is a major characteristic of biofilms (Costerton 1999). As mentioned before, these are communities of microorganisms that usually present an altered gene transcription and are frequently found in different phenotypes (Costerton 1999; Donlan and Costerton 2002). From this point of view, biofilms constitute excellent niches for the emergence of heterogeneous variants such as resistant populations and SCV. To the extent of our knowledge, this is the first report showing that biofilm formation can trigger the appearance of heteroresistance from an apparently susceptible strain. These findings suggest that heteroresistance might be a phenomenon of opportunity and not a definition, which represents a clinical concern since its prediction might not always be possible. Understand the surrounding conditions that trigger this phenomenon is crucial to advance to a correct evaluation and antibiotherapy.

This study demonstrated the existence of a sub-population resistant to colistin within a *K. pneumoniae* strain that seemed to be exclusively associated with biofilms. It is confirmed that SCV represent the sub-population responsible for heteroresistance to colistin. These findings suggest that heteroresistance may be a phenomenon closely related to biofilm formation not only in *K. pneumoniae* but at global level and that surrounding conditions like a biofilm arrangement might play a fundamental role in the onset of such behaviour. It should be highlighted the importance of screening for heteroresistance in clinical practice and to further analyse the putative biofilm-forming ability before proceed to any antibiotherapy. Nevertheless, more studies are needed and our group is already studying the triggering of heteroresistance in biofilms of other *K. pneumoniae* strains and different bacterial species to better understand the extent of this phenomenon.

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Conflict of interest. None declared.

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