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Highlights

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***In vitro* activity of colistin against biofilm by *Pseudomonas aeruginosa* is significantly improved under "cystic fibrosis–like" physicochemical conditions**

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- Colistin was tested against planktonic and biofilm CF *P. aeruginosa* cells.
- "CF-like" (anaerobiosis, pH 6.5) and "CLSI-suggested" conditions were considered.
- MIC, MBC, and MBEC values were comparatively assessed against 12 CF strains.
- The antibacterial and anti-biofilm activity was improved under "CF-like" condition.
- It is desirable to rethink the protocols used for antibiotic susceptibility testing.

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In vitro activity of colistin against biofilm by *Pseudomonas aeruginosa* is significantly improved under "cystic fibrosis–like" physicochemical conditions

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ABSTRACT

The impact of physicochemical conditions observed in cystic fibrosis (CF) lung on colistin activity against both planktonic and biofilm *P. aeruginosa* cells was evaluated. MIC, minimum bactericidal concentration (MBC), and minimum biofilm eradication concentration (MBEC) values were assessed against 12 CF strains both under "CF-like" (anaerobiosis, pH 6.4) and "standard" (aerobiosis, pH 7.4) conditions. The activity of colistin was significantly higher under "CF-like" conditions compared to "standard" ones, both against planktonic (MIC_{90}: 1 and 4 μg/mL, respectively) and biofilm ($MBEC_{90}$: 512 and 1.024 μg/mL, respectively) cells, as confirmed by scanning electron microscopy. Improved activity was not related to biofilm matrix amount. It may be necessary to adequately "rethink" the protocols used for *in vitro* assessment of colistin activity, by considering physicochemical and microbiological features in the CF lung at the site of infection. This could provide a more favorable therapeutic index, rationale for administration of lower doses, probably resulting in reduced toxicity and emergence of resistant clones.

1. Introduction

Pulmonary chronic infection is the main determinant of morbidity and mortality in patients with cystic fibrosis (CF) (Lechtzin et al., 2006; O'Sullivan and Freedman, 2009). *Pseudomonas aeruginosa* is considered to be the major pathogen infecting more than 70% of CF patients, although culture-based and molecular approaches have demonstrated the polymicrobial etiology of CF lung infections (Mahenthiralingam, 2014; Sibley et al., 2011; Zhao et al., 2012).

Antibiotic therapy – aimed at eradicating *P. aeruginosa* during early lung infection or at reducing the frequency of exacerbations during chronic infection – has played a major role in increasing median survival of CF patients during the last decades (Gaspar et al., 2013).

Regular inhalation antibiotic therapy represents an important strategy for the management of chronic pulmonary infections in CF, since it was proven successful for the maintenance of lung health during chronic infections by reducing the risk of pulmonary exacerbations and rates of pulmonary function decline (Konstan et al., 2011; McCoy et al., 2008). In fact, antibiotic administration by aerosol produces higher concentrations of the drug at the site of infection, compared to those obtained following parenteral or oral treatments, thus increasing the pharmacokinetic and pharmacodynamic indices relative to MIC (Geller et al., 2002; Gibson et al., 2006). Furthermore, aerosol administration yields low serum antibiotic concentrations, thus reducing the potential for systemic toxicity (Geller et al., 2002). Tobramycin, aztreonam lysine, and colistin are the currently approved antibiotics for inhalation treatment of *P. aeruginosa* infections in CF patients (Máiz et al., 2013).

Colistin, also known as polymyxin E, is a polypeptide antibiotic belonging to polymyxin group. It is mainly active against Gram-negative organisms where binds to the membrane causing cell death (Chambers, 2006; Storm et al., 1977). Marketed in the 1950s, it was neglected up until very recently because of its nephrotoxicity and neurotoxicity. However, the emergence of resistance to other antibiotics led to a re-evaluation of its pharmacokinetic characteristics becoming the first drug to be commonly used by inhalation for the management of chronic *P. aeruginosa* lung infection in CF patients (Jensen et al., 1987).

Conventionally, antibiotic selection is directed by the results of antibiotic susceptibility testing. However, several studies recently questioned the utility of routine antibiotic susceptibility testing in influencing the clinical outcome of CF patients, especially in the case of *P. aeruginosa* infection (Hurley et al., 2012; Smith et al., 2003). The poor clinical predictive value of antibiotic susceptibility tests might be mainly due to the inadequacy of the currently performed *in vitro* susceptibility tests in closely reflecting the anticipated mode of *P. aeruginosa* biofilm growth and the physicochemical conditions (anaerobiosis and acidic pH) observed in CF lung that could significantly affect the antimicrobial activity of antibiotics at the site of infection (Barcia-

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Macay et al., 2006; Debets-Ossenkopp et al., 2010; Falagas et al., 1997; Garrison et al., 1997; Hassett et al., 2002; Hill et al., 2005; Hunter and Beveridge, 2005; King et al., 2010). In addition, the need for new effective scores for assessing clinical outcome in CF patients makes even more questionable the predictivity of susceptibility results (Quittner et al., 2009).

Therefore, the aim of the present work was to evaluate, for the first time, the effect of "CF-like" conditions – namely anaerobic atmosphere and acidic pH – on the activity of colistin against both planktonic and biofilm *P. aeruginosa* cells. To this, the antibacterial activity of colistin – as assessed by MIC, minimum bactericidal concentration (MBC), time-killing, and minimum biofilm eradication concentration (MBEC) measurement – was comparatively evaluated both under "CF-like" conditions (anaerobiosis, pH 6.4) and CLSI-suggested ("standard": aerobiosis, pH 7.4) ones against 12 *P. aeruginosa* strains isolated from chronically infected CF patients. The role of the amount of extracellular polymeric substance (EPS) in biofilm susceptibility to colistin and the effect of colistin on biofilm ultrastructure were also assessed under both conditions considered.

2. Materials and methods

2.1. Bacterial strains and growth conditions

In vitro activity of colistin was evaluated against 12 *P. aeruginosa* strains isolated from sputum collected in CF patients admitted to the "Bambino Gesù" Children's Hospital of Rome. Strains were identified by API 20 NE (bioMérieux, Marcy-L'Etoile, France) and then selected for multidrug resistant phenotype – that is resistance to at least 2 of the following groups of antibiotics: β-lactams, aminoglycosides, and fluoroquinolones (Saiman et al., 1996) – and for the ability to form biofilm on polystyrene. Antibiotic susceptibility patterns of strains tested are summarized in Table 1.

Strains were stored at −80 °C until use, when they were plated twice onto Trypticase soy agar (Oxoid SpA, Garbagnate M.se, Milan, Italy) in order to regain the original phenotype.

All assays were carried out by using a standardized bacterial inoculum. Briefly, *P. aeruginosa* colonies grown overnight on Mueller–Hinton agar (MHA; Oxoid S.p.A., Milan, Italy) were resuspended in cation-adjusted Mueller–Hinton broth (CAMHB; Beckton & Dickinson, BD, Le Point de Claix, France) and incubated overnight at 37 °C under agitation (130 rpm). Suspension was then adjusted to an OD_{550} of 1.0 (corresponding to 4–9 × 10^8 CFU/mL) with sterile CAMHB.

Table 1
Antibiotic susceptibility profiles of *P. aeruginosa* strains tested in this study.

Strain	MIC (μg/mL)										
	AK	GM	TO	CI	LE	TZ	PM	AT	P/T	MP	IP
Pa1	6	**8**	4	0.38	**≥32**	8	8	4	6	1	3
Pa2	**≥256**	**48**	**16**	0.25	0.75	**≥256**	**≥256**	**16**	**≥256**	**≥32**	**≥32**
Pa3	**≥256**	**24**	**16**	**3**	**≥32**	8	**≥256**	0.75	4	**≥32**	**≥32**
Pa4	8	**48**	**≥32**	**≥32**	**≥32**	**≥32**	**≥256**	**≥256**	**≥256**	**≥32**	**≥32**
Pa5	**32**	**12**	4	0.5	1.5	**≥256**	**≥256**	**≥256**	**≥256**	**≥32**	**≥32**
Pa6	16	**6**	1.5	0.38	1	**≥256**	**≥256**	**≥256**	**≥256**	**≥32**	**≥32**
Pa7	**32**	**8**	4	**1.5**	1	**≥256**	**≥256**	**≥256**	**≥256**	**≥32**	**≥32**
Pa9	**≥256**	**48**	**16**	**12**	**≥32**	6	**≥256**	3	8	**≥32**	**≥32**
Pa10	**≥256**	**48**	**16**	**4**	**≥32**	**≥256**	**≥256**	**≥256**	**≥256**	**≥32**	**≥32**
Pa16	8	4	1	**4**	**≥32**	**≥256**	**≥256**	0.5	2	0.19	0.75
Pa18	**≥256**	**32**	**16**	**8**	**12**	8	8	4	8	**≥32**	**≥32**
Pa21	16	**6**	2	0.28	1.5	**≥256**	**≥256**	**≥256**	**≥256**	**≥32**	**≥32**

Bold are MIC values indicative for resistance or intermediate susceptibility, according to CLSI guidelines (CLSI, 2010). AK = amikacin; GM = gentamicin; TO = tobramycin; CI = ciprofloxacin; LE = levofloxacin; TZ = ceftazidime; PM = cefepime; AT = aztreonam; P/T = piperacillin/tazobactam; MP = meropenem; IP = imipenem.

2.2. Colistin

Starting from a powder of known potency (Sigma-Aldrich S.p.A., Milan, Italy), a colistin sulphomethate stock solution was prepared in milliQ-grade reagent water (Millipore S.p.A., Milan, Italy). The stock was 0.22-μm, filtered, and aliquoted at −80 °C until use.

2.3. Kinetic of biofilm formation

The kinetic of biofilm formation by each of 12 *P. aeruginosa* strains was spectrophotometrically monitored during a 7-day period. Briefly, 100 μL of the standardized inoculum were aliquoted in each well of a 96-well polystyrene, flat bottom, tissue culture-treated microtiter (Iwaki, Bibby srl; Milan, Italy) and statically incubated at 37 °C up to 7 days, replacing CAMHB daily. Every 24 h, biofilm samples were washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich S.p.A.; Milan, Italy), then biofilm biomass was quantified by spectrophotometric assay following staining with Hucker-modified crystal violet, and results expressed as OD_{492} (Hucker, 1921).

2.4. Evaluation of colistin activity against planktonic and biofilm cells

The *in vitro* activity of colistin against planktonic and sessile *P. aeruginosa* cells was evaluated under both "standard" (aerobic atmosphere, pH 7.4) and "CF-like" experimental conditions, which are those simulating the physical-chemical properties observed in CF lung environment (anaerobic atmosphere, pH 6.4) (Hassett et al., 2002; Worlitzsch et al., 2002). The pH value was adjusted to the desired value by adding 1 M HCl or NaOH, while anaerobic atmosphere was obtained by using AnaeroGen kit (Oxoid S.p.A.).

2.4.1. Activity against planktonic cells: MIC and MBC

Standardized inoculum was prepared in sterile 0.9% saline to reach an OD_{550} value of 0.350 (corresponding to about 1–5 × 10^8 CFU/mL) and diluted 1:10 in CAMHB at adjusted for desired pH value. MIC of colistin, tested at concentrations ranging from 0.12 to 64 μg/mL, was measured by broth microdilution technique, according to CLSI guidelines (CLSI, 2010). In order to evaluate the impact of "CF-like" condition on growth rate, viable count was carried out for each positive control (not exposed to colistin) used for MIC reading.

MBC was evaluated by plating onto MHA 10-μL aliquot from wells showing no visible growth at MIC reading. Following incubation at 37 °C for 24 h, MBC value was defined as minimum colistin concentration able to eradicate 99.9% of the starting inoculum.

2.4.2. Activity against mature biofilms: MBEC

Five-day-old biofilms, formed as previously stated, were washed twice with PBS and then exposed to colistin concentrations ranging from 2 to 1.024 μg/mL. Following 24-h exposure at 37 °C, biofilm samples were collected by scraping and then underwent 10-fold dilutions for colony count onto MHA. MBEC was measured as the minimum concentration of colistin required to completely eradicate biofilm viability.

2.5. Evaluation of glucose amount in biofilm samples

The amount of EPS produced in mature 5-day-old biofilms, formed under "standard" conditions as stated in paragraph 2.4, by each of 12 *P. aeruginosa* strains was evaluated by measuring EPS glucose content. Biofilm samples were washed twice in PBS, then collected by scraping, and finally resuspended in sterile saline. The glucose amount was then measured by colorimetric assay, according to Dubois et al. (1956).

2.6. Microscopic analysis

The effects of colistin exposure on biofilm's ultrastructure and architecture were analyzed by scanning electron microscopy (SEM). Five-day-old

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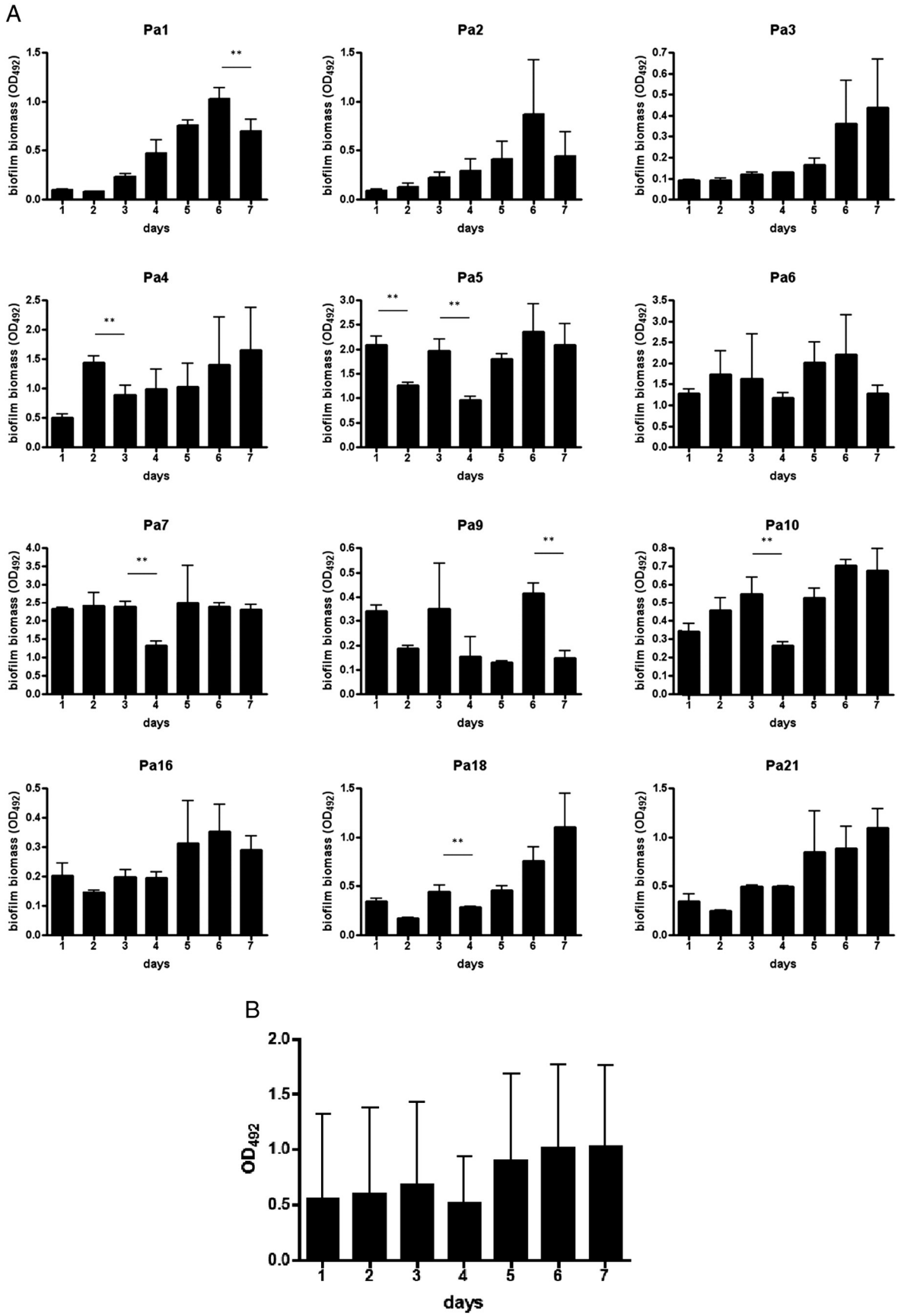

Fig. 1. Kinetic of biofilm formation by the 12 *P. aeruginosa* strains isolated from CF patients. Kinetic of biofilm biomass formation was assessed considering each of 12 strains individually (A) or as a whole (B). The amount of biofilm biomass, formed onto polystyrene during 7-day incubation under "standard" CLSI-recommended experimental conditions (aerobic atmosphere, pH 7.4), was spectrophotometrically measured (OD_{492}) following crystal violet staining. Results are means + SDs. $^{**}P < 0.01$, unpaired *t*-test.

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biofilm formed by *P. aeruginosa* Pa6 strain was allowed to form in polystyrene tissue culture 75 cm^2 flasks (Iwaki) and then exposed to colistin 64 μg/mL for 24 h under both "standard" and "CF-like" conditions.

To better visualize EPS in biofilm samples, we used cationic dye Alcian blue, according to Erlandsen et al. (2004). Briefly, biofilm samples were fixed with a solution of 2% paraformaldehyde, 2% glutaraldehyde, and 0.15% Alcian blue 8GX in 0.1 mol/L sodium cacodylate buffer (pH 7.4). Samples were post-fixed in osmium tetroxide and then dehydrated in a series of aqueous ethanol solutions (50%, 70%, 80%, 95%, and 100%). Specimens were critical point dried, mounted on aluminum stubs with conductive carbon cement, allowed to dry for 3 h, and coated with 15-nm Au film with an agar automatic sputter coater. After processing, samples were observed with a Philips XL30CP scanning electron microscope in the high-vacuum mode at 15 kV.

2.7. Statistical analysis

All experiments were performed in triplicate and repeated on 2 different occasions. Results were expressed as means ± SDs. Differences in mean amount of formed biofilm were measured by Student's t-test. Interdependency between EPS amount and MBEC values was evaluated by calculating Spearman's linear correlation coefficient. Differences in MIC, MBC, MBEC, and relative ratio values were considered as statistically different if equal at least to 2-log_2 dilution. A P value less than 0.05 was considered as statistically significant.

3. Results

3.1. Kinetic of P. aeruginosa biofilm formation on polystyrene

To define the optimal experimental conditions for testing *in vitro* colistin activity against preformed *P. aeruginosa* biofilms, we analyzed the kinetics of biofilm formed by 12 *P. aeruginosa* strains, under "standard" conditions, during a 7-day incubation. Biofilm biomass formed on polystyrene was assessed every 24 h by a colorimetric method, and results are shown in Fig. 1.

All strains were able to form biofilm on polystyrene, and most of them (Pa1, Pa2, Pa3, Pa4, Pa10, Pa16, Pa18, and Pa21) showed a time-dependent biofilm formation, with increasing biofilm levels from day 1 to day 7. However, significant variations in mean biofilm biomass levels were observed among strains throughout the study period considered (Fig. 1A). Particularly, a significant decrease in biofilm biomass formed was observed during transition from day 1 to day 2 (Pa5), day 2 to day 3 (Pa4), day 3 to day 4 (Pa5, Pa7, and Pa10), and day 6 to day 7 (Pa1 and Pa9). No significant reduction was found from day 4 to day 6 of incubation.

Considering all the strains as a whole, mean biofilm biomass levels did not significantly vary during the study period considered (Fig. 1B). However, higher absolute biofilm biomass levels were observed from day 5 to day 7 of incubation. As suggested by high SD values, biofilm biomass amount formed varied greatly among the strains tested, regardless of time point considered.

Taken together, our results suggest that 24-h exposure to colistin of 5-day-old biofilm is the optimal experimental layout to avoid underestimation or overestimation of colistin activity against preformed *P. aeruginosa* biofilms.

3.2. In vitro activity of colistin against planktonic P. aeruginosa cells

The antibacterial activity of colistin against 12 *P. aeruginosa* strains, comparatively assayed under both "CLSI-recommended" and "CF-like" conditions, is summarized in Table 2.

Overall, under "CF-like" conditions, colistin showed MIC_{50} and MIC_{90} values significantly lower than those observed under "standard" ones (MIC_{50}: 0.5 and 4 μg/mL, respectively; MIC_{90}: 1 and 4 μg/mL, respectively). With the exception for Pa1, all of strains tested resulted to be significantly more susceptible to colistin under "CF-like" conditions.

Considering the strains as a whole, experimental conditions considered did not differ for MBC_{50} and MBC_{90} values (MBC_{50}: 2 and 4 μg/mL; MBC_{90}: 8 and 4 μg/mL, for "CF-like" and "standard" conditions, respectively). Interestingly, mean killing quotient (MBC/MIC ratio) values clearly showed that the mechanism of action of colistin is dependent on experimental conditions considered, which resulted bactericidal under "standard" condition (mean MBC/MIC: 1.16), while bacteriostatic under "CF-like" condition (mean MBC/MIC: 4.8).

3.3. In vitro anti-biofilm activity of colistin against mature biofilms formed by P. aeruginosa

The activity of 24-h exposure of different colistin bactericidal concentrations against 5-day-old *P. aeruginosa* biofilms was comparatively assayed under both "standard" and "CF-like" conditions, and results are summarized in Table 3.

Considering the strains as a whole, for most of the biofilms (8 out of 12, 66%), MBEC values measured under "CF-like" conditions were lower than those obtained under "standard" ones, although the difference (1-log_2) was not statistically significant ($MBEC_{50}$: 128 versus 256 μg/mL; $MBEC_{90}$: 512 versus 1024 μg/mL, respectively). However, looking at the values obtained for each strain, colistin resulted to be significantly more active under "CF-like" conditions in 4 out of 12 (33.3%) strains tested, as shown by MBEC ratio values ($MBEC_{standard}/MBEC_{CF\text{-}like}$: 4, ≥8, 8, and 4 for Pa1, Pa6, Pa7, and Pa10, respectively). Only in the case of Pa3 strain, MBEC observed under "CF-like" conditions was higher, although not significantly, than the "standard" ones ($MBEC_{standard}/MBEC_{CF\text{-}like}$: 0.5).

MBEC/MBC values showed that transition from planktonic toward sessile phenotype caused a significant reduction of colistin activity for all strains tested, with comparable

Table 2
Antibacterial activity of colistin against planktonic cells of 12 *P. aeruginosa* strains isolated from CF patients.

Strain	MIC (μg/mL)		MBC (μg/mL)	
	Under the following conditions:		Under the following conditions:	
	"Standard"	"CF-like"	"Standard"	"CF-like"
Pa1	2	1	2	2
Pa2	4	**1**	4	4
Pa3	4	**0.5**	4	8
Pa4	4	**0.25**	4	1
Pa5	4	**0.5**	4	**0.5**
Pa6	4	**0.5**	4	1
Pa7	2	**0.5**	2	4
Pa9	4	**0.5**	4	8
Pa10	2	**0.25**	4	2
Pa16	2	**0.125**	4	**0.25**
Pa18	4	**0.5**	4	**1**
Pa21	4	**0.25**	4	2
MIC_{50}[a]	4	**0.5**		
MIC_{90}[b]	4	**1**		
MIC_{range}	2-4	0.125–1		
MBC_{50}[c]			4	2
MBC_{90}[d]			4	8
MBC_{range}			2–4	0.25–8
Mean MBC/MIC[e]	1.16	4.80		

Susceptibility tests were performed under "standard" CLSI-recommended (aerobiosis, pH 7.4) and "CF-like" (anaerobiosis, pH 6.4) conditions. Bold are MIC or MBC values differing at least 2-log_2 from those observed under "standard" conditions.

[a] MIC_{50} represents the MIC value at which ≥50% of the strains are inhibited.
[b] MIC_{90} represents the MIC value at which ≥90% of the strains are inhibited.
[c] MBC_{50} represents the MBC value at which ≥50% of the strains are killed.
[d] MBC_{90} represents the MBC value at which ≥90% of the strains are killed.
[e] MBC/MIC, killing quotient.

Table 3
In vitro activity of colistin against 5-day-old biofilms formed by 12 *P. aeruginosa* strains isolated from CF patients.

Strain	MBEC (μg/mL)		MBEC ratio ("standard"/"CF-like")
	Under the following conditions:		
	"Standard"	"CF-like"	
Pa1	256	64	**4**
Pa2	128	128	1
Pa3	64	128	0.5
Pa4	512	512	1
Pa5	256	128	**2**
Pa6	>1024	128	**≥8**
Pa7	1024	128	**8**
Pa9	64	32	**2**
Pa10	1024	256	**4**
Pa16	32	32	1
Pa18	256	128	**2**
Pa21	1024	512	**2**
$MBEC_{50}$[a]	256	128	
$MBEC_{90}$[b]	1024	512	
$MBEC_{range}$	32 to >1024	32–512	

MBEC was measured both under "standard" CLSI-recommended (aerobiosis, pH 7.4) and "CF-like" (anaerobiosis, pH 6.4) conditions. In bold are the MBEC ratio values showing a significant difference (≥2-log_2) between MBEC values obtained under 2 experimental conditions considered.

[a] $MBEC_{50}$ represents the MBEC value at which ≥50% of the strains are inhibited.
[b] $MBEC_{90}$ represents the MBEC value at which ≥90% of the strains are inhibited.

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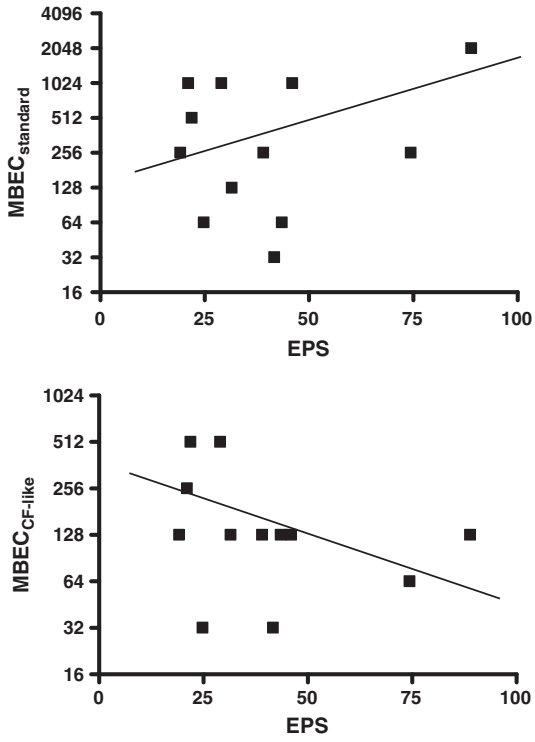

Fig. 2. Correlation between EPS amount in *P. aeruginosa* biofilms and susceptibility to colistin. EPS amount of 5-day-old biofilm samples was measured by a colorimetric assay, according to Dubois et al. (1956), and then correlated with biofilm susceptibility to colistin, as assessed by MBEC values measured both under "standard" CLSI-recommended ($MBEC_{standard}$) and "CF-like" ($MBEC_{CF\text{-}like}$) experimental conditions. Spearman's rank correlation coefficient showed no statistically significant relationship between EPS amount and MBEC values, both expressed as μg/mL.

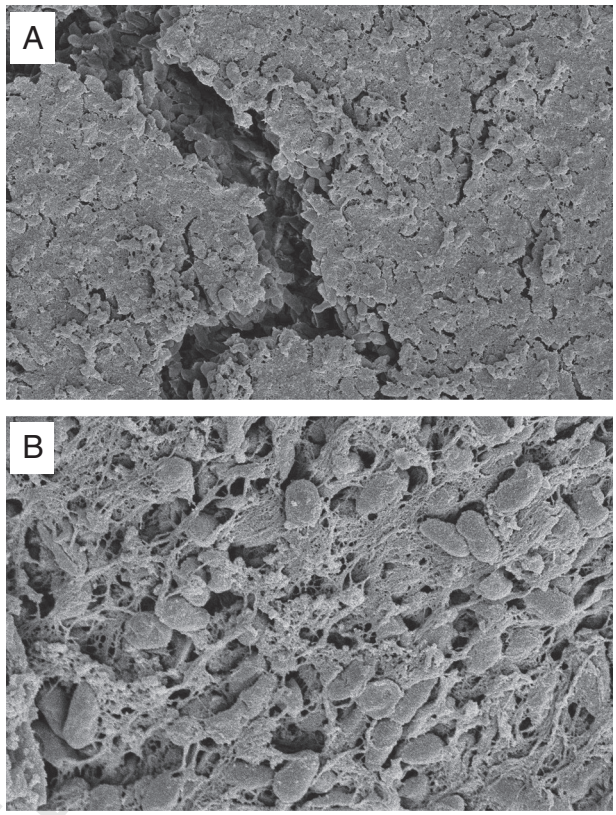

Fig. 3. SEM of 5-day-old biofilm formed by *P. aeruginosa* Pa6 under "standard" CLSI-recommended conditions. Biofilm was allowed to form onto polystyrene for 5 days at 37 °C under aerobiosis and at pH 7.4. (A) Biofilm showed a complex architecture consisting of multilayered cell structure embedded in an abundant EPS evenly distributed through the thickness of biofilm. (B) EPS showed a fibrillar organization forming a network-like structure among cells. Magnification: 10 K (A), 30 K (B).

extent for both conditions tested (MBEC/MBC: 166 ± 182.7 versus 137.7 ± 146.1, for "standard" and "CF-like" settings, respectively; $P > 0.05$, paired *t*-test) (Tables 2 and 3).

3.4. EPS amount in mature P. aeruginosa biofilm and susceptibility to colistin

No statistically significant relationship was found between the biofilm EPS amount, measured by a colorimetric method, and the biofilm resistance to colistin, as assessed by MBEC calculation, regardless of experimental conditions considered (Spearman's rank correlation coefficient: 0.067 and −0.386, for "standard" and "CF-like" experimental conditions, respectively; $P > 0.05$) (Fig. 2).

3.5. Effect of colistin on P. aeruginosa biofilm ultrastructure

The effect of colistin, under different experimental conditions used, on the ultrastructure of mature *P. aeruginosa* biofilm was microscopically assessed by SEM analysis (Figs. 3 and 4). *P. aeruginosa* Pa6 strain was selected because of the highest $MBEC_{standard}/MBEC_{CF\text{-}like}$ ratio value among the strains tested.

SEM observations of 5-day-old biofilm revealed a complex organization consisting of a multilayered cell structure embedded in an abundant EPS evenly distributed through the thickness of biofilm (Fig. 3A). At higher magnification, EPS revealed a fibrillar appearance and network organization (Fig. 3B).

Biofilm exposure to colistin 64 μg/mL for 24 h under "CF-like" conditions showed a significant reduction of both cellularity and structural complexity, compared to unexposed control (Fig. 4). The evidence for cells showing a "deflated balls-like" morphology suggested that cell lysis occurred following exposure to colistin (Fig. 4).

4. Discussion

Colistin is a polymyxin showing relevant antimicrobial activity against Gram-negative microorganisms, particularly against multidrug-resistant *P. aeruginosa* strains (Catchpole et al., 1997; Jensen et al., 1987). Although its systemic use has been suspended because of nephrotoxicity and neurotoxicity (Catchpole et al., 1997), the emergence of multidrug-resistant strains has, therefore, renewed interest in the use of colistin for the treatment of *P. aeruginosa* infections in CF patients because of the lower toxicity associated to aerosol administration and poor ability in selecting resistance (Beringer, 2001). Nebulized colistin is, in fact, currently used in many European CF centers, supported by positive results arising from some clinical trials (Frederiksen et al., 1999; Littlewood et al., 1985; Sabuda et al., 2008).

In accordance with the guidelines provided by CLSI as well as by EUCAST, susceptibility of *P. aeruginosa* to antibiotics is currently being tested *in vitro* against planktonic forms, under aerobic atmosphere, and at pH levels comparable to those measured in human serum, which is commonly close to neutral. These conditions are diametrically opposed to those which microorganisms face at the site of infection.

First, it is well known that *P. aeruginosa* grows as biofilm in CF lung and that antibiotic susceptibility of sessile cells is significantly higher – up to 1.000-fold – compared to planktonic counterpart (Moskowitz et al., 2004; Singh et al., 2000; Worlitzsch et al., 2002). Second, *P. aeruginosa* is present in the airway lumen adhered to plaques/plugs of respiratory mucus rather than on airway epithelial cells. Bacteria can penetrate in the thickened mucus and grow – in the presence of an electron acceptor, such as nitrate or arginine, for fermentation – in hypoxic/anaerobic zones generated by the raised O_2 consumption by CF epithelia (Hassett et al., 2002; Worlitzsch et al., 2002; Yoon et al., 2002). In this environment, antibiotics could be significantly affected in their activity. With this in mind, King et al. (2010) found that the *in vitro* activity of tobramycin, amikacin, and aztreonam against *P. aeruginosa* CF strains was significantly reduced under anaerobic atmosphere.

Third, pH is one of the most important factors known to affect the activity of antibiotics (Barcia-Macay et al., 2006; Debets-Ossenkopp et al., 1995; Falagas et al., 1997). It has been observed that the epithelial lining fluid of patients with CF is acidified, in part due to inflammation as the pH of the exhaled breath condensate increases significantly with

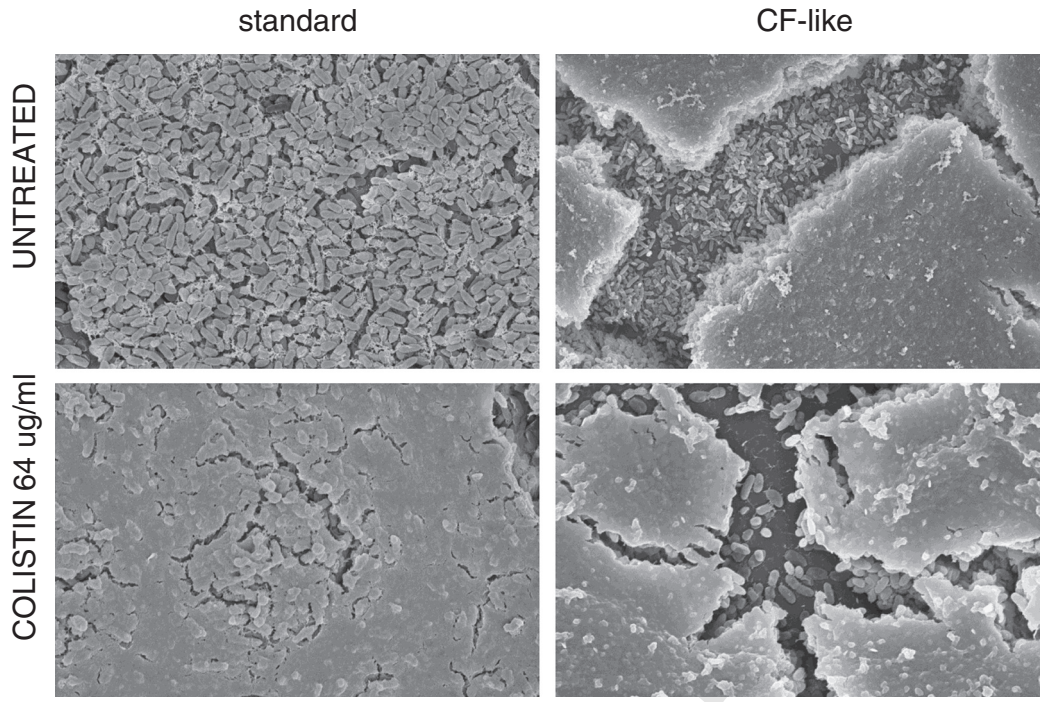

Fig. 4. SEM analysis of mature *P. aeruginosa* biofilms following exposure to colistin under "standard" CLSI-recommended and "CF-like" experimental conditions. Five-day-old biofilms formed by *P. aeruginosa* Pa6 strain were exposed to colistin 64 μg/mL for 24 h both under "standard" (aerobiosis, pH 7.4) and "CF-like" (anaerobiosis, pH 6.4) experimental conditions. Control (untreated) biofilms were not exposed to colistin. Exposure to colistin caused a reduction of cellularity and EPS disruption under "CF-like" conditions only. Magnification: 10 K.

Q4 treatment of an exacerbation (Smith and Welsh, 1992; Tate et al., 2002). A pH value equal to 6.4, significantly different from that used for antibiotic susceptibility tests, was measured in the lung tissue of patients with pneumonia (Worlitzsch et al., 2002). A scenario in which in the case of *P. aeruginosa* biofilm produces an acidic microenvironment that, although it cannot be regarded as a natural defense mechanism, is able to provide additional protection to biofilm against aminoglycosides (Hunter and Beveridge, 2005) was also considered.

In the absence of an adequate system for evaluating the *in vitro* susceptibility to antibiotics that is that simulating as much as possible physical and chemical conditions observed *in vivo* at the site of infection, the selection of antibiotics to be used in therapy is mainly empiric. This approach can result in reduced effectiveness of the antibiotic, side effects, and the emergence of resistant clones.

The present work was, therefore, aimed at evaluating the impact of "CF-like" physicochemical conditions on colistin activity against both planktonic and biofilm *P. aeruginosa* cells. Susceptibility to colistin of 12 *P. aeruginosa* strains collected from chronically infected CF patients was evaluated, both by cell viable count and microscopic assays, under "CF-like" (anaerobiosis, pH 6.4) experimental conditions, compared to "standard" ones (aerobiosis, pH 7.4).

Since colistin is used in CF patients for the treatment of chronic infection by *P. aeruginosa*, we firstly standardized the experimental model allowing us to obtain a mature and stable biofilm, which was needed for studying the activity of colistin without confounding factors (e.g., spontaneous biofilm detachment from the plastic support). Our results indicate the period between day 5 and day 6 of incubation as the only one characterized by nonsignificant changes in terms of biofilm biomass (cellularity + EPS). SEM analysis of biofilms formed by *P. aeruginosa* Pa6 strain following incubation at 37 °C for 5 days under aerobic atmosphere revealed the presence of a mature biofilm consisting of a complex multilayered structure embedded in a relevant amount of fibrillar EPS.

Based on these results, the activity of 24-h exposure at colistin was evaluated, comparatively under both "standard" and "CF-like" conditions, against 5-day-old *P. aeruginosa* biofilms.

MBEC values showed that the anti-biofilm activity of colistin is enhanced under "CF-like" conditions, although this effect resulted to be dependent on the strain considered. Particularly, for most of the strains tested (8 out of 12, 66.6%), $MBEC_{standard}/MBEC_{CF\text{-}like}$ values suggested that biofilms were significantly more susceptible to colistin in an anaerobic and acidified environment, although to different extents ($MBEC_{standard}/MBEC_{CF\text{-}like}$ range: 2 to ≥8). Improved colistin anti-biofilm activity under "CF-like" conditions was not related to the level of biofilm antibiotic resistance since mean MBEC/MBC ratio values obtained under both experimental conditions were comparable. To the contrary, the delayed *P. aeruginosa* growth generally observed under anaerobic conditions (Hill et al., 2005; O'May et al., 2006) is likely to contribute to the increased activity of colistin in "CF-like" conditions.

SEM observation confirmed these findings, showing that exposure to colistin under "CF-like" conditions simplifies biofilm architecture by causing a significant reduction in cellularity and EPS amount, compared to exposure under "standard" setting. Furthermore, the evidence for cells with characteristic "deflated ball-like" morphology indicated, for the first time, that under "CF-like" conditions, colistin preserved bactericidal activity against *P. aeruginosa* quiescent cells, already found under the "classic" setting (Cai et al., 2009; Haagensen et al., 2007).

Our results may have a relevant clinical impact if we consider that activity of other antibiotics commonly used in the therapy of CF lung infections, such as aminoglycosides and macrolides, is not affected (Gudmundsson et al., 1991) or even reduced (Garrison et al., 1997; Hill et al., 2005), in an acidified environment. Garrison et al. (1997), using an *in vitro* pharmacodynamic model in the presence of a pH value of 6.4, similar to that measured in the lung tissue in patients with pneumonia, found that bactericidal activity exhibited by clarithromycin against *Haemophilus influenzae* was significantly reduced, compared to that observed under alkaline environment. Hill et al. (2005), evaluating the activity of several antibiotics against *P. aeruginosa*, found that activity exhibited by tobramycin, amikacin, meropenem, and cotrimoxazole under anaerobic atmosphere was significantly reduced toward both planktonic and sessile (biofilm) *P. aeruginosa* cells. To the contrary, in partial agreement with our results, colistin and

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meropenem were most active against *P. aeruginosa*, regardless of the atmosphere (aerobic or anaerobic) considered (Hill et al., 2005).

The intrinsic antibiotic resistance of biofilms is probably the result of a complex physiological process. In particular, it was observed that *P. aeruginosa* responds to the anaerobic conditions by increasing the production of alginate, thus placing a physical barrier to antibiotic diffusion (Hassett et al., 2002; Worlitzsch et al., 2002). In fact, the adsorption of positively charged antibiotics, such as aminoglycosides, to the negatively charged alginate retards the penetration of the molecule through the biofilm (Gordon et al., 1988). Since colistin has a net positive surface charge, we evaluated the possible impact of EPS on polymyxin activity against biofilms under both experimental conditions tested. It was recently observed that under limited nutrient conditions, glucose dominated in the EPS material of *P. aeruginosa* mature biofilms (Myszka and Czaczyk, 2009). Therefore, the total amount of glucose present in the biofilms formed by each of 12 *P. aeruginosa* strains tested was estimated by a colorimetric assay. Although we observed opposite trend under experimental conditions tested, no statistically significant correlation was found between the total amount of glucose present in the biofilm and its susceptibility to colistin, regardless of the condition tested. It is possible that the total amount of glucose that we measured does not reflect the actual amount of alginate present in the sample. More targeted studies will be needed to explore interaction between EPS and colistin.

5. Conclusions

Our results show that colistin activity against both planktonic and sessile *P. aeruginosa* cells appears to be significantly influenced by environmental conditions and more specifically increased under acidified and anaerobic environment, similar to those found in CF lung. It would be, therefore, desirable to adequately "rethink" the current protocols used for assessing antibiotic efficacy, by considering experimental conditions, which simulate the actual physicochemical and microbiological characteristics of the CF lung ecosystem.

In this way, the choice of antibiotic would be more rational and, accordingly, more predictive of therapeutic success. In the specific case of colistin, reviewing guidelines would provide a more favorable therapeutic index, rationale for the administration of lower doses, probably resulting in reduced toxicity and emergence of resistant clones.

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