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Human airway mucus alters susceptibility of *Pseudomonas aeruginosa* biofilms to tobramycin, but not colistin

Laura Müller[1†], Xabier Murgia[2,3†], Lorenz Siebenbürger[4], Carsten Börger[4], Konrad Schwarzkopf[5], Katherina Sewald[1], Susanne Häussler[6,7], Armin Braun[1], Claus-Michael Lehr[2,4,8], Marius Hittinger[4] and Sabine Wronski[1]*

[1]*Fraunhofer Institute for Toxicology and Experimental Medicine (Fraunhofer ITEM), Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Centre for Lung Research (DZL), Member of the REBIRTH Cluster of Excellence, Nikolai-Fuchs-Straße 1, 30625 Hannover, Germany;* [2]*Helmholtz Institute for Pharmaceutical Research (HIPS), Helmholtz Centre for Infection Research, Universitätscampus E8.1, 66123 Saarbrücken, Germany;* [3]*Korea Institute of Science and Technology, KIST Europe, Campus E7.1, 66123 Saarbrücken, Germany;* [4]*PharmBioTec GmbH, Science Park 1, 66123 Saarbrücken, Germany;* [5]*Department of Anaesthesia and Intensive Care, Klinikum Saarbrücken, Winterberg 1, 66119 Saarbrücken, Germany;* [6]*Helmholtz Institute for Infection Research, Inhoffenstraße 7, 38124 Braunschweig, Germany;* [7]*TWINCORE, Centre for Experimental and Clinical Infection Research, Feodor-Lynen-Straße 7, 30625 Hannover, Germany;* [8]*Department of Pharmacy, Saarland University, Campus, 66123 Saarbrücken, Germany*

*Corresponding author. Tel: +49-511-5350-444; Fax: +49-511-5350-155; E-mail: sabine.wronski@item.fraunhofer.de
†Contributed equally.

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Objectives: In the context of cystic fibrosis, *Pseudomonas aeruginosa* biofilms often develop in the vicinity of airway mucus, which acts as a protective physical barrier to inhaled matter. However, mucus can also adsorb small drug molecules administered as aerosols, including antibiotics, thereby reducing their bioavailability. The efficacy of antibiotics is typically assessed by determining the MIC using *in vitro* assays. This widespread technique, however, does not consider either bacterial biofilm formation or the influence of mucus, both of which may act as diffusion barriers, potentially limiting antibiotic efficacy.

Methods: We grew *P. aeruginosa* biofilms in the presence or absence of human tracheal mucus and tested their susceptibility to tobramycin and colistin.

Results: A significant reduction of tobramycin efficacy was observed when *P. aeruginosa* biofilms were grown in the presence of mucus compared with those grown in the absence of mucus. Diffusion of tobramycin through mucus was reduced; however, this reduction was more pronounced in biofilm/mucus mixtures, suggesting that biofilms in the presence of mucus respond differently to antibiotic treatment. In contrast, the influence of mucus on colistin efficacy was almost negligible and no differences in mucus permeability were observed.

Conclusions: These findings underline the important role of mucus in the efficacy of anti-infective drugs.

Introduction

Airway epithelium is covered by a thin mucus layer, enabling continuous clearance of inhaled pathogens, pollutants and other environmental particles. This process is achieved by constant mucus secretion into the airway lumen and the coordinated beating of epithelial cell cilia.[1,2] Unfortunately, in diseases such as COPD, asthma or cystic fibrosis (CF) the mucociliary machinery is significantly compromised,[3–5] reducing the mucus clearance rate and providing an optimal environment for bacterial infections.[6] In CF patients, a congenital mutation of the *CFTR* gene leads to abnormal expression of the CFTR chloride channel, resulting in an overall water imbalance of the airways that ultimately leads to increased mucus viscosity and recurrent infections.[7,8]

Pseudomonas aeruginosa is amongst the most prevalent pathogens found in chronically infected CF patients. The vast majority of CF patients are treated with antibiotics from early childhood. Inhaled tobramycin therapy in particular is relatively effective in early stages of *P. aeruginosa* infection and has increased the lifespan of CF patients.[7] Besides tobramycin, common inhaled antibiotics for the management of CF infections include colistin, levofloxacin and aztreonam.[9] Upon chronicity of the infection, eradication is difficult to achieve[10] due to the different mechanisms used by *P. aeruginosa* to evade both antibiotic

treatment and the host's defence. Therefore, the actual susceptibility of *P. aeruginosa* to antibiotic treatment in terms of antibiotic concentration can differ enormously from the MIC, as determined in routine *in vitro* tests. It is noteworthy that these assays are usually performed using planktonic bacteria, rather than bacterial biofilms, and do not consider the presence of mucus either.

In CF lungs, *P. aeruginosa* forms structured bacterial communities, referred to as biofilms.[11] These communities consist of bacterial cells surrounded by a self-produced extracellular matrix, known to protect bacteria from the host's immune system and from antibacterial treatment.[12] In their native airway environment, *P. aeruginosa* biofilms are often embedded in mucus, which represents an additional barrier to the diffusion of inhaled drugs.[13,14] Mucus is a hydrogel composed mainly of water and mucin glycoproteins. Mucins are continuously secreted into the airway lumen, where they form a cross-linked, mesh-like structure with a tight pore size that impedes penetration of inhaled coarse particles.[13] Moreover, the sugar side chains of these mucins are mostly negatively charged at physiological pH and can limit the diffusion of small molecules due to electrostatic interactions.[15] This might hold true in particular for tobramycin and colistin, which are polycationic antibiotics[9] and can thus undergo electrostatic interactions with these polyanionic hydrogel matrices. Therefore, the challenge for an effective antibiotic therapy is to overcome not only the extracellular matrix of the biofilms but also the mucus layer. These barriers are barely addressed in the currently available *in vitro* models used during pre-clinical development stages of anti-infective drug candidates.

The aim of our study was to investigate the impact of airway mucus on *P. aeruginosa* biofilm susceptibility to antibiotic treatment. For this purpose, *P. aeruginosa* biofilms were grown *in vitro* in the presence or absence of human tracheal mucus and the antibacterial efficacy of colistin and tobramycin under these more *in vivo*-like conditions was investigated.

Materials and methods

An expanded version of the Materials and methods section is available as Supplementary data at *JAC* Online.

Bacterial strains and antibiotics

PAO1 (ATCC 15692) and GFP-PAO1 (Thomas Bjarnsholt, Copenhagen) were used. Tobramycin sulphate salt and colistin sulphate salt were obtained from Sigma–Aldrich, Munich, Germany.

MIC assay

MIC assays were performed according to CLSI guidelines[15a] with antibiotic concentrations ranging from 0.125 to 512 mg/L.

Ethics

Collection and preparation of human mucus was approved by the Ethics Commission of The Chamber of Medicine Doctors of the Saarland (file number 19/15). Informed consent was obtained from all patients.

Extraction and preparation of human mucus

Undiluted human tracheal mucus samples were collected with the endotracheal tube method.[16–18] Mucus samples were collected by centrifugation and stored at −20°C until further use. Samples were freeze-dried and weighed before and after the process to determine the amount of sublimated water. Before its use, the freeze-dried mucus was exposed to UV radiation for 1 h and re-hydrated with PBS using the same volume that had previously been sublimated. With this re-suspension protocol, an elastic-dominant, bacteria-free mucus hydrogel was achieved.[19]

Biofilm cultivation and susceptibility testing

In short, 96-well plates were inoculated with 100 μL of bacterial suspension and 100 μL of mucus or PBS and samples were cultured under static conditions with 100% humidity, 37°C and 0% CO_2 for 24 h. Thereafter, samples were treated with 10 μL of tobramycin or colistin with a final concentration of 100, 300 or 900 mg/L representing 100×, 300× and 900× MIC, respectively. Efficacy was assessed according to the viable bacterial load determined by dilution plating and counting of cfu.

Tobramycin activity after pre-incubation in mucus

A volume of 100 μL of mucus was mixed with 10 μL of tobramycin suspension, resulting in a final concentration of 300 mg/L in the well. After 24 h of incubation, tobramycin-containing mucus was inoculated with 100 μL of PAO1 (2×10^7/mL), leading to a final tobramycin concentration of 150 mg/L. As positive treatment control 150 mg/L tobramycin with vehicle and a negative control without tobramycin were used. After further incubation for 24 h at 37°C, treatment efficacy was analysed visually for turbidity and by dilution plating and determination of cfu.

Laser scanning confocal microscopy

Briefly, GFP-tagged PAO1 biofilms were cultured in 96-well plates (μ-plate 96 well, ibidi, Martinsried, Germany) as described above. Imaging was performed using an LSM 510 Meta confocal microscope (Zeiss, Jena, Germany). The 488 nm argon laser line was used for excitation and the BP 505–530 filter for emission. Z-stacks were performed with intervals of 0.5 μm and a range of 30 μm using the Plan-Neofluar 40×/1.3 oil DIC objective. Images were processed using the imaging software Imaris version 7.6.5, including its associated surpass module (Bitplane Scientific Software, Zurich, Switzerland). The surface of all fluorescent signals above a determined threshold was computed as bacterial biomass. The obtained values were displayed as the surface area (in μm^2) covered with a GFP-positive signal.

Tobramycin and colistin diffusion studies

Briefly, experiments were performed using Transwell® membranes (Corning, Durham, NC, USA) with a surface of 0.33 cm^2 and a pore size of 4 μm. Biofilms in the presence or absence of mucus or sham-infected mucus samples with a total volume of 100 μL were cultured on the membrane of the Transwell® inserts overnight. Inserts were placed into the companion plates and 100 μL of 600 mg/L tobramycin or colistin solution in PBS was then added to the apical compartment. The basolateral compartment was filled with 600 μL of PBS. A volume of 50 μL was sampled from the basolateral compartment after 15, 30, 60, 90, 120 and 3600 min.

HPLC measurement

Briefly, HPLC and MS/MS methods similar to those described in the literature for tobramycin[20] and colistin[21] were applied with minor modifications.

Statistical analysis

Three independent runs were performed for each experiment with technical duplicates or technical triplicates (diffusion studies). All values are given as mean ± SD or mean ± SEM (diffusion studies). Statistical analysis

was performed with the GraphPad Prism software (GraphPad 7 Software, Inc., USA) using the Mann–Whitney *U*-test. Differences were considered statistically significant at the level of $P < 0.05$.

Results

Mucus characterization

Eighty-five independent mucus samples were used in this study. The mean age of the patients was 56.6±15.7 years; 54% were male and 46% female, and 24 out of 85 were smokers (28%). Individual samples were combined to produce 10 independent mucus batches. The water content of the mucus samples was 95%±1%. Mucus samples obtained with the method described above showed different mucin concentrations (8%–22% of the solid content), pH values between 7 and 8.5, and a DNA content of <1%.[19]

Biofilm susceptibility to antibiotic treatment in the presence of human mucus

P. aeruginosa biofilms exhibited an increased tolerance to both tobramycin and colistin (Figure 1) as compared with the MIC values for planktonic bacteria, which were 0.5–1 and 1 mg/L for tobramycin and colistin, respectively, in good agreement with the values previously reported in the literature.[22,23] For instance, full eradication of the biofilms grown in PBS required a concentration 900 times higher than the MIC, irrespective of the antibiotic used. In biofilms grown in a mucus environment, a concentration-dependent decrease in viable bacterial load could still be seen for both antibiotics; however, tobramycin efficacy was significantly impaired in the presence of mucus, leading to a shift of the IC_{50} value from 100 mg/L without mucus to >900 mg/L with mucus. Thus, killing of biofilm-grown bacteria was not achieved with tobramycin under normal culture conditions in the presence of mucus.

In contrast, colistin treatment efficacy was not affected by the mucus environment, with IC_{50} values of 170 and 190 mg/L for biofilms grown in PBS and in mucus, respectively. Complete bacterial killing by colistin could be observed with treatment concentrations of 300 and 900 mg/L, respectively.

Tobramycin activity in human mucus

In the biofilm susceptibility assay, treatment with tobramycin, but not colistin, was significantly less effective in the presence of mucus. To investigate whether this effect was due to inactivation of tobramycin by mucus, tobramycin efficacy was tested after pre-incubation in mucus. In these experiments, tobramycin pre-incubated in mucus exhibited bactericidal activity against planktonic bacteria similar to that of tobramycin alone, indicating maintained activity (Figure 2). In addition, reversibility of the antibiotic tolerance to tobramycin observed in the presence of mucus was investigated by performing an MIC assay subsequent to the biofilm susceptibility assays. For this purpose, we incubated PAO1 bacteria extracted from 48-h-old biofilms formed in mucus in order to determine whether the decreased susceptibility to antibiotic treatment was persistent. The MIC for PAO1 re-isolated from biofilms previously cultured in the presence of mucus was 0.5–2.5 mg/L and thus in the same range as the control MIC (0.5–1 mg/L), indicating that susceptibility was restored and the mucus environment did not induce genetic resistance of PAO1 to tobramycin.

Biofilm structure in human mucus environment

Confocal images of GFP-PAO1 were taken to visualize potential differences in terms of biofilm structure, bacterial density and total biomass induced by the mucus environment. An increased amount of bacterial GFP signal was observed in the presence of mucus (Figure 3a and d) compared with biofilms grown in the absence of mucus (Figure 3b and d), which was not due to unspecific autofluorescence of the mucus itself (Figure 3c). These observations also correlated with the cfu counts of these samples (Figure 3e). Both methods revealed a higher bacterial count for biofilms grown in the presence of human mucus.

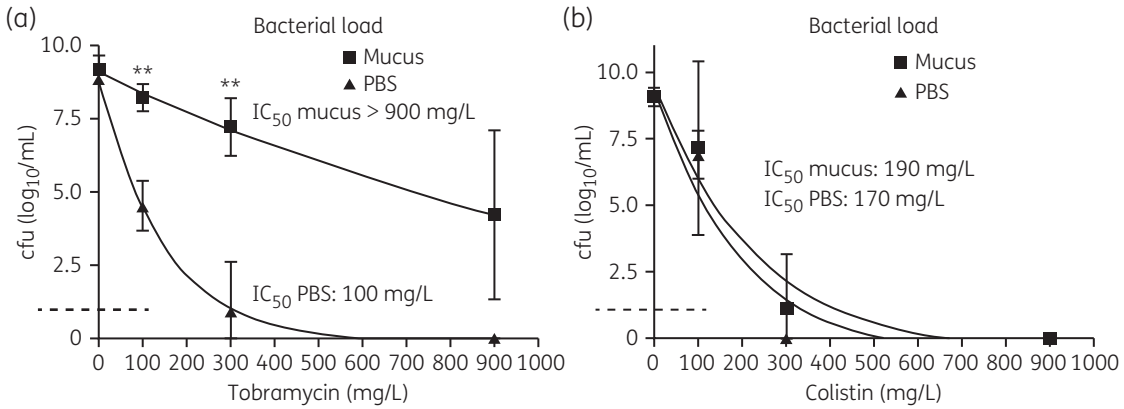

Figure 1. Biofilm susceptibility to antibiotic treatment. PAO1 biofilms grown in mucus or PBS for 24 h were treated with tobramycin (a) or colistin (b). After 24 h of incubation, efficacy was assessed by determination of cfu. The cfu counts are depicted logarithmically as regression curves showing the mean ± SD for $n = 3$ experiments, each with technical duplicates. A double asterisk indicates statistical significance at $P < 0.01$, according to the Mann–Whitney *U*-test, for comparison of mucus versus PBS at the individual concentrations. The broken line indicates the detection limit.

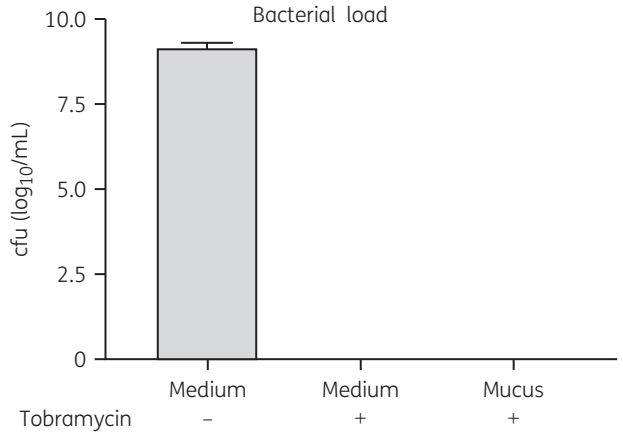

Figure 2. Tobramycin activity in human mucus. Mucus pre-incubated with tobramycin was inoculated with PAO1 and incubated for 24 h. Medium only and medium containing the same concentration of tobramycin were used as controls. Bar graphs show the mean + SD for $n = 2$ samples, each with technical duplicates.

Antibiotic diffusion through human mucus

Diffusion studies revealed significantly retarded and decreased diffusion of tobramycin through PAO1 biofilm formed within mucus (Figure 4a) compared with diffusion through pure mucus or biofilm formed in PBS. Diffusion through PBS only was used as control indicating 'free diffusion'. In all groups, fast diffusion was observed within the first 2 h, but diffusion through biofilm in mucus was significantly delayed. Equally in all groups, tobramycin diffusion decelerated over time until reaching saturation; however, the final cumulative masses varied, with mucus + PAO1 reaching the lowest value (Figure 4a), but they were near the theoretical maximal mass of 45 μg for both antibiotics. Table 1 shows the maximal cumulative mass (Y_{max}) of tobramycin, which was in a similar range for all experimental conditions. The half-life ($t_{1/2}$) of tobramycin was slightly increased for mucus only and markedly increased for biofilm in mucus (Table 1). Colistin diffusion was comparable for all groups (Figure 4b). No phase exponential association fit was performed for colistin, as standard deviations were too high. These findings are in agreement with the observed cfu reduction following antibiotic treatment.

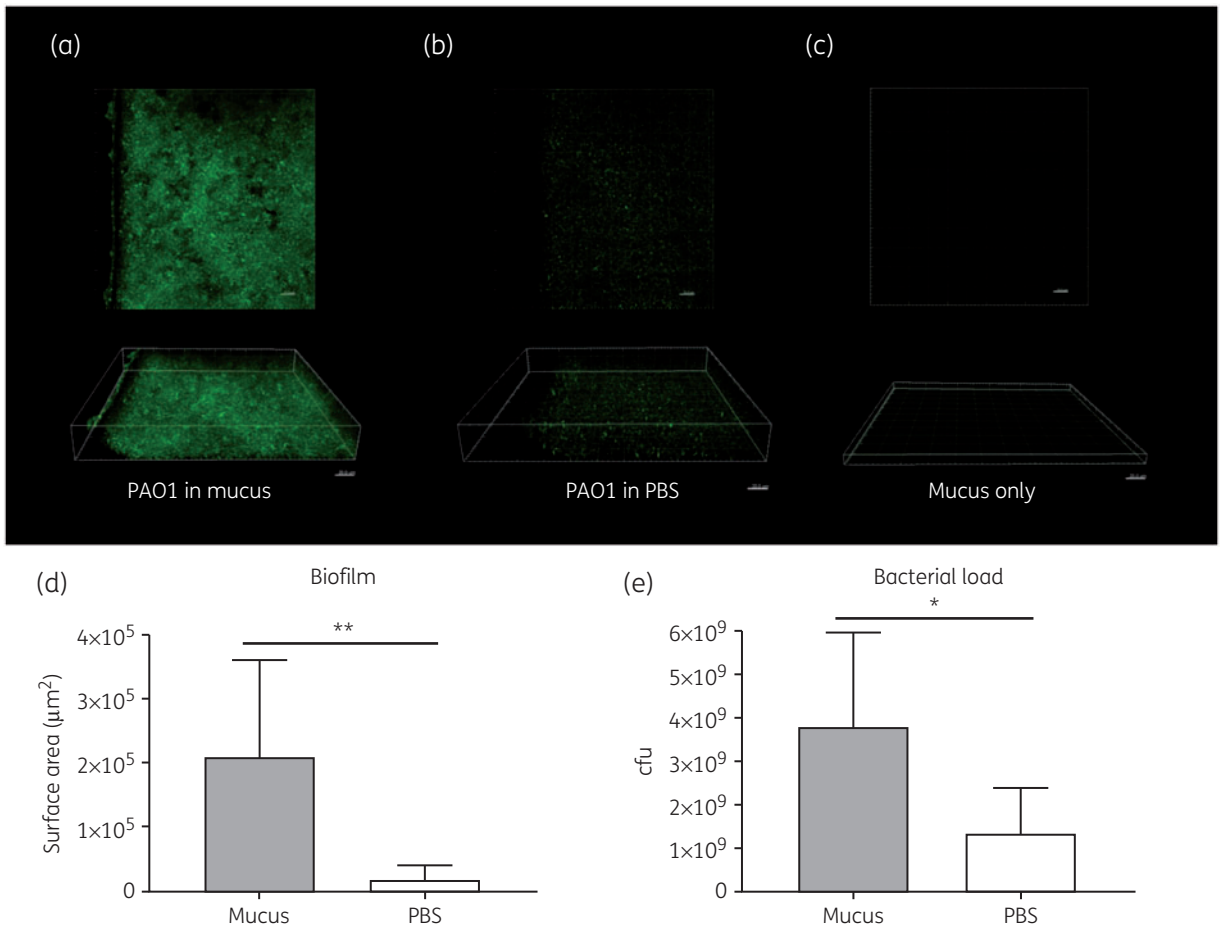

Figure 3. Biofilm structure and bacterial count in mucus. GFP-PAO1 biofilms were grown in mucus (a) or PBS (b) for 2 days and imaged by confocal microscopy. Uninfected mucus was imaged as a control (c). Fluorescence signals were quantified using Imaris software (d) and cfu were determined (e). Bar graphs show the mean + SD for $n = 3$ samples, each with technical duplicates. A non-parametric *t*-test (Mann–Whitney *U*-test) was performed for statistical analyses. $^{*}P < 0.05$. $^{**}P < 0.01$. Scale bars represent 20 μm.

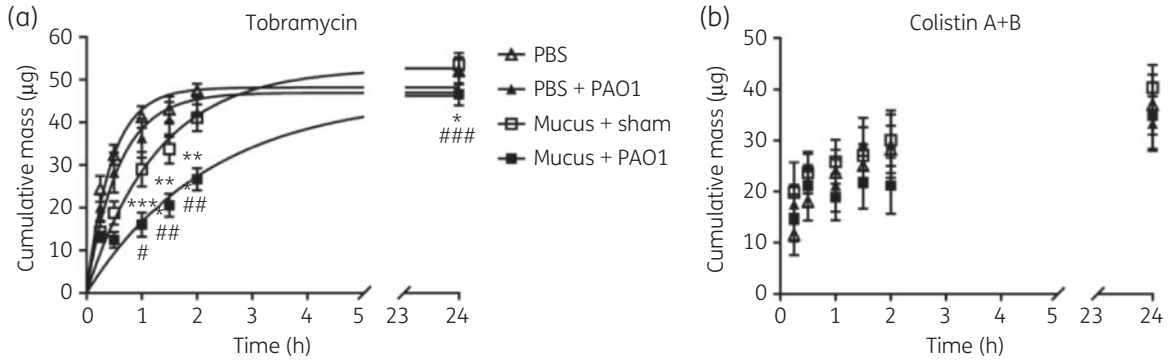

Figure 4. Tobramycin and colistin diffusion through biofilm formed in mucus. PAO1 biofilm cultured in mucus or PBS in the apical compartment of a Transwell® was treated with tobramycin or colistin. PBS only or sham-infected mucus was used as a control. Diffusion of the antibiotic was assessed by HPLC and is depicted as cumulative mass. Scatter plots show the mean ± SEM for $n = 3$ experiments, each with triplicates. A single asterisk, a double asterisk and a triple asterisk indicate statistical significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, according to the Mann–Whitney *U*-test, for comparison of biofilm in mucus with PBS. A single hash, a double hash and a triple hash indicate statistical significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, according to the Mann–Whitney *U*-test, for comparison of biofilm in mucus with mucus only. A one-phase exponential association fit was performed for tobramycin diffusion using GraphPad Prism 7, described by the equation $Y = Y_{max} \times (1 - e^{-k \times x})$.

Table 1. Y_{max} and $t_{1/2}$ of tobramycin following diffusion through biofilms in mucus

Tobramycin diffusion	PBS	PBS + PAO1	Mucus + sham	Mucus + PAO1
Y_{max} (μg)	44.75–52.13	41.11–54.28	46.65–58.94	41–51.48
$t_{1/2}$ (h)	0.22–0.43	0.24–0.66	0.65–1.16	1.19–1.98

A one-phase exponential association fit was performed using GraphPad Prism 7, described by the equation $Y = Y_{max} \times (1 - e^{-k \times x})$. Y_{max} (μg) and $t_{1/2}$ (h) of tobramycin were calculated for each group. The table shows the 95% CI for $n = 3$ transports, each with triplicates.

Discussion

Chronic airway infections with *P. aeruginosa* are a major cause of morbidity, especially among patients suffering from CF. Antibiotic treatment leads to improved lung function and life quality,[24] although permanent bacterial eradication is often not accomplished due to a variety of adaptation mechanisms of the pathogen. Biofilm formation is one of the most crucial resistance mechanisms used by many bacterial species. Different *in vitro* test systems, including pure bacterial cultures,[25] co-cultures of bacteria and epithelial cells,[26] and bacteria grown in artificial sputum medium,[27] have been used to investigate bacterial biofilm formation and the development of increased antibiotic tolerance. Bjarnsholt *et al.*[28] examined explanted CF lung and observed bacterial biofilm to be embedded in mucus with no direct contact with the airway epithelium, which was also found by Worlitzsch *et al.*[29] On the contrary, Crabbé *et al.*[30] co-cultured epithelial cells with *P. aeruginosa* and observed increased antibiotic efficacy in the presence of airway cells, suggesting that they might improve antibiotic efficacy. In the chronically infected CF lung, the viscous mucus of the airways is highly colonized with bacterial biofilms.[31] Biofilms as well as airway mucus are well known to act as a barrier to antibiotics.[13,14,32] For instance, the alginate found in the extracellular matrix of bacterial biofilms can adsorb antibiotic molecules,[32,33] and the negatively charged mucin glycoproteins offer multiple sites for electrostatic interactions,[15] in particular for polycationic antibiotics such as tobramycin and colistin.[34,35] So far, the ability to reduce the diffusion of antibiotic molecules has been shown separately for these individual elements. In CF airways, however, biofilms are formed within the respiratory mucus. Consequently, one could expect further reduction of antibiotic bioavailability due to the combined effect of the biofilm's extracellular matrix and mucus. In the present study, we investigated the susceptibility of *P. aeruginosa* biofilms to two polycationic antibiotics, tobramycin and colistin, in the presence or absence of human mucus.

Interestingly, biofilms grown in the presence of airway mucus led to a significant loss of efficacy for tobramycin, but not colistin, indicating that the mucus environment affects antibiotic tolerance of *P. aeruginosa* biofilms.

Even at tobramycin concentrations as high as 900 mg/L, full eradication of bacteria could not be achieved in our static *in vitro* model composed of *P. aeruginosa* biofilms and mucus. The tobramycin concentrations used in the present study correlate well with the maximum sputum concentrations found in CF patients, which have been reported to range from 486 to 695 mg/L.[9] The reported maximum sputum concentrations for colistin were around 40 mg/L, 2.5-fold lower than the lowest concentration used in this study, which was associated with a reduction of the cfu count, but was far from achieving full eradication of *P. aeruginosa* biofilms, either in the absence or in the presence of mucus.

The observed loss of efficacy could be due to a strong interaction between the polycationic antibiotics and the components of the biofilm and mucus,[34–36] which may have reduced bioavailability of the antibiotics. Hunt *et al.*[35] demonstrated that the diffusion of tobramycin through dialysate bags containing CF sputum was reduced due to tobramycin binding to sputum components. The reduced amount of unbound tobramycin might then lead to insufficient treatment. Similarly, Huang *et al.*[34] measured the diffusion of colistin through dialysate bags containing porcine mucins. They concluded that colistin binds to porcine mucins and thereby

loses its activity.[34] To address this issue, tobramycin was pre-incubated with human tracheal mucus for 24 h and its efficacy against planktonic *P. aeruginosa* was subsequently assessed. In this setting, tobramycin maintained its efficacy, implying that tobramycin was at least partly unbound and not inactivated by mucus. Moreover, *P. aeruginosa* re-isolated from 48-h-old biofilms was not associated with an increased MIC, indicating that no genetic resistance mechanisms were responsible for the decreased susceptibility to tobramycin. Instead, adaptive resistance mechanisms such as increased efflux pump activity, permeability changes due to alterations of lipopolysaccharides or porins, or expression of biofilm-specific genes might have resulted in increased bacterial tolerance.[9,37] Considering the mechanism of action of tobramycin (intracellular, binding to ribosomes, acting on metabolically active bacteria)[38] and colistin (extracellular, bacterial cell wall, acting independently of metabolism),[39] an increased efflux pump activity together with alterations in the outer membrane porins would impact the efficacy of tobramycin to a larger extent than that of colistin. Pamp *et al.*[40] found that the metabolic status of bacteria differs within a biofilm, leading to different antibiotic susceptibilities. Metabolically inactive bacteria might be less susceptible to tobramycin, but more susceptible to colistin.

The permeation kinetics studies of tobramycin and colistin through human tracheal mucus did not show a significant effect on the net permeation of the antibiotics. Compared with its permeation through the bare Transwell® membrane, tobramycin was just slightly, but not significantly, hindered by tracheal mucus alone, whereas no effect at all on colistin permeation through mucus was observed compared with the bare membrane. Although the experimental design applied in this study did not cover the kinetics of the first 15 min, the data suggest that tobramycin and colistin molecules are small enough to diffuse through the mucus pores and that the electrostatic interactions taking place between the polycationic antibiotics and mucins are transient and most probably are overcome fast enough not to be detected within the time span of our experiment. In contrast, the biofilm/mucus mixtures had a significant impact on the diffusion of tobramycin compared with its diffusion through mucus or biofilms alone. These results indicate that *P. aeruginosa* biofilms formed within human mucus might have a different structure that may further impact their susceptibility to tobramycin. Cattoir *et al.*[41] found that mucus controls the expression of virulence factors and influences several metabolic pathways after culturing *P. aeruginosa* strains originating from CF lungs in the presence or absence of mucus. *P. aeruginosa* has also been shown to adhere to human respiratory mucins. Vishwanath and Ramphal[42] assumed that this ability might facilitate mucus colonization. Landry *et al.*[43] suggested that human mucins influence both biofilm structure and antibiotic susceptibility. They detected that *P. aeruginosa* forms flat, homogeneous biofilms on a smooth surface like glass, but rather inhomogeneous biofilms in contact with mucins, as interactions with mucins lead to immobilization, resulting in enhanced biofilm formation and antibiotic resistance. In the present study, *P. aeruginosa* biofilms were grown in resuspended human tracheal mucus that previously had been freeze-dried. Upon resuspension, mucus keeps its native viscoelastic properties,[19] typical of cross-linked polymers. Thus, planktonic *P. aeruginosa* inoculated in human tracheal mucus could have been easily immobilized by the strict pore size of the mucin network, which has been reported to range from 100 to 400 nm in CF sputum.[5] The pores within the mucin network might act as *P. aeruginosa*-immobilizing scaffolds, while bacteria can degrade mucus components[44] and use them as nutrients[45] or even incorporate them into their extracellular matrix, leading to a compacted biomass. We observed significantly increased bacterial numbers in the presence of mucus; however, bacterial load did not further increase following 24 h of incubation, probably due to limitation of nutrients. Additionally, we compared bacterial growth in mucus, nutrient-rich LB medium and PBS and detected the highest bacterial numbers in the presence of mucus (Figure S1). Walker *et al.*[46] reported that *P. aeruginosa* uses biopolymers originating from necrotic neutrophils as a scaffold for formation of biofilms, which then display increased antibiotic tolerance.[47] Indeed, after growing *P. aeruginosa* biofilms for 48 h in the presence of mucus, we found a significantly higher amount of biomass compared with biofilms grown in the absence of mucus, which might also account for the reduced efficacy of tobramycin in our *in vitro* setting.

Biofilms are often associated with surfaces; however, it is now known that biofilms in CF lungs are not attached to surfaces, but are embedded in host material such as mucus.[28] Sønderholm *et al.*[48] inoculated agar beads with fluorescent *P. aeruginosa* to mimic the presence of a secondary matrix, suggesting that this affects biofilm physiology and consequent treatment susceptibility. Like Sønderholm *et al.*,[48] we believe that mucus could act as a secondary matrix in our system and probably also in the human lung. Palmer *et al.*[49] inoculated CF sputum with *P. aeruginosa* and observed strong bacterial growth and altered bacterial physiology. They concluded that CF sputum is a perfect bacterial growth medium for *P. aeruginosa* and influences bacterial communication and motility, which play important roles in biofilm formation. These results match well with our results. Based on the composition of CF sputum, Palmer *et al.*[50] developed a synthetic CF sputum medium. Like artificial sputum medium,[27] this preparation is mainly composed of amino acids, salts and some other components found in CF sputum, yielding a relatively low-viscosity fluid with approximately the same mean osmolality as CF sputum. Due to their composition, however, these artificial sputum media normally behave like Newtonian fluids, lacking the intermolecular interactions and covalent cross-links that give airway mucus its viscoelastic characteristic.

P. aeruginosa was found to form small aggregates with an average size ranging from 5 to 100 μm that were embedded in mucus.[28] Formation of aggregates could also be found in an agar bead model.[48] However, in the initial stages of biofilm formation that the timeframe of our experiments was able to cover—in contrast to chronic *P. aeruginosa* infection in severely diseased CF patients—bacteria were found to colonize the human mucus environment rather homogeneously instead of forming small aggregates. A more detailed confocal analysis with higher magnification would yield more information on biofilm structure.

In our system mucus is considered to be a very relevant environmental factor for biofilm formation in CF lungs; however, there are considerable differences between mucus from healthy humans and CF patients. In CF patients, hydration of secretion is impaired; consequently, respiratory mucus is dehydrated, resulting in increased viscosity. The predominant mucins MUC5AC and MUC5B are overproduced in CF lung secretion, triggered by *P. aeruginosa* LPS.[51] Chronic infection with *P. aeruginosa* leads to a

strong immune response with neutrophil granulocytes being the predominant immune cell. Vast amounts of necrotic neutrophils cause an increased DNA content in the mucus, which results in further thickening. These aspects have not been addressed in our system. An alternative method could be the use of CF sputum; however, sputum is an expectorated secretion, which is a mixture of mainly mucus and saliva in contrast to pure mucus directly originating from airway epithelium. Furthermore, we experienced mucus from explanted CF lungs to be highly colonized with different bacterial and fungal species, with high variation between patients. Therefore, we thought mucus from 'healthy' airways to be a valuable tool allowing performance of reproducible experiments.

Conclusions

In summary, a model considering mucus as the natural microenvironment for *P. aeruginosa* biofilms in human lungs has been successfully developed, suggesting that the mucus environment should be considered as a key factor in *in vivo* biofilm formation. Additionally, this might be of high relevance for anti-infective drug development. We noted significantly decreased efficacy of tobramycin, but not of colistin, against biofilms in the presence of human mucus. Biofilm formation in human mucus resulted in a more heterogeneous structure, a higher bacterial load and a significantly impaired transport rate for tobramycin, resulting in decreased tobramycin efficacy. More studies are needed, however, to further investigate the role of the mucus environment in *P. aeruginosa* biofilm formation and delineate the differential effects on antibiotic efficacy.

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Transparency declarations

M. H., L. S., C. B. and C.-M. L. were employees of PharmBioTec GmbH at the time of publication. All other authors: none to declare.

Supplementary data

An expanded version of the Materials and methods section and Figure S1 are available as Supplementary data at *JAC* Online.

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