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Short communication

Antibiotic resistance of mixed biofilms in cystic fibrosis: impact of emerging microorganisms on treatment of infection

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ABSTRACT

Cystic fibrosis (CF) is a genetic disorder associated with multispecies infections where interactions between classical and newly identified bacteria might be crucial to understanding the persistent colonisation in CF lungs. This study investigated the interactions between two emerging species, *Inquilinus limosus* and *Dolosigranulum pigrum*, and the conventional CF pathogen *Pseudomonas aeruginosa* by evaluating the ability to develop biofilms of mixed populations and then studying their susceptibility patterns to eight different antimicrobials. Monospecies biofilms formed by *I. limosus* and *D. pigrum* produced significantly less biomass than *P. aeruginosa* and displayed greater sensitivity to antimicrobials. However, when in dual-species biofilms with *P. aeruginosa*, the emerging species *I. limosus* and *D. pigrum* were crucial in increasing tolerance of the overall consortia to most antibiotics, even without a change in the number of biofilm-encased cells. These results may suggest that revising these and other species interactions in CF might enable the development of more suitable and effective therapies in the future.

1. Introduction

Cystic fibrosis (CF) is a common genetic disease involving the production of thick and sticky mucus that predisposes CF patients to frequent pulmonary infections [1,2]. *Pseudomonas aeruginosa* is the dominant pathogen colonising older patients in CF [3], often adopting a biofilm mode of growth as a survival strategy. However, recent studies have shown that standard laboratory methods may fail to identify or may misidentify other isolates that are actually present but are usually only detected using molecular biology techniques [4]. Two novel microorganisms that have been increasingly isolated from CF specimens are *Inquilinus limosus* and *Dolosigranulum pigrum*. Although available information about these organisms is scarce, their presence is very likely to occur together with conventional pathogens, creating a diverse mosaic of bacteria in the CF lung [5].

Antibiotic resistance is a well-known phenomenon in chronic infections and is an increasing concern in CF [6]. The intensive selective pressure provided by the large amount of antibiotics to which CF microbial populations are exposed is one factor contributing to such resistance. Typically, the choice of antimicrobials in CF only relies on antibiotic susceptibility testing of the traditional organism, *P. aeruginosa*. However, the wealth of species of different phenotypes and sensitivities prevailing in the airways of these patients is clearly more complex than a monomicrobial disease and it has been suggested that they can undermine the effectiveness of the treatment commonly practiced, thus resulting in a less than optimum treatment outcome for affected individuals. To the authors' knowledge, this is the first study evaluating the spectrum of antimicrobial resistance of dual-species biofilms involving classical and emerging microorganisms related to CF. The ability of these species to develop single and mixed biofilms was also assessed through biomass and culturable cells analysis and the obtained results were correlated with the susceptibility profiles found for the species involved.

2. Materials and methods

2.1. Bacterial strains, growth media and buffers

Three CF-related bacterial species were used in this study, the traditional pathogen *P. aeruginosa* PA14 as well as two emerging microorganisms, *I. limosus* M53 and *D. pigrum* CIP 104051 (Institute Pasteur Collection, Paris, France). *Pseudomonas aeruginosa* and *I. limosus* pure cultures were grown in tryptic soy broth (TSB) (EMD Chemicals Inc., Gibbstown, NJ), whereas brain–heart infusion (BHI) (EMD Chemicals Inc.) was used to culture *D. pigrum*. Since *P. aeruginosa* and *D. pigrum* require different culture media to grow,

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Table 1
Bacterial cultures and growth conditions used in this study.

Bacterial culture	Strain	Growth medium	Incubation temperature (°C)	Incubation period (h)
Pseudomonas aeruginosa	PA14	TSB/TSA	37	6
Inquilinus limosus	M53	TSB/TSA	37	48
Dolosigranulum pigrum	CIP 107041	BHI/TSA	37	72
P. aeruginosa + *I. limosus*	–	TSB/TSA	37	24
P. aeruginosa + *D. pigrum*	–	TSB or BHI/TSA	37	24

TSB, tryptic soy broth; TSA, tryptic soy agar; BHI, brain–heart infusion.

for experiments of mixed biofilms involving these two microorganisms, *P. aeruginosa* was also grown in BHI and *D. pigrum* was also grown in TSB. The temperature of incubation was 37 °C and the period of biofilm formation varied for each strain. All conditions used for single and mixed biofilm experiments are summarised in Table 1.

2.2. In vitro biofilm formation

Single and mixed biofilms were grown on a Calgary Biofilm Device (CBD) (MBEC Biofilm Technologies, Calgary, Alberta) as previously described by Ceri et al. [7].

2.3. Analysis of pre-formed biofilms

Single and mixed biofilms formed on the CBD were further analysed in terms of biomass and number of culturable cells.

2.3.1. Biomass

Peg lids were rinsed with double-distilled water and were left to dry for 10 min. Biofilm-growing bacteria on the pegs were then stained with 1% (v/v) crystal violet (Sigma-Aldrich, Oakville, ON, Canada) for 1 min and were washed twice with double-distilled water. Pegs were then decolorised with pure methanol (Sigma) and the optical density at 550 nm of the obtained solution was measured using a microtitre plate reader (Labequip Ltd., Markham, ON, Canada). At least eight replicates were run for each condition.

2.3.2. Cell culturability

The number of adhering bacteria within the biofilm was determined by breaking four pegs of the CBD under aseptic conditions. An Aquasonic Water-table Sonicator (model 250 HT; VWR International, Edmonton, AB, Canada) was used to disrupt the biofilm on the broken pegs submerged in sterile 0.9% (v/v) saline complemented with 1% (v/v) Tween 20 for 10 min (Sigma). The disrupted biofilms were subsequently serially diluted in saline and plated on tryptic soy agar (TSA) (EMD Chemicals Inc.) for viable cell counting. Selective agar media were used to plate *P. aeruginosa* [*Pseudomonas* isolation agar (PIA)] and *I. limosus* [*Burkholderia cepacia* selective agar (BCSA) supplemented with 300 000 IU/L polymyxin B and 100 mg/L ticarcillin]. TSA and PIA plates were incubated for 24 h (for *I. limosus*, TSA plates were incubated for 40 h), whereas BCSA plates were incubated for 48 h at 37 °C before enumeration of colony-forming units (CFU). All samples were run in quadruplicate.

2.4. Antibiotic stock solutions

Eight clinically relevant antibiotics were used, including tobramycin, gentamicin, levofloxacin, ciprofloxacin, clindamycin, cefotaxime, chloramphenicol and rifampicin. All antibiotics were from Sigma-Aldrich. Stock solutions of antimicrobial agents were prepared at 5120 μg/mL and then 500 μL aliquots were stored at −70 °C. Working solutions were prepared on the day of use at 1024 μg/mL in cation-adjusted Mueller–Hinton broth (BD Diagnostics, Franklin Lakes, NJ). For susceptibility testing, antibiotic concentrations ranged from 2 μg/mL to 1024 μg/mL.

2.5. Antibiotic susceptibility testing

After washing biofilms with saline, a 'challenge plate' was prepared according to Ceri et al. [7]. The minimum inhibitory concentration (MIC) was determined by reading the optical density of the challenge plate at 650 nm, with the exception of those cultures that used sheep blood in the susceptibility testing, where MIC values were determined by visual observation of the turbidity gradient on the challenge plate. This turbidity demonstrates the ability of bacteria to grow as a planktonic population in the presence of antibiotic; hence, the minimum concentration where growth inhibition occurs is equivalent to the MIC value for most organisms [7,8]. Minimum biofilm eradication concentration (MBEC) values were determined by enumerating spot plates for bacterial growth.

2.6. Statistical analysis

Data were analysed using Prism software v.4.0 for Macintosh (GraphPad Software Inc., La Jolla, CA). The ability of strains to form biofilms was assessed by one-way analysis of variance (ANOVA) tests, and Tukey's post hoc test was performed to subsequently compare pairs of columns. Results were considered statistically significant at $P < 0.05$.

3. Results

In this study, bacterial biofilms of traditional and emerging CF-related microorganisms were readily formed on the CBD, providing a valuable and reliable technology for selection of clinically effective antibiotics. Susceptibility assays were performed after all biofilms achieved a threshold concentration of 10^4–10^6 CFU/peg, which required different incubation times for each biofilm (Table 1).

The concentrations of antibiotic able to inhibit planktonic bacteria (MIC) and those required to kill biofilm-encased bacteria (MBEC) are summarised in Table 2. Most antibiotics were effective in inhibiting planktonic growth of single species at low concentrations; however, mixed planktonic populations required equal or even higher concentrations than those applied to inhibit the planktonic growth of single populations.

Only a few antibiotics were able to kill biofilm bacteria at relatively low concentrations. Generally, MBEC values were significantly greater compared with MIC data, suggesting that once established, biofilms are notoriously difficult to eradicate and high doses of antimicrobials are needed to eliminate them. Monospecies biofilms involving only *P. aeruginosa* were considerably more resistant to most antibiotics tested than those developed by other organisms.

The minimum bactericidal concentration required to kill bacteria in mixed biofilms was generally equal to the concentration needed to kill the more resistant single biofilm of the encompassed species, which was predominately *P. aeruginosa*. Results obtained

Table 2
In vitro susceptibility patterns of single-species and dual-species cultures of cystic fibrosis-related organisms to eight clinically relevant antibiotics[a].

Antibiotic	*Pseudomonas aeruginosa*	*Inquilinus limosus*	*Dolosigranulum pigrum*	*P. aeruginosa* + *I. limosus*	*P. aeruginosa* + *D. pigrum* (TSB)	*P. aeruginosa* + *D. pigrum* (BHI)
Tobramycin						
MIC	<2	128	16	64	16	8
MBEC	>1024	512	128	256	>1024	>1024
Gentamicin						
MIC	8	16	512	>1024	16	8
MBEC	128	512	>1024	>1024	>1024	>1024
Levofloxacin						
MIC	<2	<2	4	<2	128	8
MBEC	>1024	16	256	>1024	>1024	>1024
Ciprofloxacin						
MIC	<2	<2	256	>1024	<2	<2
MBEC	>1024	32	512	>1024	8	1024
Clindamycin						
MIC	>1024	512	16	>1024	>1024	>1024
MBEC	>1024	>1024	>1024	>1024	>1024	>1024
Cefotaxime						
MIC	16	<2	<2	256	32	256
MBEC	>1024	>1024	>1024	>1024	>1024	>1024
Chloramphenicol						
MIC	128	256	4	>1024	1024	128
MBEC	>1024	1024	32	>1024	>1024	>1024
Rifampicin						
MIC	>1024	<2	<2	>1024	>1024	>1024
MBEC	>1024	8	32	>1024	>1024	>1024

MIC, minimum inhibitory concentration; MBEC, minimum biofilm eradication concentration; TSB, tryptic soy broth; BHI, brain–heart infusion.
[a] MIC and MBEC values expressed in mg/L.

with selective media enabled the observation that *P. aeruginosa* is the organism prevailing within dual-species biofilms with *I. limosus*, presenting ca. 70% of the total cell number. Because there is no information about specific selective media available for *D. pigrum*, no such study was performed for this microorganism. By comparing the susceptibility patterns of *P. aeruginosa* and *D. pigrum* dual-species biofilms growing in different media (Table 2, last two columns), it was observed that antibiotics easily inhibited the growth of planktonic suspensions when TSB was used to culture mixed populations. Susceptibility profiles of these dual-species biofilms in TSB were similar to those obtained with *P. aeruginosa*, showing reduced susceptibility to antibiotics as biofilms. Biofilms growing in BHI did not follow any similar pattern of susceptibility, showing variations in both MIC and MBEC values.

Pseudomonas aeruginosa was clearly the organism that produced the greatest amount of biomass (Fig. 1a) and also showed highest CFU counts (Fig. 1b). *Inquilinus limosus* and *D. pigrum* presented statistically significantly less biomass than *P. aeruginosa* ($P < 0.001$). These species showed similar numbers of CFU (ca. 10^4 CFU/peg), however 2 log below the cell number produced by *P. aeruginosa* ($P < 0.001$) (Fig. 1b). Dual-species biofilms formed between *P. aeruginosa* and each of the unconventional species reached cell numbers similar to biofilms of *P. aeruginosa* alone (ca. 10^6 CFU/peg), however biomass was markedly reduced in mixed-species biofilms. To confirm that the observed biomass decrease was associated with the amount of extracellular matrix produced by the cells, the protein and polysaccharide content in the matrix was determined using established methods [9]. It was observed that dual-species biofilms of *P. aeruginosa* with *I. limosus* or *D. pigrum* had a reduction in the matrix of ca. 30% and 35%, respectively, compared with *P. aeruginosa* biofilms alone (Supplementary Table S1).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2012.04.020.

No statistically significant difference was found between dual-species biofilms of *P. aeruginosa* and *D. pigrum* growing in TSB and BHI, either in biomass formation (Fig. 1a) or in the number of produced cells (Fig. 1b).

4. Discussion

Although it was originally thought that only a limited number of organisms could cause symptomatic infection and lung injury in CF, it has now been shown that the microbial ecology of the CF lung is far more complex than originally thought. In this work, bacteria growing in biofilms were notoriously more difficult to eradicate than when growing planktonically. The well-known increased resistance associated with biofilms is likely to be multifactorial, depending for instance on the alteration of the metabolism of bacterial cells, on the extra barrier of protection provided by the extracellular matrix that so often encases cells within a biofilm, or even by the spatial arrangement, as well as the number of those bacterial cells that form the layers of the biofilm [10].

Single *P. aeruginosa* biofilms were more resistant to most antibiotics than those formed by emerging species. This may be attributed to the higher biomass and cell numbers obtained for *P. aeruginosa* biofilm formation compared with the other biofilms. The well-known intrinsic resistance and acquired tolerance to antibiotics of *P. aeruginosa* biofilms [11] may also support the previous results. Its large and plastic genome favours the species in providing greater adaptability to most hostile environments and to antibiotic treatment, meaning that infection with this species is more arduous to treat [12].

Conversely, emerging bacteria did not show a great ability to form biofilms on the CBD. Nevertheless, the fact that some isolates do not form in vitro biofilms does not impair the ability of these organisms to survive in the patient lung, as recently suggested [13]. Indeed, the reduced capability of *I. limosus* and *D. pigrum* to form biofilms was reflected in their sensitivity to most tested antibiotics. These organisms showed significantly less biomass and number of cells than *P. aeruginosa*, making them more vulnerable to antimicrobial agents. The fact that these organisms require an extended incubation time could help explain the slow growth, which consequently results in the lowest amount of biomass and number of produced cells by their single biofilms. In addition, the slimy character of *I. limosus* bacterial colonies may contribute to the slow growth on the peg surface of the CBD.

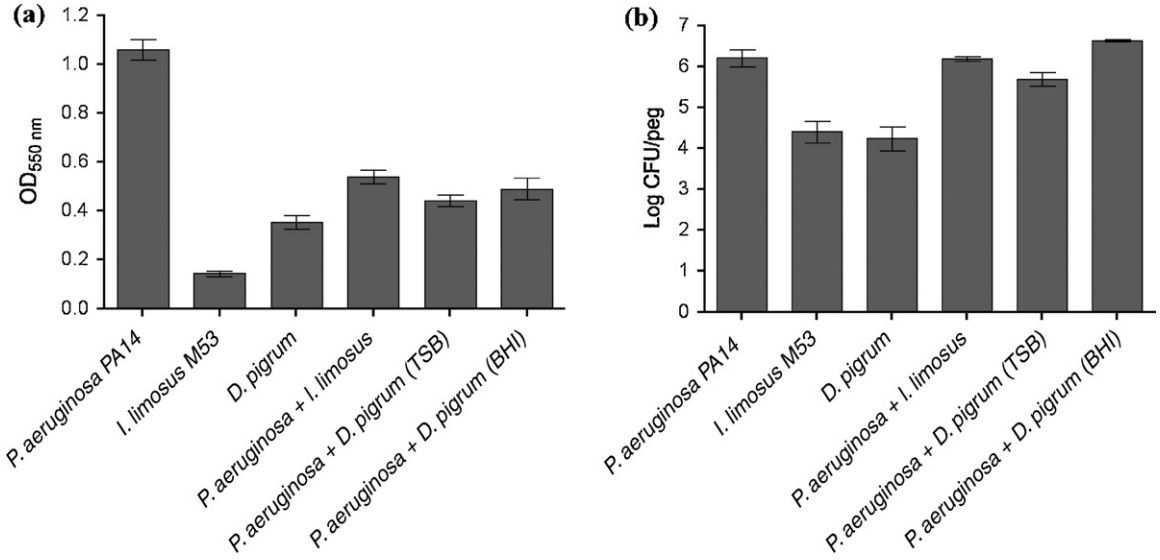

Fig. 1. (a) Biomass and (b) culturable cells obtained for single-species and dual-species biofilms after the respective period of incubation (see Table 1). The mean ± standard deviation for at least six replicates is illustrated. OD_{550}, optical density at 550 nm; TSB, tryptic soy broth; BHI, brain–heart infusion.

Most studies involving mixed biofilms in CF have only included classical pathogens such as *P. aeruginosa* and *B. cepacia* [14]. Co-infections of traditional pathogens with rare species in CF lungs remain largely unexplored, limiting the understanding of the importance of these interspecies interactions. In this study it was demonstrated that most antibiotics presented a poor activity against dual-species biofilms of *P. aeruginosa* with an emerging species. Generally, these biofilms required effective antibiotic concentrations at least equal to that used to kill the same species when in monospecies biofilms. The results obtained by selective media, showing the predominance of *P. aeruginosa* within the consortia, could be the basis for a higher contribution of *P. aeruginosa* to the antibiotic resistances presented by dual-species biofilms. It is recognised that mixed biofilms alter the metabolic activity of the consortium and hence may alter the susceptibility patterns of the population. This can reflect itself for instance in an alteration in the overall biofilm structure and extracellular matrix by both microorganisms, impairing access of antibiotics into the consortium, or by a decreased antibiotic uptake rate through the cell membrane. Here we have shown that independently of the reduced biofilm biomass formed and a decrease in matrix content, the arrangement and even the high number of biofilm-encased cells in mixed-species biofilms was enough to imply an increased resistance on those consortia. This strongly suggests that these organisms and eventually other unusual species might have a great importance in the outcome and treatment of infection in CF. Inaccurate identification of non-conventional pathogens and the disregard for the interactions between all bacteria may lead to ineffective antibiotic therapeutic strategies that could select for antibiotic-resistant pathogens.

In conclusion, although the novel species found in CF appear to be more easily treated by antibiotic therapy than the classical pathogens, they can enhance the antibiotic resistance of mixed populations where they are involved. It is clear that the complex interactions between bacteria in the host play an important role in the complex pathology of the disease and may be often responsible for the increase in antibiotic tolerance. Thus, treatment of infection in CF will probably be more effective in the future by categorising the disease as polymicrobial. It remains to be understood whether in those cases where resistance of *P. aeruginosa* CF biofilms to treatment occurs, we might in fact not be in the presence of a resistant strain of *P. aeruginosa* in the patient but rather of a polymicrobial colonisation.

Note: The data in this manuscript are available in www.biofomics.org, where it is possible to access them for research purposes.

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