BRITISH JOURNAL OF BIOMEDICAL SCIENCE
2021, VOL. 78, NO. 2, 95–97
https://doi.org/10.1080/09674845.2020.1819003

BIOMEDICAL SCIENCE IN BRIEF

Pseudomonas aeruginosa – *Candida* interplay: effect on *in vitro* antibiotic susceptibility of *Pseudomonas aeruginosa* when grown in the presence of *Candida* culture

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ARTICLE HISTORY Received 8 July 2020; Accepted 28 August 2020
KEYWORDS Cystic fibrosis; CF; *Pseudomonas aeruginosa*; *Candida albicans*; antibiotic resistance; quorum sensing

There are several clinical infection scenarios where the Gram-negative bacterium, *Pseudomonas aeruginosa* and the yeast, *Candida* spp. may co-exist in the pathological site of infection. Such co-habitation may be transient, as in the case of superficial cuts and abrasions, where the residency time is short and therefore not long enough to elicit potential physiological effects between these organisms. Conversely, there are clinical infection scenarios, including burns, diabetic foot and vascular ulcers, as well as cystic fibrosis lung infections, where these organisms are present chronically and where there is sufficient time for there to be physiological interplay between these organisms, which could have both positive and negative consequences for each organism, as a result of this interaction/relationship. Cystic fibrosis (CF) is a good model to use as an exemplar to investigate one such bacterial-yeast interaction further, namely that of antibiotic susceptibility to the predominant bacterial pathogen, *Pseudomonas aeruginosa*.

CF is the most common lethal autosomal recessive genetic condition in Caucasian populations, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This results in disruption or absence to normal chloride ion secretion in cells, due to defective or absent CFTR protein. In the lungs, this results in the production of thick sticky dehydrated mucus, which is difficult to clear, due to cessation of normal mucociliary clearance mechanisms. The volume and viscosity of this sputum overwhelms the ability of cilia to beat and collectively purge secretions from the lower airways, through intrinsic and normal host airway clearance mechanisms. This results in effective stagnation of such CF secretions at these anatomical locations [1]. Consequently, these now immobilised secretions act as a 'sink' for endogenous commensal flora from the patient, such as commensal yeasts, including *Candida* spp., as well as acting as a new home for newly acquired environmental organisms, including *Pseudomonas aeruginosa* [PA]. Through time, the microbial richness and biodiversity alters and many studies with CF lung microbiomes have demonstrated that CF lung biodiversity is important in disease progression [2]. *PA* is frequently isolated and is an important bacterial pathogen in CF, where recent data in the UK shows that it is present chronically in the lungs of 41.4% of adult (≥16 years; n = 2386/5952) and is present intermittently in 17.5% of the same adult CF population [3]. In children with CF, the occurrence is 5.9% (229/3895) and 19.4%, respectively [3].

Whilst recent advances in molecular and bioinformatics technology have allowed for the quantification of microbial diversity in the CF airways, little is yet known with regard to the physiological interactions and interplay that occurs between microbial neighbours in the CF lung, that may manifest as microbe–microbe interactions/associations, which could be potentially detrimental to the human host. Previous studies have shown that *Candida* spp. is a very common inhabitant of the CF airways, with almost near complete colonisation of CF adult patients (97%) [4], where *C. albicans* was the most frequently isolated *Candida* species (58%), followed by *C. dubliniensis, C. parapsilosis* and *C. glabrata* (39%, 9.1% & 5.2%, respectively) [4].

This study therefore aimed to examine the consequence for antibiotic susceptibility in PA (n = 8 isolates) to five commonly employed anti-pseudomonal antibiotics (ceftazidime, ciprofloxacin, colistin, meropenem, and tobramycin), when grown in the presence of *Candida* Culture Extracts (CCEs).

Three species of *Candida* were examined in this study, including *C. albicans, C. glabrata (Nakaseomyces glabrata)* and *C. parapsilosis*. Yeast organisms were obtained from the Northern Ireland Regional Mycology Reference Laboratory, Royal Victoria Hospital, Belfast,

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Northern Ireland. All *Candida* were clinical isolates. *Candida* isolates were propagated in the laboratory on Columbia Blood agar (Oxoid CM0031, Oxoid Ltd., Basingstoke, UK), supplemented with 5% (v/v) defibrinated horse blood for 48h at 37°C, under aerobic conditions and passaged a further three times, prior to use. Eight PA isolates, including six PA isolates from CF sputum and two PA blood culture isolates, were employed in this study. All bacterial isolates were part of the HSC Microbiology Culture Repository (MicroARK) housed at the Northern Ireland Public Health Laboratory, Belfast City Hospital. Their identification was confirmed employing the MALDI-TOF (BioMerieux Ltd., UK), in accordance with the manufacturer's instructions.

Candida isolates were inoculated individually into Nutrient Broth (Oxoid CM1) (200 ml), to give an initial inoculum of *circa* 10^6 colony forming units (cfu)/ml and incubated aerobically for 1 week at 37°C. Following this, *Candida* Culture Extracts (CCEs) were prepared by filter-sterilisation of the supernatant employing a Stericup-GP Sterile Vacuum Filtration system (150 mL process volume) through a 0.22-µm filter. CCEs were stored in the dark at 4°C until employed.

Sterile CCE (50mls) was added to Mueller-Hinton agar (450mls) in order to prepare 10% [v/v] CCE-supplemented agar. All PA isolates were investigated and their antibiotic susceptibility to five anti-pseudomonal antibiotics (ceftazidime (30 µg disk), ciprofloxacin (5 µg), colistin (10 µg), meropenem (10 µg) & tobramycin (10 µg)) was determined by disk diffusion assay employing Clinical and Laboratory Standards Institute (CLSI) methodology and interpretive criteria [5]. Plates were incubated aerobically for 48 h at 37 °C and zones of inhibition (mm) were recorded and compared to zone sizes of the control (with no CCE present). Each isolate was classified as sensitive, intermediately resistant or resistant, according to CLSI criteria [5].

Statistical analyses were performed using the student t-test (2-tailed; paired) comparing zones of the treatments with CCE incorporated, with the control (no CCE present). Probability values less than 5% (<0.05) were considered statistically significant.

The effect on antibiotic susceptibility when employing Mueller-Hinton agar supplemented with 10% [v/v] CCE from *C. albicans, C. glabrata* and *C. parapsilosis* is shown in Table 1. Statistically, there were significant differences in the eight PA isolates examined in mean zone sizes of the five antipseudomonal antibiotics, with the exception of *C. glabrata* and ciprofloxacin ($p = 0.36$) and meropenem ($p = 0.07$). Overall, when grown in the presence of CCEs, PA's antibiotic susceptibility increased with all antibiotics, with the exception of *C. albicans* and *C. parapsilosis* with meropenem, where antibiotic susceptibility decreased. This is in contrast to the effect on PA susceptibility seen with the other β-lactam antibiotic examined, namely ceftazidime, where antibiotic susceptibility increased significantly in the presence of each of the three CCEs examined.

Several previous reports have highlighted interplay between PA and *Candida*, where several factors have been responsible including (i) the role of cell wall components [6], (ii) quorum sensing molecules [6], (iii) promotion of colonisation with *Candida* spp. associated with increased risk of PA-associated ventilator-associated pneumonia [7], (iv) increase in fungal colonisation promoted pneumonia by PA, (v) prior *C. albicans* colonisation promoted enhanced PA clearance [8] and (vi) increased virulence with PA observed in a Zebrafish swimbladder model with *Candida* co-infection [9].

Certain important differences exist with this study that would not be normally found in the *in vivo* state within the CF airways. Firstly, our study was performed exclusively using planktonic cells, all of which were not within any chronic *Candida* and *Pseudomonas* biofilm relationship, which would be more representative of the *in vivo* CF state. Secondly, the metabolic and physiological status of the current study differed in that nutrients had not been exhausted, as would be the case in the chronic CF airway, thereby placing extra stress responses on both organisms within chronic airways *in vivo*. With these differences, it is difficult to extrapolate as to whether or not such observed antibiotic synergism with PA brought about through *Candida* secondary metabolites, would be functional *in vivo* in the CF airways. Nevertheless, given the increasing burden of antibiotic resistance seen in CF PA, such synergistic relationships are worthy of further exploration, in an attempt to reduce growing resistance burdens and potentially offer a route to antibiotic resistance reversal.

Table 1. Antibiotic susceptibility of *Pseudomonas aeruginosa* (n = 8) against five anti-pseudomonal antibiotics when grown in the presence of *Candida* Culture Extracts (CCEs) (n = 3).

Mean zone of inhibition, mm (standard deviation) [p value]				
Antibiotic (disk concentration µg)	Control (no CCE)	+ *C. albicans* CCE	+ *C. parapsilosis* CCE	+ *C. glabrata* CCE
Ceftazidime (30 µg)	29.8 (5.0)	37.3 (5.1) [0.0002]	41 (3.6) [<0.0001]	36.3 (4.8) [0.01]
Ciprofloxacin (5 µg)	21.3 (13.4)	27.3 (17.0) [0.04]	28.5 (13.0) [0.01]	23.1 (16.0) [0.36]
Colistin (10 µg)	12.5 (2.2)	21.3 (2.6) [<0.0001]	23 (2.8) [<0.0001]	19.3 (3.0) [<0.0001]
Meropenem (10 µg)	33.8 (12.3)	20.0 (11.3) [0.02]	23.5 (10.8) [0.01]	39.8 (6.0) [0.07]
Tobramycin (10 µg)	16.8 (2.0)	28.7 (2.4) [<0.0001]	28.3 (2.1) [0.0001]	31.3 (5.7) [0.001]

CCE = *Candida* Culture Extracts

In the current study presented herein, the interplay between PA and *Candida* largely resulted in increased antibiotic susceptibility of the PA isolates to ceftazidime, ciprofloxacin, colistin, and tobramycin, but not to meropenem, which showed decreased antibiotic susceptibility with *C. albicans* and *C. parapsilosis*. Further work is now required to help elucidate the underlying mechanisms accounting for increased susceptibility in PA, as well as the chemical composition of the secondary metabolites excreted by the *Candida* spp. leading to increased PA susceptibility.

Whilst this increase in antibiotic susceptibility was noted under the experimental conditions employed, such interactions between neighbouring microbial flora *in vivo* would be missed, when antibiotic susceptibility is tested using routine microbiology service-based Standard Operating Procedures (SOPs), as these are performed under highly standardised conditions in monoculture. This, in turn, raises the concern of either under-reporting or over-reporting antibiotic susceptibility when solely relying on laboratory service SOPs. Therefore, *in vitro* antibiotic susceptibility should be therefore regarded as an important, but not an absolute measure of an organism's response to a given antibiotic, as the *in vitro* scenario is unable to factor in the syntheses of variables, which the *in vivo* scenario presents.

This work represents an advance in biomedical science, as it demonstrates novel interplay involving antibiotic susceptibility, between an established respiratory bacterial pathogen and hitherto, what are regarded as commensal yeast organisms. This interplay may be exploited therapeutically in the clinical management of CF airway disease, as well as helping to further develop our understanding of complex ecological interactions within the CF lung microbiome, through such near-patient *in vitro* studies. Future studies examining potential antimicrobial synergies by combining CCEs from different *Candida* spp., as well as the effect of *Pseudomonas aeruginosa* extracts on *Candida* spp could potentially identify further interactions between *Pseudomonas aeruginosa* and *Candida* spp. with therapeutic relevance.

Disclosure statement

No potential conflict of interest was reported by the authors.

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