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Clinical Paper

Discovery of inhibition of *Burkholderia cenocepacia*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* by the Brown Rot Basidiomycete Fungus, *Postia placenta*

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ABSTRACT

Antimicrobial resistance (AMR) has now emerged as a major global public health problem. Certain bacterial pathogens, particularly Gram negative organisms associated with patients with cystic fibrosis (CF), have become resistant to several classes of antibiotics resulting in pan-resistance, which creates a clinical treatment dilemma. This study wished to explore the production of antibacterial extracellular metabolites from plant pathogenic fungi. Fungal Culture Extracts (FCEs) were prepared from 10 fungi (*Armillaria gallica, Clitocybe nebularis, Fusarium coeruleum, Fusarium oxysporum, Fusarium poae, Hymenoscyphus fraxineus, Nectria fuckeliana, Phytophthora infestans, Phytophthora ramorum, Postia placenta*), which were tested for activity against the CF pathogens, *Pseudomonas aeruginosa* (PA) (n=8), *Burkholderia cenocepacia* (n=2) and *Stenotrophomonas maltophilia* (n=2). In addition, FCE were assessed for their ability to alter antibiotic susceptibility in PA (n=8), with six antipseudomonal antibiotics (ceftazidime, ciprofloxacin, colistin, meropenem, piperacillin/tazobactam, tobramycin). None of the FCEs showed inhibitory activity to the 12 bacterial isolates tested, with the exception of the FCE from *Postia placenta*, which showed inhibition against all 12 bacteria. An antagonistic interaction was observed, where a statistically significant decrease in mean zone sizes was noted with *Armillaria gallica* (p=0.03) and *Phytophthora infestans* (p=0.03) FCEs and their interaction with the fluoroquinolone antibiotic, ciprofloxacin. Given the increase in clinical morbidity and mortality associated with chronic lung infections with *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia*, coupled with the difficulty in treating such chronic infection due to overwhelming antimicrobial resistance, any novel substance showing inhibition of these organisms merits further investigation as a potential future antimicrobial agent, with potential clinical therapeutic application.

INTRODUCTION

Antimicrobial resistance (AMR) has now emerged as a global health crisis, where it is estimated by the World Health Organisation (WHO) that in 2016, there were 490,000 persons infected with tuberculosis (TB), which was multidrug resistant[1]. Furthermore, the WHO states that AMR threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi, as well as indicating that without effective antibiotics, the success of major surgery and cancer chemotherapy could be compromised[1]. Whilst the burden of AMR is increasing with several organisms, it is particularly worrying in treating chronic Gram-negative infections in patients with cystic fibrosis (CF), where AMR has developed at an alarming rate, to the extent that there are some infections which are resistant to all classes of antibiotics presenting a treatment dilemma[2], Development of AMR in the causal bacteria of chronic infections may result in denial of lung transplantation and access to important clinical trials of new therapies.

Therefore, such clinical dilemmas create an urgent need to discover effective alternative antimicrobial agents that have proven *in vitro* activity against such bacteria and can translate into *in vivo* efficacy. This can be accomplished through accelerated drug discovery programmes, drug re-purposing and through revisiting antibiotics from fungi programmes, the so-called "Fleming II" approach. Fungi are a rich source of bioactive compounds in nature, including the antimicrobials, produced as secondary fungal metabolites[3]. and this, coupled with their biodiversity of an estimated 1.5 – 5.1 million existing species, they remain a potentially important source of discovering novel antimicrobial agents[3].

Therefore, the aim of this study was to examine the production of antibacterial substances from 10 plant pathogenic fungi

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and their *in vitro* effect on the Gram-negative CF pathogens, *Burkholderia cenocepacia*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia,* as well as examining the interaction of fungal culture extracts with conventional antibiotics.

MATERIALS AND METHODS

Description of environmental fungi and bacteria employed

Ten species of environmental plant pathogenic fungi were examined in this study (Table 1). Fungal organisms were isolated from diseased plant material. Fungal isolates were propagated in the laboratory at 22°C in Potato Dextrose Sabouraud Maltose Broth (PDSMB) consisting of equal volumes of Potato Dextrose broth (potato extract; 4g/L, glucose; 20g/L) and Sabouraud Maltose broth (Maltose; 20g/L, neo-peptone; 10g/L, $MgSO_4.7H_2O$; 1g/L, KH_2PO_4;1g/L). The identity of all species was confirmed by PCR amplification and sequencing of rRNA genes. The Gram-negative bacteria (n=12 isolates), including *Pseudomonas aeruginosa* (n=8), *Burkholderia cenocepacia* (n=2) and *Stenotrophomonas maltophilia* (n=2) 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. All isolates were originally isolated from sputum of adult patients with cystic fibrosis. All bacterial isolates were recovered on Columbia Blood agar (Oxoid

Table 1: Description of environmental macro- and filamentous plant pathogenic fungi examined in this study

Environmental fungi	Common name	Taxonomy (Phylum)	Description	Previous antimicrobial reports
Armillaria gallica	Bulbous Honey Fungus/White Rot Fungus	*Basidiomycota*	Macrofungus.Plant pathogen; Root rot of garden trees	None
Clitocybe nebularis	Clouded Funnel/ Clouded Agaric	*Basidiomycota*	Macrofungus. Saprophytic fungus found in under conifers	Antifungal activity [4]
Fusarium coeruleum	None	Ascomycota	Filamentous fungus. Plant pathogen causing dry rot of potatoes	None
Fusarium oxysporum	None	Ascomycota	Filamentous fungus. Plant pathogen causing a variety of wilt diseases	Antibiotic activity [5]
Fusarium poae	None	Ascomycota	Filamentous fungus. Plant pathogen causing Head Blight in wheat	Antibiotic activity [6]
Hymenoscyphus fraxineus	None	Ascomycota	Filamentous fungus. Plant pathogen causing Ash Dieback disease in Ash trees (*Fraxinus excelsior*)	None
Nectria fuckeliana	None	Ascomycota	Saprophytic fungus and plant pathogen causing apple canker, Nectria twig blight and coral spot in orchards. Flute canker.	None
Phytophthora infestans	Oomycete or potato blight mould	Oomycota	Filamentous fungus. Plant pathogen causing potato blight disease	Antibiotic activity [7]
Phytophthora ramorum	Oak blight	Oomycota	Filamentous fungus: Plant pathogen causing sudden oak death	None
Postia placenta	Brown rot disease	Basidiomycota	Macrofungus. Plant pathogen causing brown rot disease.	None

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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. Their identication was confirmed employing the MALDI-TOF (BioMerieux Ltd., UK), in accordance with the manufacturer's instructions.

Preparation of fungal culture extracts (FCEs)
Plant pathogenic fungi (Table 1) were inoculated in PDSMB, as described above and slowly agitated aerobically for six weeks at 22°C. Following this, FCEs were prepared by filter-sterilisation of the supernatant employing a Stericup-GP Sterile Vacuum Filtration system (150mL process volume) through a 0.22 µm filter. FCEs were stored in the dark at 4°C until employed.

Antibacterial susceptibility assays with FCEs

(i) **Direct Effect**: Inoculum (0.5 McFarland standard) of each of the 12 isolates described above were streaked individually on to the surface of individual Columbia Agar supplemented with 5% (v/v) defibrinated horse blood. The plates were labelled in sectors representing each FCE drug plus appropriate controls. FCE extracts (10 µl) were pipetted onto the media and left to dry. The plates were inverted and incubated at 37°C incubator for 48h. Any observed zones of inhibition (mm) in the region of FCE was measured and recorded.

(ii) **Indirect Effect**: Five plant pathogenic fungi from Table 1, including *Armillaria gallica*, *Clitocybe nebularis*, *Fusarium oxysporum, Nectria fuckeliana, Phytophthora infestans*[4,5,6,7] were included. FCEs were prepared individually for each of these fungi, as described above. FCE (50mls) was added to Mueller-Hinton agar (450mls) in order to prepare 10% [v/v] FCE-supplemented agar. Eight PA isolates, including six PA isolates from CF sputum and two PA blood culture isolates, were investigated and their antibiotic susceptibility to six anti-pseudomonal antibiotics (ceftazidime (30µg disk), ciprofloxacin (5µg), colistin (10µg), meropenem (10µg), piperacillin/tazobactam (110µg) & tobramycin (10µg)) was determined by disk diffusion assay employing Clinical and Laboratory Standards Institute (CLSI) methodology and interpretive criteria[8]. Plates where incubated aerobically for 48hrs at 37°C and zones of inhibition (mm) were recorded and compared to zone sizes of the control (with no FCE present). Each isolate was classified as sensitive, intermediately resistant or resistant, according to CLSI criteria[8].

RESULTS AND DISCUSSION

None of the FCEs showed inhibitory activity to the 12 bacterial isolates tested, with the exception of the FCE from *Postia placenta*, which showed activity against all 12 bacterial isolates tested (Figure 1). The effect on antibiotic susceptibility when employing Mueller-Hinton agar supplemented with 10% [v/v] FCE from five plant pathogenic fungi is shown in Table 2(a)-(f). Statistically,

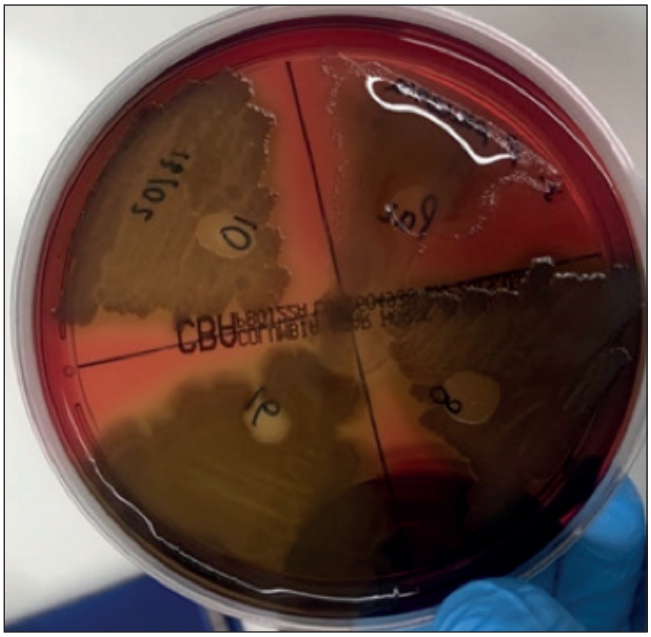

Figure 1:
Inhibition of four isolates of *Pseudomonas aeruginosa* isolated from sputum of patients with cystic fibrosis with Fungal Culture Extracts from the Brown Rot fungus, *Postia placenta*

there was significant differences in the eight PA isolates examined in mean zone sizes of the six anti-pseudomonal antibiotics with *Clitocybe nebularis*, *Fusarium oxysporum* and *Nectria fuckeliana.* [4,5,6,7] An antagonistic interaction was observed between two fungi and ciprofloxacin zone sizes, where a statistically significant decrease in mean zone sizes was noted with *Armillaria gallica* (p=0.03) and *Phytophthora infestans* (p=0.03) FCEs and their interaction with the fluoroquinolone antibiotic, ciprofloxacin, where zone sizes decreased from 25.3mm (control with no FCE added) to 19mm and 13.3mm, respectively. This is an interesting observation, in that these fungi are in some way interfering with the antibacterial properties of this fluoroquinolone by making it less antibacterial. At this stage, we are unaware of the constituent components of the fungal extracts which could account for this effect on the fluoroquinolone, but this antagonism may be due in part to the *in vitro* lowering of the pH and the release of cations (Ca^{2+}, Mg^{2+}).

Of all the environmental fungi examined, only one, namely *Postia placenta* showed to have antibacterial activity against all Gram-negative organisms tested. These bacterial pathogens were selected, as they can be difficult-to-treat clinically due to being multi- and, at times, pan-resistant to all antipseudomonal antibiotics available. Therefore, without an efficacious antibiotic available to clinically treat such cases, it is important to seek novel molecules that can inhibit the growth of these organisms.

Postia placenta, formerly known as *Rhodonia placenta*, is a brown rot macrofungus that is largely responsible for the decay of wooden structures by rapid depolymerisation of cellulose, where it is a cause of wood rot in ships, in mines

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attacking wooden pit props and in the timber of buildings[9]. Taxonomically, this basidiomycete belongs to the Phylum*: Basidiomycota* - Class: *Agaricomycetes* - Order: *Polyporales* - Family*: Fomitopsidaceae.* Pathologically, this fungus acts on wood through cellulose degrading mechanisms, including the enzymatic degradation by small cellulases and through the generation of hydroxyl free radicals, via Fenton chemistry, where Fe(II) and H_2O_2 react to form hydroxyl radicals (OH^-) [$H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + OH$] [10]. Analysis of the *P. placenta* genome revealed few conventional cellulases suggesting that much of its cellulose degradation involves the production of free radicals[10]. The production of free radicals by *Postia* may account for its antibacterial activity against bacterial pathogens, which are susceptible to such oxygen scavenging species. Additionally, other genome, transcriptome and secretome analysis of this fungus have identified other antibacterial molecules, including quinones which may also add to its antibacterial activity.

Previously, there has been a report on a novel fungal immunomodulatory protein (rFIP-ppl) from *Postia placenta*, where antitumor assays demonstrated significant cell proliferation inhibitory activity and apoptotic effects in human tumour cells, particularly on gastric tumour cells (MGC823) than against hepatoma (HepG2) cells[11]. To date, there have been no reports of extracts of this polypore fungus demonstrating antibacterial activity, therefore the antibacterial activity against the Gram-negatives described in this report are novel and worthy of further investigation.

In conclusion, FCEs from the brown rot fungus, *Postia placenta*, inhibited these important CF Gram-negative pathogens on all occasions, whilst similar FCEs from nine other pathogenic plant fungi did not show any antibacterial activity. Given the increase in clinical morbidity and mortality associated with chronic lung infections with *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Stenotrophomonas maltophilia*, coupled with the difficulty in treating such chronic infection due to overwhelming antimicrobial resistance, any novel substance showing potential in inhibiting these organisms merits further investigation as a potential future antimicrobial agent, with potential benefits in the treatment of such difficult-to-treat infections.

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CONFLICT OF INTEREST

None

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Table 2a-f: Zones of inhibition created by six antibiotics on PA strains (n=8) when grown on Muller-Hinton agar supplemented with 10% [v/v] Fungal Culture Extracts (FCEs).

The tables are colour coded depending on whether the strain showed sensitivity, intermediate resistance or resistance to the antibiotic according to CLSI criteria. If a strain susceptibility classification was altered when grown on a media including fungal supernatant when compared to the Standard Muller Hinton control (Table 2a), the zone of inhibition is outlined. Zones of inhibition where analysed using a two-tailed paired student t-test with significant p values (p=<0.05) noted in red. Tables titled according to FCE incorporated.

Key as per CLSI standards	Sensitive	Intermediately resistant	Resistant	Different from Control Plate	PA = *Pseudomonas aeruginosa*

Table 2a – Mueller-Hinton Control (no Fungal Cultural Extract added)

Isolate	Zone of inhibition (mm)					
	Ceftazidime CAZ30	Tobramycin TOB10	Colistin CT10	Meropenem MEM10	Ciprofloxacin CIP5	Piperacillin/ Tazobactam TZP110
PA CF/05/11	30	26	16	28	26	14
PA CF/96/06	28	28	8	0	10	28
PA BC/07/658	0	22	12	34	32	28
PA CF/96/49	22	24	14	32	18	18
PA CF/05/49	36	24	16	34	38	24
PA CF/96/33	34	30	18	18	10	38
PA CF/05/56	26	28	18	0	32	30
PA 91/BC/07	16	24	0	26	36	16
Mean	24	25.75	12.75	21.5	25.25	24.5

Table 2b - *Nectria fuckeliana*

Isolate	Zone of inhibition (mm)					
	Ceftazidime CAZ30	Tobramycin TOB10	Colistin CT10	Meropenem MEM10	Ciprofloxacin CIP5	Piperacillin/ Tazobactam TZP110
PA CF/05/11	46	0	20	36	28	34
PA CF/96/06	28	28	18	28	0	26
PA BC/07/658	0	20	14	10	32	0
PA CF/96/49	22	20	10	32	16	0
PA CF/05/49	28	22	12	40	32	26
PA CF/96/33	0	34	14	20	8	24
PA CF/05/56	20	32	10	36	30	36
PA 91/BC/07	18	24	0	26	32	20
Mean	20.25	22.5	12.25	28.5	22.25	20.75
Individual antibiotics (p=)	0.48	0.37	0.81	0.32	0.06	0.51
All antibiotics (p=)	0.50					

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Table 2c - *Phytophthora infestans*

Isolate	Zone of inhibition (mm)					
	Ceftazidime CAZ30	Tobramycin TOB10	Colistin CT10	Meropenem MEM10	Ciprofloxacin CIP5	Piperacillin/ Tazobactam TZP110
PA CF/05/11	36	30	14	26	0	36
PA CF/96/06	0	28	0	24	0	0
PA BC/07/658	26	24	0	20	0	28
PA CF/96/49	22	0	14	30	0	0
PA CF/05/49	ND	24	16	36	26	0
PA CF/96/33	28	32	0	16	8	26
PA CF/05/56	28	32	0	36	36	26
PA 91/BC/07	18	26	14	26	36	20
Mean	22.57	24.50	7.25	26.75	13.25	17.00
Individual antibiotics (p=)	0.96	0.72	0.19	0.39	**0.03**	0.24
All antibiotics (p=)	0.10					

Table 2d – *Fusarium oxysporum*

Isolate	Zone of inhibition (mm)					
	Ceftazidime CAZ30	Tobramycin TOB10	Colistin CT10	Meropenem MEM10	Ciprofloxacin CIP5	Piperacillin/ Tazobactam TZP110
PA CF/05/11	38	30	8	28	30	34
PA CF/96/06	26	34	0	24	0	28
PA BC/07/658	16	24	0	14	36	0
PA CF/96/49	18	24	14	32	0	0
PA CF/05/49	28	26	16	36	26	26
PA CF/96/33	30	32	0	0	0	28
PA CF/05/56	32	0	18	36	30	26
PA 91/BC/07	0	0	14	26	30	18
Mean	23.5	21.25	8.75	24.5	19	20
Individual antibiotics (p=)	0.89	0.37	0.28	0.67	0.06	0.41
All antibiotics (p=)	0.13					

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Table 2e - *Clitocybe nebularis*

Isolate	Zone of inhibition (mm)					
	Ceftazidime CAZ30	Tobramycin TOB10	Colistin CT10	Meropenem MEM10	Ciprofloxacin CIP5	Piperacillin/ Tazobactam TZP110
PA CF/05/11	36	28	10	30	28	0
PA CF/96/06	20	28	22	20	0	26
PA BC/07/658	0	22	14	26	32	28
PA CF/96/49	22	0	14	34	18	26
PA CF/05/49	26	22	0	36	0	28
PA CF/96/33	26	34	20	20	0	26
PA CF/05/56	36	0	16	38	32	28
PA 91/BC/07	20	24	14	28	36	18
Mean	23.25	19.75	13.75	29	18.25	22.5
Individual antibiotics (p=)	0.78	0.22	0.78	0.19	0.18	0.48
All antibiotics (p=)	0.48					

Table 2f - *Armillaria gallica*

Isolate	Zone of inhibition (mm)					
	Ceftazidime CAZ30	Tobramycin TOB10	Colistin CT10	Meropenem MEM10	Ciprofloxacin CIP5	Piperacillin/ Tazobactam TZP110
PA CF/05/11	0	28	0	28	28	34
PA CF/96/06	20	30	18	24	0	22
PA BC/07/658	0	24	12	28	30	14
PA CF/96/49	18	24	14	34	0	18
PA CF/05/49	ND	0	16	38	32	26
PA CF/96/33	ND	32	18	16	0	32
PA CF/05/56	36	28	0	36	28	26
PA 91/BC/07	16	22	12	26	34	16
Mean	15	23.5	11.25	28.75	19	23.5
Individual antibiotics (p=)	0.38	0.50	0.70	0.21	**0.03**	0.78
All antibiotics (p=)	0.42					

The tables are colour coded depending on whether the strain showed sensitivity, intermediate resistance or resistance to the antibiotic according to CLSI criteria. If a strain susceptibility classification was altered when grown on a media including fungal supernatant when compared to the Standard Muller Hinton control (Table 2a), the zone of inhibition is outlined. Zones of inhibition where analysed using a two-tailed paired student t-test with significant p values (p=<0.05) noted in red. Tables titled according to FCE incorporated.

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