Pseudomonas aeruginosa
In Tasmania

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Dedicated to God and to my Father
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Abstract

*Pseudomonas aeruginosa* is an organism commonly found in the environment, and one of the most common causes of infectious disease in humans. Infections caused by *P. aeruginosa* may present in many forms, reflective of the great versatility of this organism. *P. aeruginosa* infection occurs more commonly in patients with some form of immunocompromisation, and this is particularly significant in the nosocomial setting and as a cause of chronic infection in the cystic fibrosis (CF) lung.

Tasmania is an island state in the southernmost portion of Australia. It has a relatively small, isolated population with a greater balance of the population living in rural and regional areas than any other state in Australia. The state has a single tertiary care referral hospital in the capital city, Hobart and amongst the highest number of CF births per capita of anywhere in the world. Until 2003, CF patients in Tasmania did not attend any centralised clinics, and had little social or other contact with each other. These factors provided a unique opportunity to study the epidemiology of *P. aeruginosa* infections in a whole population sample.

In addition, a large number (n=184) of *P. aeruginosa* isolates from diverse clinical and environmental sources, including isolates from adult CF patients, were obtained from both within the major tertiary referral hospital in Tasmania and the wider community. Antimicrobial resistance testing was performed on all isolates by four separate methods and the results of these compared. A sub group of CF and hospital environment strains appeared to present with an increased propensity towards antimicrobial resistance and frank multi-drug resistance. Molecular epidemiological analysis of the CF strains revealed a single genotype of *P. aeruginosa* to be infecting over a quarter of the adult CF patients in the state. Isolates of the genotype concerned showed a greater propensity towards multi-drug resistance than any other cohort of *P. aeruginosa* strains included in the study, and were shown to
cause poorer clinical outcomes in infected patients. The strain was determined to be a new CF clonal complex, described as Australian Epidemic Strain 3 (AES3). The source of this strain appears to a CF summer camp which occurred more than ten years prior to this study. A further common genotype (infecting 11% of adult Tasmanian CF patients) was identified. This strain was described as the “Tasmanian CF cluster strain”.

Further molecular epidemiological analysis of P. aeruginosa strains from infected patients within the major tertiary referral hospital and the wider community, as well as environmental isolates from these and other sites showed that the AES3 and Tasmanian CF cluster strains are not common in non-CF patients, and do not have an obvious environmental source either in the hospital or the wider community.

A survey of the virulence factor genes associated with all isolates in this study was performed. This represented one of the most comprehensive studies of virulence genes over a wide range of P. aeruginosa isolates ever performed. The study found no specific difference in the prevalence of these genes between AES3 strains and other CF strains. CF strains were less likely to carry the low prevalence, horizontally transferred, exoU virulence gene. Conversely, isolates recovered from environmental sampling in the hospital intensive care and neurosurgical wards showed an increased propensity towards both antimicrobial resistance and exoU+ genotype.

A selected group of CF, non-CF clinical and environmental isolates were subjected to an assay of global cellular virulence in a novel modification of the Dictyostelium discoideum eukaryotic virulence assay employing two
D. discoideum mutants. The majority of clinical CF isolates supported the growth of D. discoideum. D. discoideum was unable to grow on any other isolates of P. aeruginosa, except one environmental isolate which supported the growth of only one of the two D. discoideum mutants tested. No difference in the capacity of clonal complex strains and unique CF strains to support D. discoideum growth and development was identified. Variations in the capacity of D. discoideum to develop beyond the amoeboid stage were noted within the CF isolates. No significant differences were noted between assays performed in the presence or absence of azithromycin, ceftazidime or tobramycin.

This was the first study of which we are aware to demonstrate the capacity of wild-type CF P. aeruginosa strains to support the growth of D. discoideum, and has provided significant findings with regard to “whole cell” virulence of this organism, and its down-regulation in the CF lung. Differences in the degree of development of D. discoideum on CF isolates may also lead to new insights into the mechanisms of virulence in such strains in the human disease setting.

The work presented in this thesis has found new information regarding the epidemiology of P. aeruginosa infections. It has also provided new information regarding the distribution of the exoU gene in hospital environmental isolates, and the association of this genotype with hospital intensive therapy wards. Finally, in describing a novel modification of the D. discoideum virulence model, and applying this model to multiple clinical and environmental isolates of P. aeruginosa, this work has added to the body of scientific knowledge regarding the expression of virulence by P. aeruginosa isolates from different clinical and environmental sources.
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Conference Abstracts


Bradbury, R., A. Champion, 2006 “Investigations into the Tasmanian Clonal Strain of Pseudomonas aeruginosa”, Proffered Paper. AIMS/AACB National Scientific Meeting, Hobart


Bradbury, R., D. Reid, A. Champion, 2005 “Molecular Epidemiology of Pseudomonas aeruginosa Infections in Tasmania”, Proffered Paper. Australian Society for Microbiology National Scientific Meeting, Canberra (PP10.1)


Bradbury, R., D. Reid, A. Champion, 2004 “Genome Diversity of Pseudomonas aeruginosa Isolates From Tasmanian Cystic Fibrosis Patients”, Proffered Paper. Australian Society for Microbiology National Scientific Meeting, Sydney (PP32.6)
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