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Preliminary feasibility and modelling of a liquid matrix *Dictyostelium discoideum* virulence assay for *Pseudomonas aeruginosa*

Alan Charles Champion[a,b], Neralee Kaye Houston[a], Richard Stewart Bradbury[c] and David William Edward Cochrane Reid[d]

[a]School of Medicine, University of Tasmania, Hobart, Australia; [b]Faculty of Science, Medicine and Health, University of Wollongong, Wollongong, Australia; [c]School of Medical & Applied Sciences, Central Queensland University, Rockhampton, Australia; [d]QIMR Berghofer Medical Research Institute, Herston, Australia

ABSTRACT

Objectives: To develop and determine the feasibility of using a liquid matrix adaptation of the *Dictyostelium discoideum* bacterial virulence assay by testing on well-characterised clinical and environmental isolates of *Pseudomonas aeruginosa*.

Materials and methods: Axenic AX2 *D. discoideum* were co-cultured with clinical and environmental isolates of *P. aeruginosa* in costar 24-well tissue culture plates for 24 h. A *P. aeruginosa* PAO1 positive control was tested in biological quintuplicate. Wells were then inspected using an inverted microscope and the degree of cytotoxic changes (sparse growth compared to control combined with rounding of cells and cytoplasmic shrinkage) on the *D. discoideum* cells was observed. A *Klebsiella aerogenes* negative control was included with each assay series.

Results: Sixty-five clinical and 20 environmental *P. aeruginosa* isolates were tested in the model. Cystic fibrosis respiratory isolates were found to be significantly ($P < 0.05$) less cytotoxic than *P. aeruginosa* from other sources. Limitations attached to the funding of this paper did not allow validation against previously employed models or animal models.

Discussion: A liquid matrix *D. discoideum* model for the analysis of *P. aeruginosa* virulence in a eukaryotic host is feasible, but further validation of the model is required before it may be employed in routine setting.

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Introduction

Over the past decade, there has been increasing interest in the use of eukaryotic models for assaying the virulence of pathogenic bacteria. Such models are intended to provide a flexible, reproducible and reliable measure of virulence without the need to use mice or other vertebrates in research. Apart from the imperative on the scientific community to ensure that animal models are used only where necessary, and where meaningful results are likely to be obtained, non-vertebrate cell models are generally far less expensive, more easily up-scaled and have a shorter generation time than laboratory animal or mammalian cell culture models.

Various non-vertebrate models have been employed for this purpose with regard to the common opportunistic pathogen *Pseudomonas aeruginosa*, including the nematode *Caenorhabditis elegans*,[1] *Galleria mellonella* caterpillars,[2] *Drosophila melanogaster* fruit flies,[3] *Arabidopsis thaliana* water cress[1] and *Acanthamoeba polyphaga*.[4] However, it is the various models employing the social amoeba *Dictyostelium discoideum* that have been employed the most extensively.[5–12]

In previous papers, the majority of wild-type clinical strains of *P. aeruginosa* have been shown to be incapable of supporting the growth of *D. discoideum* when grown on agar plates. Work by Lelong et al.[8] and Bradbury et al.[7] showed that cystic fibrosis (CF) strains have a far greater propensity to support the growth of *D. discoideum*, demonstrating decreased virulence towards eukaryotic phagocytic cells in these bacterial isolates. One of these studies also found a non-CF environmental (swimming pool) isolate of *P. aeruginosa* that showed similar attenuated virulence,[7] demonstrating that this is not a factor exclusive to CF isolates. Lelong et al.[8] showed that the virulence of CF isolates of *P. aeruginosa* may be attenuated in a *D. discoideum* model over the course of infection.

Almost all previous *D. discoideum* assays of the virulence of *P. aeruginosa* have been performed on a solid agar matrix. A liquid state *D. discoideum* virulence model has only been reported once in the literature.[13] However, this model required co-culture with *Klebsiella aerogenes*, a potentially confounding factor in results, and has not been performed again since. While useful,

CONTACT Alan Charles Champion alanc@uow.edu.au

easy and convenient, the use of solid agar medium has several limitations. The ratio of bacteria to amoebae cannot be easily controlled and therefore virulence is measured in an excess of bacteria. A cell culture supernatant liquid assay has been described by Cosson et al.[5] and also used by Ouellet et al.[12] This method measures the effects of filtered supernatants of overnight cultures of *P. aeruginosa* on *D. discoideum*, an effective system for gross supernatant virulence, but not allowing observations of the subtleties of host–pathogen interaction in co-culture.

This series of experiments was designed to test the hypothesis that a *D. discoideum* liquid matrix assay for the virulence of *P. aeruginosa* is a feasible alternative to current solid media-based models. This preliminary model was tested on numerous clinical and environmental isolates of *P. aeruginosa* and inferences regarding the overall feasibility of assay design and the relative virulence of the tested strains were drawn from the results.

Material and methods

Bacterial culture

A well-characterised collection of *P. aeruginosa* clinical and environmental strains [14] was used in this study (Table 1). All strains tested were originated from different patients or environmental sources. One of the CF isolates tested belonged to Australian Epidemic Strain 3 (AES3). None of the CF isolates tested were found to possess *exoU* according to work previously performed. [14] Strains were recovered from storage in maintenance media at –20 °C, and spread onto nutrient agar plates for incubation at 37 °C. The formula for the nutrient agar (VSM) was the same as the previously described modified SM agar.[7] A specified requirement of the supporting granting body was that no animal products be used in the course of experimentation. Hence, bacteriological peptone was replaced with vegetable peptone (Vegetone VHPL5; Oxoid, Thebarton, South Australia) for this assay.

Dictyostelium culture

Axenic AX2 *D. discoideum* spores from silica gels were washed by centrifugation in salt solution (SS)[6] and allowed to germinate in sterile axenic medium at 21 °C. Axenic medium was identical to HL5 medium except that vegetable peptone was substituted for bacteriological peptone. The media contained (per litre): 14 g vegetable peptone (Vegetone VHPL5; Oxoid, Thebarton, South Australia); 7 g of yeast extract (Oxoid LP0021, Thebarton, South Australia); 13.5 g of glucose (Sigma–Aldrich, St Louis, MA); 0.43 g of $Na_2HPO_4.2H_2O$ (Sigma–Aldrich, St Louis, MA); and 1.9 g of KH_2PO_4 (Sigma–Aldrich, St Louis, MA). Final pH of the medium was adjusted to 6.5–6.7 and it was sterilised by autoclaving for 20 min at 121 °C.

Virulence assay

Assays were carried out in Costar 24-well tissue culture plates. A single colony of *P. aeruginosa* was cultured in 10 ml VHPL5 medium overnight at 21 °C. The bacteria were washed twice in 10 ml sterile SS before being diluted to an optical density of 0.10 at 600 nm. *D. discoideum* were taken from an axenic culture (between 5×10^4 and 5×10^5), counted and washed twice in SS before being diluted to a final concentration of 2×10^8 cells/L. These concentrations for both bacteria and *D. discoideum* were adopted after titration of the system using the *K. aerogenes* control strains to determine the minimum bacterial concentration that allowed *D. discoideum* cells to reach the density critical to the onset of development (as observed by commencement of streaming) within 24 h. To start each assay, 0.5 ml of bacterial solution with optical density of 0.10 Abs600 (equivalent to approximately 3.0×10^{11} CFU/L) and 0.5 ml of *D. discoideum* were added to each well. All assays were run in parallel triplicate culture, alongside a *K. aerogenes* control. Virulence assays of the laboratory control strain, *P. aeruginosa* PAO1, were repeated in biological quintuplicate to confirm the reproducibility of the assay for a known virulent strain of *P. aeruginosa*. Each well was inspected using an inverted microscope at 24 h. Any cytotoxic effects (sparse growth compared to control combined with rounding of cells and cytoplasmic shrinkage) on the *D. discoideum* cells (Figure 1) were observed and recorded.

Statistical analysis

A Fisher's exact test was used to calculate the significance of cytotoxic effects by single source vs. all other sources combined. A result with *P* value of <0.05 was considered

Table 1. Cytotoxicity of *P. aeruginosa* from different sources on *D. discoideum* AX2 in a liquid phase virulence assay after 48-h incubation at 21 °C.

	Samples	Cytotoxic effect observed		Fisher exact test
Source of isolation	*n*	*n*	%	*P* value
CF respiratory	7	0	0	0.015
non-CF respiratory	13	6	46	0.772
Sputum trap	5	4	80	0.169
Urine	15	8	53	0.569
Skin and soft tissue	25	13	52	0.470
Hospital environment	16	8	50	0.787
Community environment	4	1	25	0.623
Total samples	85	36	45	

to be significant. SPSS software (version 10.0; SPSS Inc., Chicago, IL, USA) was employed for these analyses.

Results

D. discoideum inoculum density

Dictyostelium growth was found to be optimal when using a *K. aerogenes* inoculum with an optical density of 0.10 Abs_{600} (equivalent to approximately 0.8×10^8 CFU/mL), with bacteria being largely cleared at 24 h post-inoculation, and streaming of cells well underway at 48 hours. At lower titrations, growth was sub-optimal, and development did not occur as critical cell densities were not reached.

Reproducibility of assay

The *K. aerogenes* control repeated with every assay batch resulted in reproducible abundant growth without cytotoxicity to *D. discoideum*. *P. aeruginosa* PAO1 controls showed reproducible absence of cytotoxicity. Growth of *D. discoideum* in co-culture with *P. aeruginosa* PAO1 was variable, being equivalent to the control in three assays and slightly less than the control in two assays. In all cases, cytotoxicity was not observed. The growth compared to the *K. aerogenes* control and the cytotoxic effects of each strain of *P. aeruginosa* on *D. discoideum* were found to be reproducible in parallel triplicate.

Virulence of isolates by source

No isolates of *P. aeruginosa* caused death of *D. discoideum* AX2 at the inoculum concentrations used in this assay. Forty-four of the tested isolates (55%) had no detectable cytotoxic effect (Figure 1) on *D. discoideum* cells, whereas 36 (45%) showed cytotoxicity. CF respiratory isolates of *P. aeruginosa* ($n = 7$, including one AES3 isolate) were the only group isolates to universally demonstrate no cytotoxic effect on *D. discoideum* ($P < 0.05$). Between 25% and 80% of isolates from other sources showed cytotoxicity (Figure 1) towards *D. discoideum* in this assay (Table 1). The *P. aeruginosa* PAO1 and *K. aerogenes* controls showed reproducible results in all assays. Both allowed luxuriant growth of *D. discoideum* and did not exhibit cytotoxic effects.

Discussion

This report presents the use of an axenic liquid matrix for a *D. discoideum* eukaryotic virulence assay for bacteria in a 24-well plate system. The test is simple, fast, reproducible and efficient in space and resources. The assay results using this system show decreased virulence of CF respiratory isolates of *P. aeruginosa*, which is consistent with the findings of other studies.[7, 8] A small selection of CF isolates was studied in this paper, and it would be valuable to repeat this experiment with a large cohort of CF isolates and a longitudinal cohort from the same patient, as was previously performed in solid agar virulence assays by Lelong et al.[8]

It must be emphasised that validation of this assay against other existing *D. discoideum* assays or mammalian models has not occurred due to funding body limitations on the use of animals or animal-derived products in this research. The use of wild-type and virulence knock-out mutants of *P. aeruginosa* in the assay would further validate the assay. The purpose of this report is to present the feasibility of a liquid *D. discoideum* virulence assay and that any group wishing to continue this work should undertake the required further validation, which is not possible in this case due to funding body limitations.

In all previous models, only virulence knock-out, CF respiratory isolates and a single community environment isolate could maintain the growth of viable *D. discoideum*. All other wild-type clinical and environmental isolates tested apparently killed the eukaryotic amoebae before any host–pathogen interactions were observed. The continued viability of *D. discoideum* in this model allows subtle differences in the cytotoxicity of the bacteria being tested to be observed. Those isolates with attenuated virulence in a previously described agar plate model [7] continued to show a significant difference to virulent isolates. However, in all published agar plate models, an excess of bacterial inoculum was used. A previous mouse model study found that the infectious dose of mucoid *P. aeruginosa* for CF mice was 5×10^6 CFU/lung, whilst non-CF BALB/c mice showed 65% mortality at an infectious dose of 5×10^7 CFU/lung.[15] Using a liquid-based assay, lower inocula of bacteria, more reflective of the infectious dose of *P. aeruginosa* in the host, may be achieved.

This is the first paper to describe survival of *D. discoideum* in co-culture with PAO1. The absence of cytotoxicity was reproducible in quintuplicate. This is explainable by the low inoculum of bacteria used in each assay, which was designed to reflect the low ratio of bacteria: host cells that would be present in a genuine infection setting, rather than use excess concentrations of bacteria from previous models. It appears that at such low ratios, the virulence of *P. aeruginosa* to *D. discoideum* is much attenuated, possibly due to the quorum-sensing threshold for the bacteria not having been met. In this paper, the lowest titration of bacteria required for development of *D. discoideum*, as observed in co-culture the *K. aerogenes* control, was employed. However, future comparison of higher titrations of bacteria at the end of logarithmic phase growth would be advisable to determine at what point each isolate induces total cell death in *D. discoideum* (for those isolates in which this occurs) and if this is a linear effect or if sudden induction of virulence by quorum sensing occurs at a certain titration of bacterial

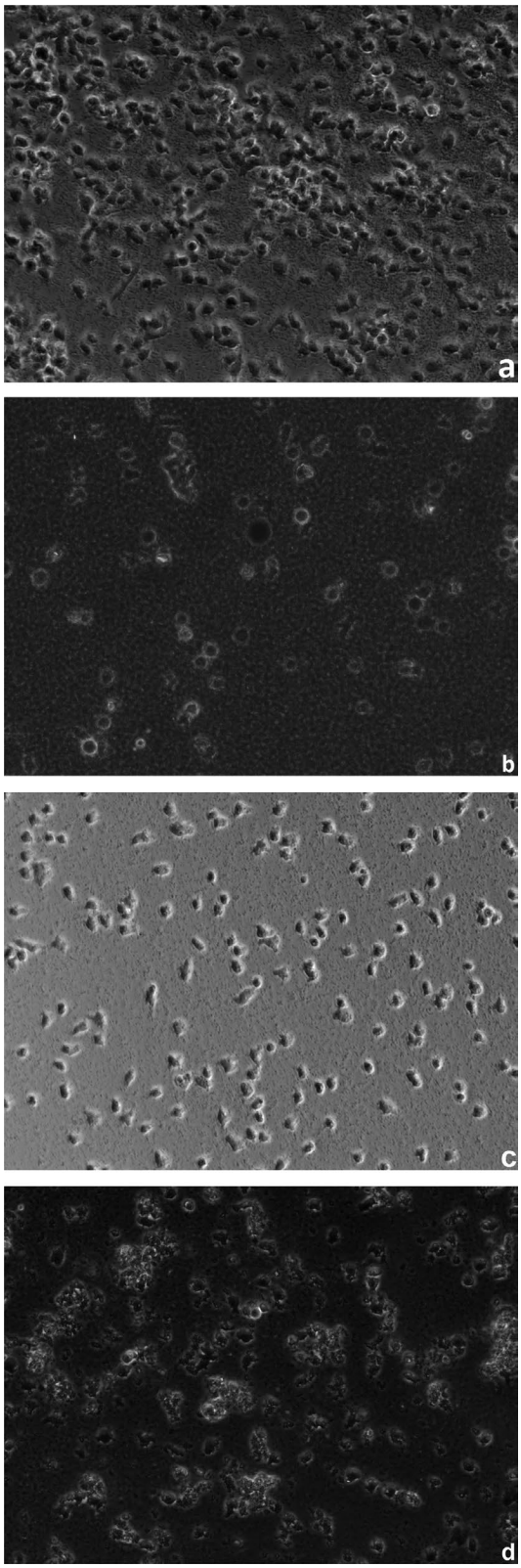

Figure 1. Effects on *D. discoideum* cells in liquid co-culture with bacteria (a) with *K. aerogenes,* no cytotoxic effect; (b) with a virulent skin and soft tissue isolate; cytotoxic effect (sparse growth compared to control combined with rounding of cells and cytoplasmic shrinkage) in co-culture; (c) with an avirulent CF strain, no cytotoxic effect; and (d) with another virulent skin and soft tissue isolate, cytotoxic effect (rounding of cells and cytoplasmic shrinkage). Assays photographed after 48-h incubation in co-culture at 21 °C, phase contrast, ×100 magnification.

cell concentration. Furthermore, a positive control of higher virulence than PAO1 should be sought to use if this low density of inoculum was to be continued in use.

Quantitative measurement of *Dictyostelium* cell numbers and cell death/cytotoxicity were not performed in this assay. The currently reported method relies on a qualitative assessment of cytotoxicity (cell rounding, etc.). It is suggested that future work should include a quantitative assessment, such as the measurement of lactate dehydrogenase in test supernatants. This would allow more specific quantification of cytotoxicity and eukaryotic cell death. Furthermore, the use of flow cytometry would allow accurate, objective and quantifiable measurement of *Dictyostelium* growth or decline during the course of testing.

A further logical expansion of this liquid culture process is teasing out the roles of various cellular and non-cellular factors by the application of expression microarrays to the co-culture model. Stopping the interaction of the co-culture at different time points would allow the mapping of virulence factor expression, cell signalling and metabolism of *P. aeruginosa* when in contact with a eukaryotic phagocyte (that is considered a model of human macrophages) over time.

We believe the liquid *D. discoideum* assay described here could be easily adapted to a range of bacteria. The ability to both quantify results and also easily observe and record cellular activity with an inverted microscope makes this system extremely useful and adaptable. In addition to growth and cytotoxicity, such observations could feasibly be expended to development, where clear differences between bacterial strains were observed, but not quantified. The use of a liquid assay also provides more versatility for further testing such as the use of expression PCRs, alteration of amoebae–bacterium concentration ratios, addition of therapeutic agents in titration to the assay, microscopic analysis of host–pathogen interactions and flow cytometry applications, to name few, which were not available with traditional agar plate virulence models.

Conclusion

We present data showing the initial feasibility of testing of bacterial virulence against eukaryotic phagocytic cells in a liquid matrix with a 24-h end point. Limitations of the funding mean that full validation of this assay against existing assays and virulence knock-out mutants should be performed. A more objective and quantitative measure of amoeboid cell growth and cytotoxic effect would also benefit further validations of this liquid matrix assays. In summary, this preliminary investigation indicates that the use of a liquid matrix *P. aeruginosa* virulence assay in co-culture with *D. discoideum* offers promise for greater versatility in manipulation of infectious dose and potential downstream investigations of virulence interactions when compared to traditional solid agar assay.

This report represents an advance in biomedical science because:

- It shows the preliminary potential of a liquid-based bacterial virulence assay
- With further validation and development, the assay has the potential to offer a new and versatile laboratory measurement of whole eukaryotic cell-based bacterial virulence interactions without the use of animals or mammalian cell lines
- This work demonstrates that such an assay may be viably performed without the use of animals or animal-derived products through the substitution of such products with commercially available alternatives.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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