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Zebrafish: An Animal Model for Testing Suitability of *Pseudomonas* Species for Bioremediation of Pesticide Influenced Soil

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ABSTRACT

Prolonged usage of chemicals in agriculture has devastated biodiversity to a large extent including microbial diversity and density. Upon intense exposure certain microorganisms acclimatize to chemical influenced soil and exploit those chemicals as their sole source of carbon and/or nitrogen. Bioremediation studies over the past decades predominantly imply on isolation of *Pseudomonas* species with the property to consume chemical contaminants as their source of energy. *Pseudomonas spp*. have adapted themselves to extreme conditions by altering their metabolic pathway. Hence these can be to some extent denoted as bio-indicators of chemical contaminants. But suggesting *Pseudomonas spp.* for field study or for bioremediation threatens us in particular with the development of antibiotic resistance and instigating secondary infections, thus restricting their application. The present study involves in possibility of *Pseudomonas* species to be considered for bioremediation by testing their ability in causing mortality in zebrafish. Isolates for the comparative study include *P. aeruginosa* (clinical isolate), *P. aeruginosa* (soil isolate with pesticide degradation property) and *P. pseudoalcaligenes* (soil isolate with pesticide's carrier molecule degradation property). Results obtained confer that antibiotic resistance and mortality caused by the clinical strain was significantly higher thus advocating an affirmative annotation for the field application of soil isolates.

Key words: *Bioremediation, Zebrafish, pesticide, Pseudomonas spp.*

INTRODUCTION

Extreme and improper use of pesticides in agricultural practices have polluted soil and environment to a large extent [1]. Upon enduring usage of toxic chemicals, microbial communities tend to get acclimatized to utilize them as sole sources of energy [2]. Of the bacterial community, *Pseudomonas* species have higher incidence of adaptation and utilization rate and hence are predominantly found in polluted zones. *P. aeruginosa* is a free-living, ubiquitous, and opportunistic bacterium. Moreover, *P. aeruginosa* is also the epithet of an opportunistic nosocomial pathogen in humans. *P. aeruginosa* is tarnished for its antibiotic resistance and is, therefore, a particularly treacherous and feared pathogen [3]. Thus their application in remediating polluted soils becomes limited due to their pathogenicity.

Current study involves in exploring the virulence rates of *P. aeruginosa*, clinical and soil isolates with pesticide degrading property, and also compared with *P. pseudoalcaligenes* of soil origin with the potency to degrade pesticide's carrier molecule. In our study zebrafish (*Danio rerio*) was used as a model organism to study the virulence caused by these strains. The zebrafish has increasingly been used for biomedical research due to its high fecundity, comprehensive molecular tools, and economic feasibility. Zebrafish as mini-hosts in terms of genetics, physiology, and structural similarity, and also has both innate and adaptive immune functions [4].

METHODOLOGY:

Isolates for the study:

Soil isolates namely, *P. aeruginosa* and *P. pseudoalcaligenes* with the potency to biodegrade Methyl parathion (commercial grade) and their carrier molecule were tested for their virulence against the clinical isolate of *P. aeruginosa*. Nutrient broth containing respective isolates was standardized using 0.5 McFarland standard [5] with appropriate controls.

Generation time for the isolates were calculated and tabulated [6,7] (Table 1). Antimicrobial susceptibility studies for selected antibiotics were detected using Kirby-Bauer technique (disc diffusion method) [8,9]: Vancomycin (Va^{30}), Ofloxacin (Of^{5}), Tobramycin (Tb^{10}), Ceftazidime (Ca^{30}), Norfloxacin (Nx^{10}), Co-trimoxazole (Co^{25}), Cefuroxime (Cu^{30}), Tetracycline (T^{30}), Cefpodoxime (Cep^{10}), Cephotaxime (Ce^{30}) and Sterile disc (S) (Table 2).

Acclimatization of Zebrafish:

Male Zebrafish (*Danio rerio*) [10] of 2 – 4 weeks old were chosen for the current study. Fishes were made to swim in 150 ml sterile distilled water and left unfed for 24 hours before subjecting to test.

Virulence testing:

Virulence rate was observed by treating the fishes with culture broth of the respective isolates (with appropriate controls) [11,12,13].

In acute testing, experimentation unit fishes (n=1) were observed under 1ml 0.5 McFarland standard of the respective isolates along with appropriate positive and negative controls [14]. In chronic testing, experimentation unit fishes were treated for every 24 hours with 1ml 0.5 McFarland standard of the respective isolates along with appropriate positive and negative controls. Cumulative results were observed. In lethal dosage testing, experimentation unit fishes were observed under increasing volume *i.e.* 1 ml to 10 ml of 0.5 McFarland standards of the respective isolates along with appropriate positive and negative controls [15,16,17,18].

In order to ensure the inoculum size and viability, absorbance of water at 625nm in a UV-vis Spectrophotometer was recorded in the experimental units before, at the time of treatment, and at the end of the experiment [19,20,21]. Fishes from positive control units, negative control units and test units were dissected and observed for microbial infection. Suspected organs were ground in sterile distilled water and cultured on Nutrient agar for confirmation of the infectious agent.

Experimentation units consist of transparent plastic disposable containers of 200 ml capacity containing 150 ml of distilled water and one pellet (0.05 g) of commercially available food, covered airtight and UV sterilized. Fishes were washed in sterile saline before introducing into the experimental unit and left for a day in the unit to get acclimatized [22,23,24]. All experiments including the positive and negative controls were done in triplicates to ensure the reliability of the results. After the experiment, dead fishes and their organs were disposed as per ICMR guidelines [25].

RESULTS:

Table 1: Generation time (in minutes)

S.no.	Isolates	Generation time
1.	*Pseudomonas aeruginosa* (clinical isolate)	19.68±0.28
2.	*Pseudomonas aeruginosa* (soil isolate)	23.28±0.38
3.	*Pseudomonas pseudoalcaligenes* (soil isolate)	21.31±0.64

Table 2: Antibiotic Susceptibility Test

S.No.	Antibiotics	Zone of Inhibition (Diameter in mm)		
		P. aeruginosa (clinical isolate)	*P. aeruginosa* (soil isolate)	*P.pseudoalcaligenes* (soil isolate)
1.	Vancomycin (Va^{30})	- (R)	- (R)	- (R)
2.	Ofloxacin (Of^{5})	33.6±0.57 (S)	35±0 (S)	27±0 (S)
3.	Tobramycin (Tb^{10})	26±0 (S)	30±0 (S)	29±0 (S)
4.	Ceftazidime (Ca^{30})	- (R)	14±0 (I)	10±0 (I)
5.	Norfloxacin (Nx^{10})	44.3±0.57 (S)	40.3±0.57 (S)	37.6±1.1 (S)
6.	Co-trimoxazole (Co^{25})	- (R)	16.6±0.57 (I)	- (R)
7.	Cefuroxime (Cu^{30})	- (R)	- (R)	- (R)
8.	Tetracycline (T^{30})	- (R)	11.6±1.52 (I)	16±0 (S)
9.	Cefpodoxime (Cep^{10})	- (R)	- (R)	- (R)
10.	Cephotaxime (Ce^{30})	10±0 (R)	15±0 (I)	15±0 (I)
11.	Sterile disc (S)	Satisfactory	Satisfactory	Satisfactory

NOTE: "-"– No zone of inhibition, "R"- Resistant, "I" – Intermediate and "S" – Sensitive

Virulence Testing:

At the end of 10 days of acute testing no mortality was observed in the experimentation units for all the three isolates. Upon cumulative observation in chronic testing, mortality was observed in experiment units treated with *P. aeruginosa* (clinical isolate) on the 5th day and *P. aeruginosa* (soil isolate) on the 7th day. No mortality was observed in the experimentation units treated with *P. pseudoalcaligenes* (soil isolate). In case of lethal dosage testing (LD), mortality was observed in the experimentation units treated with *P. aeruginosa* (clinical isolate) from LD4 to LD10 and LD6 to LD10 for *P. aeruginosa* (soil isolate). No mortality was observed in the experimentation units treated with *P. pseudoalcaligenes* (soil isolate) from LD1 to LD10. No significant variation was observed in absorbance values at 625nm during the experiments. Culture analysis was also satisfactory and confirmed the presence of the respective *Pseudomonas spp* as the infectious agent.

DISCUSSION:

With reference to the above results, *P. pseudoalcaligenes* (soil isolate) were not found to be pathogenic to zebrafishes. Whilst, *P. aeruginosa* (clinical isolate) caused higher mortality rate compared to *P. aeruginosa* (soil isolate), which may be due to escalated doubling time of the soil isolate with respect to clinical isolate. Thus soil isolates of *Pseudomonas spp.* can be considered for bioremediation of pesticide influenced soils with appropriate precautions.

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