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Phenotypic Characterization and Antibiotic Resistance of *Pseudomonas* spp. from Seafood and Aquaculture Farm Environments

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A total of 115 different *Pseudomonas* strains isolated from fish, prawn, brackish water and fresh water aquaculture farms environments were characterized based on the biochemical tests, species variations, distribution ratio, and antibiotic resistance profile. 48% *Pseudomonas* were identified as fluorescent group whereas 52% were falling in the non-fluorescent *Pseudomonas* group. Eight different species namely *Pseudomonas aeruginosa, P.fluorescens, P.putida, P.alcaligenes, P.pseudoalcaligenes, P.cepacia* (*Burkholderia cepacia*) *P.maltophilia* and *P.stutzeri* were isolated from seafood, brackish water, and fresh water environments. *P.maltophilia* and *P.stutzeri* were not detected from brackish water sources. Biochemical characterization showed diverse mode of nutritional requirements for each species as most commonly used carbon source i.e. glucose was not utilized by *P.alcaligenes, P.pseudoalcalignes,* and inositol was not utilized by *Pseudomonas* fluorescent group. All isolates were positive for indole, catalase and oxidase. Starch was not hydrolyzed by *Pseudomonas* spp. except *P.stutzeri*. Variable growth pattern was observed at 4°C and 42°C for each species. Antibiotic resistance against nine antibiotics viz. kanamycin, nitrofurantoin, chloramphenicol, oxytetracycline, gentamicin, rifampicin, neomycin, sulphamethizol, and streptomycin was determined. All isolated *Pseudomonas* spp. were resistant to nitrofurantoin and sulphamethizol. *P.maltophilia* and *P.stutzeri* were sensitive to 7 of 9 antibiotics tested.

Key words : *Pseudomonas*, antibiotic, biochemical test, aquaculture environments

Pseudomonas includes species of various economic and ecological importances and comprises the fluorescent i.e. *Pseudomonas aeruginosa, P.fluorescens, P.putida* and non-fluorescent groups i.e. *P.pseudoalcaligenes, P.cepacia* (*Burkholderia cepacia*), *P.maltophilia* and *P.stutzeri*. These species show dissimilarities in phenotypic and genotypic characteristics and are frequently isolated from the aquatic, clinical and agriculture isolates (Campbell *et al.*, 1995; Ergin *et al.*,1999; Rangarajan, *et al.*, 2001; Brown *et al.*, 2004). *Pseudomonas* spp. viz; *P.aeruginosa, P.cepacia, P.putida* and *P.stutzeri* are human pathogens and generally isolated from hospital environments. Some species like *P.aeruginosa* and *P.putida* are considered fish pathogens (Shahid et al., 2003). *Pseudomonas* spp. has been isolated from the shellfish and aquaculture environments (Ekanem *et al.*, 1997; Surendran *et al.*, 2000) and also associated for spoilage of seafood (Gram *et al.*, 1996).

Heterogeneous aquaculture pond environment offers several possible microniches for the versatile *Pseudomonas*, and play significant role in pond water and soil. *Pseudomonas* species utilize a broad range of low molecular weight compounds, complex organic compound, recalcitrant residues of decomposition as pesticides. *Pseudomonas* spp. also show other metabolic traits such as production of siderophores and bacteriocins which inhibits pathogenic bacteria and fungi. *Pseudomonas* constitutes the major micro flora in the environmental sample including the brackish water aquaculture farms and had a role in the mineralization (Herbert, 1999), and most prominent nitrate reducers in aquaculture ponds (Hargreaves, 1998).

Indiscriminate use of antibiotics and other chemicals led to development of resistant bacterial strains towards these antibiotics and chemicals in the farm environments.

In addition to its innate resistance, these organisms acquired additional resistance towards antibiotics and other chemicals due to plasmids. Antibiotic resistance in bacteria hatchery-reared larvae and post larvae of *Macrobrachium rosenbergii* have been showed by Sahul hameed *et al.*(2003). Use of oxytetracycline has caused increased bacterial resistance in shrimp farms (Nash *et al.*, 1992) and similarly, implications of antibiotics in aquaculture results in development of antibiotics resistant in microorganisms (Brown, 1989).

Materials and Methods

Brackish water, fresh water, farm sediments, fish and prawn samples were collected from the brackish water and fresh water aquaculture farms of Ernakulam and surrounding area. Crab, mussel and squid were procured from local fish market. Water samples were collected in sterile bottles, sediments in pre-sterilized Petri dishes and fish and prawn samples in sterile polythene bags. *Pseudomonas* strains were isolated by two methods; first, by Pseudomonas enrichment broth followed by Pseudomonas isolation agar (Difco) and second, by King's B media method. Isolated and purified cultures were streaked on Nutrient agar (Difco) slants for further identification and characterization.

All isolates were phenotypically characterized as per Microbiological Methods by Collins *et al.*, 1985 and Bergey's Manual of Systematic Bacteriology, Vol.I. Based on the key biochemical reactions i.e. pigment formation, oxidative/fermentative, growth at 4°C and 42°C, arginine hydrolysis, gelatin liquefaction isolates were divided in to different species. Further reactions like indole formation, oxidase, catalase, utilization of amino acids (arginine, lysine, alanine, valine) and sugars (glucose, arabinose, trehalose, xylose) were tested. The isolates were also tested for utilization of starch, growth at 0% salt (P_1N_0), and denitrification.

All isolates were assayed for antibiotic susceptibility test on Hekton Muller agar (Difco). The isolates were tested using antibiotics discs (Himedia, Mumbai, India) for their susceptibility to a set of nine antibiotics; novobiocin, oxytetracycline, rifampicin, neomycin, kanamycin, streptomycin, nitrofurantoin, chloramphenicol, and gentamicin. The results were recorded on the basis of the diameter of the inhibition zone from the zone size interpretative chart supplied by the manufacturer.

Results and Discussion

A total of 115 *Pseudomonas* isolates were isolated from different samples and based on the key biochemical tests, the isolates identified were *P.aeruginosa*, *P.fluorescens*, *P.alcaligenes*, *P.pseudoalcaligenes*, *P.cepacia* (*Burkholderia cepacia*), *P.putida*, *P.maltophilia*, *P.stutzeri*. All isolates were falling in to eight different species of *Pseudomonas* as per the Bergey's Manual of Systematic Bacteriology. The distribution of the species showed 48% fluorescent *Pseudomonas*, whereas 52% were non-fluorescent *Pseudomonas*. As showed in Table 1, distribution ratio of *Pseudomonas* species from seafoods, brackish and fresh water aquaculture environments samples, *P.aeruginosa* was the predominant species, showing 23% of total isolates followed by *P.alcaligenes* (18%), *P.fluorescens* (17%) and *P.cepacia* (17%). The distribution ratio of *P.aeruginosa* from the hospital environments reported high at 91% (Ergin *et al.*, 1999). Our observations agreed with the result of Noble *et al.* (1994), the isolation rate of *P.stutzeri* was 2.0%. The fluorescent species were predominant in fresh water samples whereas, non-fluorescent species were dominant in brackish water samples.

Table 1. Distribution of *Pseudomonas* spp.

Sl. No.	Pseudomonas Species	Isolation	Distribution Rate (%)
1	*P.aeruginosa*	23	20.00
2	*P.fluorescens*	17	14.79
3	*P.alcaligenes*	18	15.65
4	*P.pseudoalcaligenes*	15	13.04
5	*P.cepacia*	17	14.78
6	*P.putida*	15	13.04
7	*P.maltophilia*	7	6.08
8	*P.stutzeri*	3	2.62
	Total	115	100.00

Table 2. Biochemical characteristics of *Pseudomonas* spp.

Biochemical Tests	*P.aeruginosa* (23)*	*P.fluorescens* (17)*	*P.alcaligenes* (18)*	*P.pseudoal caligenes* (15)*	*P.cepacia* (17)*	*P.putida* (15)*	*P.maltophilia* (7)*	*P.stutzeri* (3)*
Fluorescent pigment	23	17	0	0	0	15	0	0
Pyocynin	23	0	0	0	0	0	0	0
HL	Oxi[a] 23	Oxi 17	Alk[b] 18	Alk 15	Oxi 16	Oxi15	Alk 7	Oxi 3
Indole	23	17	18	15	17	15	3	3
Catalase	23	17	15	14	17	15	7	3
Cytochrome oxidase	23	15	15	15	17	15	2	3
Gelatin	2	15	2	0	14	0	5	0
Starch	0	0	0	0	0	0	0	2
Growth At 4°C	3	13	1	0	2	12	0	1
Growth At 42°C	22	2	15	3	16	0	0	2
Growth at 0%Nacl	4	3	12	10	10	13	7	3
Arginine	23	17	0	14	0	14	5	0
Alanine	20	17	15	12	15	0	5	0
Valine	0	16	0	0	1	0	7	3
Arabinose	3	15	4	2	0	0	7	0
Glucose	23	17	1	0	15	13	7	3
Inositol	0	0	1	0	14	2	6	0
Trehalose	2	15	0	0	15	0	6	0
Xylose	19	14	0	0	8	13	6	1
Denitrification	23	14	1	0	3	0	0	3

*No. of isolates tested, [a] Oxidative, [b]Alkaline

Biochemical characteristics of *Pseudomonas* spp. as showed as fluorescent pigment was produced by all the isolates of *P.aeruginosa, P.fluorescens* and *P.putida* whereas pyocynin was observed in *P.aeruginosa* isolates. Dissimilarities in biochemical characteristics, a simple characterization based on analysis of proteinase K resistant whole cell protein pattern and eight phenotypic tests which distinguishes most environment isolates of fluorescent *Pseudomonas* species with others was suggested by Sorensen et al. (1992).

In case of sugars, *P. pseudoalcaligenes* could not utilize glucose, arabinose, trehalose, inositol and xylose whereas, these sugars were utilized by *P.maltophilia.*

Almost all *Pseudomonas* spp. were positive for cytochrome oxidase, catalase, indole production and HL reaction. Starch was not utilized by *Pseudomonas* isolates except *P.stutzeri.* Denitrification was not observed in the isolates of *P.pseudoalcaligenes, P.putida, P.maltophilia* and *P.alcaligenes.* Arginine was hydrolyzed by the fluorescent group i.e. *P.aeruginosa, P.fluorescens* and *P.putida* (Table 2). Alanine was utilized by all identified species except *P.putida* and *P.stutzeri* and valine was utilized by *P.fluorescens* and *P.matlophilia.* Valine assimilation reaction was negative for *P.aeruginosa, P.alcaligenes, P.pseudoalcaligenes,* and *P.cepacia,* trehalose, glucose and xylose were utilized by the most of fluorescent species i.e. *P.aeruginosa, P.aeruginosa,*and *P.putida.* In agreement with Gavini *et al.* (1989) variable growth pattern was observed for at zero % Nacl, showing the wide environmental adaptability of *Pseudomonas* species. All isolates of *P.maltophilia* and *P.stutzeri* showed growth in zero% (P_1N_0) shows the fresh water origin of

these organisms. The nutritional reactions of *P.alcaligenes* were varied in previous works Picket *et al.* (1986) and Shaw *et al.* (1982) and the variation in nutritional requirements were further addressed by Tamaoka *et al.* (1984). Result observed in this study, most of the sugar reactions were negative for *P.alcaligenes* and in case of *P.pseudoalcalignes*, characteristics were more similar to those described by Gavini *et al.* (1989), except assimilation of alanine and arginine was observed in our study. There was slight anomaly in result, as two isolates of *P.putida* and *P.cepacia* did not utilize glucose as sole carbon source. *P.maltophilia* utilized all amino acids and sugars used for the tests.

Most of the characteristics of the *P.stutzeri* agreed with the previously published studies of *P.stutzeri* (Pelleroni *et al.*1984, and Homes *et al.*, 1986), especially the denitrification and ability to use starch as a sole carbon source. All the strains negative to arginine and alanine in this study in agreement with Gilardi, 1978. and only variation was observed in for lysine assimilation. Most of the other nutritional characteristics studied in this analysis agreed with those given by Stanier *et al.*, 1966, and Palleroni *et al.*, 1970.

Antibiotics are in general not well investigated from ecotoxicological perspective, but recent studies show that many of them are moderately to highly toxic to aquatic life (Holten *et al.*, 1999; Halling *et al.*, 2000, Wollengerger *et al.*, 2000).Because of the widespread use of antibiotics, the resistance profile of the microorganisms are changing, as evidenced by the increasing resistance among bacterial population from aquatic and other environments (Campbell *et al.*,1995; Ergin *et al.*,1995; & Shrivastava *et al.*,2003). A field survey conducted by Greilaurd *et al.*, (2003) in the Thailand shrimp farms, at least 13 different antibiotics were used in the culture ponds. In our study *P.aeruginisa* were resistant to almost all antibiotics tested except gentamicin. All isolated *Pseudomonas* spp. except *P.aeruginosa* were sensitive to gentamicin and neonycin (Table 3). *P.aeruginosa* is naturally resistant to β-lactam, including cerphalosporins, quinolone, chloramphenicol and tetracycline, mainly because of low permeability of their cell wall. Campeau etal.1996, examined gram negative bacilli in the aquatic sources (USA) for the resistance to antibiotics and found that six of nine antibiotics, tested by disc diffusion method. The most common group of resistant bacteria was *P.fluorescens*. A high level of resistance by *P.aeruginosa* towards imipenem and ceftazidine were observed (Campeau *et al.*, 1996 and Kato *et al.*, 2001). All *P.fluorescens* isolates were resistant to oxytetracyclin, nitrofurantoin, chloramphenicol, sulphamethizol, 17% isolates were resistant to kanamycin and 41% were resistant to streptomycin. Despite the varia-

Table 3. Antibiotic resistance assay of *Pseudomonas* Isolates

Sl. No.	Species	No of isolates	Percentage of isolate resistance to antibiotics[a]								
			G	O	R	N	K	S	Nf	C	Sm
1	*P.aeruginosa*	23	0	100	100	60	100	100	100	100	47
2	*P.fluorescens*	17	0	100	100	0	17	41	100	100	100
3	*P.alcaligenes*	18	0	22	100	0	0	0	100	33	88
4	*P.pseudoalcaligenes*	15	0	13	100	0	0	0	100	23	75
5	*P.cepacia*	17	0	52	100	0	0	100	100	100	100
6	*P.putida*	15	0	100	100	0	13	26	100	100	100
7	*P.maltophilia*	7	0	0	0	0	0	0	100	0	100
8	*P.stutzeri*	3	0	0	0	0	0	0	100	0	100

[a]G, Gentamicin; O, Oxytetracycline; R, Rifampicin; N, Neomycin; K, Kanamycin; S, Streptomycin; Nf, Nitrofurantoin; C, Chloramphenicol; Sm, Sulphamethizol

tions in the origin of isolates, our findings support the other observations of Campbell *et al.*, 1995 and Miranda *et al.*, 2002, antibiotic resistance in *P.fluorescens* and *P.putida* from agriculture and aquatic sources. Resistant pattern to other strains showed, 22% of *P.alcaligenes* isolates were resistant to oxytetracycline, 33% resistant to chloramphenicol, and 88% were resistant to sulphamethizol. Similarily, in case of *P.pseudoalcaligenes*, 13% isolates were resistant to oxytetracycline, 23% resistant to chloramphenicol, and 75% resistant to sulphamethizol. These results show that *P.alcaligenes* and *P.pseudoalcaligenes* share close homology in antibiotic pattern, though they differ in phenotypic characteristics. Almost all the isolates of *P.cepacia* and *P.putida* were sensitive to kanamycin. *P.maltophilia* and *P.stutzeri* were sensitive to seven of nine antibiotics tested and were resistant to nitrofurantoin and sulphamethizol. Use of antibiotics and detection from the seafood and aquatic environments can pose potential risk to farm workers and consumers, health of cultured shrimp, and the environments. The widespread detection of antibiotic resistant in bacteria may cause difficulties to treat bacterial infections in cultured shrimp, and additionally great health risk to humans.

This study demonstrates that *Pseudomonas* spp. phenotypic characters vary with the source of the samples and also shows the versatile nature of *Pseudomonas* spp. from seafood and aquaculture environments. The significant high frequencies of antibiotic resistance to certain antibiotics show the level of antibiotic in the environment and also high percentage of antibiotics resistant in some *Pseudomonas* spp. with no history of antibiotic use in the aquaculture farms of the area, suggests that antibiotic resistance can be developed due to factors other than the presence of drug in the corresponding environments. Minimizing and controlling antibiotic use in aquaculture farms should help reduce the risk of antimicrobial resistance in *Pseudomonas* and other bacteria. Further studies are necessary to determine the potential public health significance of important members of resistant bacteria in seafood and aquaculture.

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