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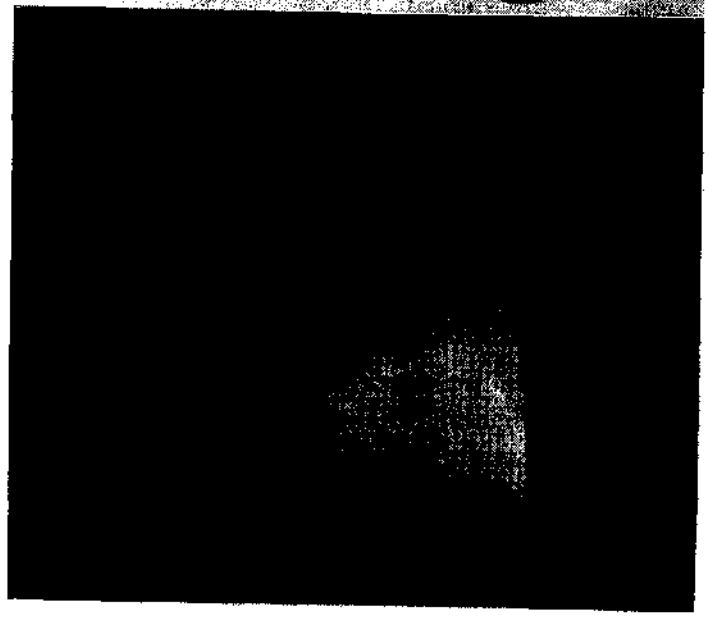

Studies on the oxidative positive gram negative rods in the perennial and pokkali fields around Cochin

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ABSTRACT

Out of the three observations made during the period May and June 1997, totally 13 isolates of oxidase positive gram negative rods were encountered from water and sediment samples of perennial and pokkali fields. Three strains were producing green fluorescent pigment. Fluorescent pigment production was enhanced by dipotassium hydrogen phosphate when compared to glycerol and magnesium chloride. Five cultures showed antagonistic activity against all the test pathogens, namely, Vibrio anguilarum, Mycobacterium and Cytophaga and were found highly resistant to penicillin.

Introduction

It has been shown by several workers that the normal bacterial flora of fish is a direct reflection of bacterial population of the environment they inhabit (Bauman *et al.*, 1971).It has been observed that as many as 37 varieties of bacterial flora are encountered in fresh water and 40 types in the marine ecosystem. *Pseudomonas* species are frequently associated with fish and are found on fish eggs, skin, gills and intestine (Inglis and Hendrie, 1993). As *Pseudomonas* species are so widespread and numerous, they may at times become involved in disease processes and act as secondary invaders of fish due to stress and other factors. Some species are reported as primary pathogens, principally, *Pseudomonas anguillisceptica, Pseudomonas fluorescens* and *Pseudomonas putida* are also found to cause septicaemia in finfish, brownspot disease in shrimps and bacterial necrosis in molluscs.

The *Pseudomonas* species are reported to be having antimicrobial activity against other microorganisms (Laine *et al.*, 1996). Padilla (1990) has reported that the bacteriocin of *Pseudomonas* spp. strain R10 was active *in vitro* against several enteropathogenic bacteria.

In the present study an attempt was made to obtain the oxidase positive nonfermentative, gram negative rods of perennial and seasonal ponds around Cochin and identify and isolate the pathogenic *Pseudomonas* species. Also, the characterization of the bacteria, including morphology, growth characters, biochemical activities, utilization of organic compounds for growth, sensitivity to antimicrobial agents and antagonistic activities were studied based on standard procedures (Shewan *et al.*, 1960).

Materials and methods

Samples from pokkali and perennial ponds located near Narakkal in Vypeen island were collected during the period from May and June and the hydrological parameters were determined. Samples were subjected to quantitative and qualitative analysis as per the scheme given (Table 1)

Table : 1. Pattern of analysis of samples for isolation of *Pseudomonas* spp.

water and
sediment samples
↓
enrichment in
malachite green broth
↓
isolation by pour plate method
Seawater +TTC
Pseudomonas agar + TTC
Cetrimide agar + TTC
↓
selection of *Pseudomonas*
by cytochrome oxidase test positive
pattern of glucose metabolism
in H&L test
↓

Oxidative		Alkaline	No reaction
green pigment	no pigment	no pigment	no pigment
Pseudomonas (Group I)	*Pseudomonas* (Group II)	*Pseudomonas* (Group II)	*Pseudomonas* (Group IV)

Total plate count and identification of the colonies are carried out using three media namely, seawater agar, *Pseudomonas* agar, for fluorescein (Hi Media) and cetrimide agar (Hi Media). Addition of Triphenyl Tetrazolium Chloride (TTC) in the selective media reduces the tetrazolium salt by bacterial oxidative enzymes and leads to the formation of a water insoluble red coloured compound, Formazan, which helps in identifying the colonies.

Identification of colonies was done based on two basic tests namely,

the oxidase test and Hugh and Leifson's oxidation fermentation test. Oxidase test is carried out using the cyto-chrome oxidase reagent-N',N',N', tetramethyl paraphenylene diamine dihydrochloride in which a filter paper is dipped and the culture is streaked using a loop. Immediate appearance of a deep purple colour indicated the positive reaction. Hugh and Leifson's medium is prepared and distributed into tubes, which is sterilized and stab inoculated to find out the glucose metabolism.

Composition of Hugh and Leifson's medium

Seawater agar: Peptone-1%, Agar-agar-2%, Ferric phosphate- 0.01%, Aged seawater-100ml, pH-7.2, 15 lbs., 30mins.

Pseudomonas agar: Tryptone-1%, peptone-1%, agar agar-1.5%, Dipotassium hydrogen phosphate-0.15%, magnesium sulphate-0.15%, distilled water-100 ml; pH-7.2+/- 0.2%. 15lbs, 15mins.

Cetrimide agar: Beef extract-1%, peptone-1%, sodium chloride-0.5%, cetrimide-0.03%, agar-agar-1.2%, distilled water-100ml, and pH at 25°C 7.3+/-0.1.

Hugh and Leifson's glucose: Peptone-1%, sodium chloride-0.5%, glucose-1%, agar-agar-0.3%, distilled water-100ml, phenol red-1cc/100cc of 0.1% solution , dipotassium phosphate-0.3%.

The following tests were conducted to arrive at the species level identification:

1. Growth at 5°C and 37°C
2. Growth at different salt concentrations.
3. Hydrolysis of organic compounds like starch, arginine, casein, gelatin etc.
4. Utilization of citrate as sole carbon source.
5. Penicillin sensitivity.

Because of importance of *Psendomonas* spp in bacterial denitrification processes, utilization of nitrogenous compounds like urea, asparagine, cysteine, glutamic acid, aniline ammonium chloride, ammonium oxalate etc were tested in peptone water containing glucose and the respective nitrogen compound.

Antibiotic sensitivity studies: The sensitivity tests were carried out as a means to arrive at the species level identification. Penicillin (15 mcg per disc), Kanamycin (30mcg per disc), Ampicillin (30mcg per disc) etc. were used to study the sensitivity effects of these antibiotics.

Antagonistic activity studies: Cross streaking method and agar diffusion method were used toughened out the inhibitory effects of the pathogens.

Well plate method: The culture centrifugate was swabbed on the SWA agar medium and wells were cut in the medium to which pathogen broth cultures were added. Presence or absence of growth indicates the inhibitory action of *Pseudomonas* on the test pathogens. In the cross streak method, the test pathogens were streaked across the *Pseudomonas* culture in the petri plate, containing medium.

Results and discussion

It was found that the optimum count of forming units were obtained in dilutions of 10^3 for water in both perennial and seasonal ponds and at 10^4 for sediment., cetrimide agar media did not support the growth of environmental bacteria. It was found that surface water gave more count compared to sediment. In the present study conducted in early monsoon and monsoon period, high numbers of *Pseudomonas* occurred in the selective medium, *Pseudomonas* agar, F. Maximum bacterial populations were observed during monsoon months and the primary environmental factors supporting the pathogenic *Pseudomonas* included moisture, temperature, acidity, organic and inorganic matter supplied.

The fertility of the pokkali field was indicated by the colour of fermentation and hydrogen sulfide production in TAB BART Bio indicator medium, as the colour was more in pokkali water sample. 31 motile gram negative rods were found to be oxidase positive, out of which 13 were identified to be non fermentative based on oxidation fermentation characters in Hugh and Leifson's glucose medium.

In the H&L medium, seven isolates (22.58%) were found to show oxidative type of metabolism and four (12.9%) showed alkaline. Two (6.45%) of the isolates did not show any change. These isolates were

identified to be belonging to different groups (*Pseudomonas* Group I, Group II, Group III and Group IV) based on further evidences like presence of fluorescent pigment.

Three of the isolates were showing green fluorescent pigment. It is well known that the fluorescent pigment production property is unstable and is dependent on the nature of the medium for its manifestation, which was found to be enhanced by the addition of dipotassium hydrogen phosphate to the medium.

Six of the cultures (46.15%) were found to produce hydrogen sulphide in cysteine medium. Out of the 13 cultures 38.46% exhibited moderate growth as well as poor growth. 15.38% exhibited no growth at all. Poor growth in 5°C indicated *P. fluorescens, P. putida and P. anguillisceptica.* Growth at 37°C was there for P.*fluorescens* and *P.putida.* But *P.anguillisceptica* was unable to grow at 37°C. Inspite of these results it is seen that temperature alone cannot be used for the polarly flagellated gram negative rods because of their normally wide temperature range.

Most of the isolates were found to be growing well in peptone glucose medium without sodium chloride and in media containing 5%NaCl. Higher concentrations of sodium chloride (7% and 10%) did not support the growth of bacteria. Another feature was the absence of fluorescence in the medium. At 7% NaCl concentration only one isolate was showing good growth, which was identified as *Alteromonas piscicida.* Isolates incapable of growing at higher concentrations of NaCl were designated as *P. anguillisceptica.*

Proteolytic and amylolytic activity of the isolates were tested and the results showed that 53.84% of the isolates liquefied gelatin and 76.92% fermented arginine. Amylolytic activity of the isolates was poor since only one isolate hydrolysed starch.

The utilization of citrate as sole carbon source also formed important criterion in identifying the species. 69.73% were capable of using citrate. Species level identification is given in the Table 2.

Table 2 - Species identification tests for identification of *Pseudomonas* spp.

Culture No.	Growth at different concentrations of Sodium Chloride				Gelatin liquefaction	Fluorescence	Casein	Starch hydrolysis	Arginine	Citrate as carbon source	Identified genera
	at 0% NaCl	at 5% NaCl	at 7% NaCl	at 10% NaCl							
1	+	+	-	-	-	-	-	-	+	+	*Pseudomonas putida*
3	+	+	-	-	-	-	-	-	+	+	*Pseudomonas putida*
4	-	-	-	-	-	-	-	-	+	-	*Pseudomonas putida*
5	++	+	-	-	-	+	+	+	+	+	*Pseudomonas fluorescens*
6	-	-	-	-	-	-	-	-	-	-	*Alcaligenes faecalis*
7	++	+	-	-	-	-	-	-	+	+	*Pseudomonas putida*
8	+	++	-	-	-	-	+	-	+	-	*Pseudomonas fluorescens*
9	++	+	-	-	-	-	+	-	-	+	*Pseudomonas anguilliseptica*
18	+	+	-	-	-	-	+	+	-	-	*Alteromonas piscida*
25	++	+	-	-	-	+	+	-	+	+	*Pseudomonas fluorescens*
26	+	-	-	-	-	-	-	-	+	+	*Pseudomonas putida*
28	++	+	-	-	-	-	+	-	+	+	*Pseudomonas fluorescens*
29	+	+	-	-	-	-	-	-	+	+	*Pseudomonas fluorescens*

Utilization of nitrogenous compounds: After the incubation period, nitrate reduction as tested and ammonia production was the dominating reaction, which is at its maximum in inorganic substances like ammonium chloride and ammonium oxalate (100% in both). The nitrate reduction capability was recorded high when ammonium chloride was used as a substrate. These results indicated that the involvement and importance of *Pseudomonas* spp. in the nitrogen cycle. Although denitrifiers are usually distributed in the aquatic environment as pure cultures, only 5% of the bacterial species are endowed with the ability to

liberate free nitrogen from nitrate or nitrite in the presence of abundance of organic matter (Zobell, 1946). Most of the *Bacillus*, *Alealigenes*, *Pseudomonas* , *Serratia* and *Vibrio* were found to be extremely active nitrate reducers. Ammonification and peptonisation are mainly done by Pseudomonadales and Eubacteriales. Chandrika (1984), reported that *Pseudomonas*, the veteran degrader of organic matter in the marine ecosystem dominated the pre monsoon during 1974-75.

Antibiotic sensitivity tests: Sensitivity studies with three antibiotics showed that all isolates were resistant to penicillin and ampicillin while 46.15% were sensitive to kanamycin. It can be inferred that the predominant flora of bacteria comprising *Psendomonos spp.* were quite resistant to antibiotics.

Antagonistic activity studies: By the well plate studies it was found that 38.73% of the isolates were actively inhibiting *Vibrio angullarum* and 53.84% were inhibiting *Cytophaga*, the test pathogens. 96.15% of the isolates were found to be inhibiting both *Edwardsiella* and *Mycobacterium* (Table 3). Also well plate method was found to be giving good results compared to cross streak method. It appears that *Pseudomonas* is a natural competitor, which can be use to control other pathogens. Padilla (1990) has reported that the bacteriocin of *Pseudomonas* spp. strain R10 was active *in vitro* against several enteropathogenic bacteria.

Table 3 Antagonistic activity of selected *Pseudomonas* spp on some test pathogens (well plate method)

Organism	Test pathogens			
	Vibrio anguilarum	*Cytophaga*	*Edwardsiella*	*Mycobacterium*
Pseudomonas putida	+	+	+	+
Pseudomonas putida	+	+	+	+
Pseudomonas putida	-	+	-	
Pseudomonas fluorescens	-	+	-	+
Alcaligenes faecalis	-	-	-	-
Pseudomonas putida	-	-	-	-

Pseudomonas anguillisceptica	-	-	-	-
Alteromonas piscicida	-(+ in one well)	-	-	-
Pseudomonas fluorescens	-	-	+	-
Pseudomonas putida	+	+	+	+
Pseudomonas fluorescens	Slight growth in one well	-	-	-
Pseudomonas fluorescens	Slight growth in wells	+	+	+
Pseudomonas fluorescens	+	+	+	+

Most number of isolates occurred in surface water compared to sediment and it was found that the selective medium gave more number of *Pseudomonas* isolates. The presence of fluorescence under U-V light was anguillisceptica important character in identifying isolates, in the present study. Another important observation was that bacterial denitrification is one of the important functions of pseudomonadales and inorganic nitrogenous compounds were found to be degraded more quickly when compared to organic compounds. Also, the pathogenic *Pseudomonas* were found to be mesophilic in nature and proteolytic activity was more compared to amylolytic activity. In the present study, it was seen that, most isolates were quite resistant to antibiotics like penicillin and ampicillin. Inhibitory activity was well demonstrated against some important test pathogens like *Vibrio*, *Cytophaga*, *Edwardsiella*, and *Mycobacterium*.

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