PCR BASED GENETIC ANALYSIS OF *AEROMONAS HYDROPHILA* ISOLATED FROM THE AQUACULTURE SYSTEMS

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Dedicated To My Beloved Mom…

…who has been my inspiration .

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CERTIFICATE

Certified that the dissertation entitled **"PCR BASED GENETIC ANALYSIS OF *AEROMONAS HYDROPHILA* ISOLATED FROM THE AQUACULTURE SYSTEMS"** is a record of independent bonafide research work carried out by **DIVYA, P. R. (MC-62)** during the period of study from September 2000 to August 2002 under our supervision and guidance for the degree of **Master of Fisheries Science (Mariculture)** at the Central Marine Fisheries Research Institute, Kochi, and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

I hereby declare that this thesis entitled "PCR-BASED GENETIC ANALYSIS OF *AEROMONAS HYDROPHILA* ISOLATED FROM THE AQUACULTURE SYSTEMS" is based on my own research and has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition .

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सारांश

जलकृषि तंत्र से पृथक्कृत *ऐरोमोनास हाइड्रोफिला* वियुक्तों के बीच जननिक विषमजातीय एवं बहुजातीय सम्बन्धों के विश्लेषण हेतु पी.सी.आर. पर आधारित आर.ए.पी.डी. तकनीक का उपयोग करते हुये वियुक्तों का आण्विक चरित्र-चित्रण किया गया. इस तकनीक के कई फायदे जैसे कि साधारण, आसान, तेज व जाति वियुक्तों के बीच अधिक विभेदन क्षमता प्रदर्शित हुये. पाँच यादृच्छिक प्रारम्भकों का प्रयोग करते हुये जीवाणु वियुक्तों के डी.एन.ए. के पी.सी.आर. वर्धन से 46 बैंड प्राप्त हुये जो कि एगेरोस जेल वैद्युत कण संचलन पर भिन्न एवं समंकन योग्य थे. प्रत्येक वियुक्त के लिये भिन्न प्रतिमान से आर.ए.पी.डी. परिच्छेदिका की बहुरूपकता साफ जाहिर हुई. वर्धित खण्डों के बीच बहुरूपकता का स्तर 95% प्रतिशत था जिससे कि लक्षणप्ररूप से सजातीय *एरोमोनास हाइड्रोफिला* जाति के वियुक्तों के बीच संजीनिक विषमजातीयता जाहिर होती है. प्रारंभक का चुनाव महत्वपूर्ण पाया गया क्योंकि आर.ए.पी. डी. की विभेदन क्षमता वर्धन के लिऐ प्रयुक्त प्रारंभक के अनुसार बदलती देखी गई. वियुक्तों की जननिक पहचान का गुणांक जननिक दूरियों के मुकाबले साधारणतया अधिक था. वियुक्तों के बीच पारस्परिक जननिक सम्बन्धों को दर्शाने वाले जातिवृत्तीय वृक्ष भी बनाये गये. आर.ए.पी.डी. विश्लेषण *एरोमोनास हाइड्रोफिला* का आण्विक जननिक चरित्र-चित्रण एवं जननिक विषमजातीयता के विश्लेषण हेतु वांछनीय तकनीक पाई गई.

ABSTRACT

Molecular genetic characterization of *Aeromonas hydrophila* isolates from aquaculture systems was carried out to evaluate the genetic heterogeneity and phylogenetic relationship among them, using PCR based RAPD technique. This technique was found to have many advantages like being simple, easy, rapid and highly discriminatory between isolates within the species. PCR amplification of the DNA from the bacterial isolates using five random primers produced 46 amplicons, which were distinct, as scorable bands on agarose gel electrophoresis. Polymorphism of RAPD profile was evident with a unique pattern for each isolate. The level of polymorphism among the amplified fragment was 95% indicating genomic heterogeneity among the isolates within the species of *A.hydrophila*, which were homogenous phenotypically. Choice of the primer was found to be important as the discriminatory ability of RAPD varied with the primer used for amplification. Two of the amplicons shared by all the isolates were identified which could serve as species specific markers. Cellular protein profile of the isolates generated through SDS-PAGE did not reveal any polymorphism as all the isolates presented uniform pattern indicating its usefulness as a tool for species level identification. Coefficients of genetic identities between the isolates were generally high compared to the genetic distances. Phylogenetic tree depicting the relative genetic relationship among the isolates was also made. RAPD analysis was found to be a desirable tool for molecular genetic characterization and for evaluation of genetic heterogeneity in *A.hydrophila*, which remain hidden by other techniques like morphological and biochemical characterization and cellular protein profile.

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CONTENTS

1. Introduction

INTRODUCTION

Aquaculture has grown significantly in economic value worldwide during the past decades. However, the aquaculture industry faces several problems that have to be solved before it can achieve significant growth as an industry. Disease is one of the major threats faced by this industry.

The role of the motile aeromonad, *Aeromonas hydrophila* as an etiological agent in fish and shellfish disease is highly significant. Although the outcome of disease by this secondary pathogen mostly depends on the presence of a primary pathogen, *A.hydrophila* is well recognized as a primary pathogen too (Miyata *et al.,* 1995).

Aeromonas hydrophila (Chester 1901) Stanier 1943, is gram negative, facultatively anaerobic, motile, waterborne bacteria with wide distribution. Since 1960, the potential of these organisms to cause diseases in human has been recognized and they have long been known to be pathogenic to cold and warm-blooded fauna. In humans and other mammals, *A.hydrophila* has been reported as the causative agent of numerous manifestations associated with gastroenteritis, systemic infections and bacterial endocarditis. In amphibians, reptiles and fishes, these organisms have reportedly been associated with necrotic septicaemic and ulcerative diseases (Austin and Austin, 1993; Inglis, *et al.,* 1993; Olafsen *et al.,* 1993). Though the exact role of *A. hydrophila* in disease remains in a state of flux, it is accepted as an opportunistic pathogen of homeothermic and poiekilothermic hosts.

To determine the health risks in aquatic environment associated with the exposure to *A.hydrophila*, epidemiological tracking is required. Conventional methods used for bacterial identification are tiresome, time consuming and not fit for mass scale screening of

bacteria. Microbial molecular genetics is gaining popularity in recent times as an essential tool in the classification of microbes. Highly discriminatory molecular typing methodologies allow rapid and sensitive differentiation within even a single serotype. These methodologies include plasmid profile analysis, ribotyping, DNA fingerprinting, cellular Fatty Acid Methyl Ester (FAME), Multilocus Enzyme Electrophoresis (MEE) etc.

Arbitrary/Random primed amplification of polymorphic DNA (AP-PCR/RAPD) has been increasingly reported as a method for the genetic characterization of microorganisms. Polymerase Chain Reaction (PCR) is the technique for *invitro* amplification of DNA, which lies between two regions of known sequence. AP-PCR/RAPD-PCR is one of the techniques useful for bacterial identification. The underlying theory in AP-PCR/RAPD-PCR is that arbitrary sequence is used as single primers that target complimentary genomic sequence in order to generate a genetic profile.

The rapid technique which was developed by Williams *et al.*, (1990) can be used to produce simple and reproducible DNA fingerprints. This is made possible using randomly designed short primers. Both genomic variations between bacterial species and genetic polymorphism between bacterial strain could be identified as the difference in the molecular size and number of DNA fragments obtained.

In the present study, a phylogenetic analysis of *Aeromonas hydrophila* isolates from aquaculture systems was carried out using the RAPD method and cellular protein profiling to evaluate genetic heterogeneity and phylogenetic relationship among them.

2. Review of Literature

REVIEW OF LITERATURE

The genus *Aeromonas* comprises of mesophilic motile and psychrophilic non-motile gram negative ubiquitous bacteria. Worldwide studies have demonstrated that *Aeromonas spp.* are universally distributed and widely isolated from environmental and food samples.

Miyata *et al.*,(1995) has reported *Aeromonas hydrophila* as both an opportunistic pathogen and a primary pathogen. Ever since *Aeromonas hydrophila* was recognized as a significant pathogen of humans and many aquatic organisms, much of the efforts were dedicated to develop methods for the correct identification of the species belonging to the genus *Aeromonas.* A complete review of methodologies for the isolation, identification and enumeration of *Aeromonas* spp. from clinical, environmental and food samples has been done by Joseph *et al.*,(1988).

For many years, the taxonomy of *Aeromonas* spp. was confusing and after significant revision, it now appears reasonably ordered by the work of Joseph *et al.*,(1994). The latest taxonomy of the genus has depended on a complex mixture of phenotypic and genotypic data. Bergy's Manual of Systematic Bacteriology (Popoff,1984),on the basis of phenotypic characteristics recognizes the 4 species in the genus *Aeromonas*: *A.hydrophila, A.caviae, A.sobria and A.salmonicida.*

A.hydrophila is a frequently encountered pathogen of human as well as aquatic and terrestrial animals (Janda and Duffey, 1988 ; Paniagua *et al.*,1990 and Anguita *et al.*,1993). Since more and more *A.hydrophila* strains are being found from different samples, development of sensitive and specific methods for their genetic characterization and rapid identification is very important. Many of the classical microbial procedures for the detection of *Aeromonas* spp. are laborious and time consuming or don't allow quantitative assessment

of these organisms. A number of modern molecular biology techniques are available for this purpose. This include techniques like DNA hybridization probes, DNA sequencing, Restriction Endonuclease Analysis (REA), PCR amplification of concerned sequences with specific primers, Amplified Fragment Length Polymorphism (AFLP), PCR based finger printing using random primers etc. However, Mazurier *et al.*,(1992) has suggested that many of them are not suited for large-scale routine use. Technical sophistication is the main constraint towards the adoption of many of these techniques for routine analysis. However, PCR based fingerprinting is suited for molecular genetic characterization.

PCR based Molecular Characterization of Microbes.

PCR is a technique for the *invitro* amplification of DNA, which lies between two regions of known sequence. American Biochemist Kary B. Mullis conceived the idea of PCR in 1983 and was later developed into a technique by Mullis and his associates at the Cetus Corporation in Emeryville, California. This technology has proven to be a revolutionary method, which gives scientist the great advantage of generating a large number of target DNA sequences from trace amounts of DNA material. Since it's introduction was first reported by Saiki *et al.*,(1985), PCR has already become a wide spread technique in research laboratories. RAPD is one of the many modifications of PCR principle, which can be used for molecular genetic characterization of the microbes.

RAPD–PCR

In 1990, this new method for the rapid fingerprinting of genomic DNA through the Random Amplified Polymorphic DNA (RAPD) was described simultaneously by Welsch and Mc Clelland (1990) and Williams *et al.*,(1990). Their work was based on the PCR principle. This technique is best suited for molecular genetic characterization of the microbes. This technique using

random primers is relatively simple, easy, and has wide genome coverage. It has the advantage of not requiring any information on the DNA sequence of the bacteria in question (Pattanayak *et al.*,2001). Only nanogram quantities of the DNA are required for this type of fingerprinting as against other methods of fingerprinting.

The RAPD technique was used by Miyata *et al.*,(1995) to analyze the genetic differentiation of *Aeromonas salmonicida* sub spp. *salmonicida* and *A. hydrophila* isolated from U.S.A., U.K. and Japan. Reproducible profiles of the genomic DNA fingerprints were generated by PCR amplification using single, randomly designed primer. The RAPD profiles of non-motile aeromonads were identical. However, profiles of motile aeromonad, *A. hydrophila* differed between isolates. Genetic homogenity in *A. salmonicida* and genetic variety in *A.hydrophila* was revealed through this study.

Okaey *et al.*, (1996) used the RAPD-PCR to investigate the differentiation of the genus *Aeromonas* at the genomospecies level. In most cases, each strain gave a unique fragment, illustrating genetic heterogeneity at the genomospecies level. However, some homogeneity in fragment sizes was seen among strains within a genomospecies. This study therefore complemented and supported the current classification of Aeromonas into genomospecies. The potential of RAPD-PCR to differentiate between the genomospecies of *Aeromonas* was also proved through their experiment.

Single locus, multiplex and seminested PCR procedures were experimented by Ozbas *et al.*(2000) for the detection of *Yersinia enterocolitica* and *A.hydrophila* in raw milk samples. PCR results were compared with conventional cultural procedures. These results indicated that PCR analysis of raw milk could be used as rapid diagnostic method for both these pathogens.

Martin Kearley *et al.*, (1994) reported numerical analysis and the application of RAPD-PCR for the differentiation of *Vibrio* strains. RAPD

fingerprinting results showed that the regional strains were significantly different from their reference strain *Vibrio splendidus biovar* or *Vibrio ordallii*. Fast identification of species and strains of *Vibrio* by amplification of polymorphic DNA was also been done by Martinez *et al.*, (1994).

Hoi *et al.*, (1997) made a comparative study of ribotyping and RAPD-PCR for characterization of *Vibrio vulnificus*. Both the results showed that the collection of strains under study were genetically heterogeneous. They could also come to the conclusion that the RAPD-PCR was not appropriate for developing a typing scheme for *V. vulnificus*. However, this method can be applied for the identification of individual strains or to the sub typing of ribotypes because of its high sensitivity.

Genetic relatedness among environmental, clinical and diseased-Eel *Vibrio vulnifcus* isolates from different geographic regions by ribotyping and RAPD-PCR was reported by Covadonga *et al.*, (1998). On the basis of their result, it was proposed that RAPD-PCR was a better technique than ribotyping for rapid typing in the routine analysis of new *Vibrio vulnificus* isolates.

AP-PCR approach is sensitive to minor modifications of reaction parameters. However, optimization of the arbitrarily primed polymerase chain reaction (AP-PCR) for intraspecies differentiation of *Vibrio vulnificus* was done by Vickery *et al.* ,(1998).

Warner and Oliver, (1999) conducted the RAPD analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *Vibrio* species. This method clearly differentiated between members of the genus *Vibrio*, and between isolates of *Vibrio vulnificus*. All of the *Vibrio vulnificus* isolated from clinical specimens produced an additional band, which was only occasionally found in environmental strains. This suggests that this band may be correlated with the ability of a strain to produce infection in humans.

Goarant *et al.*, (1999) conducted the molecular typing study on *Vibrio* strains implicated in shrimp disease outbreaks in New Caledonia and Japan by using AP-PCR. It allowed rapid identification of isolates at the genomo species level and studies of intraspecific population structures of epidemiological interest. Clusters identified within the species *Vibrio penaeicida* were related to their area of origin, allowing discrimination between Japanese and New Caledonian isolates.

Cluster analysis of AP-PCR generated fingerprints of *Vibrio vulnificus* isolates from patients fatally infected after consumption of raw oysters was evaluated by Vickery *et al.*,(2000). Their analysis revealed significant genetic heterogeneity among these strains suggesting that *Vibrio vulnificus* has high degree of variation in it's genomic organization. Multiple pathogenic strains with greatly diverse genomic arrangements caused the infection rather than a single type of infective strain.

Mazurier *et al.*,(1992) developed a method to obtain reproducible DNA fingerprints from *Campylobacter* by PCR based amplification without the need to isolate total DNA. RAPD profiles were generated with three randomly designed 10-mer primers. Depending on the primer, the analysis of RAPD profiles resulted in different levels of discrimination between the strains. Clear correlations were observed between results of RAPD analysis and serotyping.

Lawrence *et al.*,(1993) used the 10-mer primer OPM-01 to generate RAPD profile by PCR for 91 strains of *Listeria monocytogenes* from raw milk, food, veterinary, medical and environmental sources. They could find that annealing at low stringency and introducing 1-min ramp time between annealing and extension temperatures enhanced the reproducibility.

Arbitrary products produced by PCR have been shown to detect polymorphisms in DNA from bacterial isolates of the same species, in a study

conducted by Brousseau *et al.*, (1993). A range of *Bacillus thuringiensis* isolates were typed by using randomly designed primers.

RAPD based fingerprinting of 21 serovars of *Bacillus thuringiensis* representing different serotypes was performed by Pattanayak *et al.*,(2001) using 19 random decamer primers. Pair wise genetic similarity analysis revealed very low similarity values, indicating high genetic divergence. The high genetic divergence may be due to the presence of plasmid and their conjugal transfer mechanism. The dendogram generated based on RAPD data, grouped the serovars of Bt into 2 major clusters.

A less complex PCR-based typing method for *Mycobacterium tuberculosis* was outlined by Ross and Dwyer (1993). Linton *et al.*,(1994) reported the rapid discrimination of *M.tuberculosis* strains by RAPD analysis. Investigations of the epidemiology of tuberculosis have been hampered by the lack of strain-specific markers that can be used to differentiate the isolates of *M.tuberculosis*. The development of a rapid protocol for RAPD analysis in 8 hours helps in faster identification and is an alternative to Restriction Fragment Length Polymorphism (RFLP) analysis.

Molecular determination of infection source of a sporadic *Legionella pneumonia* case associated with hot spring bath was evaluated by Miyamoto *et al.*,(1997). They used 5 molecular methods including repetitive element PCR, AP-PCR, Restriction Endonuclease Analysis (REA) and macro restriction endonuclease analysis by pulse field gel electrophoresis.

Inter-species differentiation of benign *Theilerias* by genomic fingerprinting with arbitrary primers was developed by Chansiri *et al.*,(1998). The method was simple for sensitive diagnosis in characterization of the parasites, since it does not require prior DNA sequence information to construct species-specific probes or primers.

Genomic sub typing of *Edwardsiella ictaluri* isolated from diseased channel cat fish by AP-PCR was done by Bader *et al.*, (1998). AP-PCR combined with Enterobacterial Repetitive Intergeneric Concensus PCR primer was used for subtyping the isolates. Biochemical analysis showed homogeneity of isolates and was not useful for discriminating between isolates.

MIKOLAJCZAK *et al.*,(1999) reported the molecular characterization of unicellular marine *Cyanobacteria* from the Baltic Sea using DNA sequence analysis and RAPD-PCR. The results were correlated with the classical determination relying on morphological features. RAPD-PCR was used to investigate the DNA sequence polymorphism. A unique band from RAPD pattern was cloned, sequenced and developed into a probe to compare the strains by Dot-Blot hybridization.

RAPD analysis conducted by Magarinos *et al.*,(2000) have shown the existence of 2 geographically linked clonal lineages in the marine bacterial fish pathogen *Photobacterium* damsela *subsp. piscicida*. The RAPD analysis using various oligonucleotide primers differentiated the European strains from the Japanese strains.

A comparison of AP-PCR, antibiotic resistance and plasmid profile of *Salmonella enteritidis* isolated from fish was conducted by Radu *et al.*,(2000). The AP-PCR result showed that the collection of isolates were genetically very heterogenous. Their results also demonstrated that AP-PCR method was more sensitive than plasmid profiling and antibiotic resistance patterns with respect to the individualization of the isolates.

Eight strains of *Desulfosporosinus meridiei* sp. *nov.*, a spore forming sulfate reducing bacterium were clustered into 2 main groups based on phylogeny, RAPD-PCR patterns, and nutritional characteristics (Robertson *et al.*2001).

RAPD-PCR/AP-PCR has also successfully been used to type *Lactococus lactis* (Cancilla *et al.*, 1991), *Haemophilus somnus* (Myers *et al.*, 1993), *Helicobacter pylori* (Akopyanz *et al.*, 1992), *Staphylococcus aureus* (Saulnier *et al.*, 1993), *Streptococcus uberis, Klebsiella pneumonia* and *Esherichia coli* (Bassam *et al.*, 1992), *Histoplasma capsulatum* (Kersulyte *et al.* (1992), pathogenic fungi *Aspergillus fumigatus* (Brown, A. *et al* 1992.), *Candida albicans* (Lehmann, *et al.*, 1992) and Aphanomyces *astaci* (Huang *et al.*,1994).

Protein profiling for characterization of microbes

Mc Lean *et al.* (1993) suggested that cellular protein profiles have wide applications in characterization, classification and identification of bacterial isolates.

Coveney *et al.* (1987) characterized and compared four lactic streptococcal bacteriophages on the basis of structural protein analysis along with morphology and DNA homology.

In a study by Daly & Stevenson (1990), water extracted proteins from 9 geographically diverse strains of *Renibacterium salmoninarum* were compared by SDS-PAGE. Extracts from seven of these strains as well as the type strain ATCC-33207 were similar in having a major protein of 57Kda and a minor protein of 58Kda. While one of the remaining strains (char strain) did not contain the 58Kda protein, the other strain (MT-239) lacked both the proteins, thus helping in their characterization.

Niemi *et al.* (1993) made use of the protein profile resolved by the SDS gel electrophoresis for identifying the faecal *Streptococcus* from the environmental samples.

Ragni *et al.* (1996) characterized 6 mosquitocidal *Bacillus thuringiensis* strains by protein profiling. While five of them showed same protein profile and

mosquitocidal activity, the sixth strain showed a different protein profile as well as a novel mosquitocidal activity.

Mycoplasma gallisepticum, was found to have protein bands of 40-67 KDa while *M.gallinaraum* and *M.synoviae* were found to have identical protein bands of 35-43 and 60-94KDa respectively (Thongkamkoon *et al.*1996). Khan *et al.*,(1996) worked out the protein profile of the whole cell protein of 42 strains of *Pseudomonas aeruginosa* by SDS-PAGE and found the presence of 45 protein bands of different molecular weights. Individual isolates had 37 to 42 protein bands ranging in molecular weight from 340 KDa to 14.3 KDa.

Nakayama & Masashi (1997) carried out protein profiling of halophytic Archaea, *Halobacterium halobium*. The two-dimensional SDS-PAGE of the whole cell extract revealed the existence of 242 different bands.

A study of the profile of cell wall proteins of different species of thermophilic *Lactobacilli* by Gatti *et al.*, (1997) showed that a protein of approximately 50KDa was characteristic for all the strains of *L.helveticus* and two proteins of about 20 and 30 KDa were typical to *L.debrueckoi*. He concluded that the SDS-PAGE analysis of cell wall proteins could be used for differentiating between the two species.

The protein profile of three members of the genus *Brachyspira* viz. *B.hyodysenteriae, B.innocens* and *B.pilosicoli,* were compared with that of *B.aalborgi* using SDS-PAGE, by *Ochiai* et al., (1997). The profile of *B. aalborgi* was different from others, except for the two heavy protein bands of 49.4 and 52.3 Kda in the *B.innocens*

Hadrys *et al.*,(1992) observed that RAPD is able to reveal intraspecies genetic diversity which remain hidden with other methods of characterization especially the cellular protein profile. Hanninen *et al.*,(1995) could not find any intraspecies variations between 28 finfish isolates of *A.salmonicida* in their cellular protein profile resolved through SDS-PAGE where as 15 RAPD patterns were resolved from analysis using two primers.

3. Materials and Methods

MATERIALS AND METHODS

Aeromonas hydrophila used in the study were isolated in the Microbiology laboratory of CMFRI. They were tested for the confirmity with the accepted definition.

3.1. Isolation of *Aeromonas hydrophila*

Fish samples, water and sediment were collected from the aquaculture systems for the isolation of bacteria, following the aseptic procedures. Isolation of *Aeromonas hydrophila* was done using selective medium, Rimler Shotts agar (HI MEDIA).

Approximately 1gram sample (intestine and gills) from fish were aseptically transferred to a sterilized glass mortar, ground well with pestle and mixed well with 99 ml of sterile distilled water taken in a conical flask. In the case of sediment, 1 g of sample was transferred to a conical flask and shaken well. Water samples were membrane filtered and the filter paper was put in sterile 99ml distilled water dilutions and shaken in bacteriological shaker for 30 minutes. After thorough shaking, serial dilutions were made according to the standard procedures.

From the samples thus prepared, 1ml was transferred to 10cm sterile glass petridishes and pour plated using Rimler Shotts agar (Shotts and Rimler, 1973). Then the plates were incubated at 37°C for 24-48 hours. Yellow colonies without black centers facilitate the rapid identification of *Aeromonas hydrophila* in Rimler Shotts media.

Selected colonies were subcultured in nutrient agar slants and peptone water as stock culture. Subculture was also done at different stages of the study, to carry out different biochemical tests. If the streak plate has more than one morphological type of colony, each type was subjected to the

confirmation steps. The identity of the isolates was confirmed with morphological characteristics and biochemical tests.

3.1.1. Morphological characterization

Morphological characterization including motility test and gram staining reactions were carried out.

1.Hanging drop method

A drop of young culture was transferred to the center of the coverslip. A cavity slide was placed over the coverslip and then inverted so that the drop of culture will be hanging in the centre of the cavity slide. Microscopic examination was carried out to find the motility. *A.hydrophila* is found to be motile.

2.Gram's stain

A thin smear of the culture was prepared, air-dried and then heat fixed. The primary staining with crystal violet was followed by the addition of mordant iodine. The crystal violet- iodine complex formed is decolourized with 95% alcohol. Saffranin was added as counterstain to the smear. The bacteria are classified into gram positive, gram negative, gram variable and gram unreactive forms. *A.hydrophila* is gram-negative.

3.1.2. Biochemical tests

Pure cultures of *Aeromonas hydrophila* were maintained in nutrient agar slants and peptone broth at room temperature for carrying out differential biochemical tests.

1.Oxidase test

For conducting the oxidase test, freshly prepared N', N', N', - Tetramethyl paraphenylene diamine dihydrochloride was poured on a filter paper strip and the culture is streaked on it using a sterile glass rod. Immediate appearance of a deep purple colour indicated the presence of oxidase enzyme. *A.hydrophila* is oxidase positive.

2.Trehalose fermentation

Oxidase positive colonies are then tested for trehalose fermentation.Trehalose fermentation is determined by inoculating a tube containing 3-10 ml of 0.5% trehalose in purple broth base with a colony from nutrient agar and incubating at 35°C for 24±2 hours. A change in colour of the medium from purple to yellow is considered positive for trehalose fermentation test, indicating the presence of *A.hydrophila*.

3.Catalase test

Hydrogen peroxide was taken in a test tube and a loop containing bacterial culture was dipped into it. The catalase positive reaction is shown by dense bubble of oxygen coming out of the loop, whereas negative reaction fails to form the oxygen release. *A.hydrophila* is catalase positive.

4.IMVIC tests

a) Indole test: Trypton broth media was inoculated with one loopful of 24-hour nutrient broth culture and incubated for 2-4 days . Into the tube was added 5cc of Kovac's reagent. The appearance of deep cherry red ring in the reagent layer indicated the presence of indole. *A. hydrophila* is indole positive.

b)Methyl red- Voges Proskauer test:

MR-VP broth was inoculated with the bacteria and incubated for 3-4 days, at room temperature and then the MR-VP test were carried out

MR test: 5 drops of 0.04 % solution of methyl red was added to the cultured MR-VP broth, mixed well and read at once. A red colour is positive while yellow colour signifies a negative test.

VP test: 0.6 ml of 5% solution of α-naphthol in ethanol and 0.2 ml of 40% KOH are added to 1ml of MR-VP cultured broth. Pink colour in 2-5 minutes and deepening to magenta or crimson in 30 minutes indicates VP positive and negative if it remains colourless.

C) Citrate utilization test: The citrate utilization was detected by inoculating the cultures on slants made from Simmons Citrate Agar. After incubation period at room temperature, the positive reaction is shown by the development of blue colour in the medium, indicating positive result for *A.hydrophila.*

5.Nitrate reduction test

The ability to reduce nitrate was tested in ordinary peptone broth containing 0.3% potassium nitrate inoculated with the culture. Turbidity was checked after the incubation period and the nitrate reduction was tested with Nitrate reagent (α-Napthylamene-Reagent A and Sulphanilic acid-Reagent B mixed in equal proportion while doing the test). *A. hydrophila* gives positive reaction for this test.

6.Production of Ammonia or Ammonium salts.

Peptone broth was prepared, sterilized and inoculated with a loopful of culture. Tubes were incubated for 3-4 days after which two drops of Nessler's reagent was added to the tube. The presence of ammonia in the

medium reacting with Nessler's reagent is shown by quick development of yellow, orange or brown colour. The intensity of colour indicates the concentration of dissolved ammonia. *A.hydrophila* gives positive result with ammonia production.

3.1.3. Antibiotic sensitivity test

Resistance of *A.hydrophila* to Ampicillin and Novobiocin was conducted with young and active cultures (24 hours). The test cultures were streaked on the Agar media and antibiotic discs were placed on the surface of the medium and the plates were incubated. *A.hydrophila* is resistant to Ampicillin and Novobiocin.

3.2. Aeromonas hydrophila isolates used for the molecular characterization.

In all, 18 bacterial isolates, which were classified as *A.hydrophila* on the basis of the morphological characteristics, biochemical tests and antibiotic sensitivity tests were used for the molecular genetic characterization. Of the 18 number of isolates, 12 isolates were from fish, 4 from water and 2 from sediment. These isolates were designated serially from Ah_1 to Ah_{18}.

3.2.1.Preparation of nutrient broth cultures

Nutrient broth cultures of the isolates were made to facilitate the isolation of DNA from them. Nutrient broth used for the culture contained 1.5% salt. Incubation was done at 37°C for 24 hours.

3.3.Isolation of bacterial DNA

3.3.1.Principle

The commonly used protocols in bacterial DNA isolation consists of lysis of cell wall using lysozyme/ detergent, followed by incubation with a proteolytic enzyme like Proteinase-K followed by phenol/ chloroform/ isoamyl alcohol extraction. Such procedures effectively remove the contaminating proteins but are not effective in removing exo-polysaccharides that are produced by many bacterial genera and which can interfere with the activity of molecular biological enzymes such as restriction endonucleases and ligases. This problem can be solved by CTAB extraction prior to phenol extraction.

3.3.2.Reagents required

All the chemicals and enzymes used were of molecular biology grade.

1) TEG Buffer (pH8)
2) Lysozyme (Sigma, USA)
3) 10%SDS
4) Neutral phenol
5) Chloroform: Isoamyl alcohol (24:1)
6) 3M Sodium acetate
7) Absolute ethanol
8) 70%Ethanol
9) Deionised water
10) Nacl
11) TE buffer
12) CTAB
13) Proteinase-K.

3.3.3.Procedure:

- Inoculate a 10ml liquid culture media with the bacterial strain. Incubate for overnight.
- Centrifuge 1.5ml of the culture in a microcentrifuge for 2 minutes or until compact pellet forms. Discard the supernatant.
- Resuspend the pellet in 567μl of TE by repeated pipetting.
- Add 30μl of 10% SDS and 3μl 20mg/ml proteinase-K to give a final concentration of 100μg/ml proteinase-K in 0.5% SDS. Mix thoroughly and incubate at 37^0C for 1hr.
- Add 100μl of 5M NaCl and mix thoroughly.
- Add 80μl CTAB/ NaCl solution. Mix thoroughly and incubate for 10 min at 65^0C.
- Add equal volume of chloroform/ isoamyl alcohol mix thoroughly and spin 4 to 5 min in a centrifuge. A white interface should be visible after centrifugation.
- Extract the aqueous phase with phenol / chloroform isoamyl alcohol and spin in a microcentrifuge for 5 min. Transfer the supernatant to a fresh tube. Add 0.6 volume of isopropanol to precipitate the nucleic acids.
- Transfer the spongy white DNA precipitate to a fresh tube containing 70% ethanol.
- Spin at 10, 000 rpm for 5 min at room temperature.
- Carefully remove the supernatant and briefly air- dry the pellet.
- Redissolve the pellet in 100μl TE buffer and use for further analysis.

Several modifications are tried on the above protocol in an attempt to reduce the extraction time and expensive chemicals like CTAB, Proteinase-K, etc. Bacterial cell wall lysis was done using TEG (pH8) with lysozyme (5mg/ml) replacing expensive Proteinase-K. This is followed by the denaturation of proteins with SDS at room temperature and further extraction with neutral Phenol and Chloroform to augment protein precipitation. The supernatent was re-extracted with neutral Phenol and the DNA was directly precipitated from the resulting aqueous phase. The dried DNA pellet was re-hydrated with sterile deionised water and used for further studies.

The qualitative integrity of DNA sample isolated are checked by 0.8 % agarose gel electrophoresis, detected by subsequent ethidium bromide staining and viewed under UV Transilluminator. The DNA quantification was done by comparing the intensity of the sample DNA with known concentration of molecular weight markers run along with the sample.

Based on the quantitative and qualitative analysis of DNA using agarose gel electrophoresis, dilution of the template DNA for RAPD-PCR is decided. Dilution is made in such a way that the final concentration of template DNA for RAPD-PCR is approximately 50 ng/µl.

3.4. Random Amplified Polymorphic DNA - Polymerase Chain Reaction (RAPD-PCR).

3.4.1.Primers used

A panel of10 numbers of decamer random primers from M/S Operon Technologies, designated as OPA-01 to OPA-10 was used for PCR amplification of the bacterial DNA template.

3.4.2. RAPD PCR: Standardization

Standardization of the optimum concentration of different components in PCR was done by varying the concentrations. Varying concentration of $Mgcl_2$ ranging from 1mM to 2mM in assay buffer was attempted and the one with 1.5mM concentration was found to give better results. Similarly varying pH ranging from 8.5 to 9.5 was attempted and the concentration with pH 9 is found to give better amplification. Likewise the concentration of Taq polymerase and dNTP were also standardized as 1.25μM and 200μM respectively. The concentration of template DNA and primer were also optimized as 50ng and 7.5 pico moles respectively for each PCR reaction. Master mix for PCR was prepared under aseptic condition inside a laminar airflow environment. PCR assay was carried out in a final volume of 25μl. Based on the amplification efficiency, the five primers were shortlisted for regular screening of all the samples.

3.4.3. PCR cycles

Thermal cycling was performed with Perkin-Elmer thermocycler (GeneAmp.PCR System2400). Each of the 45 PCR cycles standardized for this work consisted of denaturation of DNA at 92° C for 1 minutes, primer annealing at 37°C for 1 minutes and primer extension at 72°c for 1 minutes. All PCR samples were subjected to an initial denaturation step at 94°C for 2 minutes and a final extension at 72°C for 10 minutes. PCR products were stored at -20°C until electrophoresis was performed.

3.4.4. Electrophoresis

The amplified DNA products were resolved through agarose gel electrophoresis.

Reagents required:

1) Agarose

2) 1x TEB (pH 8)

0.89M TRIS Hcl.

0.02M EDTA

0.89M Boric acid

3) Loading buffer

Glycerol 2ml.

Bromophenol blue (0.5%) 1ml.

1x TEB

4) Standard DNA marker (λDNA cut with HindIII/EcorI)

5) Ethidium bromide (1μg/ml.)

Procedure:

An agarose gel of 1.5% strength in 1X TEB was casted. Four microlitres of PCR products were mixed with 2 μl of loading buffer and loaded into the gel. The electrophoresis was carried out at 80 voltage for two hours, The gel was stained in ethidium bromide for twenty minutes and documented by gel documentation system.

3.4.5. Analysis of RAPD Data

The scorable bands produced by each of the primers, resolved through agarose gel electrophoresis, were scored in a binary matrix. (1/0 ie.1 to represent the presence and 0 to represent the absence of the band at an RAPD locus). These data were analyzed using the POPGENE 32 Software according to the procedures suggested by Baranek *et al., (2001)* for the analysis of RAPD data. Various parameters like overall amplicon frequency at each locus, number and percentage of polymorphic loci, Nei's original measures of genetic identity as well as genetic distances were estimated using the above software. Dendogram in the phylogram form was also generated using Nei's similarity coefficients by the unweighted pair group method using arithmetic averages (UPGMA Method), modified from NEIGHBOR procedure of PHYLIP Version 3.5

3.5.Bacterial protein isolation

Aeromonas hydrophila were harvested from 2 ml broth culture during the post logarithmic phase by spinning at 10K rpm, at 4°C, for 10 minute in 1.5ml eppendorf tubes in a refrigerated high speed centrifuge. The supernatant was drained off and 100 µl of TEG buffer (pH8) containing 5mg/ml lysozyme was added to each of the pellets and vortexed in a vortex mixer. This was incubated at 4°C for 15minutes, mixing gently every five minutes. The cell suspension was then centrifuged at 10000 rpm, at 4°C, for 10 minutes and the supernatant was collected in eppendorf tubes and stored at -20° C for further use.

3.6. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.6.1. *Principle*

SDS is an anionic detergent, which binds strongly to, and denatures proteins. The protein-SDS complex carries net negative charges, hence move towards the anode and the separation is based on the size of the protein.

3.6.2.Standardization of SDS-PAGE

The method in the present study was on the lines of Laemmli *et al.*, (1970). The percentage of separating gel was a critical parameter in all electrophoretic separations using discontinuous system of buffer along with stacking gel. Separating gel of 12.5%, 11.5%, 11% were tried, and the ideal percentage was found to be 11.5% concentration. This was selected for the present study. It was prepared from 30% stock of Acrylamide and Bisacrylamide monomers along with 6% of stacking gel. The concentration of protein sample to be loaded was also standardized for ideal resolution.

Gel cassette was prepared, sealed with 2% agar and then the components of separating gel buffer were mixed gently and poured into it. Immediately a few drops of butanol were dropped over the gel layer to prevent the meniscus formation and the gel was left undisturbed for 30 minutes. When the gel was polymerized, butanol over layering is removed and washed with double distilled water. Later the stacking gel mixture was prepared and was poured over the separating gel. A gel comb was placed in the stacking gel making sure that there is no air bubbles trapped in it and allowed to set for 30 minutes. The comb was removed from the solidified gel without disturbing the shape of the well. Then the gel was placed on the electrophoretic apparatus with electrode buffer ensuring that no air bubbles were entrapped at the bottom of the gel. The electrodes were then connected to the power pack.

3.6.3. Preparation of Samples

To 85µl of each of the above samples, 65µl of sample buffer is added. Simultaneously 10µl of SDS protein molecular weight marker from Banglore GENEI was mixed with 60µl of sample buffer. The samples were then boiled strictly for 5 minutes and the marker for 1 minute.

The prepared sample was loaded into the wells of the stacking gel and layered with running buffer in order to avoid disturbance to the sample. A constant voltage of 60 volts was applied until the dye front crosses the stacking gel and it was later increased to 140 volts and the electrophoresis was carried out till the dye front reaches the bottom of gel.

The gel, after completion of electrophoresis was washed gently with tap water to remove excess SDS and fixed in fixative for 2 hours. Then it was stained for Coomassie brilliant blue R-250 for a period of 2 hours.

The molecular weights of standard SDS-PAGE molecular marker used were 205KDa, 97.4KDa, 68KDa, 43KDa, 29KDa. RF value of the standard marker was calculated. A semi-log graph was drawn using the R_f values. The R_f values of unknown samples were calculated and extrapolated using the standard graph to determine the molecular weight.

4. Results

RESULTS

Aeromonas hydrophila were isolated from the fish samples and aquatic environment following the standard procedures. Primary isolation was done by streaking into the plates of Rimler-Shott's media. The isolates, which had heavy growth of yellow colonies without any black centres in this media, were selected. In primary characterisation the colonies that are gram negative, rod shaped, motile, oxidase positive, fermentative, resistant to Ampicillin and Novobiocin, were identified as Aeromonads. Detailed examination using differential tests and API 20E confirmed *A.hydrophila*. Eighteen isolates, which were confirmed to be *A.hydrophila*, based on these morphological and biochemical tests were designated serially from Ah_1 to Ah_{18} and were used for molecular characterization.

DNA Recovery

Isolation of total bacterial DNA was done following the original method developed by Murray and Thompson, (1980) as well as with modifications. The modified protocol without proteinase-K has given good result, yielding quality DNA of about 20μg from a 2ml bacterial culture. Qualitative analysis of the isolated DNA to check the yield and integrity by agarose gel electrophoresis

revealed that DNA remained intact, without any shearing and was in sufficient quantities for the envisaged work.

Molecular Characterization

Molecular genetic profiles of the 18 isolates of *A. hydrophila* carried out using RAPD-PCR as well as cellular protein profiling are presented below.

RAPD-PCR

The PCR conditions were optimized from a number of standardization trials with varying concentration of ingredients as well as thermal cycles. Concentration of $MgCl_2$ in assay buffer was found to be optimum at 1.5mM. Similarly, Taq polymerase and dNTP's were also standardized at 1.25 units and 200µM respectively. The annealing condition in the PCR cycle was optimized at 37°C. Identity and number of the amplicon bands decreased rapidly as the annealing temperature was increased to 55°C.

Initial screening of the PCR amplification using a panel of 10 primers (OPA-01 to OPA-10) gave amplification by only seven of them. These primers, which produced amplicons, scorable as bands, on resolving by agarose gel electrophoresis were OPA-01, OPA-03, OPA-04, OPA-05, OPA-06, OPA-07and OPA-10. Five of these seven were only selected for the final screening of the isolates, because they generated several reproducible bands. These primers were OPA-01, OPA-03, OPA-04, OPA-05 and OPA-10.

The amplification of the DNA from each of the 18 isolates with the above five primers produced a total of 46 amplicons, which were consistent and appeared as distinct bands on agarose gel after electrophoresis. The molecular weight of the amplicons ranged from 2.5 kb on the higher side to approximately 0.2kb on the lower side as determined from the relative mobility of marker DNA viz. λ DNA cut with HindIII /EcoR I. The RAPD pattern generated by each of the primers is presented below in detail.

OPA-01

All the isolates were screened with OPA-01. This primer produced 12 amplicons, which were scorable as distinct bands in the gel. The RAPD fingerprints generated for each of the isolates using OPA -01 are presented in Fig.1. Majority of the amplified fragments were polymorphic. While ten fragments showed variation between isolates, two were amplified in all the isolates. The amplicons shared by all the isolates were of 0.94 kb and 1.37 kb size. These are species specific amplicons and can serve as molecular markers for species identification. The fragments showing variation between isolates within the species were 2.5kb, 1.3kb, 1.2kb, 1.1kb, 1kb, 0.83kb, 0.8kb, 0.7kb, 0.6kb, 0.54kb and 0.4kb. DNA fragments of 0.8 kb, 0.7kb, 0.6 kb and 0.4kb were shared by majority of the isolates ie.,by14,14, 11, and 13 isolates respectively. While the 2.5kb and 1.0 kb fragments were found in 6 and 5 isolates, the 1.1kb and 0.54 kb fragments were found in seven isolates each. The least shared fragments were

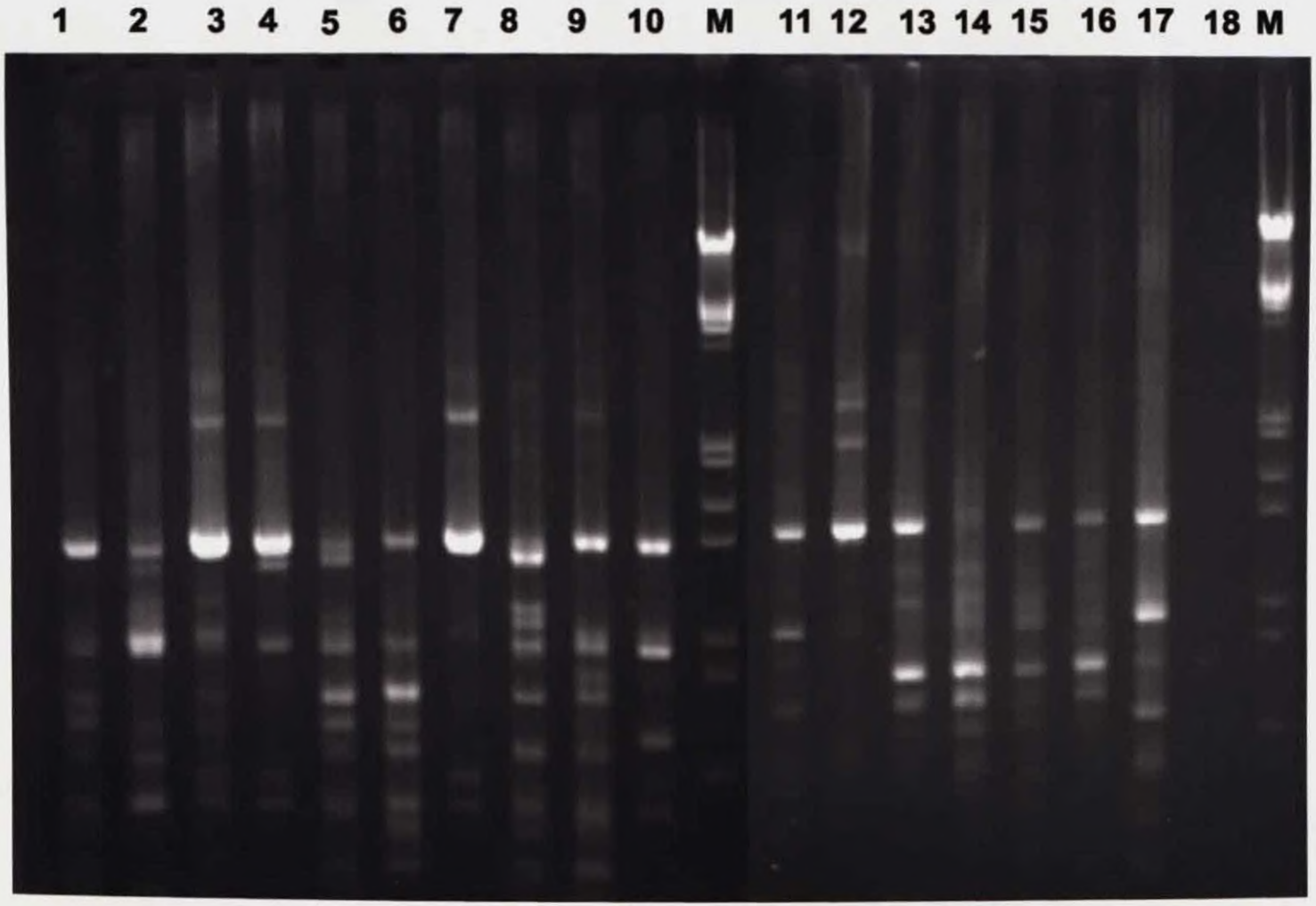

Fig.1 RAPD pattern of 18 isolates of *Aeromonas hydrophila* generated by primer OPA-01
Lane 1 - 18: Ah1 - Ah18 isolates; M : Marker

0.83 kb and 1.2 kb having amplified only in two and four isolates respectively. Frequency of each of the amplicons produced by OPA-01 over all the isolates are presented in the Table2. The frequency ranged from 0.111 to 1.00

OPA-03

Seven amplicons were only generated by OPA-03. These amplicons were within the range of 1.584 kb to 0.83 kb. One of these, viz. 0.83 kb was shared by maximum number of isolates. While the 1.584-kb fragment was shared by 11 of the isolates, the sharing of others were of a lower order. Frequency of the amplicons ranged from 0.111 to 0.722. Fig.2 presents the RAPD fingerprints of isolates generated by primer OPA-03.

OPA-04

A total of 10 amplicons were generated by OPA-04 ranging in size from 2.5 kb to 0.55 kb. Though the number of amplification varied in individual isolates, the amplicon of 1.584 kb was shared by all except three. The amplicons of 2kb, 1.9 kb, 0.8 kb, 0.7 kb and 0.55 kb were found to be common in majority of the isolates ie., 12 to 14 number of the isolates. The amplicons generated by OPA-04 from each of the 10 isolates as resolved by agarose gel electrophoresis is presented in Fig.3. Frequency of each of the amplicons over all the isolates are presented in Table 2. The frequency ranged from 0.333 to 0.889.

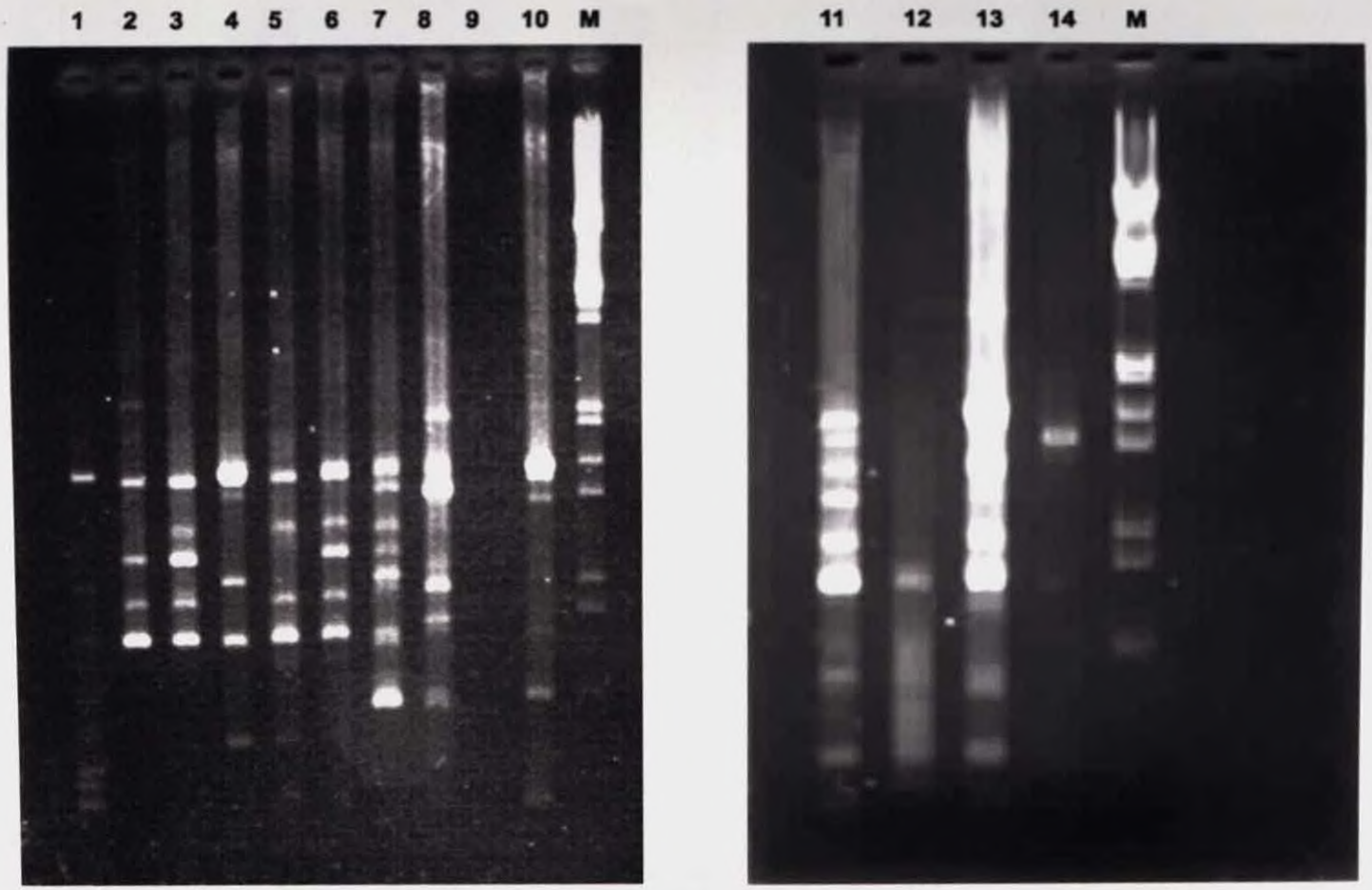

Fig.2 RAPD pattern of 14 isolates of *Aeromonas hydrophila* generated by primer OPA-03
Lane 1 - 14: Ah5 - Ah18 isolates; M : Marker

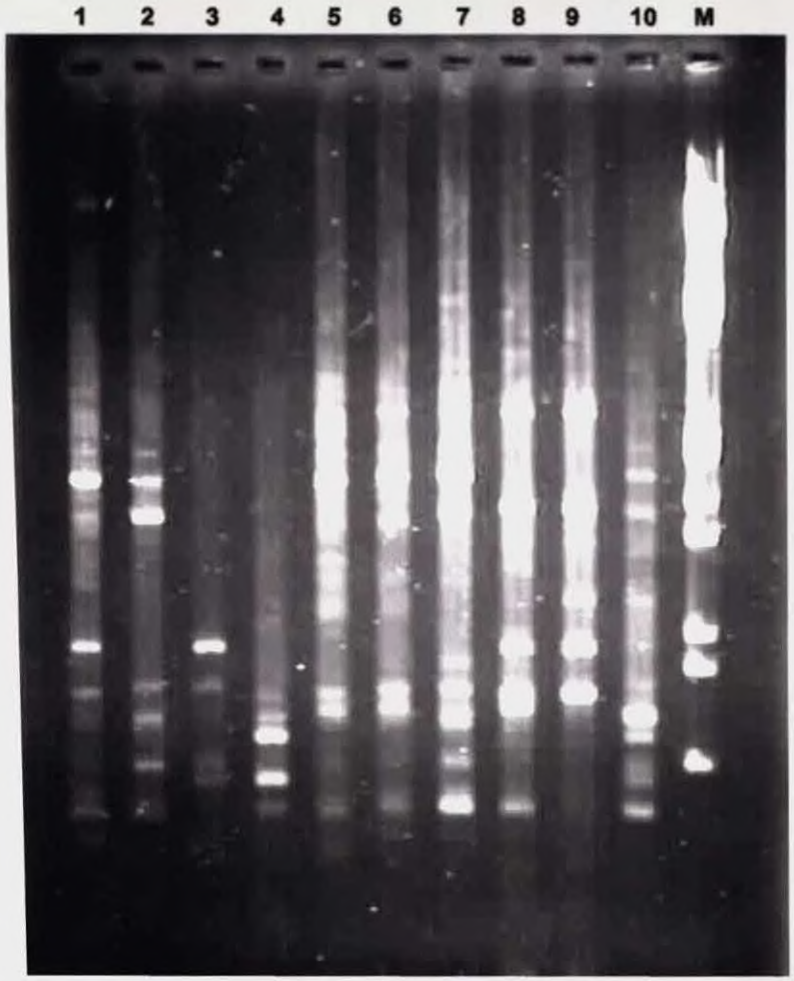

Fig.3 RAPD pattern of 10 isolates of *Aeromonas hydrophila* generated by primer OPA-04
Lane 1 - 10: Ah1-Ah10 isolates; M : Marker

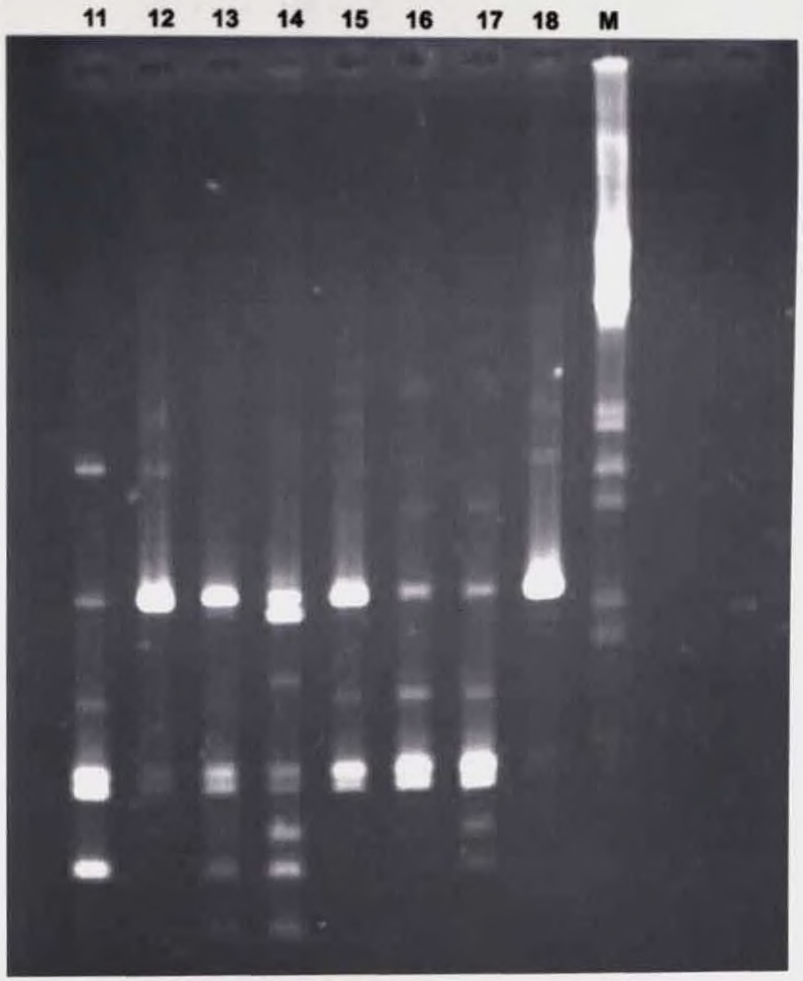

Fig.4 RAPD pattern of 8 isolates of *Aeromonas hydrophila* generated by primer OPA-05
Lane 11 - 18: Ah11 - Ah18 isolates; M : Marker

OPA-05

Primer OPA-05 produced the least number of amplicons among the primers used. Of a total of 6 amplicons, four were below 0.5 kb size only. The remaining was of 1 kb and 0.7 kb size. While the 1 kb and 0.7 kb fragments were shared by 11 isolates, the 0.45 kb and 0.20 kb fragments were amplified in 12 isolates each and 0.3 kb was shared by 10 isolates. The 0.564 kb fragment was shared by 15 isolates. However, none of the amplicons were shared by all the isolates. Frequency of the amplicons was high and ranged from 0.611 to 0.833. The amplicons generated by OPA-05 from each of the isolates as resolved by agarose gel electrophoresis is presented in Fig.4.

OPA-10

The primer OPA-10 with 11 number of amplicons was second to only OPA-01. These 11 bands were within the range of 1.9 kb to 0.2kb. While none of the amplicons were shared by all the isolates, the 0.947kb amplicon was common to 16 of them. The 1.9 kb and 1.58 kb amplicons were shared by 11 & 12 isolates respectively. Others were shared only by a few number of isolates. As can be seen from the Table 2, frequency of the different amplicons varied from 0.055 to 0.833. The RAPD fingerprints generated by each of the isolates using primer OPA-10 is presented in Fig.5.

In all, the amplification of the DNA from the 18 isolates with the above five primers produced a total of 46 amplicons, which were consistant and scorable as

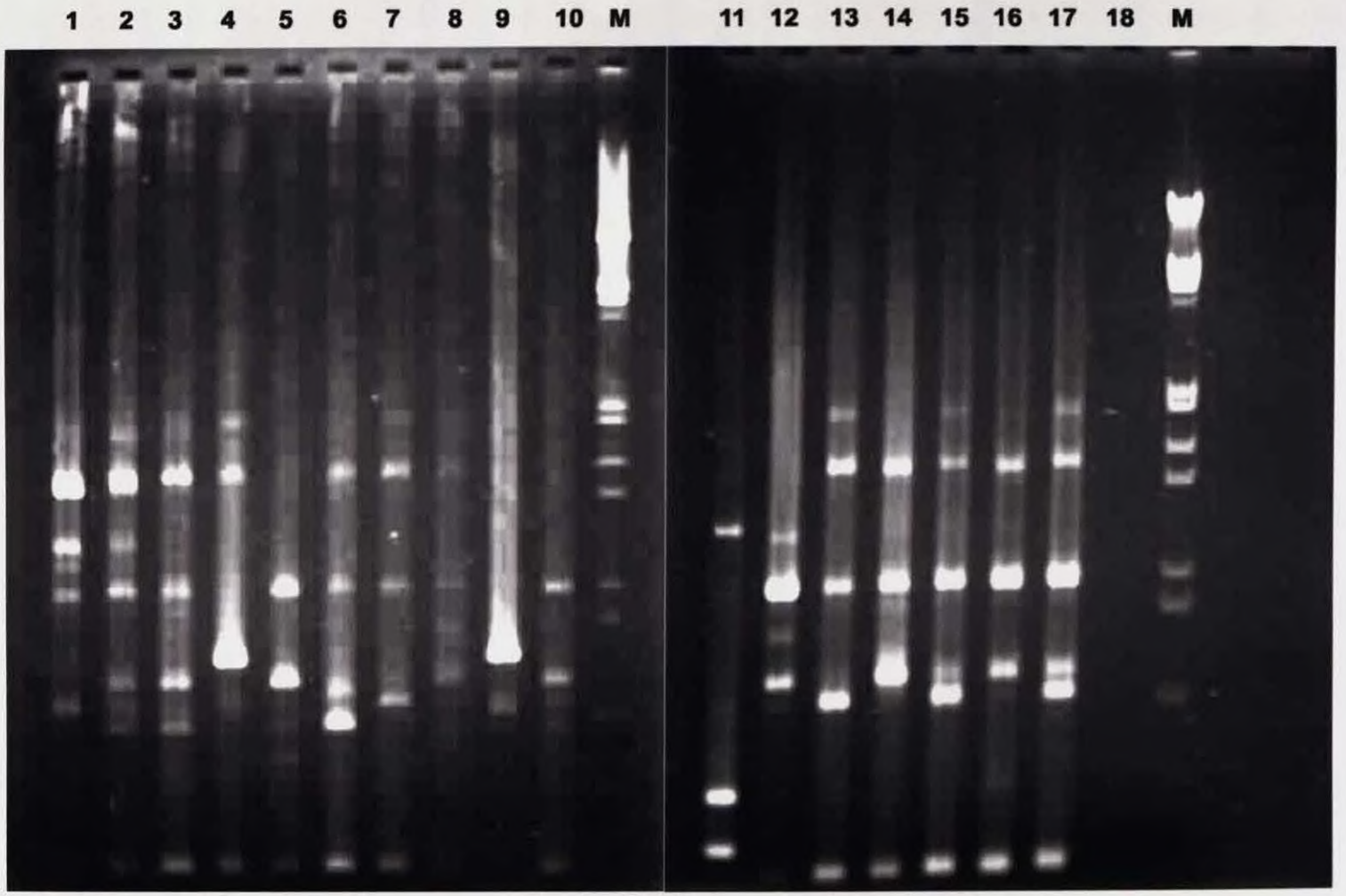

Fig.5 RAPD pattern of 18 isolates of *Aeromonas hydrophila* generated by primer OPA-10
Lane 1 - 18: Ah1 - Ah18 isolates; M : Marker

distinct bands on agarose gel after electrophoresis. Informations on amplicons from different primers are summarized in Table1. The molecular weight of these amplicons ranged from 2.5 kb on the higher side to approximately 0.2kb on the lower side.

Polymorphism, Similarity index, Genetic distance and Phylogenetic relationship.

The data pertaining to random amplified DNA fragments produced by all the primers across the 18 isolates were analyzed by the POPGENE 32 Software and the resulting estimates are presented below.

Polymorphism of the RAPD pattern was quite apparent among the different isolates. RAPD fingerprint pattern was unique for each of the isolates with all the primers. Comparison of the fragment at each of the 46 loci indicated that all but 2 fragments were polymorphic. The two non-polymorphic loci were of OPA-01. However, persual of the banding pattern shown in Table1 indicate that there were a number of fragments, which were homologous among many of the isolates. The POPGENE analysis indicated that the overall polymorphism was 95.65%. All the primers except OPA-01 were highly discriminatory. The two monomorphic loci of OPA-01 with fixed allele viz.1.33kb and 0.94 kb are species specific and so can serve as molecular markers of *A.hydrophila*.

The average similarity index between isolates within species, considering all the amplicons resulting from all the primers estimated as Nei's original measures of genetic identity and genetic distance between the 18 isolates are shown in Table 3. Persual of the table shows that though the coefficient of genetic identities ranged from 0.413 to 0.848, it was of high magnitude in most of the cases. Estimates of genetic distances were of lower magnitude. Of the 153 pair wise genetic identity estimates, ninety-eight percentage (98%) were higher

than 0.5 and sixty-three percentage (63%) of the estimates were more than 0.6 in magnitude. Genetic similarity less than 0.5 were observed only in 1.96-% cases ie. only 3 out of a total of 153 pair wise comparisons.

Dendograms in the phylogram form, depicting the phylogenetic relatedness between various isolates and Nei's genetic similarity coefficients, generated by the unweighted pair group method using arithmetic averages (UPGMA, modified from NEIGHBOR procedure of PHYLIP Version3.5) is presented in Fig.7.

Examination of the dendrograms shows that the isolates were grouped into two major clusters. While 13 of the isolates (Ah_1 to Ah_8, Ah_{13}, Ah_{14}, Ah_{15}, AH_{16} and Ah_{17}) were clustered together into a major group, 4 isolates (Ah10, Ah11, Ah 12 and Ah18) were in a second cluster with a single isolate(Ah_9) forming a solitary cluster in between the two.

Cellular Protein Profile

Soluble cellular protein extracted from the isolates was subjected to SDS-PAGE analysis. All the isolates revealed 13 protein bands, the molecular size of which ranged from 100 KDa to 28 KDa. No polymorphism was evident as all the isolates revealed uniform profile. Cellular protein profile of the isolates resolved through SDS-PAGE electrophoresis is presented in Fig.6.

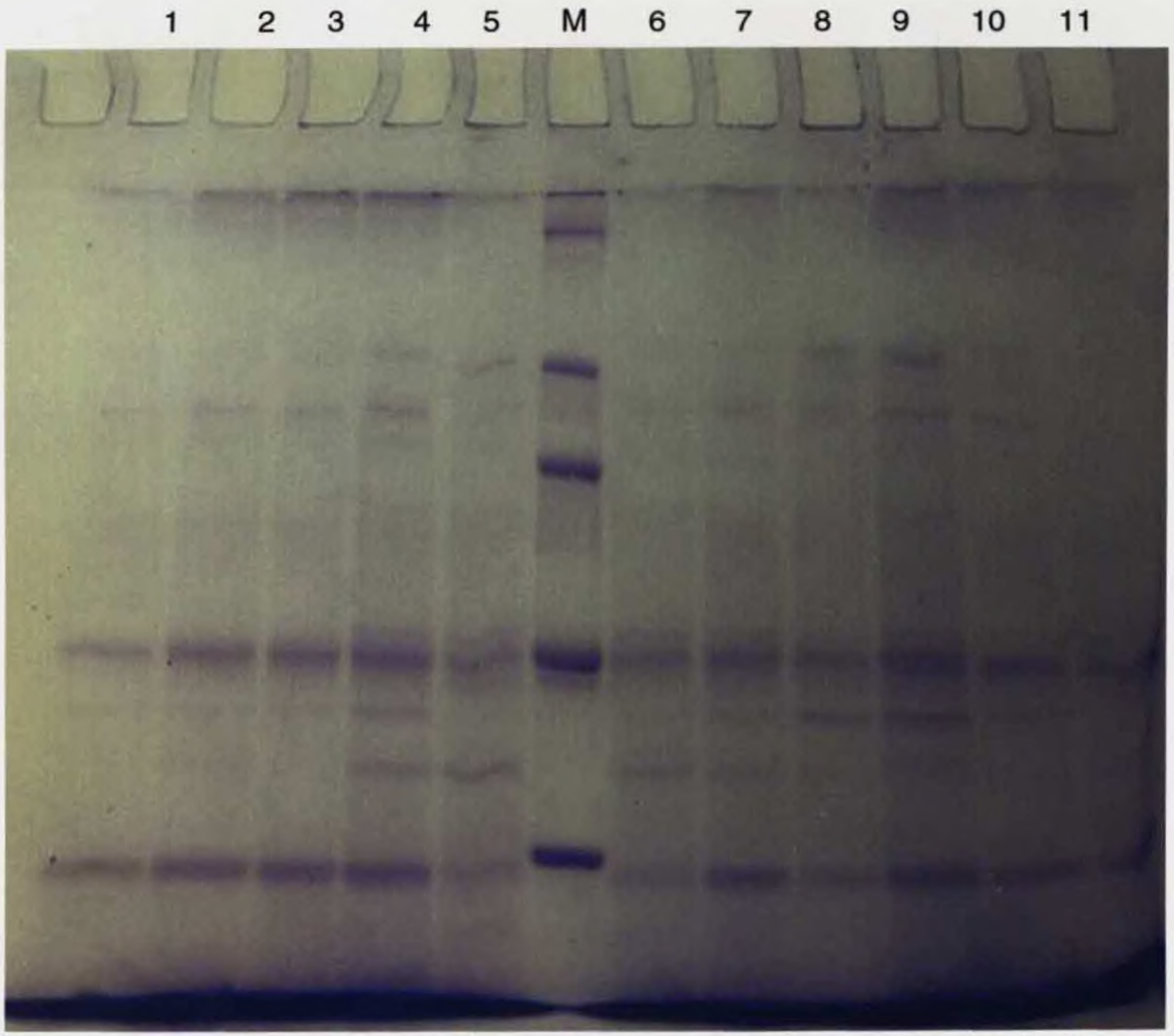

Fig. 6. Cellular protein profile of *A. hydrophila* resolved through SDS-PAGE Electrophoresis
Lane 1-11: Ah1, Ah2, Ah3, Ah4, Ah6, Ah7, Ah13, Ah14, Ah17, Ah18, Ah12 isolates
Lane M: Marker

Fig. 7. Dendogram based on the RAPD data of *Aeromonas hydrophila* isolates showing genetic relatedness among them.

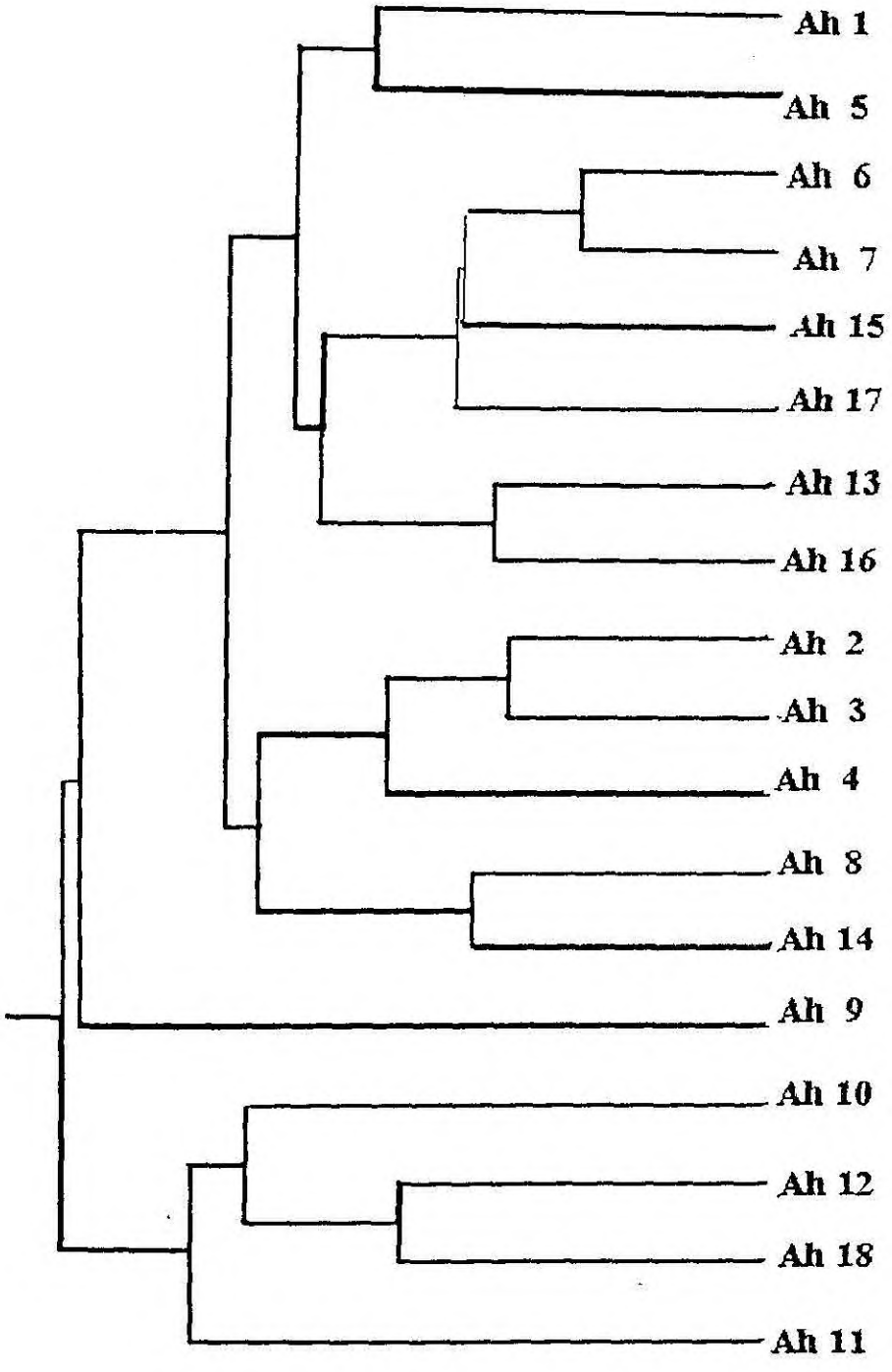

Table 1. Summary of amplicons generated by random primers.

Primer No:	Primer sequence	No. of amplicons	Amplicon size range	Amplicons sharing	
				size of amplicons (bp)	No. of isolates sharing this band
OPA-01	CAGGCCCTTC	12	(400-2500)bp	2500	6
				1370	18
				1200	4
				1100	7
				1000	5
				940	18
				830	2
				800	14
				700	14
				600	11
				540	7
				400	13
OPA-3	AGTCAGCCAC	7	(830-1584)bp	1584	11
				1300	6
				1200	6
				1100	6
				1000	2
				940	7
				830	12
OPA-4	AATCGGGCTG	10	(550-2500)bp	2500	7
				2000	12
				1900	13
				1584	16
				1100	6
				900	11
				800	13
				700	12
				600	6
				550	14
OPA-5	AGGGGTCTTG	6	(200-1000)bp	1000	11
				700	11
				564	15
				450	12
				300	10
				200	12
OPA-10	GTGATCGCAG	11	(200-1900)bp	1900	11
				1584	12
				1000	4
				947	16
				831	1
				700	2
				600	9
				564	6
				400	3
				300	1
				200	1

Table 2. Amplified DNA fragment frequency across all the *A.hydrophila* isolates

Primer/Amplicon	1	2	3	4	5	6	7	8	9	10	11	12
OPA -01	0.3333	1.000	0.2222	0.3889	0.2778	1.000	.1111	.8333	.8333	.6111	.4444	.7222
OPA -03	0.6111	0.3333	0.3333	0.3333	0.1111	0.3889	0.7222					
OPA-04	0.3889	0.6667	0.7222	0.8889	0.3333	0.4444	.8333	.6667	.3333	.8333		
OPA-05	0.6111	0.687	0.8333	0.6667	0.6111	0.7222						
OPA-10	0.6111	0.6667	0.2222	0.8333	0.0556	0.1111	0.5000	0.3496	0.1667	0.0556	0.0556	

Table 3. Nei's original measures of Genetic Identity and Genetic Distance between the *A. hydrophila* isolates

Pop ID	Ah 1	Ah 2	Ah 3	Ah 4	Ah 5	Ah 6	Ah 7	Ah 8	Ah 9
Ah 1	****	0.7174	0.6087	0.5652	0.7174	0.7391	0.6304	0.6087	0.5217
Ah 2	0.3321	****	0.8043	0.7174	0.7826	0.7174	0.6087	0.6739	0.4565
Ah 3	0.4964	0.2177	****	0.7391	0.6304	0.6087	0.6304	0.6522	0.6087
Ah 4	0.5705	0.3321	0.3023	****	0.6304	0.5652	0.6304	0.5652	0.5217
Ah 5	0.3321	0.2451	0.4613	0.4613	****	0.7609	0.6522	0.7174	0.5870
Ah 6	0.3023	0.3321	0.4964	0.5705	0.2733	****	0.8478	0.6957	0.6522
Ah 7	0.4613	0.4964	0.4613	0.4613	0.4274	0.1651	****	0.5870	0.6739
Ah 8	0.4964	0.3947	0.4274	0.5705	0.3321	0.3629	0.5328	****	0.6522
Ah 9	0.6506	0.7841	0.4964	0.6506	0.5328	0.4274	0.3947	0.4274	****
Ah 10	0.5328	0.5705	0.6931	0.6098	0.4274	0.4613	0.4964	0.6098	0.4613
Ah 11	0.6931	0.5705	0.8842	0.6931	0.6506	0.6098	0.5705	0.6931	0.6931
Ah 12	0.4613	0.4964	0.6098	0.6931	0.4964	0.6098	0.6506	0.6098	0.6098
Ah 13	0.3023	0.3947	0.4274	0.4964	0.3947	0.3629	0.4613	0.4274	0.6506
Ah 14	0.6506	0.3947	0.2451	0.4964	0.3947	0.4964	0.5328	0.2451	0.4964
Ah 15	0.4613	0.4964	0.5328	0.6931	0.4964	0.2733	0.2451	0.4613	0.6098
Ah 16	0.4132	0.2772	0.3064	0.5024	0.3996	0.4132	0.4468	0.4327	0.6583
Ah 17	0.4274	0.3947	0.4274	0.4274	0.3947	0.2451	0.2733	0.4964	0.5705
Ah 18	0.3321	0.6506	0.6931	0.6098	0.5705	0.6098	0.7376	0.6098	0.6098

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Table 3. continued

Pop ID	Ah10	Ah 11	Ah 12	Ah 13	Ah 14	Ah 15	Ah 16	Ah 17	Ah 18
Ah 1	0.5870	0.5000	0.6304	0.7391	0.5217	0.6304	0.6615	0.6522	0.7174
Ah 2	0.5652	0.5652	0.6087	0.6739	0.6739	0.6087	0.7579	0.6739	0.5217
Ah 3	0.5000	0.4130	0.5435	0.6522	0.7826	0.5870	0.7361	0.6522	0.5000
Ah 4	0.5435	0.5000	0.5000	0.6087	0.6087	0.5000	0.6051	0.6522	0.5435
Ah 5	0.6522	0.5217	0.6087	0.6739	0.6739	0.6087	0.6706	0.6739	0.5652
Ah 6	0.6304	0.5435	0.5435	0.6957	0.6087	0.7609	0.6615	0.7826	0.5435
Ah 7	0.6087	0.5652	0.5217	0.6304	0.5870	0.7826	0.6397	0.7609	0.4783
Ah 8	0.5435	0.5000	0.5435	0.6522	0.7826	0.6304	0.6487	0.6087	0.5435
Ah 9	0.6304	0.5000	0.5435	0.5217	0.6087	0.5435	0.5177	0.5652	0.5435
Ah 10	****	0.6087	0.6957	0.5000	0.5435	0.5652	0.5395	0.6739	0.6087
Ah 11	0.4964	****	0.6522	0.5000	0.5000	0.5652	0.5832	0.5435	0.6087
Ah 12	0.3629	0.4274	****	0.6739	0.5435	0.5652	0.6269	0.6304	0.7391
Ah13	0.6931	0.6931	0.3947	****	0.6087	0.7174	0.7926	0.6957	0.6739
Ah14	0.6098	0.6931	0.6098	0.4964	****	0.7174	0.7798	0.6522	0.5435
Ah 15	0.5705	0.5705	0.5705	0.3321	0.3321	****	0.7707	0.7609	0.5652
Ah16	0.6170	0.5392	0.4670	0.2325	0.2488	0.2604	****	0.7052	0.6706
Ah17	0.3947	0.6098	0.4613	0.3629	0.4274	0.2733	0.3493	****	0.6304
Ah18	0.4964	0.4964	0.3023	0.3947	0.6098	0.5705	0.3996	0.4613	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

5. Discussion

Discussion

Molecular genetic characterization of the field isolates of *A. hydrophila* using PCR -RAPD technique was carried in the present study to evaluate the genetic heterogeneity and phylogenetic relationship among them.

The potential of RAPD analysis for characterization of *A.hydrophila* was assessed. RAPD technique carried out in this study was found to have many advantages over the conventional methods as well as other molecular techniques. It is simple, easy and rapid, compared to the phenotypic characterizations and identification methods, which relies on time-consuming techniques that have limited discriminating power. On the other hand RAPD has high discriminating power.

PCR-RAPD technique doesn't require any prior information on the sequence of the DNA being characterized. However, it has wide genomic coverage unlike amplification using specific primers and therefore has higher discrimination. In the present study also RAPD profile was found to be highly discriminatory between all the isolates. The polymorphism of the amplified fragment was 95.65% with only two amplicons being shared by the isolates.

The genomic approaches like DNA hybridization and ribotyping used for the identification and typing of bacterial strains are useful for taxonomic studies and identification to sub species level. However, they are not sufficient to estimate the extent of genetic heterogeneity within the species or strains. AP-PCR/ RAPD-PCR generates fingerprints with high discriminating ability so that it can be used to compare various isolates within species. The very high discriminating power of RAPD-PCR for comparison at species level and within species has been stressed by Goarant *et al.*,(1999).

The PCR based RAPD technique required the least quantity of DNA among the various techniques. We were able to generate reproducible RAPD profiles with as little as 50ng of DNA per PCR reaction, in our study. The advantage of RAPD over other methods of fingerprinting in respect of sample quantity, result quality and sensitivity has been stressed by Miyata *et al.*,(1995). They observed that the required sample quantity for RAPD is less than one hundredth of the amount required for usual methods.

The advantages of this method for the characterization of *Listeria monocytog*enes, *Vibrio vulnificus and Bacillus thuringiensis* have already been reported by Lawrence *et al.*,(1993), Covadonga *et al.*,(1998) and Pattanayak *et al.*,(2001).

In the present study the protocol for DNA isolation developed by Murray and Thompson (1980) was modified and experimented. The modified protocol without the use of proteinase-K has given good result, yielding quality DNA of about 20 µg from a 2-ml bacterial culture. RAPD technique is so simple that it even eliminates the need for pure DNA, a requirement for other fingerprinting methods (Mazurier et al, 1992).

PCR is influenced by several factors like Mg^{2+} concentration and PCR conditions, DNA extraction methods, batch to batch variation in primer synthesis, ratio of DNA template concentration to primer concentration, the model of thermocycler used and concentration of Taq DNA Polymerase. We have optimized the random primed PCR amplification of *A. hydrophila* DNA at concentrations of 1.5mM, 1.25 units and 200µM for $Mgcl_2$, Taq polymerase and dNTP's respectively. Annealing conditions were optimized at 37°C. Increasing the annealing temperature to 55°C, to see whether background banding could be reduced, showed decline in number and identity of amplicon bands. Therefore, the annealing temperature was optimized at 37°C.

Though PCR amplification was tried with 10 numbers of decamer random primers only seven of them produced scorable amplicons. However, only 5 of them rendered consistent bands with good amounts of discrimination. The discriminatory level of RAPD varied with primer used. Therefore, the choice of primer is important.

The DNA fingerprint based on RAPD profiles revealed considerable amounts of polymorphism by way of variations in size and number of amplified fragments. Each isolate produced a unique RAPD pattern leading to absolute polymorphism. Comparison of individual amplicons also revealed considerable polymorphism. Polymorphic fingerprint pattern between isolates reflects the intra species genomic heterogeneity in *A.hydrophila*. Molecular characterization of *A.hydrophila* strains from Japan by Miyata *et al.,* (1995) also lead to the same observation. This correlates with the observation by other workers that motile aeromonas are genetically diverse (Mac Innes and Trust, 1979) and antigenically very heterogeneous (Mulla and Millership, 1993).

Estimates of the coefficients of Nei's genetic identities were generally high as all the isolates belong to the same species. However, none of the coefficients reached unity indicating the genetic variance existing in the species. The estimates of the coefficients of genetic distance also confirm this fact. The phylogenetic tree also illustrates close relationship within the species with certain amount of genetic diversity.

Considerable genetic heterogeneity among the isolates were revealed even though they belong to the same species and were phenotypically homogenous as revealed by morphological and biochemical characterizations. This is quite possible. Phenotype is depended on the coding region of DNA, the sequence changes are subjected to natural selection and culling. Whereas, major portion of the DNA in the cell is non coding regions which can accumulate genetic variation as they are not subjected to natural selection and therefore

accumulate variations which can be detected by nucleic acid base techniques and not by morphological or biochemical characterizations or isozyme or cellular protein profiling.

Cellular protein profile resolved through SDS- PAGE could not reveal any intraspecies variation as the profiles of all the isolates were similar. A similar condition was reported in *A.salmonicida* by Hanninen *et al.*, (1995) who got 15 RAPD patterns from 28 Finnish strains using 2 primers even though SDS pattern was uniform for all these strains. The homogeneity of the cellular protein profile among all the isolates within the species indicates its usefulness as a tool for species identification.

The presence of homogeneity in biochemical characterization and protein profile and heterogeneity in RAPD profile could be due to the occurance of protein isoforms or isozymes. While the isoforms and isozymes cannot not be differentiated in biochemical tests, the variations in DNA sequences causing them can be detected during molecular characterization. It could be also on account of differences in plasmids which can vary highly in field isolates through mutations to acquire drug resistance and through conjugational transfer mechanism or transposon like inverted repeats flanking the toxin genes, which help in high frequency of DNA rearrangements but are not detected in conventional techniques. Pattanayak *et al.*, (2001) suggested plasmid variation as a possible reason for the existence of genetic divergence in *Bacillus thuringiensis*.

Search for species specific molecular markers from among a large number of amplicons, to assist the detection *A.hydrophila* species pointed towards two presumptive amplicons generated by OPA-01. This amplicons of 940bp and 370bp were shared by all the 18 isolates. This may correspond to the conserved regions in the genome and can serve as species-specific markers. Thus, from the point of view of the species identification, OPA-01 is most

desirable among the primers used in the study. Screening with more primers can give rise to more of such markers.

The occurrence of large number of RAPD genotypes in the species indicating intra-specific genetic diversity which remain hidden with other methods of characterization, especially cellular protein profiling, reflect the potential and sensitivity of this approach for population genetic and systematic studies. Hadrys *et al.*, (1992) has made a similar observation after carrying out RAPD studies in crabs.

Pattanayak *et al.*,(2001) after comparing the results of earlier attempts to identify, characterize and discriminate between strains of *B. thurinigiensis* by M13 finger printing, DNA hybridization using variable region of 16S rDNA, PCR amplification of 16S to 23S ribosomal intergenic spacer sequence, AFLP fingerprinting and RAPD- PCR by Miteva *et al.*,(1991) Te Giffel *et al.*,(1997), Bourque *et al.*, (1995) and Pattanayak *et al.*, (2000, 2001) respectively, has concluded that fingerprinting based on M13, AFLP and RAPD were only able to detect high levels of diversity. He has also come to the conclusion that genetic relatedness and grouping of serovars based on RAPD analysis was similar to that of AFLP analysis. AFLP although very sensitive and powerful needed higher sophisticated technique and therefore recommended RAPD for characterization and classification in broad perspective to complement serotyping.

The present study indicate that while RAPD is an attractive choice for genetic characterization and evaluation of intraspecies diversity of A. *hydrophila*, the species specific amplicons and cellular protein profile can serve as species specific identification tools.

6. Summary

Summary

- Molecular genetic characterization of *A.hydrophila* isolates from aquaculture systems was carried out using PCR – RAPD technique.

- DNA from 18 isolates were amplified with 10 numbers of Operon decamer random primers, of which five were found to generate reproducible RAPD patterns with several bands on resolving with agarose gel electrophoresis.

- PCR amplification of the DNA with these primers resulted in 46 amplicons which were consistent and reproducible.

- RAPD was found to have high level of discriminatory ability between isolates of *A. hydrophila*.

- Choice of the primer was found to be important as the discrimination ability of RAPD varied with the primer used for amplification.

- The usefulness of RAPD-PCR for the evaluation of genomic diversity was demonstrated.

- Two species specific amplicons generated by OPA-01 were identified which could serve as species-specific markers.

- RAPD profile revealed considerable genomic heterogeneity within the species, which were otherwise homogenous in morphological and biochemical characterization.

- Polymorphism of the RAPD profile was evident. Each isolate had a unique pattern.

- Variations were evident in respect of the size and number of amplicons generated by the isolates. The overall polymorphism was 95.65 percent.

- No polymorphism was observed in the cellular protein profile as all the isolates had uniform pattern.

- The homogeneity of the cellular protein profile among all the isolates within the species indicates its usefulness as a tool for species identification.

- Nei's coefficient of genetic identities between the isolates estimated from POPGENE software were generally high, but did not reach unity and coefficients of genetic distances were generally moderate.

- Phylogenetic tree of the 18 isolates depicting the genetic relationship among them was constructed. While thirteen of the isolates were grouped into a major cluster, only four were in another cluster with a single isolate placed between the two.

- RAPD was found to be an attractive technique to evaluate interspecies genomic heterogeneity, which may remain hidden, by other techniques of characterization like morphological, biochemical and cellular protein profile.

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