MOLECULAR CHARACTERISATION OF BACTERIAL PATHOGENS OF FINFISH AND SHELLFISH

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BY

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JULY 1999

Dedicated to

my dear father

CERTIFICATE

Certified that the dissertation entitled **"Molecular characterisation of bacterial pathogens of finfish and shellfish"** is a bonafide record of work done by **Ms. B. Madhavi** under our guidance at the Central Marine Fisheries Research Institute during the tenure of her **M.F.Sc (Mariculture)** Programme of 1997-1999 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

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DECLARATION

I hereby declare that this thesis entitled **"Molecular characterisation of bacterial pathogens of finfish and shellfish"** 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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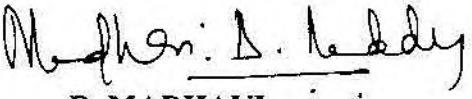

B. MADHAVI

सारांश

पखमछलियों और कवचप्राणियों के जीवाणु रोगजनकों (Bacterial Pathogens) के आण्विक अभिलक्ष्णा (मोलिकुलार कारक्टराइजेशन) किया गया। जीवाणु जातियाँ विब्रियो पारेहीमोलिटिक्स, विब्रियो फिश्चेरी, विब्रियो ऑग्वीलारम, विब्रियो कोलेरा, ऐयरोमोनास हाइड्रोफिल, एयरोमोनास सालमोनिसीडा, स्टेप्टोकोकस, एशरिकिआ कोली और सालमोनेल्ला टाइफी पर अध्ययन किये थे। अभिलक्षण का अध्ययन इन जीवाणुओं के प्लासमिड डि.एन.ए., कोशकीय प्रोटीन और आन्टिबयोग्राम के आधार पर किया था। अग रोस जेल इलक्ट्रोफोरेसिस के परिए विघटित प्लासमिड प्रोफाइल प्रत्येक जीवाणुओं के विशेष पाटेर्न दिखाया।

जीवाणुओं के बीच की विभिन्नता उनमे निहित प्लासमिडों की संख्या या प्लासमिड डी. एन. ए. के भार के अनुसार थी। विभिन्न जीवाणुओं के बीच प्रतिजैविकों पर प्रतिरोध शक्ति में भी गहरी भिन्नता देखी गयी। तीन जातियाँ यानी एयरोमोनास साल्मोनिसिडा, एयरोमोनास हाइड्रोफिला और स्टेप्टोकोकी जाति से कोशकीय प्रोटीन अलग करके एस.डी.एस.-पी.ए.जी.इ. के ज़रिए विघटित किया तो इनके प्रोटीन प्रोफाइलों में सुस्पष्ट भिन्नता देखी गयी। जब एयरोमोनास हाइड्रोफिला प्रोफैल में 29-30 प्रोटीन बान्डस प्रकट हूए, तो एयरोमोनास सालमोनिसिडा में अतिरिक्त 10 और बान्ड ज्यादा देखे गये।

एयरोमोनास हाइड्रोफिला के विभन्न प्रभेदों के प्रोटीन प्रोफाइलों की तुलना एक ही जाति के प्रभेदों के बीच समजातता की उच्च अवस्था दिखायी। ऐसा महसूस हुआ कि दोनों, यानी प्लासमिड और प्रोटीन प्रोफाइल्स जीवाणुओं की विभिन्न जातियों के पहचान, वर्गीकरण और पुष्टि केलिए एक अतिरिक्त साधन के रूप में उपयुक्त किया जा सकता हैं।

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CONTENTS

1. INTRODUCTION

1. INTRODUCTION

Microbial molecular genetics is gaining popularity in recent times as an essential tool in the classification of the microbes. Numerical taxonomy of bacteria, unless supported by molecular taxonomy is not acceptable in the modern times. Proper understanding of the DNA and protein profiles of bacteria can be used as an efficient and sensitive tool for rapid identification of bacteria whereas, the conventional methods are tiresome, time consuming and not fit for mass scale screening of bacteria.

Plasmids are circular DNA molecules, that lead an independent existence in the bacterial cell. Plasmids carry one or more genes, and often, these genes are responsible for some of the useful characteristics displayed by the host bacterium. They have the capacity to replicate autonomously and are stably inherited. The plasmid DNA generally code for different functions of bacteria like antibiotic resistance, drug resistance, virulence degradative enzymes, fertility factors, production of colicins etc.

The plasmid profile, with respect to their number and molecular weight are specific to individual species. Thus, the plasmid profile can serve as a fingerprint for each species. Plasmid profile has its application in the characterization, classification and confirmation of the bacteria. They act as a supplement to the traditional taxonomy. The restriction fragment length pattern of total chromosomes and plasmid DNA generated by endonucleases helps in the sub-typing of strains and sub-species within a species.

Plasmids being closely associated with epidemiology of bacterial pathogens, their profile can best be used as an epidemological marker for the molecular epidemology.

The role of plasmids in genetic engineering is of immense importance. They are the most widely used cloning vectors in gene manipulation. A proper study of the molecular weight, restriction pattern and nucleotide sequence of these plasmids help in their better utilization in cloning. Generally, modified natural plasmids of bacteria are used as cloning vehicles.

The main function of plasmid is drug resistance. Resistance to a large number of drugs have been reported to be mediated by plasmids. A single plasmid may confer resistance to a group of drugs. The property of resistance to antibiotics and drugs offered by plasmid is of much importance in aquaculture, wherein the intensive and super-intensive culture is accompanied by application of antibiotics as a prophylactic measure. If a bacterium achieves antibiotic resistance through plasmid, it may soon produce multiple copies of it by autonomous replication and then transfer them to other bacteria by normal gene exchange processes like conjugation, transformation, transduction etc, and making the entire population resistant to that antibiotic. The antibiotic resistance gained by fish pathogens can have an effect on the human population also (Inglis *et al.* 1993).

Protein profile has also gained an equal importance in molecular genetics. Proteins are the most abundant macromolecules in a cell, constituting half of the dry weight of most organisms. They are the macromolecules through which genetic information on the DNA are expressed, and are most versatile in function.

The protein profile, specific to the organism, can be used in the identification and classification of bacteria (McLean *et al.* 1993). A variety of functions like drug resistance, antigenicity etc. in the bacteria, are effected through the medium of cellular proteins.

Intra-cellular and extra-cellular bacterial proteins are specific to each bacterium, in their number and molecular weight. This specificity is being utilized when we prepare the fingerprint of bacteria based on the protein profile. This process of fingerprinting helps in characterization, typing, antigenic and genomic analysis and identification of bacterial pathogens. Classification of bacteria and bacteriophages and positioning them in taxonomical order with the help of protein profile is reported to be very reliable (Shieh *et al.*1986).

With so much of importance being conferred on the plasmid and protein profile of bacteria, they must be worked out for each bacterial strain, and constantly monitored for any variations in them, over the period of time. Hence, the present work was taken up to characterise some of the bacterial pathogens of shellfish and finfish based on their plasmid and protein profiles and antibiotic resistance. The bacteria selected for the study were, *Aeromonas salmonicida* causing furunculosis, *A.hydrophila* causing infectious abdominal dropsy, *Vibrio harveyi, V.parahaemolyticus,* and *V.anguillarum* causing vibriosis, *V.cholera* and *Salmonella typhi* causing food poisoning, *Streptococcus* spp. causing septicaemia and exopthalmia and *Escherichia coli* causing faecal pollution.

2. REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Plasmids are relatively small, circular DNA molecules that can exist independently of host chromosomes and are found in many bacteria and in some eukaryotes. They have their own replicating origins, can autonomously replicate and are stably inherited (Prescott *et al.* 1990).

Plasmids carry few genes usually less than 25 to 30. Their genetic information is not vital to the host, as the bacteria that lack them also can function normally. But they are found to control many functions of the host bacteria like conjugation, resistance to drugs, virulence etc. Bacteria may possess single copy plasmids that produce only one copy per host cell or multi copy plasmids that can produce even more than 40 copies per cell (Prescott *et al.* 1990). Depending on the size on the DNA, plasmids are called as large plasmids if their size is in the range of 60-120Kb and small plasmids if they 1.5-15Kb in size. While the large plasmids are usually conjugative F & R plasmids, the small plasmids are non- conjugative but can be mobilised for transfer by a conjugative plasmid in the same cell.

2.1. ROLE OF PLASMIDS

Plasmids perform a variety of duties in the bacteria. Based on their functions, they are classified as

a) F-plasmids
b) R-plasmids
c) Col.plasmids
d) Metabolic plasmids
e) Virulence plasmids

- F-plasmids play a major role in conjugation, hence also called as fertility factors.
- R-plasmids confer antibiotic resistance to the bacteria, hence called resistance factors.
- Col-plasmids have genes for the synthesis of bacteriocins, hence known as colicins.
- Metabolic plasmids carry genes for enzymes that degrade substance such as aromatic compounds, pesticides, sugars etc and induce legume nodulation in *Rhizobium* .
- Virulence plasmids make the pathogen harbouring it more pathogenic by confering it the ability to resist host's defence mechanism or through toxin production (Presscott *et al.* 1990).

Crosa *et al.* (1977) analysed plasmid DNA complement of high and low virulent strains of fish pathogen *V.anguillarum,* and detected a correlation between enhanced virulence and presence of a 50 mega dalton plasmid.

Wolf. Watz. (1988) detected plasmid encoding for the virulence in *Yersinia* spp. The virulence plasmid found in all the three pathogenic strains of Yersinia namely *Y.pestis, Y.pseudotuberculosis, Y. enterocolitica* were of 70-75Kb in size.

All the strains of *Vibrio anguillarum* examined by Pederson & Larsen, (1995),and Austin *et al.* (1995) possessed a virulence plasmid of 67Kb. A 65Kb plasmid (pJMI) has been found to be involved in the virulence and iron sequestration by the bacteria of *V.anguillarium* 775, that causes vibriosis in marine cat fish by Walter (1984), and Schmidt *et al.*(1991).

Singer *et al.* (1992) observed that the virulence plasmid pJM1 of *Vibrio anguillarum* mediates the restriction system that prevents conjugal transmission of plasmid DNA from *E.coli* donors into *V.anguillarum* 775 (pJM1).

Plasmid encoded mechanism for manganese oxidation by bacteria was studied by Colwell *et al.* (1986). Some of the self transmissible plasmids provides marine bacteria with superior survivability in the polluted environment, since they are able to transfer plasmid DNA coding for ecologically advantageous function such as detoxification of heavy metals, oxidation of manganese etc. Gregong *et al.*(1982) found that manganese oxidizing heterotrophic bacteria lost the capacity along with the loss of plasmid when maintained in laboratory, indicating the role of plasmid DNA in this aspect. The role of plasmids in the oxidation of manganese by *Bacillus* spp was studied by Van-waas bergen *et al.* (1993).

Zyskind *et al.* (1983) isolated chromosomal replication origin (Oric) from plasmid of *V.harveyii* and this was found to be functional in *E. coli.*

Oil degrading bacteria isolated from oil spills on industrial bay and an off shore oil field and grown on liquid enriched of crude oils and poly nuclear aromatic hydrocarbons by Devereuk *et al.* (1982) showed the presence of plasmids in 21% of strains from crude oil and 17% strains from poly nuclear aromatic hydrocarbons. Multiple plasmids were observed in 50% of strains with plasmids. Plasmids appear to be significant in adaptation of *Pseudomonas* spp to chronic petroleum pollution.

Amund (1984) isolated four strains of *Acinetobacter lwoffi* from water and sewage samples which metabolises hydrocarbons. All of them haboured multiple

plasmids of molecular weights ranging from 0.99 – 71.6 mda indicating their role in metabolising hydrocarbons.

Belland *et al.*(1989) identified 17 plasmids, encoding proteins, in *A.salmonicida* in the size range of 12 to 90 KDa.

Expression of virulence plasmid encoded virulence determinants was discussed by Wolf watz (1991), and observed that the three virulent species of Yersinia contained homologous virulence plasmids that encode a number of temperature inducible Ca (2+) regulatory proteins.

Floodgate (1991) found a direct correlation between the number of plasmids and the speed of crude oil degradation by bacteria. Research on plasmids in waste water bacteria showed that most of them had multiple (2-4) plasmids.

Study of the plasmid profile of bacteria from dystrophic lakes by Schuett (1988) indicated marked differences in the size of plasmids according to the extreme ecological environments.

Studies conducted by Boettcher *et al.*(1994) on sepiolid squid symbiont, *V.fischeri* showed that plasmids in this bacteria may carry genes that are important for the survival of these strains outside the symbiotic association.

Plasmids were found to perform other functions such as Cystiene biosynthesis in *Cyanobacterium.* Nicholson *et al.* (1995) observed that the plasmids of 8 and 48.5kb size are involved in the cystiene biosynthesis by *Synechococcus* spp.

An aromatic degradative plasmid TOM measuring 70 to 100Kb was isolated from *Burkholderia (Pseudomonas) capcia* G4 by Shields *et al.* (1995).

In acido-thermophilic Archaebacterium, *Thermoplasma*, temperature tolerance was found to be effected by plasmid as per the observations of Yasuda *et al.*(1995).

Noonen and Trust (1995) observed that *A.salmonicida* possesses plasmids of varying sizes that exhibit a high degree of conservation and can encode antibiotic resistance elements

Plasmid mediated histamine biosynthesis was observed in *V.anguillarum* by Barancin *et al.* (1998). Plasmids were also found to mediate in iron uptake by *V.anguillarum* and to grow under conditions of iron limitation(Tolmasky *et al.* 1985).

Mercury resistant bacterial strains of Chromobacterium, *Erwinia*, *Bacillus* spp were found to be capable of growth in presence of 50 μm HgCl, and this mercury resistance is controlled by plasmid DNA (Trevors 1987).

Chakrabarti *et al.* (1994) observed that characteristics like bio-degradation, polymyxine resistance and low level halophilism are controlled by a plasmid in *V.parahamolyticus*..

Belland & Trust (1989) reported that in *A.salmonicida*, pAsa plasmids direct the synthesis of four polypeptides.

2.2. IMPORTANCE OF PLASMID PROFILES

Plasmid profiles are characteristic for each species. Hence this can be taken as a fingerprint in identifying the bacteria. Reud & Boyle (1988) used the same to identify *Edwardsiella ictaluri* that causes enteric septicemia in channel cat fish.

Wilk (1989) reported that plasmid profile along with serological and biochemical properties are helpful in isolating *V.anguillarum* strains from diseased fish.

Zhao & Aoki (1992) reported that a plasmid of 5.1Kb size is specific to *Pasteurella piscicida* which causes Haemophilus influenza, and that it could be used as a finger print of the bacteria.

Plasmid profiling was reported to be one of the methods used in epizootological work especially in the isolation of *A.salmonicida* from salmonids affected with furunculosis (Soerum & Kvello, 1993).

Dalsgaard (1994) characterised a typical *Aeromonas salmonicida* using plasmid profile.

Plasmid profile is a very effective tool in the epidemology as a marker. Borrego (1996) observed that plasmids in all the strains of *Vibrio* P_1, (causing brown ring disease in cultured manila clams) possessed a large 49.2Mda plasmid, which can be used as an epidemological marker. (Nielsen *et al.* 1993).

Dahlberg *et al.*(1997) reported that plasmid types isolated from different habitats and from different sampling occasions showed little similarity indicating high variation.

Williems *et al.* (1993) made a nested PCR approach with primers based on conserved plasmid sequences and used it for the detection of *Coxiella burnetti* in clinical samples.

The use of plasmids as cloning vectors for transformation of bacteria was emphasized by Hackette and Das sarma (1989).

Wilk (1988) classified *V.salmonicida* was classified into four types based on plasmid profiles. *V.anguillarum* which was classified into serovar 01 and serovar 02 based on plasmid contents. He used the same technique for clasification of *V. salmonicida* also.

Olsvik (1989) observed that plasmid profiling was simple and easy to perform, useful in characterizing strains of pathogenic bacteria like *Salmonella typhimurium*. Profiles of species specific plasmids were useful for confirmation or as a supplement to traditional phenotypical identification for *Vibrio salmonicida* and *Aeromonas salmonicida sub sp. salmonicida* strains. The restriction endo-nuclease pattern of total chromosome and plasmid DNA was also shown to be useful for characterizing strains of same species.

2.3. PLASMID PROFILE:

The plasmid profile of the different species of bacteria worked out by various workers are presented below.

Potts *et al.* (1984) reported three plasmids of approximately 0.9, 10, 12Kb in *Cyanobacterium* of LPP group.

Plasmids were isolated from halophilic bacteria by Hong Y.K. (1985), and their molecular weights were recorded. The molecular size of the plasmids in *Vibrio* spp 14, *Alkaligenes* spp. 63, *Pseudomonas* spp. 11, *Flavobacterium* spp. 38, *Bacillus* spp. 16, *Alkaligene* spp. 52 reported by him were 7.2 Kb, 6.4 Kb, 6.85 Kb, 8.5 Kb, 8.75 Kb and 6.8 Kb respectively.

Cook. *et al.* (1985) found a circular extra chromosomal DNA of 10.5 Kb length in *Euglena gracilis* that constitutes 1% of its total cellular DNA.

Castets (1986) observed that the unicellular facultatively heterotrophic cyanobacterium *Synechocystes 6803* harbours four plasmids of 2.5, 5.2, 50 and 100Kb size respectively. Rebiere (1986) observed one mega plasmid of upto 1000Kb in uni-cellular *Synechococcus PCC 7942* and a large plasmid of upto 400Kb in all the seven unicellular and four filamentous cyanobacterial strains studied by him. Three plasmids pSy 09, pSy 10 and pSy 11 were reported from *Synechocystes* sp *NKBG 042902* (Matsunga *et al.* 1990)

Vachhani *et al.* (1992) observed two plasmids in non heterocystous filamentous cyanobacterium *Plectonema boryanum* strain.

Red algae *Gracilaria chilensis,* harbours a circular GC_2 plasmid of 3.8kb,and its complete DNA sequence was identified by Villemur (1990), Moon *et al.* (1995).

Goff *et al.*, (1990) found that 25% of red algae observed had two or more plasmids . Moon *et al.* (1995) reported that *Gacilariopsis lemaneioformis* harbours two plasmids of 4.4 and 3.5 Kbp, with a high copy number per cell.

Moon (1997) found that the red algae *Porphyra pukhra* had two large plasmids of 6859 and 6427 bp and three smaller plasmids of 1896, 2100 and 2102 bp.

Plasmids have been found to occur in the thermophilic bacteria. Fee & Mather (1988) isolated plasmids of 6 MDa and 47MDa from *Thermus thermophilus.* Charbonnier *et al.* (1992) discovered a plasmid of 3.45Kb (pGTS) from Hyperthermophilic *Archaebacterium* that grows in temperature range

of 68-101.5^{0}C. *Thermoplasma acidophilum* (*Acidothermophilic archae bacteria*) had a plasmid pTAI of 15.2Kb (Yasuda,1995).

Erauso *et al.* (1994) found that the Thermococcale species *Pyrococcus abyssi* harbours a plasmid of 3.5 Kb called pGT5.

Hackett (1989) discovered that *Halobacterium* plasmid pH SB1 is specific to the *Halobacterium* species. Rosen shine *et al.* (1989)observed that three isolates of *Halobacterium volcanii* had one plasmid each but these plasmids lacked homology.

Seven genera of the order Siphonocladales and 2 genera of Clado phorales showed low molecular weight plasmids of 1.5 to 3Kb. (Laclaire *et al.* (1997).

Navarro *et al.* (1995) detected a 37MDa plasmid in most of the fresh water sediment isolates of *Nitrobacter*.

Erwina herbicola ATCC 21998 was found to have two plasmids pVQ1 and pVQ2 of molecular weights 7.4 and 8.0Kb. (Koul *et al.* 1997).

Amund (1984) found that *Acinetobacter lwoffi* do harbour multiple plasmids of various molecular weights (0.99-71.6 M.dalton).

Plasmids were isolated from the bacteria, pathogenic to finfish and shell fish. Lobb & Rhoades (1987) isolated two plasmids of 5.7Kb and 4.9Kb from different strains of *Edwardsiella ictaluri*, the causative of enteric septicemia of channel catfish.

Pseudomonas like bacteria isolated by Burton *et al.* (1990) from polluted waters were found to carry plasmids of molecular masses between 35 and 312 Md.

Pasteurella piscicida isolates from *S.quinqueradiata* had one mega plasmid of 110 Kbp and two small plasmids of 3.5 and 5.1Kb. Zhao & Aoki, (1992) reported that 5.1 Kb plasmid is specific to all *Pasteurella piscicida.*

Magarinus (1992) found a common plasmid band of 20 and 7 MDa in all *Pasteurella piscicida* studied but European strains were found to have an additional 50MDa plasmid. Species specific plasmids designated as $p2P_1$ and p2 P1-4 with 964bp and 477 bp respectively were isolated from *Pasteurella* pisc*icida* (Aoki *et al.* 1997).

Dalsgaard (1993) found four different plasmid profiles in all the strains of *Cytophaga psychrophila* studied.

Bast *et al.* (1988), Toranzo *et al.* (1991) and Soerum *et al.* (1993) observed that the strains of *Aeromonas salmonicida,* the bacterial pathogen producing furunculosis of salmonid fish have uniform plasmid patterns with 4 plasmids of molecular weight 4.2, 3.6, 3.5, 3.3 MDa and a large plasmid of 50-56 MDa or more.

Dalsgaard (1994) and Pederson *et al.*(1996), found that *A.salmonicida* isolates have 2-3 plasmids each and all of them share a common small sized plasmid .The *A.salmonicida* of Atlantic coast was found to have 4-6 plasmids with 4 smaller plasmids between 4.3 to 8.1Kb. and those of Pacific coast were having 6 plasmids in the range 4.2 to 8.9Kb.

Wards *et al.* (1988,1991) found PV 01 plasmid of 30Kb in *V.ordalii.* Two plasmids were observed in *V.vulnificus* . Amaru *et al.*(1988) and Sorum *et al.* (1990) observed that *Vibrio salmonicida* isolated from cod and atlantic salmon have 61, 21,

3.4 and 2.8 mega dalton plasmids. A 61 Md. plasmid was found exclusively in *V.salmonocida* strains off Northern Norway.

A 48Mda plasmid was isolated from all strains of *Vibrio anguillarum* serovar 01. While serovar 02 strains had none. Myhr *et al.* (1991) Sorum *et al.* (1993) found *V.salmonicida* to have 3 plasmids of 21, 3.4, 2.8 Mda.and *V.anguillarum* and *V.ordelli* strains isolated off Atlantic and Pacific coast to have only a 47Kb plasmid.

2.4. PLASMIDS AND ANTIBIOTIC RESISTANCE

Dixon (1988) reported that the genes coding for antibiotic resistance these plasmids may be extra-chromosomal or chromosomal.

Genetic investigations have proved that resistance to a large number of antibiotics was encoded by plasmid DNA. These plasmids orginally called R-factors are now called as R-plasmids.

The resistance plasmids were originally observed in the clinical isolates of *Shigella* strain in Japan during 1950s. (Brown *et al.* 1988). About 70 to 80% of these strains showed multiple resistance.

The bacteria acquire plasmids as a tolerance mechanism to the changing environment, be it a drug or an antibiotic. R-plasmids are self-transmissible, hence conjugation is a regular practice which transfers the plasmid from one bacteria to the other causing rapid spread of the drug resistance. Generally, only single R-factor exist due to the retarding effect of extra DNA on cell growth.

Primary work on R-plasmids was done by Baya *et al.* (1986). He collected samples from uncontaminated open ocean areas ,polluted areas and domestic sewage

water. Bacterial isolates from these samples were tested for resistance to 9 different antibiotics and for the presence of plasmid DNA. Bacterial isolates from toxic chemical wastes most frequently contained more plasmid DNA and had more resistance to antimicrobial agents than did isolates from domestic sewage water or from uncontaminated open ocean water.

Aoki *et al.* (1971) detected both transferable and non-transferable R-plasmids, encoding tetracycline resistance in *A.salmonicida*. Of the 124 field isolates of *A.salmonicida* resistant to various chemo-therapeutants, only two isolates showed the presence of transferable R-plasmids (Aoki *et al.* 1983).

In *A.salmonicida*, Aoki (1988) detected R-plasmid encoded resistance to any one of the following antibiotics *i.e* chloramphenicol, kanamycin, sulfa monomethonine, tetracycline. Inglis *et al.*(1993) reported the presence of transferable R-plasmids encoding OTC resistance in more than a quarter of strains of *A. salmonicida* observed by him, which was supposed to be due to quick plasmid transfer by which resistance to antimicorbial drugs is spreading quickly among *A.salmonicida*. Studies by Noonan *et al.* (1990) revealed that *A.salmonicida* has plasmids of various sizes which exhibit high degree of conservation and can encode antibiotic resistance elements.

Chemotherapy and drug resistance study was done by Aoki (1992). He found that application of various kinds of chemotherapeutic agents in the treatment of bacterial infection in fish farms of Japan caused the incidence of drug resistant strains of pathogenic fish bacteria *viz. A.hydrophila, A.salmonicida, Edwardsiella tarda, Pasteurella piscicida,* non- haemolytic *Streptococcus* sp and *V.anguillarum.*

The multiple drug resistant strains of fish pathogens carrying R-plasmids have been widely distributed in fish farms.

In 1993 Kim & Aoki conducted MIC test of 12 chemotherapeutic agents on 175 strains of *P.piscicida*, collected from yellowtail cultured in different areas of Japan during 1989-1991. Almost all strains carried transferable R-plamids encoding resistance to at least one of the following antibiotics namely kanamycin, sulphamono methionine and tetracycline. There was homology among the DNA of transferable R-plasmids. They also suggested that R-plasmids with multiple drug resistance was retained within *P.piscicida* without any change in their DNA structure subject to geography or year. Kim and Aoki (1993) found R-plasmids encoding resistance to chloromphenicol in *P.piscicida Psp 9351.*

Multiple drug resistance in *V.anguillarum* strains isolated from Ayu farms by Zhao *et al.* (1992) was found to be due to R-plasmid.

A transferable R-plasmid encoded with resistance to florfenicol was identified in *E.coli* (FF resistant strains). These plasmids encoded for resistance to chloramphenicol, kanamycin, sulphomono methionine and tetracycline also (Kim *et al.*1993).

Cooper *et al.* (1993) and Starliper *et al.* (1993) conducted experiments on Romet-30 the only drug approved by the U.S Food & Drug Administration for use against enteric septicemia of channel catfish caused by *Edwardsiella ictaluri.* Recently several isolates obtained from these areas had naturally occurring resistance to Romet-30. The isolates were found to possess a 55Kb plasmid that encodes resistance to this drug. This R-plasmid was found to be identical in profile

to that of 55 kb plasmid of *E.coli* (strain 1998). The R-plasmid of both species confer resistance to Romet-30, tetracycline and tetrramycin.

The drug resistant strains of *E.tarda* carried an R-plasmid which controlled for resistance to chloramphenicol, tetracycline and sulphonamide (Aoki, *et al.* 1989).

Kontny and Thielebeule (1988) reported that R-plasmid of *A.hydrophila* code for resistance against oxytetracycline, chloramphenicol, streptomycin, ampicillin, kanamycin, gentamycin & trimethoprim. Eleven out of 15 *A.hydrophila* strains had transferable R-plasmids while others had non-transferable multiple antibiotic resistance plasmids.

Chowdhary and Inglis (1994) detected a transferable R-plasmid in *Aeromonas spp.* resistant to oxytetracycline.

Amita Jain *et al.* (1993) found correlation between plasmid pattern the drug susceptibilty pattern in *Shigella dysenteriea.*

Kirby (1978) found that, in *Eubacteria,* many characteristics including resistance to antibiotics and other agents were determined by plasmids. In *Streptomyces rimosus,* crosses provided additional evidence that extrachromosomal genes are involved in antibiotic production.

Dasgupta *et al.* (1980) showed that several of the resistance determinants could be a single plasmid and that in certain instances, at least high levels of resistance to streptomycin (200μm/ml) erythromycin and methicillin could be accounted to the plasmid gene in *Staphylococcus.* It has been noted that the *Staphylococcus* strains which have multiple antibiotic resistance due to R-plasmid usually do not possess bacteriocin plasmid and conversely a strain (*eg. S.aureus* ML

106) that carried a bacteriocin plasmid had only a restricted range of antibiotic resistance.

Contradictory results were obtained by Toranzo *et al.* (1991). He could not observe any correlation between plasmid contents and drug resistance.The same was also reported by Castro *et al.* (1992) and Giles *et al.* (1995).

2.5. PLASMID ISOLATION PROCEDURE

Plasmid isolation can be done by a number of methods each having its own advantages and disadvantages. In all these methods, the relatively small size and covalently closed circular nature of plasmid DNA is exploited in their isolation. The equilibrium centrifugation in CsCl–ethidium bromide gradients has been in vogue to prepare large amounts of plasmid DNA, since the early years. The process involves separation of plasmid and chromosomal DNA by equilibrium centrifugation in CsCl-*ethidium bromide gradient and it depends on difference* between the amounts of ethidium bromide that can be bound to linear and close circular DNA molecules. This being expensive and time consuming, alternative methods have been developed (Maniatis *et al.*, 1989).

The isolation method for plasmid devised by Kado & Liu was considered to be the best one for detecting plasmids (Fujita *et al.* 1993, Foo *et al.* 1985).

Kado and Liu (1981) devised a procedure for the detection and isolation of plasmids of various sizes (2.6 to 350 Md) that were harboured in species of *Agrobacterium, Rhizobium, Escherichia, Salmonella, Erwinia, Pseudomonas* and *Xanthomonas.* The method utilised the molecular characteristics of covalently closed circular DNA that is released from cells under conditions that denature chromosomal

DNA by using SDS (pH 12.6) at elevated temperature. Protein and cell debris were removed during extraction with phenol-chloroform. Under these conditions, chromosomal DNA concentrations were reduced or eliminated. The clarified extract was used directly for electrophoretic analysis. This procedure also permitted the selective isolation of plasmid DNA that can be used directly in nick translation, restriction endo nuclease analysis, transformation and DNA cloning experiments.

Birnboim and Doly (1979) and Ish-Horowicz & Burke (1981) developed plasmid isolation procedure based on alkaline lysis. Here, the bacterial cell wall lysis is further enhanced by the enzymatic digestion with lysozyme, and alkaline lysis was carried out in ice cold conditions. Thereafter, the plasmid DNA was concentrated by precipitation with absolute ethanol in the presence of salt solution.

A rapid technique to detect plasmid from broth culture or single colonies of *Edwardsiella ictaluri* within two to those hours by agarose gel electrophoresis was developed by Lobb and Rhoades (1987).

The protocol developed by Maniatis *et al.* in 1989 was a modification of Birnhoim and Doly (1979), Ish-Horowig and Burke (1981). The method involves cell wall lysis by lysozyme by vortexing the bacterial pellet and dissolving it in TEG buffer. The plasmid is then precipitated by adding ethanol and vortexing the mixture.

The technique of plasmid isolation developed by Goyal (1992) was a simplified method and was used in plasmid distribution study in *Cynobacterium.* The method involves direct agarose gel electrophoresis of heat treated, ethanol precipitated, plasmid preparation from the cleared lysates without requiring ultra centrifugation. This method is sensitive and can be effectively used to determine the

number of plasmids and their molecular weight from agarose gel pattern. The results compare well with those obtained by the caesium-chloride, ethidium bromide equilibrium centrifugation techniques.

2.6. PROTEIN PROFILE

The protein meaning 'first' or foremost is the most abundant macro molecule in cells and contribute to over half the dry weight of most organisms. They are the instruments by which genetic information is expressed. There are thousands of different kinds of proteins in the cell, each one carrying out a specific function determined by the gene encoding each one of them. They are not only the most abundant but are also extremely versatile in function (Lehninger, 1984).

With the advent of biotechnology, protein profiles have gained much importance. They have wide applications in characterization of bacteria (Mc Lean *et al.* 1993), isolation, classification and identification of bacteria from infections antigenic and genomic analysis. Resistance was found to be associated with proteins which inturn are supposed to be under the control of plasmids.

Balske *et al.* (1987) analysed that the cellular protein profiles of *Mycoplasma hyopneumoniae, M.hyorhinis* and *M.flocculare* by using SDS-Gel electrophoresis. They reported similarity in the protein profile of *M.hyopnuemaniae* and *M.flocculare* whereas that of *M.hyorhinis* was different. *M. hyopneumonia* had high molecular weight antigens of 108, 102, 93, 89, 87KDa and low mol.wt proteins of 74, 58, 45, 44 & 38 KDa as specific to it.

Mycoplasma gallisepticum was found to have protein bands of 40-67 KDa while *M.gallinarum* and *M.synoviae* were found to have identical protein bands of

35-43 and 60-94 KDa 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 molecule weight from 340 KDa to 14.3 KDa.

Protein profiling of halophilic Archaea, *Halobacterium halobium* was carried out by Nakayama & Masashi (1997). 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 50 KDa 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 can 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 52.3KDa. in the *B.innocens.*

2.7. IMPORTANCE OF PROTEIN PROFILE

Protein profile has a wide range of applications. The most important among them being characterization and classification of microbes based on the variations in protein profile. Based on the protein analysis Shieh *et al.* (1986) could

classify bacteriophage *Streptococcus lactus* and *S. cremoris* from cheese whey into two groups, D5 9-1/F4-1 or group G72-1/I37-1. The usefulness of protein profile based on SDS-PAGE along with electron micrography for the classification of the bacterio phage *Staphylococcus aureus* from canine as well as human into serogroups have been reported by Adesiyun *et al.* (1992).

Hellwig *et al.* (1988) compared the outer membrane protein and surface characteristics of four adherent and one non-adherent mutants of *Bordetella avium* and showed that the adherent phenotypes had identical protein profiles while the non-adherent organisms lacked at least five of the proteins present on adherent organism.

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 Streptococcal from the environmental samples. They isolated 371 presumptive faecal *Streptococci* from environmental

samples and clustered them according to their protein profile. Cluster could be tentatively identified with the help of the profile of reference strain.

Electrophoretic protein profile analysis was used by Brando (1995) in confirming the identification of three strains of *Mycoplasma mycoides* sub species *mycoides* SC type, isolated from milk of sheep with mastitis and pneumonia.

Using SDS-PAGE, Sampedro (1995) identified three outer membrane polypeptides (200, 46 & 25KDa), which are encoded by virulence plasmid, from *Yersinia enterocolithica 09* grown in brain-heart infusion medium at 37^0C. But when this was grown in tissue culture medium RPMI 1640, it expressed 5 additional polypeptides (170, 135, 118, 100, 98Kda) but lost the 25KDa band, and this pattern resemble the profile displayed by *Yersiniae* when grown in-vivo.

Borrelia garinii (NE11H) isolated from *Ixodes ricinus* haemolymph, expressed four major proteins of 33, 32, 23 and 22 KDa. During in vitro culture, it lost the expression of 22 and 23 KDa proteins, but when reintroduced into tick midgut, it regained the expression (Hu. *et al.* 1996).

Ragni *et al.* (1996) characterized 6 mosquitocidal *Bacillus thuringiensis* strains by protein profiling. While five of them showed same protein profile and mosquitoidal activity, the sixth strain showed a different protein profile as well as a novel mosqitocidal activity.

Protein profiles do have a bearing on the drug resistance, antigenicity and genomic function, on many a occassions. Bandin *et al.* (1992) had done the analysis of membrane proteins and their antigenic properties in a group of 14 geographically diverse strains of *R.salmoninarum*. Eleven isolates, including the type strain

ATCC 33209 shared similar protein profile with a major component of 57KDa, while 3 strains showed a common pattern with a major protein of 30KDa. He detected antigenic heterogeneity with two groups distinctly recognizable.

Wiedemann & Heisig (1994) studied the quinolone resistance as well as the protein profile of gram negative bacteria. He observed that the reduced drug accumulation was associated with alterations of the outer membrane protein profile.

Nielsen *et al.* (1994) isolated *A.salmonicida* sub sp. *salmonicida* from diseased salmonids in Denmark, Norway, N.America and Scotland. These isolates were characterised with regard to protein patterns, antibiotic resistance and exo-protease activity. Whole cell and outer membrane protein analysis revealed 3 different profiles in *A.salmonicida*. The molecular size of the 8 outer membrane proteins were 49, 40, 38, 37, 33, 31, 30 and 29KDa. One of the isolates had the outer protein profile deficient in 38KDa. Strains with 37KDa outer membrane protein showed multiple low level antibiotic resistance towards cephalothin, penicillin, chloramphenicol, tetracycline and quinolones. In addition, these strains were protease deficient and unable to degrade cattle and trout serum proteins and had delayed degradation of casein. The strains with 37KDa produced almost new pathological effects, while the normal protein profile strain produced typical furuncles.

A total of 17 *V.harveyi* isolates were examined by Pizzuto (1995) for virulence in *P.monodon* larvae and were classified by total soluble protein profiles generated by SDS-PAGE under reducing conditions. Two isolates out of seventeen proved to be virulent. Most isolates fell within two protein groups. Group I was

characterised by a 42KDa protein and contained 8 isolates including both the virulent isolates. Group II was characterised by a 40KDa polypeptide and contained 7 isolates. Further, 2 isolates could not be assigned to either groups, suggesting high genetic diversity within *V.harveyi*. The two virulent strains classified within group I did not demonstrate a high genetic association.

Discrimination of virulent and avirulent *Streptococcus suis capsular type 2* isolated from different geographical origins was done by Quessy *et al.* (1995) using protein profile in relation to virulence. It showed that the protein profiles of cellular fractions were similar in both virulent and avirulent isolates except the three Canadian strains for which a 135KDa protein was not detected. Culture supernatants revealed presence of a 135KDa protein in all strains except the three Canadian strains. In addition, a 110KDa protein was present in 14 of the 16 virulent strains and not in avirulent strains. This 110KDa protein therefore, appeared to be a reliable virulence marker and a good candidate for sub-unit vaccine.

Protein profiles were used by Tamassy *et al.* (1995) in the epidemology of *Helicobacter pylore* infection in various populations, along with DNA-RNA hybridization.

The variations in the profile of the cellular proteins of the bacteria, say addition or deletion of a single band or multitude of such bands are usually accompanied by correlated variations in an important function. Kontusaari & Forsen (1988) found the involvement of two cell surface proteins in the production of slime by *Streptococcus lactis* spp. *cremoris* (T5). Isolates of T5 from Finnish cultured milk villi produced two variants, a T5/30 obtained at elevated growth temperature of

30^0C and strain T5/NS obtained spontaneously at growth temperature of 17^0C. This spontaneous change of T5 to T5/NS phenotype brought about the complete loss of two cell surface polypeptides and a decreased expression in other four, Viz. 70000, 54000, 47000 and 40000Da respectively, which have found to be located in the cell wall of two slime forming encapsulated strain. The polypeptides with molecular weights of 42000 & 26000 found in strain T5 and T5/30 but absent in T5/NS were supposed to have some role in slime production.

The outer membrane from *Haemophilus (Actinobacillus) pleuropneumoniae* grown under iron-replate and iron-restricted conditions by Niven *et al.* (1989) on analysing within SDS-PAGE and immunoblotting, showed that iron restriction resulted in the appearance of two or more novel polypeptide in the range of 96-102KDa and an increased amount of 79KDa. polypeptides. Derneer *et al.* (1989) stressed the role of 105Kb & 76Kb polypeptides in adaptation to iron restricted conditions.

Chang *et al.* (1992) observed that the isolates of *Borrelia burgdorferi* strains gained an additional protein of 22KDa after reintroduction to *Ixoves ricinus.*

Singh *et al.* (1994) showed that a 53 KDa protein from *V.cholerae* (classical strain 0395) was involved in intestinal colonization.

Sinha and Haeder (1996) studied the impact of heat, salinity and L-methionine-DL-sulfoximine (MSO) on growth and total protein profile of *Cyanobacterium, Anabaena spp.* Protein profile as resolved by SDS-PAGE, after heat stress on *Anabaena* species revealed a decline in the synthesis of several proteins but at the same time, synthesis of a new set of proteins of approximately

60-65KDa was induced after 12-14 hours of incubation and they were eliminated completely after 96hrs of incubation. Most of the protein bands disappeared in the culture at 500mM NaCl. But when cultures are treated with 100 mM NaCl, the cultures elicited new proteins at around 29, 32, 40 and 70KD. The result indicates that different stressors exert specific effects on Cyanobacterial protein synthesis.

Protein profiles are deeply affected by UV radiation. SDS-PAGE analysis of total protein profile of the cells treated with UV-B showed a linear decrease in the protein content with increase in UV-B exposure time. Complete elimination of most of the protein bands occurs after 90-120 minutes of UV-B exposure in *Nostoc carmium* and *Anabaena* sps., whereas the same occurs only after 150 minutes of UV-B treatment in *Nostoc commune* and *Scytonema* spp. (Sinha *et al.* 1995).

3. MATERIALS AND METHODS

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3.1. BACTERIA:

The bacterial pathogens used in the present study were obtained from the Biotechnology Laboratory of CMFRI and from the Department of Environmental Science, CUSAT. They are:

1. *Vibrio parahaemolyticus* (VP)
2. *Vibrio cholera* (VC
3. *Vibrio fischeri* (VF)
4. *Vibrio anguillarum*(VA)
5. *Escherichia coli* (EC)
6. *Salmonella typhi* (ST)
7. *Aeromonas hydrophila*
    - 7.1. *Aeromonas hydrophila* Strain I (AH_1)
    - 7.2. *Aeromonas hydrophila* Strain II (AH_2)
    - 7.3. *Aeromonas hydrophila* Strain III (AH_3)
    - 7.4. *Aeromonas hydrophila* Strain IV (AH_4)
8. *Aeromonas salmonicida* (AS)
9. *Streptococci*
    - 9.1. *Streptococcus* sp. Isolate I (S_1)
    - 9.2. *Streptococcus* sp. Isolate II (S_2)
    - 9.3. *Streptococcus* sp. Isolate III (S_3)
    - 9.4. *Streptococcus* sp. Isolate IV (S_4)
    - 9.5. *Streptococcus* sp. Isolate V (S_5)

Strains designated as AH_2 to AH_4 and S_1 to S_5 were field isolates.

3.2. MAINTENANCE OF BACTERIAL CULTURE IN THE LABORATORY

All the bacterial strains except the *Streptococcus* were maintained on Nutrient agar slants with appropriate salt concentrations required by each of them as shown below.

Bacteria	NaCl Conc.	Incubation temp.
1. *Aeromonas hydrophila*	0.5%	35^0C
2. *Aeromonas salmonicida*	0.5%	37^0C
3. *Salmonella typhi*	0.5%	37^0C
4. *Escherichia coli*	0.5%	37^0C
5. *Vibrio cholera*	1%	37^0C
6. *Vibrio anguillarum*	2%	37^0C
7. *Vibrio parahaemolyticus*	2%	37^0C
8. *Vibrio fischeri*	0.5%	37^0C

All the isolates of *Streptococcus* species were maintained in peptone water at 35^0C which had the following composition.

Peptone water:

Peptone	- 1%
Potassium Nitrate	- 0.2%
Aged sea water	- 100 ml

3.3. PREPARATION OF BROTH CULTURES

Nutrient broth culture of each of the above bacterial strains were made for easier isolation of plasmids. Broth cultures were all incubated for 24 hours at their respective temperatures. The salt concentration of the nutrients broth was same as that of the nutrients agar slants.

3.4. ANTIMICROBIAL SUSCEPTIBILITY TEST

i) Plates were prepared with Muller Hinton Agar (M 173) for use in the Bauer-Kirby method for rapidly growing aerobic organisms.

ii) Pure culture was used as inoculum. Three to four similar colonies were selected and transferred into about 5ml of nutrient broth and incubated at 35^0C for 2-8 hours till light to moderate turbidity developed.

iii) A sterile cotton swab was dipped into the properly prepared inoculum and rotated firmly against the upper inside wall of the tube to expell the excess fluid. The entire agar surface of the plate was streaked with the swab three times turning the plate 60^0C between each streaking.

iv) The antibiotic discs were applied using aseptic technique. The discs were deposited with center at least 24 mm apart.

v) The plates were then incubated immediately at 37^0C and examined after 24 hrs. Only zones showing complete inhibition were measured and the diameters of the zones were recorded in millimeter.

The results were interpreted by using the zone size interpretative chart (Bauer *et al.* 1996: Performance standards for antimicrobial disk susceptibility

tests, 1993). The antibiogram of the various bacterial strains was prepared for correlating the antibiotic sensitivity with plasmid profile.

3.5. ISOLATION OF PLASMIDS

Plasmids were isolated from all the strain of bacteria under investigation to be further resolved by agarose gel DNA electrophoresis.

3.5.1. Reagents required

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

i) Lysozyme (SIGMA, USA).

ii) *TEG buffer (pH : 8)*

iii) Alkaline lysis buffer

1% SDS

0.2N NaOH (Prepared from a stock of 10% SDS and 2N NaOH).

iv) 3M Potassium acetate (pH : 5.2)

v) Neutral phenol

vi) Chloroform – Isoamyl alcohol (24:1)

vii) 3M sodium acetate (pH : 5.2)

viii) Absolute ethanol

ix) 70% ethanol

x) *TE buffer (pH : 8)*

10 mM Tris-HCl

1 mM EDTA

3.5.2. Procedure

The procedure adopted in the present study was a modification of the procedure developed by Maniatis *et al.* (1989).

The bacteria were harvested from the broth culture during the post logarithmic phase by spinning at 10Krpm, 4^0C for 10 minutes in 1.5 ml eppendorf tubes in a refrigerated high speed centrifuge. The supernatant was carefully drained out and the pellet was suspended in 100 µl of TEG buffer (pH 8) containing lysozyme (5mg/ml). The cell suspension was vortexed in a vortex mixture and incubated at 4^0C for 10 minutes. After the bacterial cell wall lysis in TEG buffer containing lysozyme, 200µl of alkaline lysis buffer containing 0.2 M NaOH and 1% SDS was added. The solution was mixed gently and incubated at 4^0C for 15 minutes. The solution was gently shaken to mix the contents at every five minutes. The nuclear DNA and proteins got denatured during alkaline lysis and the solution became viscous. To that viscous solution, 150µl of 3M potassium acetate (pH 5.2) was added and kept at 4^0C for 10 minutes. The contents were mixed well. A network of precipitated proteins and nuclear DNA was formed. After 10 minutes the preparation was centrifuged for 15 minutes at 10Krpm, 4^0C. After centrifugation, the clear supernatant containing plasmid DNA was collected in another microcentrifuge tube. To that, equal volume of neutral phenol was added to precipitate any proteins present in the solution. The solution was mixed by gentle shaking and kept undisturbed for 10 minutes to precipitate the proteins. The preparation was then centrifuged at 10Krpm, 4^0C for10 minutes. Protein layer got precipitated in the aqueous – organic interphase. The aqueous phase was carefully

pipetted out and transferred to another eppendorf tube and the neutral phenol extraction was repeated again. To the aqueous phase collected, equal volume of chloroform – isoamylalcohol was added to remove traces of phenol and other impurities, if any. The mixture was shaken well and centrifuged at 10Krpm, 4^0C for 10 minutes. The aqueous phase was transferred to another eppendorf tube. The quantity was measured and $1/10^{th}$ volume of 3M sodium acetate was added and mixed well. To the above mixture, 2-2.5 volume of absolute ethanol was added. The mixture was shaken well and kept at 20^0C overnight for precipitating plasmid DNA. *The ethanol precipitated preparation was centrifuged at 10Krpm, 4^0C for* 15 minutes. The supernatant was discarded carefully and the precipitate was washed with 70% ethanol to dissolve the salt (sodium acetate). The solution was then centrifuged at 10Krpm, 4^0C for10 minutes, the supernatant was discarded completely, and the pellet was air dried. When the pellet was completely free of moisture, it was dissolved in minimum quantity of TE buffer (pH 8). The plasmid DNA thus obtained was stored at - 20^0C.

3.6. DNA ELECTROPHORESIS

Plasmids isolated from the bacteria were subjected to agarose gel electrophoresis to resolve the plasmids according to their size.

3.6.1. Reagents required

i) Agarose

ii) 1 x TEB (pH 8.0)

0.89 M Tris –HCl
0.02 M EDTA
0.89 M Boric acid

iii) Loading buffer

Glycerol – 2 ml

Bromophenol blue (0.5%) – 1 ml

1x TEB – 7ml

iv) Standard DNA marker (λDNA cut with Hind III/EcorI)

v) Ethidium bromide (1μgm/ml)

3.6.2. Procedure

Preliminary trials of plasmid DNA electrophoresis with different percentages of agarose gel were carried out and 0.8% agarose gel was found to be most suitable for the resolution of plasmid DNA. Therefore, 0.8% agarose gel prepared in 1xTEB was used for routine screening of the plasmid DNA and into that 5μl of the sample was loaded along with a standard DNA marker (λ DNA cut with Hind III/EcorI). The electrophoresis was continued for about four hours.

The gel was then stained in ethidium bromide in darkness for 20 minutes. The stained gel was dipped in distilled water to remove excess stain. Then the gel was viewed by using a UV transilluminator. Plasmid DNA appeared as reddish orange bands and the approximate molecular weight of the plasmid was determined by comparing it with standard DNA marker.

3.7. ISOLATION OF BACTERIAL PROTEINS

The proteins from the following bacteria were isolated and analysed by sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE).

3.7.1. The Bacteria used for protein isolation.

1) *Aeromonas salmonicida* (AS)

2) *Aeromonas hydrophila* (AH_1)

3) *Aeromonas hydrophila* (AH_2)

4) *Aeromonas hydrophila* (AH_3)

5) *Aeromonas hydrophila* (AH_5)

6) *Streptococcus* sp.Isolate I (S_1)

3.7.2. Bacterial Protein Isolation

All the bacterial strains of *Streptococcus* spp. and *Aeromonas* spp. were broth cultured in 2 ml, and were harvested from the broth culture during the post logarithmic phase by spinning at 10 Krpm, 4^0C, 10 min. in 1.5 ml eppendorf tubes in a refrigerated high speed centrifuge.

The supernatant was drained off and to each of the pellets 100 ml of TEG buffer (pH 8) containing 5mg/ml lysosyme was added and vortexed in a vortex machine. This was incubated at 4^0C for 15 min. mixing gently every five minutes.

The cell suspension was then centrifuged at 10 Krpm, 4^0C, 10 min. and the supernatant was collected in eppendorf tubes of 0.5 ml and stored at -20^0C for further use.

3.7.3. Preparation of Samples

To the 70 µl of each of the above samples, 60 µl of sample buffer is added. Simultaneously 10 µl of SDS protein molecular weight marker from GENEI, Bangalore was mixed with 60 µl of sample buffer. The samples were then boiled strictly for 3 minutes and the marker for 1 minute.

3.8. SODIUM DODECYL SULPHATE POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

3.8.1. Principle:

SDS is an anionic detergent, which binds strongly to the protein and denatures it. The number of SDS molecules bound to a polypeptide chain is approximately half the number of amino acid residues in that chain. The protein SDS complex carries a net negative charge, hence moves towards the anode and, the separation is based on the size of the protein.

3.8.2. Standardization of SDS-PAGE:

The method used in the present study was similar to Laemmli *et al.* (1970) with some modification. 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 to choose, 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 samples to be loaded were also standardised for ideal resolution.

3.8.3. Reagents:

1) **Stock acrylamide solution** : (30%)

Acrylamide : (29.1 gm)

Bis acrylamide : (0.9 gm)

The mixture was dissolved in minimum water and made up to 100ml using double distilled water. The mixture was filtered using Whatman No.1 filter paper and stored in amber coloured bottles in a refrigerator.

2) Gel buffers:

a) Separating Gel buffer

1.5MTris –HCl – pH – 8.8

18.75 ml of 2M Tris was taken and made upto 25ml after adjusting the pH to 8.8 by using 2N HCl.

b) Stacking gel buffer.

0.5 M Tris – 3.028 gm.

The pH was adjusted to 6.8 using 2M HCl and solution was made up to 50 ml using double distilled water.

3) Electrode buffer (1litre)

0.05 M Tris HCl – 6.057 gm

0.383 M glycine – 28.527 gm/pH 8.3

0.1% SDS – 1 gm.

4) 10% SDS stock

5) Polymerizing agent.

Ammonium per sulphate – 10% (freshly prepared)

TEMED

3.8.4. Composition of 11.5% gel.

i) Separating Gel.

Stock Acrylamide solution (30%)	-	11.5 ml.
Separating gel buffer	-	6 ml
Water	-	12.07 ml
10% SDS	-	300 µl
TEMED	-	30 µl
APS	-	100 µl

ii) Stacking gel

Stock Acrylamide solution (30%)	-	2 ml.
Stacking gel buffer	-	2.5 ml
Water	-	5.4 ml
10% SDS	-	100 µl
TEMED	-	10 µl
APS	-	40 µl

Gel cassette was prepared 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 prevents 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 comb plate was placed in the

stacking gel making sure that there are 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.

3.8.5. *Preparation of Samples for Loading*

Stocks of sample buffer were prepared.

Sample buffer (10 ml)

Glycerol	-	2 ml
β- mercaptoethanol	-	1 ml
Stacking gel buffer	-	1.8 ml
Bromophenol blue	-	0.6 ml from 0.1% stock
SDS	-	1 ml
Distilled water	-	3.6 ml

3.8.6. Electrophoresis

The prepared samples were 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, as the dye front reaches the bottom of gel.

3.8.7. Staining:

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

Stain	-	(500 ml)
0.2% Coomassie brilliant blue	-	1 gm
50% methanol	-	250 ml
7% Acetic acid	-	35 ml
Double distilled water	-	*215 ml*

After staining for 2 hrs the gel is washed with tap water and placed in destainer.

Destainer:

10% Methanol

7% Acetic acid.

After proper destaining, the gel was observed for protein bands.

3.8.8. Determination of molecular weight:

The molecular weights of standard SDS-PAGE molecular marker used were 97.4KDa, 68KDa,43KDa, 29KDa, 14.3KDa RF values of the standard marker *were calculated by the formula*

$$R_f = \frac{\text{Solute front}}{\text{Dye front}}$$

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

4. RESULTS

4.1. PLASMID PROFILE

4.1.1. *Vibrio*

Plasmid DNA isolated from four *Vibrio* species *viz. V. parahaemolyticus, V. anguillarum, V. cholera and V. fischeri* were resolved by agarose gel electrophoresis and were visualised on a UV-transilluminator after staining with ethidium bromide. The plasmid DNA profile of these species as resolved in the gel are presented in plates 1 and 2.

As can be seen from the plate 2, *V. parahaemolyticus* contained only a single plasmid. The molecular size of this plasmid was determined to be about 21kb, from the comparison of the relative mobility of the plasmid DNA *vis* a *vis* that of standard molecular weight DNA markers electrophorosed along with it.

On the other hand, *V. anguillarum* and *V. fischeri* harboured two plasmids each. However these species differed in their profile with respect to the molecular weight of the plasmid DNAs. Both of them carried a plasmid of 21kb each. While the second one was a mega plasmid in *V. cholera* and it was only a small plasmid of 6kb in *V. anguillarum.*

On the contrary, *Vibrio cholera* carried three plasmids, of which one was a 21kb plasmid like that of others presented above. While one of the remaining two was a mega plasmid, the other one was a smaller plasmid of 2kb only .

Plate 1 : Plasmid DNA of *Vibrio cholera* and *Vibrio fischeri*

Lane I, II : ***V. cholera***

Lane IV : **Standard DNA marker**

Lane V, VI : ***V.fischeri***

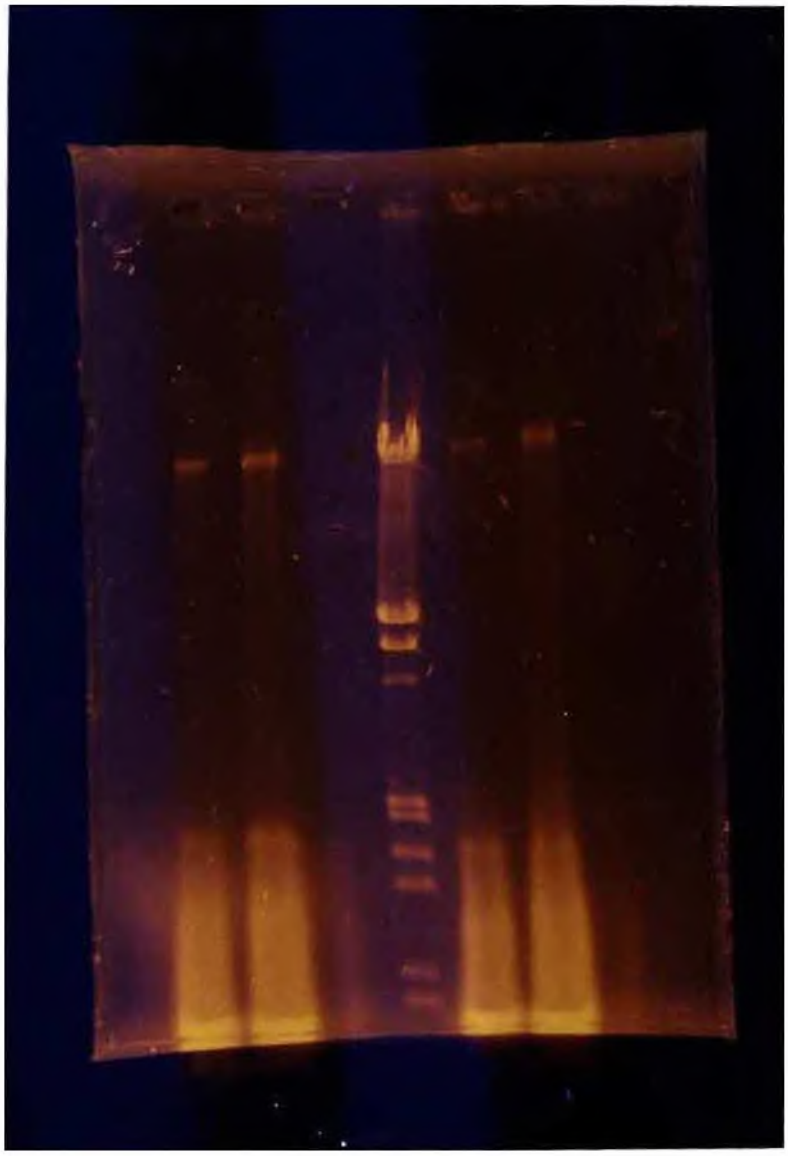


Plate 2 : Plasmid DNA profile of *Vibrio anguillarum* and *Vibrio parahaemolyticus*

Lane I : Standard DNA marker

Lane II : *V.anguillarum*

Lane III, IV, V : *V.parahaemolyticus*

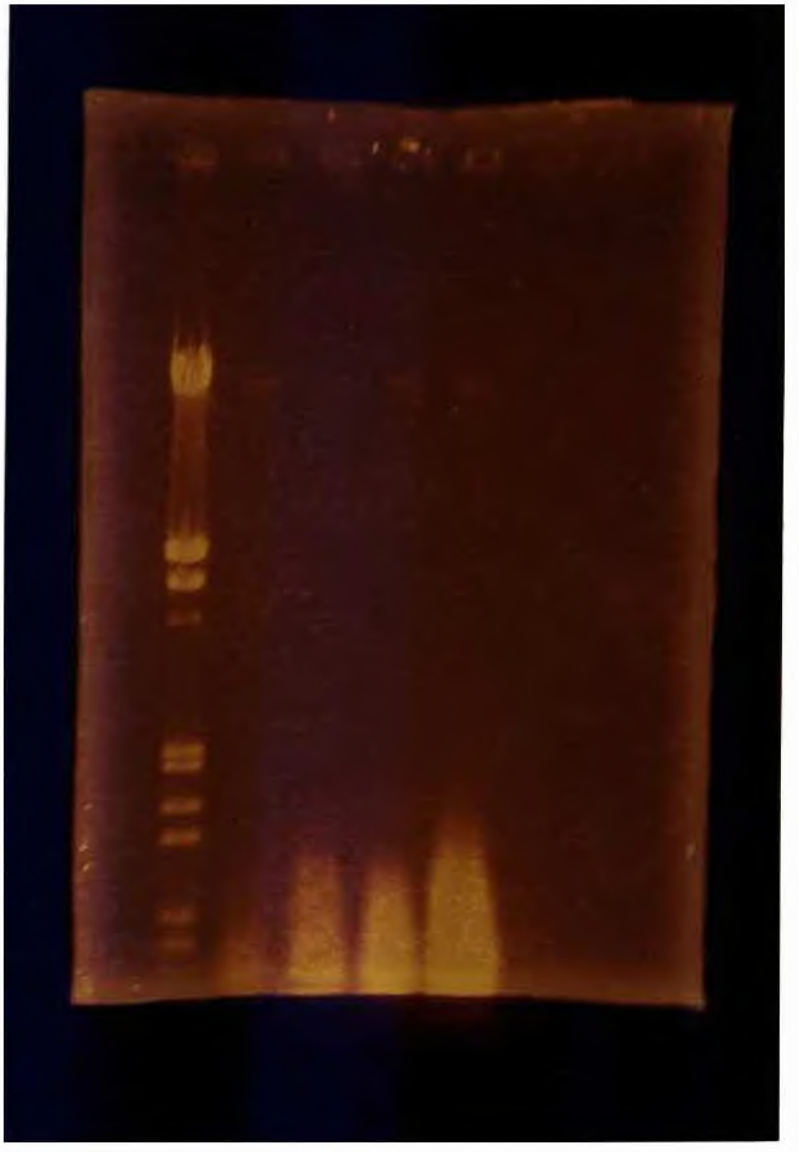


TABLE I
PLASMID NUMBER AND SIZE IN *VIBRIO* SPP.

SL. NO	BACTERIAL STRAINS	NO OF. PLASMIDS	SIZE OF PLASMIDS
1.	*Vibrio fischeri*	2	1.9kb, 21kb
2.	*V.cholerae*	3	2kb,21kb,mega plasmid
3.	*V.parahaemolyticus*	1	21kb
4.	*V.anguillarum*	2	6kb, 21kb

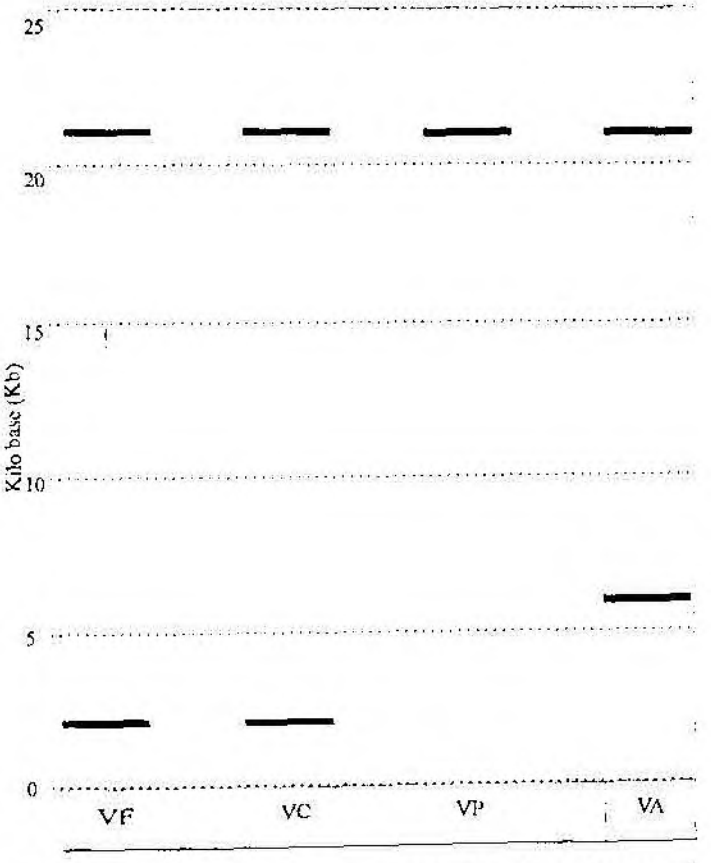


Fig.1. Relative positions of plasmid DNA in species of *Vibrio*.

4.1.2. *Aeromonas*

Plasmid DNA profile of the reference strain of *A. hydrophila* (AH_1) and *A. salmonicida* (AS) are presented in the plate 4 and plate 3 respectively. While *A. salmonicida* was harbouring multiple plasmids of 21kb, 2.1 kb and 1.4kb size, *A. hydrophila* (AH_1) was carrying only a single plasmid of 21kb size.

Plasmid DNA from three field strains of *Aeromonas hydrophila* were also electrophoretically analysed in an attempt to catalogue the extent of homology in the plasmid profile among different strains of the same species. The DNA profile resolved for the field strains of the *Aeromonas hydrophila* species *viz.* AH_2, AH_3 and AH_4 are presented in plates 5, 4 and 6 respectively.

As can be seen from the above plates, while two of the strains i.e. AH_2 and AH_3 had only a single plasmid of 21kb like that of the reference strain, one of the field strains (AH_4) carried multiple plasmids *viz.* a 21kb plasmid and two small plasmids of 2kb and 1.3kb. The relative molecular size of the plasmids of the different strains are summarised in the table II and their relative position in the agarose gel are represented in Fig. 2.

4.1.3. *Streptococci*

The plasmid pattern of the five field isolates of *Streptococci* designated as S_1 to S_5 are represented in plate 7. As can be seen from the above plate, there were considerable variations in the profiles. While the S_4 carried a single plasmid of 21kb size, all others carried more number of plasmids. The number of plasmids harboured by S_1, S_2, S_3 and S_5 were 4, 3, 4 and 2 respectively.

Plate 3 : Plasmid DNA of *Aeromonas salmonicida* (AS)

Lane I : Standard DNA marker

Lane II, III, IV, V : *A.salmonicida.*

Plate 4 : Plasmid DNA profile of *Aeromonas hydrophila* reference strain (AH_1) and field strain (AH_3).

Lane I, II : ***A.hydrophila*** **(AH_1)**

Lane III : **Standard DNA marker**

Lane V, VI : ***A.hydrophila*** **(AH_3).**

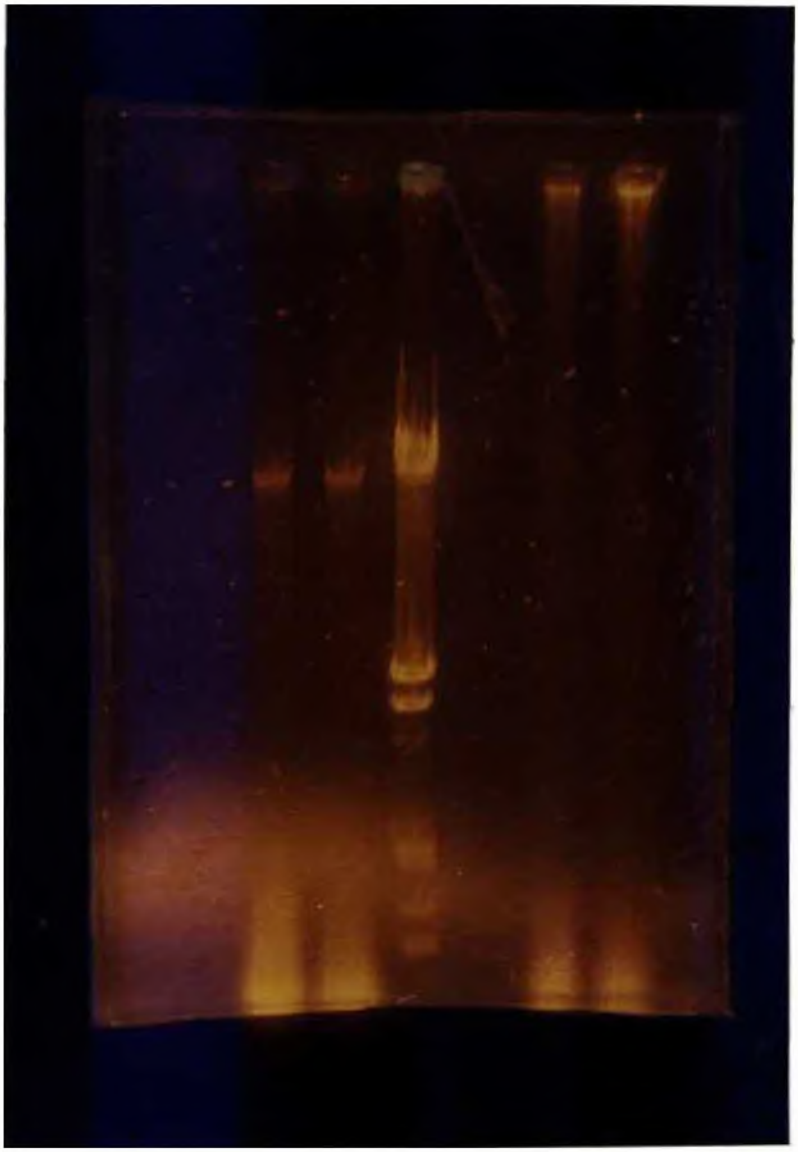


Plate 5 : Plasmid DNA profile of *Aeromonas hydrophila* field strain (AH_2).

Lane I : Standard DNA marker

Lane II, III, IV, V : *A.hydrophila* (AH_2).

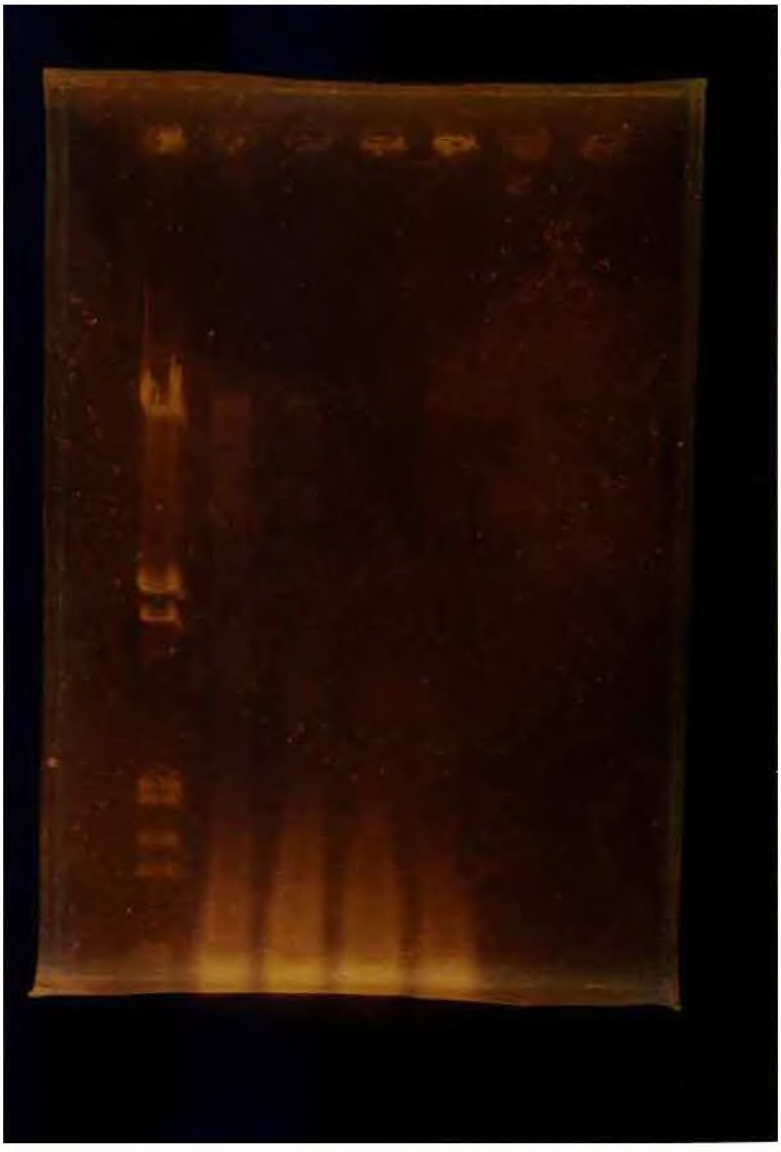


Plate 6 : Plasmid DNA profile of *Aeromonas hydrophila* field strain (AH_4).

Lane I	:	**Standard DNA marker.**
Lane II, III, IV, V	:	***A.hydrophila*** **(AH_4).**

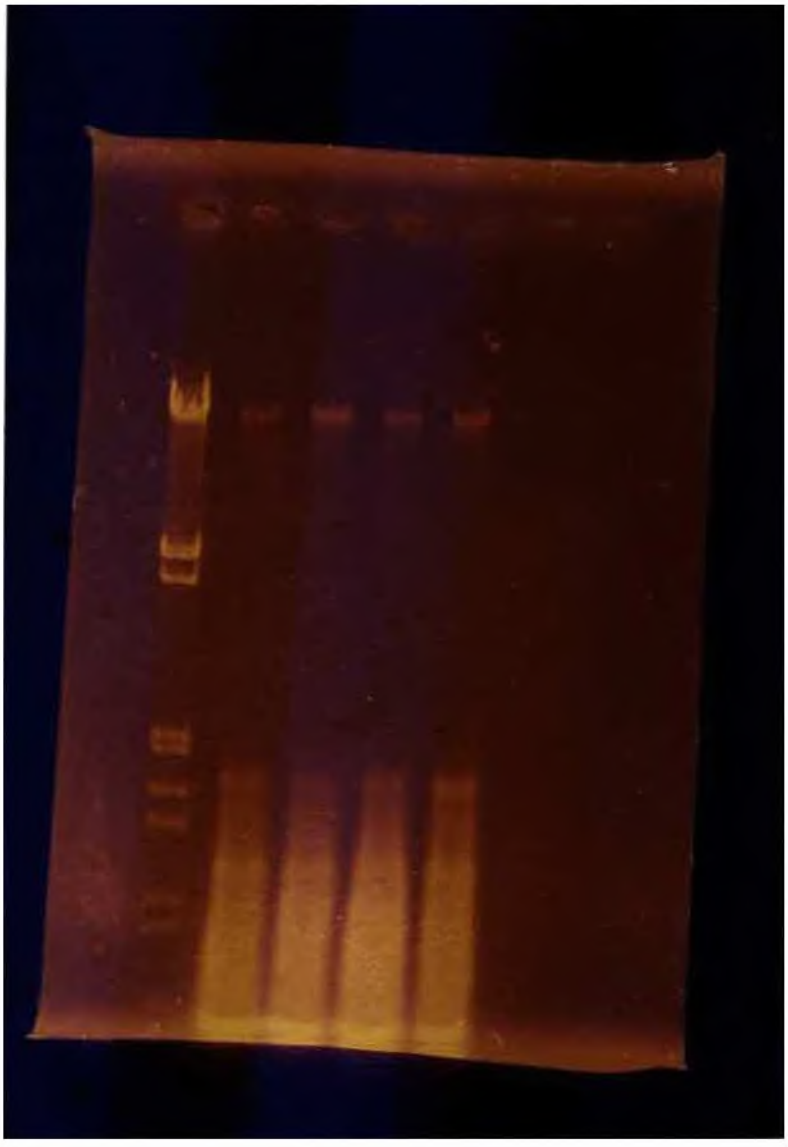


TABLE II

PLASMID DNA NUMBER AND SIZE IN *AEROMONAS* SPP.

Sl. NO	BACTERIAL STRAINS	NO OF PLASMIDS	SIZE OF PLASMIDS
1.	*Aeromonas hydrophila* AH_1	1	21kb
2.	*A. hydrophila* AH_2	1	21kb
3.	*A. hydrophila* AH_3	1	21kb
4.	*A. hydrophila* AH_4	3	21 kb, 2kb, 1.3kb
5	*A. salmonicida* AS	3	21kb,2.1kb,1.4kb

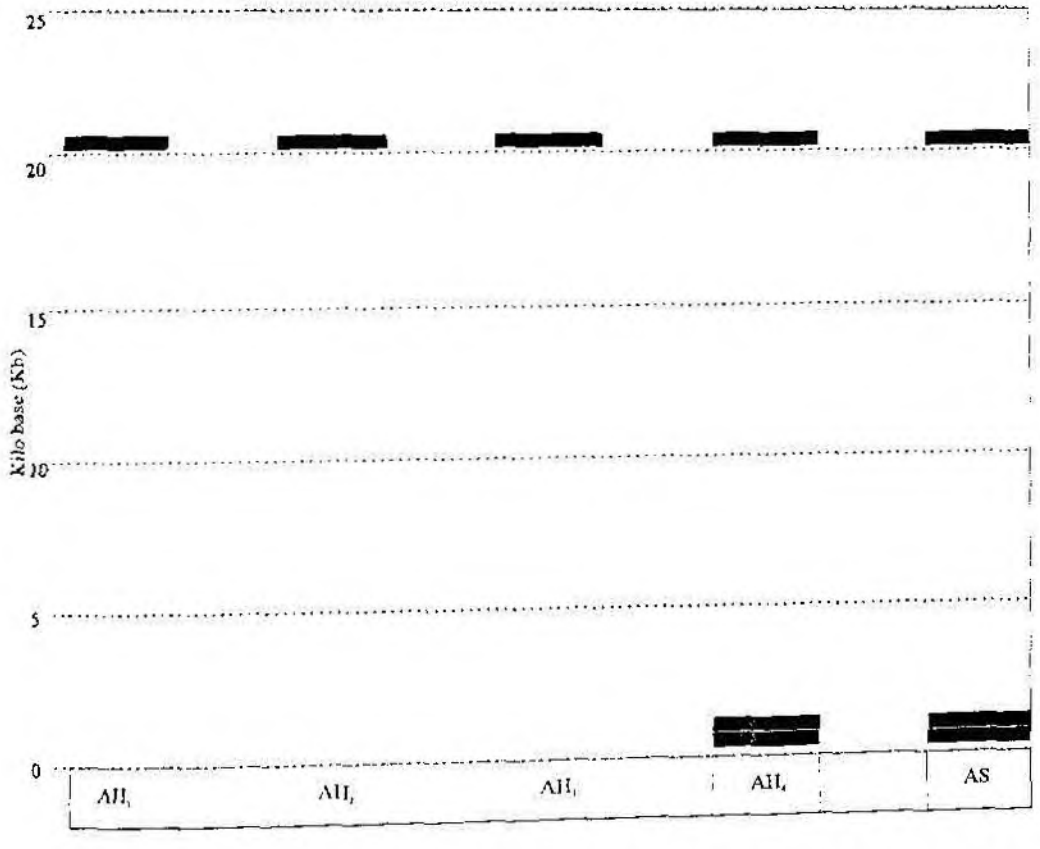


Fig.2. Relative positions of plasmid DNA in isolates of *Aeromonas* sp.

Plate 7 : Plasmid DNA profile of five isolates of *Streptococcus* spp.

Lane I	:	***Streptococcus* isolate I (S_1)**
Lane II, VI	:	***Streptococcus* isolate II (S_2)**
Lane III, IV	:	***Streptococcus* isolate III (S_3)**
Lane V	:	**Marker**
Lane VII	:	***Streptococcus* isolate IV (S_4)**
Lane VIII	:	***Streptococcus* isolate V (S_5)**

TABLE III
PLASMID NUMBER AND SIZE IN ISOLATES OF *STREPTOCOCCUS* SPP.

S.No.	Name of strains	No. of plasmids	Size of plasmids
1.	*Streptococcus* sp S_1	4	2kb, 4kb, 5kb, 23kb
2.	*Streptococcus* sp. S_2	3	2kb, 22kb, mega plasmid
3.	*Streptococcus* sp. S_3	4	2kb, 4kb, 5kb, 21kb
4.	*Streptococcus* sp. S_4	1	21kb
5.	*Streptococcus* sp. S_4	2	21kb, mega plasmid

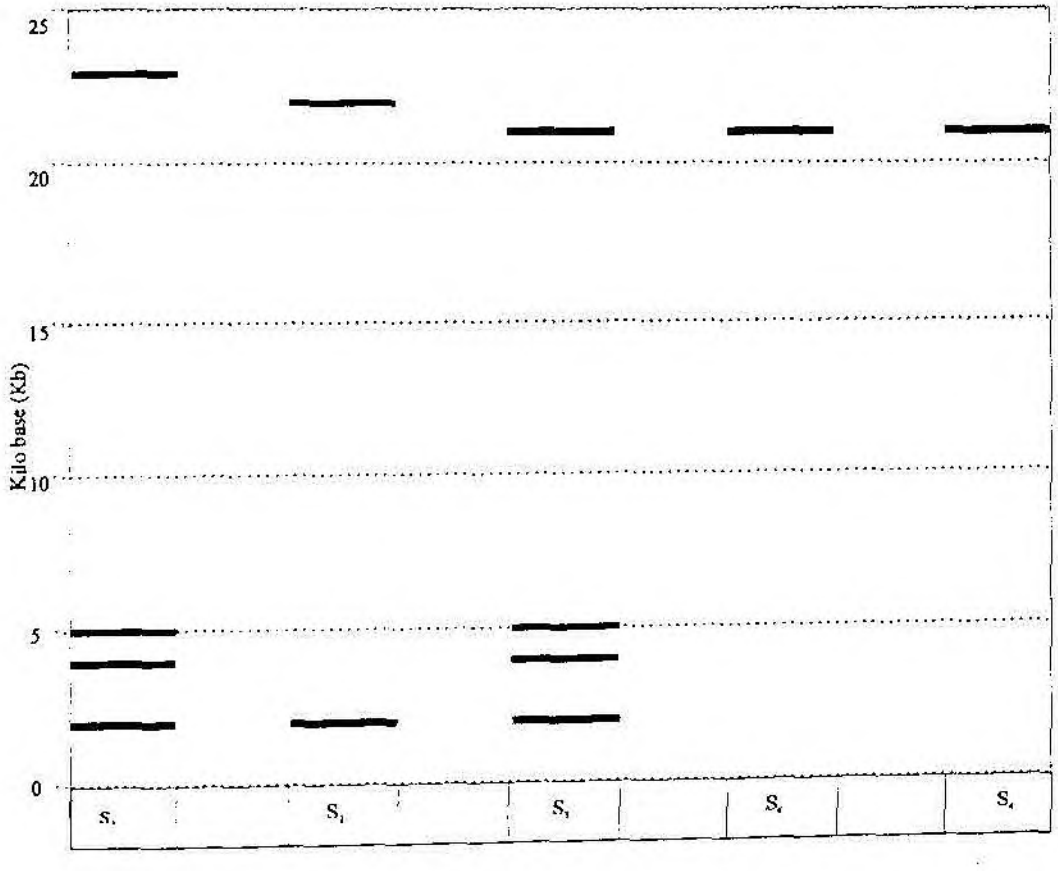


Fig.3. Relative positions of plasmid DNA in five isolates of *Streptococcus* sp.

The molecular weight of the 4 plasmids of S_1 determined with the help of molecular weight markers were 23kb, 5kb, 4kb and 2kb (Lane I, plate 7).

The S_2 carried a mega plasmid, whose molecular weight could not be determined with the help of the marker, in addition to two other plasmids of 22kb and 2kb (Lanes II, VI, plate 7).

Molecular weight of the four plasmids isolated from S_3 were 21kb, 5kb, 4kb and 2kb (Lanes III, IV, plate 7).

Of the two plasmids carried by S_5, one was a mega plasmid positioned high above the molecular weight markers and the other was adjudged to be of 21kb size (Lane VIII, plate 7).

4.1.4. *Salmonella typhi*

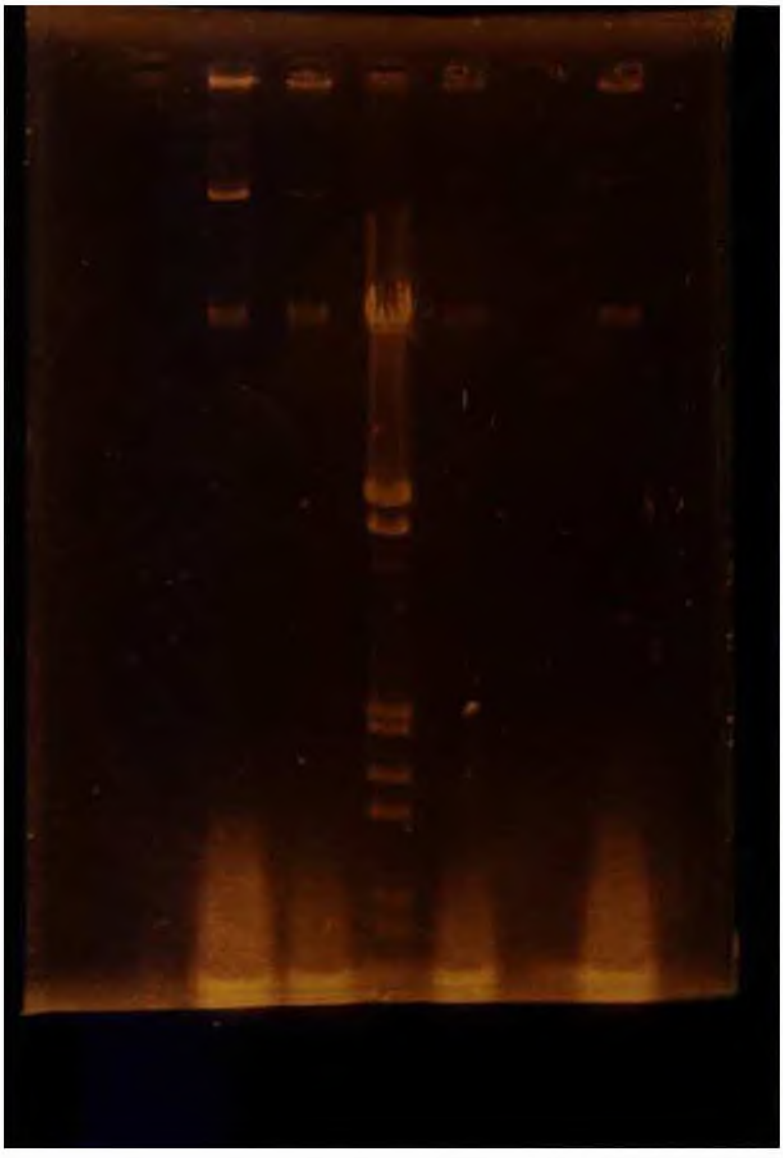
The two plasmids isolated from *S. typhi* and resolved by electropheresis are presented in Lane I & II of plate 8. As can be seen from the above plate, while one of the plasmid was of 21kb, the other was a mega plasmid.

4.1.5. *Escherichia coli*

The plasmid profile of *E. coli* is presented in Lane IV&VI & Plate 8. While one of the two plasmids was positioned in alignment with the 21kb molecular weight marker, the other one was a mega plasmid, whose size could not be determined with the help of marker.

Plate 8 : Plasmid DNA profile of *Salmonella typhi* and *Escherichia coli.*

Lane I, II	:	***S.typhi***
Lane III	:	**Standard DNA marker**
Lane IV, VI	:	***E. coli.***



4.2. ANTIBIOTIC RESISTANCE

The bacterial pathogens under investigation were checked for their susceptibility to a panel of antibiotics by disc diffusion method and an antibiogram was prepared by interpretation of the zone of inhibition into susceptible (S), intermediate (I) or resistant (R), using the zone size interpretative chart. The results are presented below.

4.2.1. *Vibrio*

The four different species of *Vibrio* showed marked differences in their resistivity pattern. The antibiograms of the *Vibrio* species under study are presented in Table IV.

Vibrio parahaemolyticus showed resistance to only oxacillin and penicillin-G, whereas it was sensitive to ampicillin, amoxycillin, bacitracin, chloro tetracycline, erythromycin, gentamycin, kanamycin, neomycin, oxytetracycline, polymyxin-B, streptomycin and tetracycline.

Vibrio cholera was also resistant to only two antibiotics *viz.* ampicillin and oxacillin. It was sensitive to amoxycillin, chloramphenicol, chlorotetracycline, erythromycin, gentamycin, kanamycin, neomycin, novobiocin, oxytetracycline, penicillin, polymyxin-B, streptomycin and tetracycline.

Vibrio fischeri was resistant to ampicillin, amoxycillin, bacitracin, erythromycin, oxacillin and penicillin, and was sensitive to chloramphenicol, chloro tetracycline, gentamycin, kanamycin, neomycin, novobiocin, oxytetracycline,

TABLE – IV

ANTIBIOGRAM OF *VIBRIO* SPP.

	VIBRIO			
	V.parah-aemolyt-icus	*V.cholera*	*V.fischeri*	*V.anguillarum*
AMPICILLIN	S	R	R	R
AMOXYCILLIN	S	S	R	S
BACITRACIN	S	S	R	S
CHLORAMPHENICOL	S	S	S	S
CHLOROTETRACYCLINE	S	S	S	S
ERYTHROMYCIN	S	S	R	R
GENTAMYCIN	S	S	S	S
KANAMYCIN	S	S	S	S
NEOMYCIN	S	S	S	S
NOVOBIOCIN	S	S	S	R
OXACILLIN	R	R	R	R
OXYTETRACYCLINE	S	S	S	S
PENICILLIN-G	R	S	R	R
POLYMYXIN-B	S	S	S	R
STREPTOMYCIN	S	S	S	S
TETRACYCLINE	S	S	S	S

polymyxin-B, streptomycin, tetracyclin and was intermediately resistant to bacitracin and erythromycin.

Vibrio anguillarum exhibited resistance to ampicillin, erythromycin, novobiocin, oxacillin, penicillin-G and polymyxin-B. It was sensitive to amoxycillin, chloramphenicol, chloro tetracycline, gentamycin, kanamycin, neomycin, oxytetracycline, streptomycin and tetracycline.

4.2.2. *Aeromonas*

The antibiogram of *A. salmonicida* and *A. hydrophila* are presented in Table V. *A. salmonicida* was resistant to 9 of 16 antibiotics tested *i.e.* ampicillin, amoxycillin, bacitracin, erythromycin, novobiocin, oxacillin, penicillin, streptomycin and tetracycline.

The antibiogram of the reference strain of *Aeromonas hydrophila* (AH_1) differed from that of *A. salmonicida*, in that the former was not resistant to streptomycin and tetracycline. Two of the field strains *i.e.* AH_2 and AH_3 also showed a similar antibiogram as that of AH_1. The antibiotic resistance pattern of the field strain AH_4 was similar to that of *A. salmonicida* with which it had homology of plasmid profile also.

4.2.3. *Streptococci*

The antibiogram of the *Streptococci* (S_1-S_5) are presented in Table VI. The S_4 was resistant to only three antibiotics namely ampicillin, oxacillin and penicillin G. However, the others were resistant to 8-9 out of 16 antibiotics tested.

TABLE V
ANTIBIOGRAM OF *AEROMONAS* SPP.

	AEROMONAS SPP				
SPECIES	AH_1	AH_2	AH_3	AH_4	AS
AMPICILIN	R	R	R	R	R
AMOXYCILLIN	R	S	R	R	R
BACITRACIN	R	R	R	R	R
CHLORAMPHENICOL	S	S	S	S	S
CHLOROTETRACYCLINE	S	S	S	S	S
ERYTHROMYCIN	R	R	R	R	R
GENTAMYCIN	S	S	S	S	S
KANAMYCIN	R	R	R	S	S
NEOMYCIN	S	R	S	S	S
NOVOBIOCIN	R	R	R	R	R
OXACILLIN	R	R	R	R	R
OXYTETRACYCLINE	S	S	S	S	S
PENICILLIN – G	R	R	R	R	R
POLYMYXIN - B	S	S	S	S	S
STREPTOMYCIN	S	S	S	R	R
TETRA CYCLINE	S	S	S	S	R

TABLE – VI

ANTIBIOGRAM OF *STREPTOCOCCUS* SPP.

STREPTOCOCCUS					
	S_1	S_2	S_3	S_4	S_5
AMPICILLIN	R	R	R	R	R
AMOXYCILLIN	R	R	R	S	R
BACITRACIN	R	R	R	S	R
CHLORAMPHENICOL	S	S	S	S	S
CHLOROTETRACYCLINE	S	S	S	S	S
ERYTHROMYCIN	R	R	R	S	S
GENTAMYCIN	S	S	S	S	S
KANAMYCIN	R	R	R	S	R
NEOMYCIN	S	S	S	S	R
NOVOBIOCIN	R	R	R	S	R
OXACILLIN	R	R	R	R	R
OXYTETRACYCLINE	S	S	S	S	S
PENICILLIN-G	R	R	R	R	R
POLYMYXIN - B	S	S	S	S	S
STREPTOMYCIN	S	S	S	S	S
TETRACYCLINE	S	S	S	S	S

TABLE - VII

ANTIBIOGRAM OF *S.TYPHI* AND *E.COLI.*

	Salmonella typhi	*E.coli*
AMPICILLIN	R	R
AMOXYCILLIN	S	S
BACTRICIN	R	R
CHLORAMPHENICOL	S	S
CHLOROTETRACYCLINE	S	S
ERYTHROMYCIN	R	R
GENTAMYCIN	S	S
KANAMYCIN	S	R
NEOMYCIN	R	R
NOVOBIOCIN	R	R
OXACILLIN	R	R
OXYTETRACYCLINE	R	S
PENICILLIN – G	R	R
POLYMYXIN - B	R	S
STREPTOMYCIN	S	S
TETRA CYCLINE	S	S

They were all resistant to ampicillin, amoxycillin, bacitracin, erythromycin, kanamycin, novobiocin, oxacillin and penicillin G.

4.2.4. *Salmonella typhi*

The species *Salmonella typhi* showed resistance to 9 of 16 antibiotics *viz.* ampicillin, bacitracin, erythromycin, neomycin, novobiocin, oxacillin, oxytetracycline, penicillin-G, polymyxin-B and was sensitive to amoxycillin, chloramphenicol, chlorotetracycline, gentamycin, kanamycin, streptomycin and tetracycline.

4.2.5. *Escherichia coli*

The *E.coli* showed resistance to ampicillin, bacitracin, erythromycin, kanamycin, neomycin, novobiocin, oxacillin, penicillin-G and was sensitive to amoxycillin, chloramphenicol, chlorotetracycline, gentamycin, oxytetracycline, polymyxin-B, streptomycin and tetracycline.

4.3. CELLULAR PROTEIN PROFILE

Analysis of the cellular soluble proteins was carried out by using the technique of SDS-PAGE (Laemmli, 1970). The bacterial species studied were *Aeromonas salmonicida, Aeromonas hydrophila* and *Streptococcus* spp. The SDS-PAGE profile of these bacteria were compared to find out species specific patterns if any. In addition to the reference strain of *A. hydrophila* (AH_1), the other three field strains of the same species viz AH_2, AH_3 and AH_4 were also studied and their protein profile compared with the reference strain to investigate the homology within the species.

Plate 9 : Cellular protein profile of field strains of *A.hydrophila*

Lane I	:	***A.hydrophila* strain II (AH_2)**
Lane II	:	***A.hydrophila* strain III (AH_3)**
Lane III	:	***A.hydrophila* strain I (AH_1)**
LaneIV	:	**Marker**
Lane V	:	***A.salmonicida* (AS)**

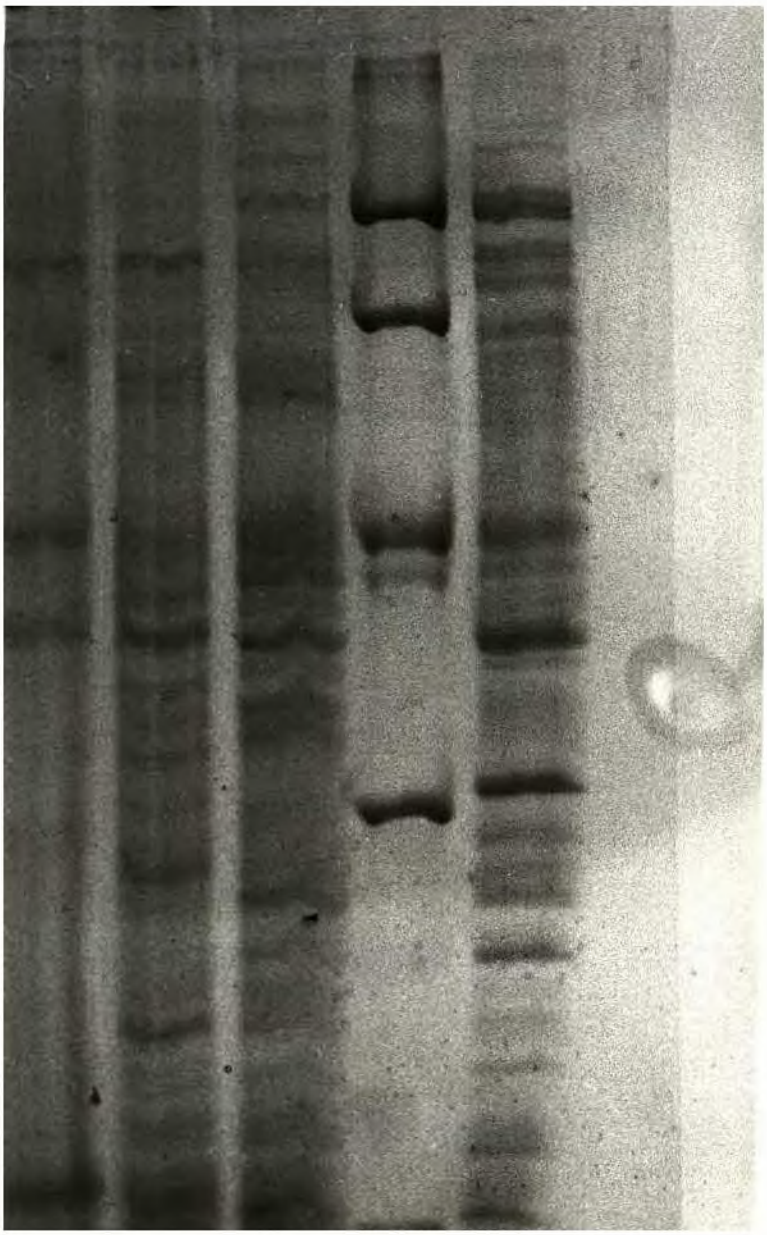


Plate 10 : Comparison of protein profile of *A.hydrophila* and *A.salmonicida*

Lane I	:	***A.hydrophila*** **(AH_1)**
Lane II	:	**Marker**
Lane III	:	***A.salmonicida*** **(AS)**

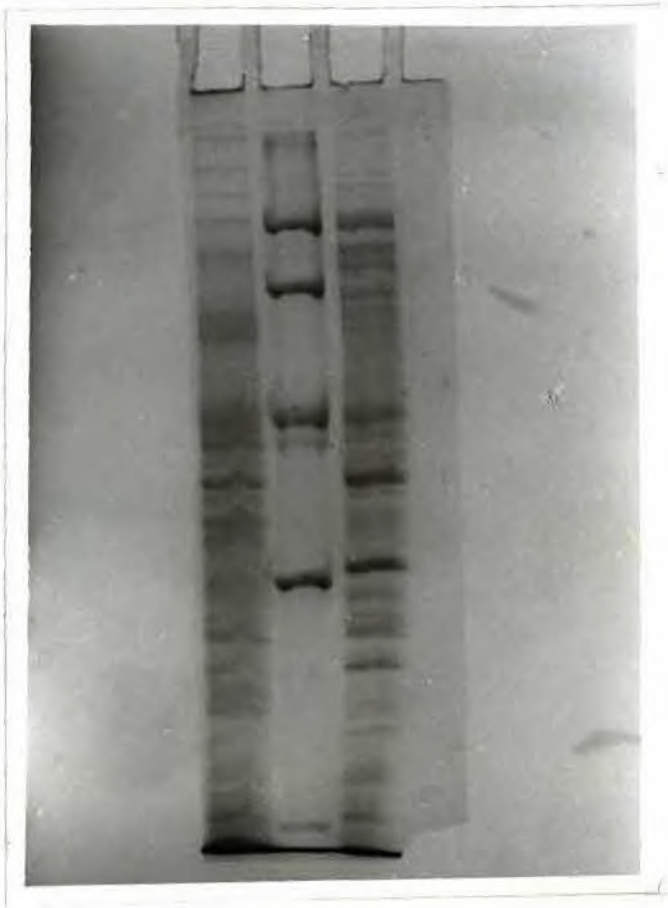


Plate 11 : Cellular protein profile of *Streptococcus* spp.

The strains of *A. hydrophila* (AH_1, AH_2, AH_3) had almost similar protein profile, all the proteins being within the range of 97.4KDa and 29KDa. About 29 bands were observed in AH_1, AH_2, and AH_4 whereas in AH_3 30 bands were present. While three of the above bands were observed to be located above that of the 97.4KDa molecular weight marker in the gel, one band was observed exactly on the same line of 97.4KDa marker. Like wise, two bands were present between 97.4KDa and 68KDa and one was on the same line of as 68KDa marker.

Similarly, three bands were observed between 43KDa and 68KDa and one was on the same line of 43KDa marker. In the case of AH_3, the additional band was of 42KDa. Six protein bands were seen between 43KDa and 24KDa and one on the same line as that of 20KDa. Eleven protein bands were seen between 24KDa and 14KDa marker.

The SDS-PAGE analysis of *Aeromonas salmonicida* has shown remarkable difference in protein profile with that of *A. hydrophila*. About thirty nine protein bands were observed and among these, ten were additionally expressed in *A. salmonicida* alone with varying molecular weights. The molecular weights of such additionally expressed proteins were as follows:

- One protein band above the 97.4KDa molecular weight marker and near to the cathodic end.
- Two protein bands between 97.4KDa and 68KDa.
- Two protein bands just below the 68KDa marker. Three protein bands between 43KDa and 68KDa.
- One intensely expressed protein band with molecular weight of 30KDa just above the 29KDa.

5. DISCUSSION

5. DISCUSSION

The present study was undertaken for the molecular characterization of bacterial pathogens that have been classified into different species on the basis of phenotypic characters. The characterization was attempted with respect to the plasmid DNA profile, protein profile and antibiotic sensitivity pattern of these bacteria.

The bacterial species taken up for the study of plasmid profile and antibiogram patterns were, *Aeromonas hydrophila, A. salmonicida, Vibrio cholera, V. anguillarum, V. parahaemlyticus, V. fischeri, Streptococcus Spp., Salmonella typhi* and *Escherichia coli.* Plasmid profiles of the different field strains belonging to *A. hydrophila* and *Streptococcus* were also studied and compared to find out the extent of homogeneity between the strains within species. Protein profile of *A.salmonicida, A.hydrophila* and *Streptococcus* were worked out using SDS-PAGE and were compared to find out species specificity of protein profiles. Similarly the fields strains belonging to *A.hydrophila* were also compared to find out the extent of homology in the protein profile between the strains within the species.

Various protocols reported in the literature were attempted in the present study for the isolation of plasmids from the bacterial cells. Of these, the protocol suggested by Maniatis *et al.* (1989), was found to be simpler. It involves the initial lysis of cell wall by lysozyme followed by precipitation of plasmid by ethanol and vortexing it. The protocol used in the present study for isolation of plasmid DNA is

a modified version from Maniatis *et al.* (1989) as per the availability of facilities in the laboratory.

5.1. *Vibrio*

The study indicated that the plasmid profile of each one of the four species of *Vibrio* under study *viz. V. cholera, V. anguillarum, V. fischeri* and *V. parahaemolyticus* was distinctly different from the other. However, it is interesting to note that all them shared the 21Kb plasmid. While *V. parahaemolyticus* harboured only this plasmid, others carried and additional plasmid each. While the size of this plasmid in *V. anguillarum* was 6Kb, their size in *V. cholera* and *V. fischeri* were 2 Kb and 1.9 Kb respectively. It could be that while the 21 Kb plasmid is shared by all members of the genera species level variations in the plasmid profile is caused by the smaller plasmids.

The antibiogram of these species also showed difference. For instance, while *V. parahaemolyticus* and *V. cholera* showed resistance only to two antibiotics each out of 16 antibiotics tested, *V. fischeri* and *V. anguillarum* showed resistance to 6 antibiotics each.

Further studies involving plasmid curing, restriction fragment analysis of the plasmid DNA, cloning of these fragments and transformation of recipient cells with cloned plasmid DNA etc. can only generate sufficient direct evidence to assign the antibiotic resistance to the specific portions of the plasmids.

5.2. *Aeromonas*

While *A. salmonicida* carried three plasmids of 21Kb ,2.1Kb and 1.4 Kb in size, the reference strain of *A. hydrophila* had only a single plasmid of 21 Kb, there being clear cut difference between the plasmid profile of the two.

However, while two of the three field strains of *A. hydrophila* had single plasmid like the reference strain, the third strain almost resembled *A. salmonicida* with plasmids of 21 Kb, 2 Kb and 1.3 Kb in them. The presence of the low molecular weight plasmids in field strains can be attributed to acquisition of these plasmids by natural conjugation.

The resemblance in the plasmid profiles of *A. salmonicida* and AH_4 strain of *A. hydrophila* cause difficulty in differentiating between them on the basis of plasmid profile alone. However, the protein profiles of both *A. salmonicida* and *A. hydrophila* were distinctly different to be noticed easily and thus could be used to distinguish between the two. While the techniques like RFLP and RAPD of the plasmid DNA are called for, to bring to light the desired amount of the variations in them, the protein profiles resolved by SDS-PAGE brings to light high level of protein polymorphism that can be directly used as bacterial fingerprint. As for example Shieh *et al.* (1986) could classify bacteriophage *Streptococcus lactus* and *S. cremoris* from cheese whey into two groups *viz.* D5 9-1/F_4-1 or group G7 2-1/I37-1 based on protein profile resolved by SDS-PAGE.

The antibiotic sensitivity pattern showed variation among the different strains. While *A. salmonicida* was resistant to 9 out of 16 antibiotics tested, the field strains of *A. hydrophila* with a similar plasmid pattern also showed resistance to 9

antibiotics (viz ampicillin, bacitracin, erythromycin, novobiocin, oxacillin, penicillin G, streptomycin and tetracycline).

The *A. hydrophila* strains which had only single plasmid were resistant to 8 antibiotics each out of the 16 tested.

It has been reported by Noon and Trust (1995) that *A. hydrophila* possesses highly conserved plasmids that encode antibiotic resistance elements. In the present study, the antibiogram pattern showed variations among the different strains which followed the same trend as that of the variations in the plasmid profile in these strains.

5.3. *Streptococcus*

The plasmid DNA of five different isolates of *Streptococcus* spp. from diseased fish when resolved by agarose gel electrophoresis, revealed considerable variation in the profile. Variations were observed in the number as well as the size of the plasmid DNA.

The antibiotic sensitivity pattern of these *Streptococci* were also interesting. While S_4 carrying single plasmid of 21 kb was resistant to only 3 antibiotics out of 16 tested, the others viz. S_1, S_2, S_3 and S_5 showed resistance to 7-9 antibiotics, thereby indicating a possible correlation between the number of plasmids and the antibiotic resistance.

Four distinct plasmid DNA patterns were observed. While three of them had multiple plasmids, one each was found to harbour two and single plasmids. The *Streptococci* designated as S_1 and S_3 had almost similar profile. Both carried 4 plasmids. While they exhibited homology in the molecular weight of the three

minor plasmids viz. 5 kb, 4kb and 2kb, the molecular size of the major plasmid of S_1 was 23kb as against the 21kb of S_3. The antibiotic resistance also showed a concurrent variation. While both were resistant to ampicillin, amoxycillin, bacitracin, erythromycin, kanamycin, novobiocin, oxacillin and penicillin the S_1 which carried the larger plasmid was resistant to novobiocin also.

The similarity between S_2 and S_5 was that both carried a mega plasmid each. While the S_2 contained two more plasmids of 22 kb and 2kb, the S_5 contained only one more plasmid of 21kb. The S_2 which carried a larger plasmid than that of the S_5, had additional resistance to kanamycin also.

In contrast, the S_4 with a single plasmid of 21Kb was resistant only to three of the antibiotics tested. It could be seen that there has been an increase in the resistance to antibiotics concurrently with the increase in the plasmid number or the molecular size of the commonly shared plasmid.

Studies conducted by Aoki *et al.* (1990) in yellow tails of Japan which were treated with antibiotics for Streptococcal infection, showed the evolution of drug resistant Streptococcal strains. Resistance to these drugs was found to be encoded by transferable R-plasmids.

5.4. *Salmonella typhi*

Salmonella typhi had two plasmids, *viz.* a 21Kb plasmid and a mega plasmid. It was found to be resistant to 7 out of 16 antibiotics tested.

5.5. *Escherichia coli*

E.coli which was resistant to 8 antibiotics of 16 tested had two plasmids, a mega plasmid and a 21Kb plasmid. The number of plasmids and antibiotic

sensitivity of *E. coli* was similar to that of *Salmonella typhi* except for polymyxin resistance additionally shown by *S.typhi.*

An attempt has been made in the present work for the molecular characterization of bacterial pathogens that have been already classified into various species and strains based on the phenotype characters. The plasmid DNA profile, cellular protein profile and antibiotic resistance pattern have been worked out for a number of bacterial species. In conclusion, the present study has revealed the species specific molecular characteristics of some of the bacterial pathogens. The plasmid profiles of the various bacteria resolved by agarose gel electrophoresis showed species specific patterns. The differences among the speices were either in the number of plasmids carried by it and or the molecular weight of the plasmid DNAs. The cellular protein profiles as resolved by SDS-PAGE showed high degree of species specificity. Distinct differences in the number and molecular weight of the proteins were exhibited by the three species studied. High degree of homology in the protein profile was also observed between the strains within species. The specificity of the molecular characteristics make them a reliable tool for confirming the identity of the bacterial species.

6. SUMMARY

6. SUMMARY

- Molecular characterization of various species of bacterial pathogens, were carried out.
- The characterization was based on plasmid DNA, cellular protein profile and antibiotic sensitivity.
- The species studied were *A.salmonicida, A.hydrophila, V.cholera, V.parahaemolyticus, V.anguillarum, V.fischeri, Streptococcus, E.coli* and *S.typhi.*
- Both the species of *Aeromonas* tested viz. *A.salmonicida* and *A.hydrophila* were found to carry a common plasmid of 21Kb.
- *Aeromonas salmonicida* carried two small plasmids of 2.1Kb and 1.4Kb, in addition to the common plasmid of 21Kb.
- The reference strain and two of the field strains of *A.hydrophila* carried only single plasmid (21Kb), whereas the third field strain harboured three plasmids viz. 21Kb, 2Kb and 1.3Kb.
- The four different species of *Vibrio, V. cholera, V. parahaemolyticus, V. fischeri, V. anguillarum* shared a common plasmid of 21Kb, while *V. fischeri* and *V. cholera* in addition had 1.9kb and 2kb plasmid respectively. *V. anguillarum* had a 6kb plasmid.
- The five different isolates of *Streptococcus* though showed variation in number of plasmids,was found to harbour a common plasmid in the range 21-23Kb.
- *Salmonella typhi* was found to harbour one mega plasmid and a 21Kb plasmid.
- *Escherichia coli* had two plasmids viz. a 21Kb plasmid and a mega plasmid.

- Antibiogram of all the bacteria under the study viz. *Aeromonas* spp, *Streptococcus* spp, *Vibrio* spp, *E.coli* and *S. typhi* was prerared by checking their antibiotic sensitivity against 16 antibiotics .
- A.*salmonicida* and AH_4 strain of *A.hydrophila* which carried multiple plasmids were found to be resistant to more number of antibiotics when compared to other strains of *A.hydrophila* which had only a single plasmid.
- Resistance to antibiotics in four species of *Vibrio* was highly varying. *V. parahaemolyticus* and *V.cholera* were resistant to only two antibiotics each, *V. anguillarum* and *V. fischeri were resistant* to 6 antibiotics.
- While one of the five isolates of *Streptococci* which carried a single plasmid was resistant only to 3 of the antibiotic tested, the other strains with higher plasmid number and molecular weights were resistant against 8-9 antibiotics.
- *E. coli* and *S. typhi* showed resistance to 9 and 8 antibiotics respectively out of 16 tested.
- Profile of the bacterial cell proteins of *A. salmonicida, A. hydrophila and Streptococcus* was carried out by SDS-PAGE.
- The protein profiles resolved were found to be species specific. It was found that the protein profile could be used to differentiate the two species.
- The protein profile of all the strains of the species, *Aeromonas hydrophila* were also worked out to study the homology within the species and it revealed high degree of homology between the strains.

7. REFERENCES

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