DISCUSSION

Motile *Aeromonas* spp. are normal inhabitants of soil and freshwater (Urriza et al., 2000) and are commonly isolated both in surface and ground waters as well as in waste water (Legnani et al., 1998). In freshwaters that are highly polluted, they can be the predominant bacteria (Larsen and Willeberg, 1984). Although no evidence exists to support a role for direct water borne transmission of *Aeromonas* in gastrointestinal disorders, at the present time, such a possibility cannot be ignored (Kannan and Nair, 2000).

Water samples analysed in the present study were directly collected from the river. They therefore present an ideal model for the study of the natural distribution of the *Aeromonas* species. Burke et al. (1984) studied the seasonal distribution of *Aeromonas* species in unchlorinated domestic water supply, and concluded that the recovery of *Aeromonas* species was not dependent on environmental temperature and it showed peak during winter.

Due to the potential pathogenic nature of these mesophilic aeromonads, it is suggested to monitor the presence of *Aeromonas* in recreational waters. *Aeromonas* spp. in domestic water supplies may be an important source of non-gastrointestinal infection in-patients with immunological abnormalities (Trust and Chipman, 1979). Such infections have been reported that after exposure to water contaminated with *Aeromonas* spp. and patients with hepatobiliary disease are also susceptible to infection with *Aeromonas* spp. Schubert (1991), suggested that water destined for human consumption should be systematically examined for *Aeromonas* spp. and this should be adopted as an index of hygienic quality.
Higher level (4.08 and 3.90 log cfu mL\(^{-1}\) respectively) of distribution of *Aeromonas* spp. as well as *A.hydrophila* in water sample was encountered in site 3. It is important to note that the site 3 receives sewage and the recreational activity also found to be extensive and the people from near by villages drink the untreated water. The species of *Aeromonas* was heavily loaded when sewage and wastewater mix with river water. The concentration of *Aeromonas* spp. in the aquatic environment appears to be related to the presence of nutrients and organic substances (Vander Kooij and Hijnen, 1988).

Likewise Araujo *et al.* (1991) studied the distribution of *Aeromonas* spp. in various levels of pollution, and they recovered \(7.2 \times 10^7\) cfu mL\(^{-1}\) in Besas river and \(5.0 \times 10^3\) cfu mL\(^{-1}\) in Durero river which are highly polluted with urban and industrial wastewaters. The distribution of *Aeromonas* spp. in the two rivers indicated that there was a marked effect of pollution load on the distribution of *Aeromonas* species. They have also recorded \(5.8 \times 10^7\) cfu mL\(^{-1}\) of *Aeromonas* spp. from Barcelona sewer, which is located in a highly industrialized area.

Borell *et al.* (1998) reported that (64.4 %) of untreated drinking water samples were positive for *Aeromonas*. Treated drinking water was the only type of water that did not exceed 10 \% of positive samples. According to World Health Organization (1996) most drinking water treatment processes appear to be able to reduce the concentration of *Aeromonas* to below 1 cfu. 100mL\(^{-1}\), even though as a result of regrowth, treated water can contain large amounts (up to \(10^3\) cfu. 100mL\(^{-1}\)). On the other hand, Havelaar *et al.* (1992) found in samples from the Netherlands that *Aeromonas* strains isolated from human diarrheal stools were not very similar to strains isolated from drinking water. In spite of
these disparate data, *A. hydrophila* was recently placed on the U.S. Environmental Protection Agency (EPA) Contaminant Candidate List (Environmental Protection Agency, 1998).

Although this organism is recognized as the dominant freshwater fish pathogens, limited surveillance data suggest that aeromonad wound infections occur at a relatively low incidence, ie 0.7 per million population (Dufour, 1986). River Amaravathy is a perennial river and is extensively utilized by the people of near by villages for drinking purpose. Without knowing the health problems of using untreated water, people directly consume the river water. The reason for this is that there is no treatment plant installed in that area. Drinking water supplies have previously been shown to be a source of mesophilic *Aeromonas*, their presence attributes to ineffective disinfection at the treatment plant, post-treatment infiltration (Havelaar et al., 1990).

In station 1 which receives cleaner water from the reservoir, the occurrence of *Aeromonas* spp. as well as *A. hydrophila* was recorded. Because of the autochthonous nature, *Aeromonas* spp. is present in cleaner water also. It is possible that the low levels of pollution may be encouraging the growth of the autochthonous aeromonad population. (Vander Kooij and Hijnen, 1988). Hanninen and Siitonen (1995) reported that *Aeromonas* are able to grow even when concentrations of nutrients are low, such as in drinking water after treatment. Araujo et al. (1991) reported that *A. caviae* and *A. hydrophila* could grow in filtered wastewater. Such finding may indicate a possibly improved survival and / or multiplication of *A. hydrophila* in cleaner water. Numerical taxonomy study showed that in water samples *A. caviae* was found to be dominant in stations 3 and 4 and the least occurred species was *A. encheleia*.  

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During rainy season the sewage mixes with rain water and enters into the river. In other seasons also the inflow of the sewage into the river was noticed due to lack of sewage collection ponds around this river. Monfort and Baleux (1990) and Poffe and Op de Beeck (1991) studied the relationship between the Aeromonas densities and sewage spills and found elevated densities of aeromonads (ie \(10^5 - 8\) 100mL\(^{-1}\)) following sewage spills. Thus sewage inlet in station 3 may be the main source for the higher incidence of Aeromonas species when compared to other stations.

About 3.30 - 3.9 log cfu mL\(^{-1}\) of A. hydrophila in the water samples was recorded in River Amaravathy. In River Gomati, Pathak et al. (1988) reported lesser quantity (1.1 x \(10^3\) to 1.6 x \(10^4\) cfu. 100mL\(^{-1}\)) of A. hydrophila. They have also stated that the lowest isolation rates were found during the monsoon season and the monsoon rains also probably contributed to the decline in isolation rates of A. hydrophila.

Studies of fresh and estuarine waters in north - eastern United States have clearly indicated a seasonal cycle in the isolation rates of A. hydrophila, which have been attributed to temperature variation (Rippey and Cabelli, 1980; Kaper et al., 1981). Counts were highest in the summer months and lowest in the winter. In contrast, in the south - eastern Unites States, the highest densities of A. hydrophila were encountered in the spring, followed by a decline during the summer than resurgence in autumn (Hazen, 1979).

In our study, the water from station 4 is used for irrigation and the water from the irrigated land returns back to the river through seepage with suspended matters. Falcao et al. (1998) observed highest percentage of Aeromonas in stream water (50 %) followed by irrigation water (33.4 %) and reservoir water (16.6 %). The water used for irrigation may
be a source of infection for humans by using vegetables grown with contaminated waters. So particular attention should be given to the water used for irrigation also. Run-off from agricultural land has been implicated as a source of indicator bacteria in waters in rural areas (Tiedemann et al., 1988), but the importance of run-off as a source of Aeromonas is not known.

The present investigation did not show any significant correlation between temperature and the distribution of Aeromonas densities. A study conducted by Rhodes and Kator (1994) revealed that there was no significant correlation between common water quality parameters used to establish trophic status and aeromonads densities in lake. In contrast to the above report, temperature - dependent variations in the counts of A.hydrophila have been observed in water thermally polluted by a nuclear reactor and in water exhibiting thermal gradients resulting from natural geothermal effluents (Hazen and Fliermans, 1979). The highest densities of A.hydrophila in geothermal effluents were found between 23.3 and 21°C.

In the case of distribution system the Aeromonas count could be controlled by adapting water treatment technique and hence water treatment is must for all the municipal water distribution system. In this regard Fernandez et al. (2000) suggested that due to heat susceptibility of A.hydrophila, additional heat decontaminating methods for municipal supply water such as pasteurization might be performed.

The results of the present investigation revealed that A.hydrophila have their natural distribution related to the amount of pollution inlet. To our knowledge this is the first report of the seasonal distribution of A.hydrophila in river sediment. Even though the water and sediment samples were collected from polluted area, none of the parameters
except microbiological and dissolved oxygen showed any significant correlation. Cavello et al. (1999) conducted qualitative and quantitative analyses of the microbial flora of Mar Piccolo basin, which receives a considerable amount of sewage and industrial waste. The highest bacterial densities, in water and in sediment samples were found in summer and the lowest in autumn. The predominant genus was *Aeromonas*.

In India chlorination is the method, which is usually practised in drinking water supplies. Although chlorination is a proven bactericidal treatment for drinking water supplies, maintenance of chlorine within the distribution system network is insufficient on its own to control the levels of aeromonads (Gavriel et al., 1998). In the chlorinated water supply (low levels of residual chlorine) of Chennai City, India, *Aeromonas* was found in 37.9% of the samples (Alavandi et al., 1999). The Netherlands is the only European country to have established an indicative limit of 20 cfu.100 mL⁻¹ in the water production plant and 200 cfu. 100 mL⁻¹ during distribution (Legnani et al., 1998). Hence, suitable control measures should be made to control this opportunistic pathogen.

The fishing activity was found to be regular near station 2, and in station 1 and 3 the fishing was done rarely. In station 4 there is no fishing activity, since water in that area is fully utilized for irrigation. Even though chlorination was not effective in complete elimination of *Aeromonas* spp, it will be helpful in reducing the number of population. The people living in the villages near the river banks should be advised not to use the river water directly without prior treatment. The potential health hazard is therefore mainly related to the use of untreated or improperly disinfected water.
Sediment samples analysed in the present investigation harbour consistent number of Aeromonas spp. as well as *A. hydrophila*. *A. hydrophila* can multiply and survive for long periods of time in soil, maintaining their virulence properties. It may be possible for these microorganisms in the soil to be transmitted to susceptible inducible either indirectly (through contaminated hands, vegetables or drinking water) or directly (through hands). Thus it is suggested that the soil may represent as an important reservoir for *A. hydrophila* and, may represent a potential health hazard when these microorganisms are present (Brandi *et al.*, 1996). *A. sobria* was the dominant species next to *A. hydrophila* in sediment sample and the highest occurrence was recorded in station 3.

**PREVALENCE OF AEROMONAS HYDROPHILA IN FRESHWATER FISH**

*A. hydrophila* is especially important as a pathogen of fish, typically producing a fulminate septicemia (Dooley and Trust, 1988). Ugajin (1979) reported the incidence of *Acinetobacter* sp, *Aeromonas* sp, *Bacillus* sp, *Pseudomonas* sp, *Serratia* sp and *Staphylococcus* sp in the intestines of freshwater salmonid fish. According to their observation *Aeromonas* was the predominant species. The fish samples were collected in station 2, where the industries discharge their treated or untreated wastes into the river and extensive fishing is done in this area. A total of 262 fishes were analyzed, out of this 134 (51.1%) specimens were found to harbour *A. hydrophila*.

The incidence was observed throughout the study period and all the fish samples analysed during October 1998 and December 1999 showed positive to *A. hydrophila*. An experiment conducted by Boulanger *et al.* (1977) also revealed a higher percentage (62%) of incidence of *A. hydrophila* in fishes than the results recorded in the present
investigation. It is thought that these organisms invade only when the host resistance is lowered by environmental stress factors such as high organic load and over crowding (Sindermann, 1979).

The fish samples analysed during the monsoon season (June, July, August and September) of 1999 showed maximum incidence (65.7%), followed by postmonsoon (October, November, December and January) with 60.7% and 57.5% during monsoon of 1998. From the above observations it is clear that the fish samples collected during the monsoon season was found to harbour more number of *A. hydrophila*. George post (1987) reported that there is usually no seasonal occurrence in fish culture facilities located on a relatively constant temperature and water flow and this may also suits for natural running fresh water.

In River Gomati about 56.1 % and 57.1 % of fish investigated during the monsoon and winter seasons respectively showed incidence of *A. hydrophila* (Pathak *et al*., 1988). The maximum incidence was recorded in monsoon season and this may be due to the fact that the temperature enhances the bacterial growth as well as secretion of mucous, a lipoprotein, facilitating bacterial colonization on fish body (Ramteke, *et al*., 1992).

Among the five species analysed, about 58.3 % of *Sarotherodon mossambicus* was found to harbour *A. hydrophila*, followed by *Catla catla* (54.75 %). *S. mossambicus* is the dominant fish species in this river having good consumer demand and is available throughout the year.

Among the various body parts analysed, intestinal region showed higher (50.8 %) incidence of *A. hydrophila*. Among the species of *Aeromonas* other than *A. hydrophila*,
A. enchelieia was the dominant species and the maximum incidence was in the intestinal region. Sugita (1995) reported five different species of Aeromonas from the intestinal tracts of river fish, with a maximum incidence of A. veronii followed by A. caviae and A. hydrophila. The organisms are usually transmitted orally except in those instances when fish have skin or gill abrasions and the organism may enter through these routes. The relatively higher number of A. hydrophila was observed in the intestinal part and this may be due to its enteropathogenic nature. Apun et al. (1999) reported that the intestines of all the analysed fish species harboured the most number of different bacterial species than any other body parts and A. hydrophila was one of the dominant species in that region and they stated that mishandling of the fish could lead to the transmission of the pathogen to humans.

The organism multiplies in the intestine or at the site of invasion and is spread throughout the body by the blood stream. Pettibone et al. (1996) isolated 74 strains of Aeromonas from skin, intestine, kidney and liver of 16 brown bullhead (Ictalurus nebulosus). The presence of aeromonads in the internal organs of fishes to the total exclusion of other bacteria is a reflection of the virulence of these organisms compared to other aquatic microflora. Once the organism enters the blood stream, it could spread to various organs and regions in the body. It is possible that some of these bacteria may initiate necrosis and abscesses that may progress to ulceration due to their strong enzymatic activity (Karunasagar et al., 1995).

About 39.5 % and 35.8 % of the gill and body surfaces respectively were found to carry A. hydrophila. In contrast to our results, Pathak et al. (1993) reported that recovery
of *A. hydrophila* was found to be higher in water exposed organs viz gill, arborescent organ and skin than visceral organs and blood. They have also explained that the increased density of *A. hydrophila* on water exposed organs may be attributed to the availability of nutrients from mucous secreted on the surface of these organs and organic contents in water. Nayak *et al.* (1999) isolated *A. hydrophila* from diseased Indian major carps and the organs taken for their study are skin, kidney, liver, gill and spleen.

Factors that enhance the ability of these bacteria to survive in chemically polluted environments have public health significance and should be assessed. Control of motile aeromonad disease by immunization has not been successful; this is because of the large number of serotypes of *A. hydrophila* (Esteve, 1995). Based on the results, it is concluded that definite identification of strains at species level could help to expand our knowledge regarding the distribution and role in fish pathogenesis of these bacteria. If we know the incidence level of each species and their pathogenicity towards humans and aquatic animals, it is easy to fix treatment or control measures accordingly. *A. hydrophila* produces haemolysins, aerolysin, cytotoxin, and enterotoxin and such factors could be expected to play a role in the fish disease. Hence particular attention should be given to control *A. hydrophila* and its extracellular products in relation to fish diseases.

**NUMERICAL TAXONOMY OF AERO MONAS SPECIES**

With the increasing awareness of the potential pathogenicity of aeromonads, it is becoming more important to detect and identify these organisms in environmental, aquacultural, clinical and food samples. There have been many recent studies into the complex taxonomy and classification of the genus *Aeromonas* based on DNA
hybridization studies. These methods are however, expensive, need considerable expertise and specialized equipment and are not yet suitable for routine diagnostic laboratory analysis.

Phenox defined by numerical taxonomy are polythetic. No single character is indispensable or sufficient to group membership. Possible problems in numerical taxonomy can be overcome by the choice of suitable tests (Sneath and Sokal, 1973). Several of the tests included in the identification matrix have been found in earlier studies as useful for the differentiation of aeromonads. Miniaturized tests may be a further alternative, which is less time and material consuming (Kampfer and Altwegg, 1992).

By introducing several new phenotypic tests and reference strains for newly described genomic species it was possible to use numerical taxonomy for grouping and identifying Aeromonas strains at the genomic species level. The numerical analysis of Aeromonas strains of different sources was in most cases helpful in the identification to the genomic species level.

**Phenon 1**

These strains displayed most of the reactions reported for *A.hydrophila*, and the strains produced acid from salicin and it is utilized as their sole source of carbon. Esteve (1995) reported *A.hydrophila* strains of salicin negative for acid production and at the same time their strains used salicin as their sole carbon source. The percentage of positive responses in tests for the use of L-histidine as carbon source and for hydrolysis of elastin was lower. Austin *et al.* (1989) also reported lower percentage of L-histidine positive strains. Similar to our observation, Kaznowski (1997) also reported the negative response of strains towards growth at 40.5°C and utilization of lactose as sole carbon source.
Phenon 2

A. sobria strains with positive for indole, glucose, acid from sucrose and growth at 37°C; they do not use L-arabinose, L-arginine and L-histidine as source of carbon has been previously reported (Austin et al., 1989). Positive reactions in tests for production of acid from L-arabinose and hydrolysis of esculin was reported by Esteve (1995) and only 50 % of their strains hydrolysed esculin, but in our study all the A. sobria strains hydrolysed esculin. Although the acid production from L-arabinose is not included in the description of A. sobria by Popoff (1984), this character has been generally considered to be negative for the species (Holt et al., 1994). Some differences were noted in the frequency of phenotypic characters in comparison with the literature data and it could be due to different sources of isolates or different geographical areas. The use of molecular methods in addition to phenotypic characteristics for final identification of Aeromonas isolates with the genomic species seems necessary.

Phenon 3

Unidentified Aeromonas species in phenon 2 contained strains of sucrose negative and it fulfils all the basic characters of Aeromonas (growth at 0 % NaCl, absence of growth at 6 and 8 % NaCl and MacConkey agar, acid from D-mannitol, hydrolysis of gelatin, starch and tween 80). For further confirmation DNA - DNA hybridisation has to be carried out.

Phenon 4

This phenon (A. jandaei) was formed at 96 % S, whereas Esteve (1995) reported only 86.2 % S and it includes 2 subgroups. Our results were in good accord with
group 'a' and these groups were differentiated each other by the responses against elastin and growth at pH 4.5. The characters observed in this study were in general accord with the description of *A. jandaei* by Carnahan *et al.* (1991), although discrepancies included positive and variable response in tests for production of acid from sucrose and D-cellobiose. The identification of *Aeromonas* strains to the genomic species level is difficult because strains belonging to different hybridisation groups are biochemically very similar (Kaznowski, 1997).

**Phenon 5**

A negative reaction in lysine decarboxylases, Voges-proskauer and positive tests for L-arabinose, D-cellobiose and salicin fermentation and hydrolysis of esculin are good accord with the results of Kaznowski (1997). The same author reported 85 % S of *A. caviae* strains which was found to be lower than this present study (92 % S). In comparison with the strains examined by other authors (Austin *et al.*, 1989 and Kaznowski *et al.*, 1989), this phenon was negative for Simmon's citrate reaction. Here also we recorded Simmon's citrate negative strains in this phena and similarly, Esteve (1995) also reported the same type of reaction in the strains clustered in this phena.

**Phenon 6**

*A. encheleia* formed at 90.5 % S was reported by Esteve (1995) and this similarity level was lower than our report (96 % S). The characters that matched with the previous reports are production of indole and present arginine dihydrolase activity. The negative reactions in tests for growth at 42° C, Simmon's citrate and lysine and ornithine decarboxylases activities.
The distinguishable characters in each phena are, gas from glucose, was found to be negative in phena 4 and phenon 3 alone showed growth at pH 4.5. Sucrose negative was observed in strains belonging to phena 2 and all other strains were sucrose positive. The strains belonging to all the 4 phena showed rhamnose negative, but the strains clustered in phena 5 showed rhamnose positive. Likewise L-arabinose and lactose was used as a sole carbon source by the strains belonging to phena 4. In this phena except L-rhamnose and citrate all other sugars are used as a sole carbon source.

ANTIBIOTIC RESISTANCE OF *A. HYDROPHILA* ISOLATED FROM WATER, SEDIMENT AND FISH

The results of antibiotic resistance between *A. hydrophila* depicts that all the strains isolated from water, sediment and fish were resistant to bacitracin, methicillin and novobiocin. Wang and Silva (1999) reported that all the strains isolated from processed channel catfish were resistant towards bacitracin, but Shome and Shome (1999) observed only 45.5% of the *A. hydrophila* strains isolated from marine and freshwater fish showed resistance to bacitracin.

All the *A. hydrophila* strains recovered from water, sediment and fish showed resistance to methicillin and similar finding was observed by Motyl et al. (1985), who reported that all *A. hydrophila* strains of human origin were resistant to methicillin. In contrast, Pettibone et al. (1996) recorded only 54% of the strains isolated from brown bullhead were resistant to this antibiotic. However, Kampfer et al. (1999) reported that no significant differences could be observed between clinical and non-clinical *Aeromonas* genomic species. These reports revealed that geographical and socio-economical parameters could influence antibiotic resistance patterns within *A. hydrophila* population.
More than 95% of the strains isolated from the three sources were resistant to erythromycin and rifampicin. Chang and Bolton (1987) found that more percentage of Asian isolates of *A. hydrophila* was resistant to tetracycline and rifampicin than Australian isolates and they also explained that local selective pressures could influence the antibiotic resistance. The strains isolated from healthy and diseased fishes from Malaysia were found to be resistant to rifampicin (Ansary *et al.*, 1992).

All the strains isolated from water sample were found to be kanamycin resistant and in the case of sediment sample about 82.5% of the strains were resistant against kanamycin. In contrast, Ramteke *et al.* (1993) and Pettibone *et al.* (1996) have not noticed any kanamycin resistant strain, whereas the investigation of Ansary *et al.* (1992) supported the existence of kanamycin resistant strains, which were about 38.2%. We have observed that 74.6% of the isolates from fish showed resistance to kanamycin. Shome and Shome (1999) reported very low percentage of strains isolated from freshwater and marine diseased fishes extended resistance to kanamycin. Thus the increased occurrence of antibiotic resistant *A. hydrophila* strains in fish body as well as in water is of considerable significance in concern with risk to fish fauna and public health.

About 96.5% of the strains recovered from water sample showed resistance towards neomycin, whereas the isolates of the sediment and fish samples exhibited only 90% and 90.3% resistance respectively. The variation in the drug resistance may well be related to the source of the *A. hydrophila* isolates and the frequency and type of antimicrobial agents prescribed for treating *Aeromonas* infections in different geographical areas (Son *et al.*, 1997).
The least percentage of chloramphenicol resistant *A. hydrophila* strains was noticed in all the three types samples (8.6, 5.0 and 6.5 % respectively). Similar findings have been recorded from Malaysian and American fish isolates (Ansary *et al.*, 1992; Pettibone *et al.*, 1996). Resistance towards chloramphenicol, erythromycin, kanamycin, nalidixic acid, streptomycin, sulphamethoxazole-trimethoprim and tetracycline has been noticed among *A. hydrophila* strains isolated from *Tilapia mossambica* (Son *et al.*, 1997).

A detailed consideration of the common mechanisms of resistance and their frequency of occurrence among important pathogens may assist the design of new chemotherapeutic agents to which microorganisms could adopt only with much greater difficulty.

Chloramphenicol resistance is an extremely rare trait in *Aeromonas* spp. and low level of resistance to chloramphenicol in *Aeromonas* spp. has been shown to result from decreased permeability (Jones and Wilcox, 1995). In our study about 8.6 % of the isolates from water exhibited resistance to chloramphenicol, followed by strains from fish (6.5 %) and sediment (5.0 %). From the above results it could be inferred that the chloramphenicol resistant strains were more in water sample. Montoya *et al.* (1992) reported a single strain which was resistant to chloramphenicol; however, Chang and Bolton (1987) found 8 % of strains resistant to chloramphenicol. In the case of *A. hydrophila* strains isolated from two European rivers, only 5 % of the strains showed resistance against chloramphenicol (Urriza *et al.*, 2000).

Gentamycin resistant strains isolated from fish were 20.0 %. Ansary *et al.* (1992) reported that about 23.5 % of the *A. hydrophila* strains isolated from healthy and diseased fish expressed resistance to this antibiotic, which was slightly higher than the resistance
encountered in our findings. However, Ramteke et al. (1993) reported that none of the A. hydrophila strains from fish and environmental samples were resistant to gentamycin.

MAR A. hydrophila were reported from environmental sources as well as freshwater fish (Pathak et al., 1993; Pettibone et al., 1996). Totally 24 patterns of multiple antibiotic resistance was exhibited by A. hydrophila strains isolated from water. The most frequently observed pattern was (B E K M Ne No Pb R Te Tr V). About 29.3 % of the strains exhibited MAR index ranging from 0.51 to 0.60. The release of MAR organisms through faeces may ultimately pave way for the contamination of fish and shellfish in the aquatic environment (Grabow et al., 1973; 1976).

The isolates of sediment samples depicted 34 different antibiotic resistance patterns. The most frequently occurred pattern was B E K M Ne No Pb R V. Eighteen isolates (45 %) exhibited MAR index between 0.51 to 0.60. Detailed guidelines for antibiotic use in all major animal species have been developed and these promote the use of old narrow spectrum antimicrobial agents over new broad-spectrum compounds (Wegener and Moller, 2000).

About 82 different antibiotic resistance patterns were recorded among fish isolates. The most frequently observed pattern was B E K M Ne No Pb R Te Tr V, which behaved similar to strain taken from water. This type of resistance pattern was expressed by 7.3 % of strains. The MAR frequency between 0.51 - 0.60, was expressed by 32.6 % of the isolates. Ansary et al (1992) reported 6 different antibiogroups with the smallest antibiotic combination showing resistance to 3 antibiotics and the largest combination to eight antibiotics. MAR index value 0.2 or above is said to be originated from high risk
sources. In this study the strains isolated from all the three samples showed MAR index value above 0.2. This clearly indicated that, the existence of MAR indexing bacteria in water, sediment and fish would be a serious health hazard to the natural flora of the aquatic ecosystem as well as to the users.

The Gram negative bacteria can indeed transfer drug resistance not only to cells of the same species but also to bacteria of different species or even different genera (Franklin and Snow, 1975). These mechanisms ultimately support a pathogenic bacterium to survive at any environmental conditions as well as in the intestinal tract of animals and human. Survival of such MAR A. hydrophila in the food of animal origin may act as a potential source of resistant bacteria for humans. The full extends and significance of this phenomenon has yet to be defined.

Surveillance of resistance is essential for the development of a detailed understanding of the relationship between drug use and spread of resistance. Surveillance systems should operate in real time be comparable between different laboratories and countries and include samples from all relevant sources - including animals, food and human beings. Surveillance should be supported by research on the mechanism and the genetic background resistance (Wegener and Moller, 2000).

HAEMOLYTIC AND PROTEOLYTIC ACTIVITY OF A. HYDROPHILA STRAINS

About 62 % of A. hydrophila strains isolated from water samples showed haemolytic activity. Araujo et al. (1991) studied the haemolytic activity of A. hydrophila strains isolated from water samples. Their results revealed that about 83.4 % of the isolates had haemolytic activity. The extracellular preparation (ECP) from the high
virulence *A. hydrophila* isolates produced 100 % mortality in all fish within 18 h of injection, while the ECP (Extracellular Products) from the low virulence isolates produced 100 % mortalities after 96 h (Sirirat *et al*., 1999).

Krovacek *et al.* (1989) reported that all the *A. hydrophila* isolates recovered from well water were positive for haemolysin and protease production. By considering the emergence of enterotoxigenic *A. hydrophila*, they suggested that it would be advisable to include these organisms when monitoring the hygienic quality of drinking water. Handfield *et al.* (1996) stated that greater proportion of *A. hydrophila* (64 %) water isolates exhibited haemolysin production and it was not possible to confirm a seasonal difference in the rate of isolation of haemolysin producing strains.

Karunasagar *et al.* (1995) examined the haemolytic activity of *A. hydrophila* strains isolated from Epizootic Ulcerative Syndrome (EUS) affected and healthy fishes and about 80 % of the *A. hydrophila* strains produced haemolysin. They have also stated that the *Aeromonas* induce dermonecrosis and these are perhaps manifestations of proteolytic and haemolytic activities. It is possible that the primary causative agent, which is unknown at this time, may be lowering the defence system of the fishes making them susceptible to invasion by virulent strains of *Aeromonas* present in the environment.

About 94.2 % of haemolysin positive *A. hydrophila* strains isolated from processed channel catfish were reported by Wang and Silva (1999). Granum *et al.* (1998) reported haemolytic positive strains from tap water, raw fish and fermented fish and it is very interesting to note that all *A. hydrophila* strains were positive for haemolysin production.
Investigation regarding the haemolytic activity (78 % strains showed haemolysin positive) and production of protease by *A. hydrophila* strains isolated from fishes showed that the haemolytic activity appeared extracellularly during early stages of growth, reaching a peak just before an increase in proteolytic activity. When trout was injected intramuscularly with *A. hydrophila* cells known to have protease and haemolytic activity blanching occurred at the site of injection within 12 h followed by extensive oedema and pronounces internal muscle destructions (Handfield *et al.*, 1996).

In sediment sample about 40 *A. hydrophila* strains were tested for their haemolytic activity and 20 (50 %) of them were found to be positive for haemolytic activity. There was no report on the haemolysin positive *A. hydrophila* strains in sediment samples. Most of the studies are based on the haemolysin production of *A. hydrophila* strains from water, fish and other clinical sources. Sediment is one of the main component in water bodies like river, lake, pond etc. Presence of virulent *A. hydrophila* strains in sediment should not be ignored.

A total of 150 *A. hydrophila* strains from fish sample were studied for the haemolysin production and about 68 % of the stains showed haemolytic activity. The suspected enterotoxigenic strains of *A. hydrophila* were tested for both haemolysin and cytotoxin production and as a result all the strains were found to be positive (Burke *et al.* 1981) in Western Australia. Oliver *et al.* (1981) tested 40 *Aeromonas* strains isolated from fishes to haemolysin production and most of the tested strains were found to be positive.

Haemolysin production of *A. hydrophila* strains isolated from healthy and infected fishes in Malaysia has been reported by Yadav *et al.* (1992). The authors also found that
there was no association between high haemolysin producers and the source of the bacterium. In this regard, we have observed the haemolysin production of *A. hydrophila* strains isolated from water, sediment and fish and even though there was not much difference, the percentage of haemolysin producing strains differed from source to source. The maximum number of strains showing haemolytic activity was from fish sample that was followed by strains isolated from water (62%) and sediment (50%).

Cahill (1990) studied the haemolysin production in *Aeromonas* spp. isolated mostly from clinical, environmental and fish samples and out of 100 strains tested 78 were found to be positive for haemolysin production. He also emphasised that, a picture is emerging of the production of single toxins with multiple activities such as enterotoxicity, cytotoxicity and the ability to haemolyse erythrocytes. Different strains appear to produce their own variations of these toxins, which may account for reports of a number of separate toxins with distinct properties.

The *Aeromonas* strains isolated from healthy and moribund fishes was found to be virulent to fish, and the agglutination test was carried out against antisera (Mittal et al., 1980). There are several factors, which play a major role in the production of haemolysin and the knowledge about that factors gives precautionary measures regarding the haemolysin producing *A. hydrophila* strains in food.

Protease enzyme was produced by haemolysin producing *A. hydrophila* strains isolated from water, sediment and fish. Strains produced protease enzyme ranging from 131-152 μg mL⁻¹. The maximum (152 μg mL⁻¹) protease enzyme production was recorded in strain (Ah1) recovered from fish (intestinal region) sample. The water from
station 3 is used for drinking purpose without any treatment and existence of haemolysin positive strains in this area definitely will cause health problem to the people who are consuming the water. The haemolysin positive and protease enzyme producing \textit{A. hydrophila} strains isolated from fish sample should not be ignored, because for most of the people the fish is an important item in their daily food.

Shome \textit{et al.} (1999) studied both haemolysin and protease enzyme production of six \textit{A. hydrophila} strains isolated from diseased Indian major carps. The results clearly indicated that all the six strains produced both haemolysin and protease enzyme. A 52 KD protein has been reported to be a haemolysin gene product (Angka \textit{et al.}, 1995), which represents as S - layer surface protein and was considered to be one of the virulence factors (Tajima \textit{et al.}, 1992).

Immune response to infections due to the bacteria belonging to the genus \textit{Aeromonas} have been broadly investigated in fishes with the aim of finding a vaccine against a number of disease syndromes in which \textit{Aeromonas} involved. In contrast, few studies have been carried out on human beings suffering from \textit{Aeromonas} enteritis. In this area Crivelli \textit{et al.} (2001) isolated \textit{Aeromonas} strains from naturally acquired \textit{Aeromonas} diarrhoea and all the \textit{Aeromonas} strains produced strong haemolytic activity.

We have observed the differences in the production of protease enzyme among the \textit{A. hydrophila} strains isolated from water, sediment and fish. This difference may be partly due to the ecological source of the organism being used. The initial source of the strains was unknown (ie) the source of the wild type strains isolated from water may be from faeces / sewage. Such variation in ecological background is known to influence the growth and virulence characteristics of the species (Mateos \textit{et al.}, 1993).
Apparently, *Aeromonas* strains, which are able to infect the human gastrointestinal tract, are a selection from the great variety of environmental strains and various factors are required for virulence. Such strains are rare in drinking water but their occurrence cannot be completely excluded. It is advisable, therefore to control *Aeromonas* concentrations in drinking water as much as possible by limiting the concentration of nutrients, residence time and - when possible - temperature (Havelaar *et al.*, 1992).

**SUSCEPTIBILITY OF *A. HYDROPHILA* STRAINS TO CHLORINE**

At present the river water is directly supplied to the village (around fifty thousand people) without any treatment. If the treatment plant is installed in future, it should be implemented with prior knowledge about the effective concentration of disinfectant used in that plant to control pathogenic bacteria. So this study is focused on the effective concentration and exposure time of chlorine (commercial bleach) to control *A. hydrophila* in the water distribution system. Chlorination is the simplest, economical and regular practice in many parts of India.

Haemolytic and protease producing four strains (Ahwl, Ahw2, Ahw3 and Ahw4) isolated from water samples were used in this study. The Ahwl strain isolated from station 1 was completely eliminated at 0.3 ppm after 150 minutes and this strain was found to be more susceptible than other strains. Tsai *et al.* (1997) studied the susceptibility of the three strains to residual chlorine at 0.1 ppm and the strains isolated from oyster and waste water effluent were killed after 60 and 120 minutes respectively. Whereas the strain isolated from clinical specimen decreased by half log cycle after 120 minutes at 0.1 ppm. The cell number was significantly decreased when the concentration of chlorine was increased to 0.4 ppm.
Ahw2 (isolated from station 2) survived (0.48 log cfu mL$^{-1}$) at 0.4 ppm concentration for 120 minutes and after 150 minutes, the cells were completely eliminated. Slade et al. (1986) isolated *A. hydrophila* from 20 municipal domestic water supplies (Haqi and Halat Amar) and the waters that from Haqi were effectively treated such that no *A. hydrophila* or coliforms were recovered. Halat Amar water was chlorinated to eliminate coliforms, but some samples did contain *A. hydrophila*. Chlorination processes which is sufficient to destroy coliforms would appear not always to be so effective in eliminating *A. hydrophila*.

Burke et al. (1984) found that chlorination of metropolitan water supplies eliminated *E. coli* but not *Aeromonas* spp. The above report clearly draws an attention regarding the elimination of *A. hydrophila*. The concentration of disinfectant in many treatment plants is fixed mainly based on the tests against faecal coliforms and they failed to concentrate on other pathogenic organisms like *A. hydrophila*. A high degree of correlation was found between *A. hydrophila* and coliforms in water which is unchlorinated, suggesting that the presence of *A. hydrophila* in treated water may be an indicator of contamination by coliforms and *A. hydrophila* is a useful indicator of pollution and public health safety of freshwater.

The strain Ahw3 recovered from relatively polluted water (station 3) survived in 0.45 ppm even after 150 minutes, while all other strains were completely killed at this concentration. It is well known that the stability of chlorine compound is influenced by temperature. In this way Sisti et al. (1998) studied the effect of temperatures ($5^0$ C and $25^0$ C) on chlorination to control *A. hydrophila*. They come out with the result that at
lower temperature free chlorine was able to reduce the number of surviving bacteria by 1 or more log compared to the results obtained with the same dose at 20°C. The concentration at which the strains were exposed is 0.31mg mL\(^{-1}\). At this concentration, only 50 % of *A.hydrophila* cells were killed in 10 minutes.

When compared to the above said results, in this present study, 50 % of the *A.hydrophila* strains were killed in different concentration and different exposure time and it varied between strain to strain. In Ahw1 and Ahw2 50 % of the cells were killed at 0.35 ppm after exposing 90 minutes. In the case of Ahw3 it was in 0.35 ppm after 150 minutes and for Ahw4 the 50 % of the cells were eliminated at the same concentration but after exposing to 120 minutes.

The survival of *A.hydrophila* in chlorinated water is characterized by an initial rapid decline in viable cells followed by a second phase of slow inactivation. It would seen reasonable to suggest that this latter phase could depend on the presence of lower concentrations of active chlorine as a consequence of rapid consumption of free chlorine in the first minutes by both bacterial flora and to a lesser extend, organic matter. If mechanisms of resistance exist in *Aeromonas* spp. it is suggested that these are not transmittable but rather, are only transiently protective, which might be due to increased aggregation or clumping of cells or to microbial adhesion to surfaces or the ability to form capsules as in other microorganisms (Ridgway and Olson, 1982).

Under favourable conditions, deposition of bacterial cells into water distribution systems may result in proliferation and subsequent colonization of the surfaces. This could dramatically reduce the effectiveness of conventional methods of disinfection
such as chlorination to achieve the destruction of any attached cells on contaminated pipe surfaces, especially in the system where the residual disinfection level is not maintained (Assanta et al., 1998).

Gavriel et al. (1998) demonstrated that the maintenance of chlorine within the distribution network is insufficient on its own to control the levels of aeromonads. In their study, of the four reservoirs, which were positive for Aeromonas in more than 10% of samples, only one reservoir had a total chlorine concentration below 0.2 mg l$^{-1}$. Of the 17 reservoirs that maintained residual chlorine in all samples tested, 10 were found to be positive for Aeromonas on at least one occasion. These results suggest that continual presence of chlorine residual is not sufficient to ensure prevention of Aeromonas recovery.

Fernandez et al. (2000) recovered A. hydrophila from chlorinated tap water and explained that A. hydrophila would be different from that of indicator organisms used to evaluate water microbiological quality in Argentina. They suggested that the stability of available free chlorine in a solution depend on (i) chlorine concentration (ii) presence and concentration of catalysts, (iii) pH of the solution, (iv) temperature of the solution and (v) presence of organic material.

In the present study, the effective concentration to kill all the inoculated cells and the exposure time was determined. At 0.5 ppm all the A. hydrophila strains inoculated were found to be eliminated after exposing to 150 minutes. The strains included in this study were recovered from water samples with different characteristics and behavior of each strain was differed in each concentration.