Discussion
DISCUSSION

Infections account for most illness, with pathogens employing ingenious mechanisms to establish disease. Diarrhoeal diseases in certain individuals have been associated with excretion of organisms as pure or predominant cultures, serological responses to the organisms and resolutions of symptoms and pathogenicity with the appearance of the organisms from the stool. WHO predicts that there will be about five million deaths in children younger than five years by 2025, 97% of these will be in the developing countries and mostly caused by infectious diseases and diarrhoea will continue to play a prominent role (WHO, 1998). One of the major challenges in the gastrointestinal diseases is the recent increase in the number of probable aetiological agents.

Isolation of diarrhoeagenic bacteria from different sampling areas

In developing countries, pathogens have been identified in 65% of stool samples from children with acute diarrhoea. Many viruses and bacterial pathogens in the intestine have been identified, of which rotavirus and enteropathogenic *E. coli* (EPEC) are the most common and the dominant normal flora in the intestinal tract of human and animals. Also the following group of bacteria viz., *Salmonella, Shigella, Aeromonas, Vibrio, Proteus, Enterobacter, Klebsiella, Citrobacter, Morganella, Serratia, Yersinia, Pseudomonas, Edwardsiella, Plesiomonas* and *Providencia* have been reported to cause diarrhoea (Bravo et al, 2003). In the present study, the percentage of diarrhoeagenic organisms isolated from the stool samples are *E. coli*, species of *Aeromonas, Proteus, Pseudomonas, Salmonella, Shigella, Staphylococcus, Streptococcus* and *Vibrio* The occurrence of these aetiological agents remains a serious public health concern. Among the bacterial genera isolated, *E. coli* was recorded as the prominent, followed by the species of *Vibrio, Salmonella, Aeromonas, Shigella, Pseudomonas, Streptococcus, Proteus* and *Staphylococcus*. Of these diversified bacterial pathogens, *E. coli* was found one of the commonest causative agents of diarrhoea. The maximum percentage of *E. coli* was recoded as 58.57% (p<0.05),
while the minimum incidence of *Staphylococcus* spp. with 5.43% was recorded. The higher percentage of incidence of *E. coli* along with the diarrhoeagenic organisms showed the significant health threat to the children in the study area. In many instances the isolation of *E. coli* from stool samples of asymptomatic individuals was similar to that of the diarrhoeal cases (Cravioto *et al.*, 1991). However, its association with several outbreaks and in volunteer studies has unquestionably confirmed the role of *E. coli* as an important aetiologic agent leading to diarrhoea (Okeke *et al.*, 2003).

Another predominant bacterial genera was *Vibrio*, which has been shown as a major cause of epidemic diarrhoea (Fasano, 2000). A significant level of incidence of *Vibrio* spp. was recorded in all the sampling areas (p<0.05). This may be due to the tendency of this organism to cause severe diarrhoea, thus making infected individuals more likely to seek medicinal attention (Oyofo *et al.*, 2002). In a recent outbreak of cholera in Chandigarh, India, it was recorded that 58.5% of children have been affected with acute diarrhoea (Taneja *et al.*, 2003). *Aeromonas* spp. can now be considered a relatively common enteropathogen (Bottarelli and Ossiprandi, 1999). In the present study, a significant proportion of incidence (30.54%) (p<0.05) of *Aeromonas* spp. was observed and hence, it may be considered as one of the causative agents of diarrhoea in children. Maltezou *et al.* (2001) revealed that *Aeromonas* was the third frequently isolated bacterial agent from children with acute diarrhoea in the area of Athens, in a proportion comparable to that of *Salmonella* and other agents. In our study, *Aeromonas* stood in the fourth position among the diarrhoeagenic causative agents encountered in children diarrhoea.

Incidence of *Salmonella* and *Shigella* species were recorded as 33.47% (p<0.05) and 10.46% (p<0.05) respectively. WHO (1995) reported that 15% of the deaths due to diarrhoea in children younger than 5 years was due to *Salmonella*. Maltezou *et al.* (2001) reported that 47% of the children diarrhoeal episodes caused by *Salmonella* and *Aeromonas* species (33%) accounted for all the episodes in Athens. Nzeako and Okafor
(2002) reported that 11% and 1.8% of *Salmonella* and *Shigella* species respectively were recorded from children infected with diarrhoea in Nigeria. In the present investigation also a significant percentage of *Salmonella* spp. was observed in the four sampling areas. It may persist as a major public health threat in all the conditions. In this study, we have also recorded the incidence of these organisms in a minor elevation in all the study areas. No significant range of these pathogens have been observed. However, no incidence of these organisms have been noticed in Kovilpalayam.

*Staphylococcus* species is a bacterium whose etiological role in diarrhoea has not been well established (Urbino et al., 2003). In the present study also the incidence of *Staphylococcus* spp. was recorded as considerably lower with 5.43%.

*Proteus* species are most common flora of human intestinal tract, along with *E. coli*, *Klebsiella* and etc. *Proteus* has been isolated from multiple environmental habitats, including long-term care facilities and hospitals. Similarly *Pseudomonas* spp., also one of the diarrhoeagenic bacterium, the incidence of this genus was considerably lower. In this study, there was no significant level of *Proteus* and *Pseudomonas* spp. observed in all the four sampling areas.

The proportions of incidence of most of the bacterial genera were predominantly recorded in the months of March to October. Also the prevalence of the diarrhoeagenic bacterial species were observed in significant level in all the four sampling areas. Understanding the cause of less severe forms of diarrhea, within a specific geographic area may enable primary care paediatricians to make the appropriate treatment decision which allow a better distribution of monetary resources including the application of preventive measures. This study provides information about the occurrence and prevalence of bacteria associated with diarrhoeal episodes and is significant incidence was recorded among children living in urban and rural area of Tamil Nadu, South India.
Incidence of *A. hydrophila*

The gravity of the present investigation focused on the incidence of *A. hydrophila* in diarrhoeal samples of children, collected from different sampling areas. It must be stressed that several pathogenic aspects of aeromonads are still waiting to be clarified. Among them, the most important one is the role of *Aeromonas* spp. as causative agent of enteric diseases in human (Abeyta *et al.*, 1986; Janda and Abbott, 1998). This hypothesis is supported by some case-control studies, in which a mechanism of enteropathogenicity mediated by an enterotoxin, has been demonstrated and the relationship between diarrhoea in human and the presence of the bacterium has been established. Moreover, the relatively high frequency of intestinal healthy *Aeromonas* carriers could also be considered as indirect evidence for the absence of virulence. In the present investigation, the association of *A. hydrophila* as one of the significant genera in children diarrhoeal samples was well established. It was recorded that 16.7 % of the samples have been found positive for *A. hydrophila*. A significant percentage of incidences of *A. hydrophila* was observed in all the four sampling areas. This clearly supports the wide spread distribution of this enteropathogen through out the sampling areas. In a study conducted in Thailand, Pitarangsi *et al.* (1982) observed higher rate of incidence of *A. hydrophila* in patients with diarrhoea. Initially *A. hydrophila* has been considered as a primary pathogen (Ljungh *et al.*, 1977; Dubey *et al.*, 1981; George *et al.*, 1985; Deodhar *et al.*, 1991), in contrary, Qadri *et al.* (1991), Palumbo *et al.* (1992), Collee *et al.* (1997) and Chang *et al.* (1997) reported that *A. hydrophila* is an opportunistic as well as secondary pathogen. The prevalence of different strains of *A. hydrophila* is likely to vary with geographical locations as *A. hydrophila* and *A. veronii* biovar *sobria* are the dominant species in Australia and Thailand (Altwegg and Geiss, 1998).

*Aeromonas* associated diarrhoeal infection has been reported from all over the world, but the incidence is relatively low in developed countries as compared to that in developing countries (Altwegg and Geiss, 1989; Sharma *et al.*, 2005). The prevalence of
different strains of *A. hydrophila* is likely to vary with geographical locations. *A. hydrophila* and *A. veronii* biovar *sobria* are the dominant species in Australia and Thailand (Altwegg and Geiss, 1998). World wide study on the incidence of *A. hydrophila* also provide the widespread sharing of this organisms. Kuijper *et al.* (1987), Khardori and Fainstein (1988), Koe *et al.* (1991), Teka *et al.* (1999), Nzeako and Okafor (2002), Urbino *et al.* (2003) and Aslani and Hamzeh (2004) reported 3.7 %, 10 %, 5.2 %, 5.5 %, 13 %, 2 % and 56 % of incidence of *A. hydrophila* respectively from Netherland, Australia, Malaysia, Bangladesh, Nigeria, Columbia and Cuba.

In India, *Aeromonas* associated diarrhoea has been reported from Mumbai (Deodhar *et al.*, 1991), Kolkata (Chatterjee and Neogy, 1972), Goa (Verenkar *et al.*, 1995), Vellore (Bhat *et al.*, 1974), Pondicherry (Sujatha and Rao, 1993), Chennai (Alavandi *et al.*, 1998; Komathi *et al.*, 1998; Alavandi and Anandhan, 2003) and Coimbatore (Subashkumar *et al.*, 2006a). Sinha *et al.* (2004) reported 17.7 % of incidence of *A. hydrophila* during the year 2000 and increased incidence was observed during 2001, with 28.1 %. This might be because of the poor hygienic as well as sanitation practices, which may lead the increased distribution of this organism among various locations.

During the summer, the percentage incidence of *A. hydrophila* was found maximum among all the four sampling areas. Moyer (1987), Janda *et al.* (1994), Kannan *et al.* (2001) and Seetha *et al.* (2004) also reported the increased incidence of this organism during summer. These reports clearly indicate that the prevalence of this organism is higher during summer months. In contrast, Teka *et al.* (1999) investigated the seasonal incidence of this organism among children affected with diarrhoea and they observed that the incidence of this organism has been recorded during September and October as well. But, none of the area have been recorded as positive for the incidence of this organism during September and October. Based on the
several researcher’s reports, it is concluded that summer is the favourable season for the increased incidence of this organism.

Based on our findings, we also came to a conclusion that this organism is widely distributed in the study areas and this is one among the diarrhoeagenic bacteria, followed by *E. coli*, *Vibrio* and *Salmonella*. The percentage of incidence of this organism in all the four study areas also envisaged about the poor hygienic conditions, one of the factors which may lead to diarrhoea among children.

**Antibiotic resistance of *A. hydrophila***

*Aeromonas* spp. has been reported to be intrinsically resistant to ampicillin (Joseph *et al.*, 1979; Aziz *et al.*, 1986; Sinha *et al.*, 2004). Chaudhary *et al.* (1996) reported a growing incidence of multidrug resistant *A. hydrophila* isolated from clinical and environmental sources worldwide. The variation in the drug resistance may be related to the source of *A. hydrophila* and the frequency of antibiotics prescribed for treating *Aeromonas* infections in different geographical area (Radu *et al.*, 1997) and increasing the drug resistance among the strains. Generally, it was observed that the majority of the strains exhibited a multidrug resistance. This increased drug resistance presents a significant threat to management of *Aeromonas* mediated diarrhoea (Cahill, 1990). Based on the reports it was suggested that β-lactam agents should be avoided in the treatment of *Aeromonas* infections, the similar statement was declared by Goni-Uriza *et al.* (2000) and Vila *et al.* (2003).

Many classes of antimicrobial agents, such as chloramphenicol, tetracycline and trimethoprim – sulfamethozazole (Motyl *et al.*, 1985; Koheler and Ashdown, 1993) aminoglycosides, cephalosporins and imipenem (Clark, 1992) have been reported to be active against aeromonads. Until now, there was no well designed, prospective and randomized antimicrobial agent against various infections (Ko *et al.*, 2003). However, the list of rationale antimicrobial agents for *Aeromonas* infections is shorten as our
knowledge of the mechanisms of β-lactam resistance is increasing, due to the production of multiple inducible, chromosomally encoded β-lactamases (Jones and Wilcox, 1995; Walsh et al., 1995; Hayes et al., 1996; Bravo et al., 2003). We have observed that most of the isolates showed resistance towards β-lactam antibiotics, such as penicillins and cephalosporins. Similar findings have been reported by Bizani and Brandelli (2001) and Bravo et al. (2003) for A. hydrophila isolated from diarrhoeal sources.

Ko et al. (1996), Nwosu and Ladapo (1999) and Joseph and Camahan (2000) reported that the second and third generation of cephalosporins were most active against A. hydrophila. In contrast, we have observed the cephalosporins like cefadroxil, cefazoline, cefotaxime, cephaloridine, ceftazidime, cefoperazone and ceftizoxime were not effective against 80 to 90.2 % of A. hydrophila strains.

It was observed that all the strains of A. hydrophila were resistant to methicillin, which was similar to the findings of Motyl et al. (1985), who reported all A. hydrophila strains of human origin were resistant to methicillin. In contrast, Pettibone et al. (1996) observed only 54 % of the strains were resistant to methicillin. However, Kampfer et al. (1999) reported both the clinical and non-clinical Aeromonas isolates did not exhibit significant resistance, although the clinical isolates showed a few more positive results with respect to antibiotic resistance.

Most of the strains of A. hydrophila showed variable resistance to non β-lactam antibiotics (Bizani and Brandelli, 2001), and susceptible to aminoglycosides (Jones and Wilcox, 1995). In the present investigation, nettilin was observed as susceptible to all the A. hydrophila. It was also observed that 40 % of A. hydrophila were resistant to amikacin, which is similar to the findings of Rashad and Kareem (1995) and Thangavel (2004). About 15 % of the strains exhibited resistance to gentamicin, which was very closer to the findings of Thangavel (2004) and Subashkumar et al. (2006a). Vasiakar et al. (2002) and Thayumanavan et al. (2003) also observed 33 % and 3.6 % of the
water and fish isolates respectively resistant to these antibiotics. In contrast, Ramteke et al. (1993) and Taher et al. (2000) reported that none of the strains isolated from fish and environmental samples were resistant to gentamicin. It clearly indicates that the increasing level of gentamicin resistant *A. hydrophila* was increasing in our study area.

It was observed that 45 % of *A. hydrophila* were resistant to nalidixic acid. But Goni-Uriza et al. (2000) and Vasaikar et al. (2002) reported 72 % and 33 % of nalidixic acid resistant respectively. In contrary, Rashad and Kareem (1995) did not find nalidixic acid resistant strains. We have also observed higher frequency of neomycin resistant strains with 70 %. The findings of Radu et al. (1998), Sinha et al. (2004) and Thangavel (2004) clearly support our results. In the present study, it was observed that 57.14 % of strains were resistant to neomycin, while Rahim et al. (1984) reported that all the strains were susceptible to neomycin.

Ko et al. (1996) found 25 % of *A. hydrophila* strains were resistant to tobramycin, which was lower than our results (35 %). Newer antibiotics like lincomycin and clarithromycin were not effective against *A. hydrophila*. It was observed that 32.5 % of the strains have resistant to co-trimoxazole. This is also contrast to the findings of Goni-Uriza et al. (2000), who reported 14 % and 67 % of the strains resistant to this antibiotic. In the present study, 40 and 67.5 % of diarrhoeal strains were susceptible to tetracycline and co-trimaxazole respectively, which is further potential drug of choice for the treatment of *Aeromonas* infection (Ko et al., 2003).

Several studies have indicated the excellent *in vitro* activity of quinolones against *Aeromonas* species (Burgos et al., 1990; Koehler and Ashdown, 1993). The present study also demonstrated that quinolones such as ciprofloxacin (65 %), sparofloxacin (35 %) and norfloxacin (5 %) confirms the different range of resistance among the diarrhoeagenic *A. hydrophila*. Many of the investigators have reported that the quinolones are widely used for the treatment of *Aeromonas* infection (Liao and Cappell, 1989; Gonzalez-Barca
et al., 1997; Grobusch et al., 2001; Orlando et al., 2001). Ko et al. (2003) have suggested the therapeutic potential of ciprofloxacin monotherapy for *Aeromonas* infection. In the present investigation, the prevalence of an increasing potential of ciprofloxacin resistant strains were recorded. It was observed that all the strains were resistant to bacitracin, which supports the findings of Ansary et al. (1992) and Radu et al. (1997). About 93% of the isolates were susceptible to polymyxin-B and the findings of Ramteke et al. (1993), Ko et al. (1996), Thayumanavan et al. (2003), Hatha et al. (2005) and Subashkumar et al. (2006a) were strongly supported our findings.

Since chloramphenicol is considered as the wide spectrum and most commonly used antibiotics, it was observed that 12.5% of the strains exhibited resistance to chloramphenicol. Only very few reports are available in this context. Chang and Bolton (1987), Jones and Wilcox (1995), Goni-Uriza et al. (2000), Vasaikar et al. (2002), Vivekanandhan et al. (2002) and Vila et al. (2003) found chloramphenicol resistant strains of *A. hydrophila* from clinical and environmental sources. Whereas Thayumanavan et al. (2003) did not observe any strains with such type of resistance.

This investigation mainly revealed that the amikacin, gentamycin, nettilin, tobramycin, co-trimoxazole, chloramphenicol, imipenem and polymyxin-B are the effective drugs against *A. hydrophila*. Current trends of using more number of antibiotics against these organisms pave the way for development of antibiotic resistant strains. The emergence of resistance among aeromonads will be increased by the frequent usage of antibiotics in hospital settings (Chaudhary et al., 1996). Local selective pressure also influences the antibiotic resistance level. Increased level of resistance among clinical isolates and water isolates of *A. hydrophila* to commonly used antibacterial agents has been observed (Ko and Chung, 1995), when these antibiotic resistant strains from the hospital environments escape into the community and may cause serious health hazards when enters suitable host system.
**MAR index of A. hydrophila**

Multiple antibiotic resistant (MAR) bacterial pathogens are the serious problem nowadays faced by the clinicians. When the antibiotic resistant bacteria enters into the environment, and hybridize with normal bacterial strains, will results in the transfer of resistant (R) plasmids, and this will be a serious problem in chemotherapy. The Gram-negative bacteria can indeed transfer drug resistance not only to the cells of the same species, but also to bacteria of different species or to even different genera (Franklin and Snow, 1975). The potential importance of bacteria acting as a reservoir of plasmid coding for antibiotic resistance has been reported by Linton et al. (1981). Development of resistance against novel, chemically synthesized agents not resembling natural metabolites or growth factors is more difficult to explain although the high risk rate of cell division in most microbial populations obviously facilitates ‘high speed’ evolution. The indiscriminatory use of antibiotics in chemotherapy is one of the ways for the arising of MAR bacterial pathogens.

Bacteria resistant to β-lactam and non β-lactam antibiotics, may occur by selective pressure or because of antibiotic abuse by humans or over use in animals (White et al., 2000). In the present study, it was observed that all the strains isolated from children diarrhoeal samples showed multiple antibiotic resistances. A MAR index value of 0.2 or above is said to be originated from high-risk source of contamination (Krumperman, 1985). In the present study, all strains showed a MAR index value more than 0.2, which implies that the organism might have been originated from high risk-sources like sewage, animal husbandry waste, biomedical waste, faecal contaminated drinking water. Survival of such MAR A. hydrophila strains in the food of animal origin may become contaminated with bacteria of intestinal origin and consequently animals may act as a potential source of resistant bacteria for humans. Multiple antibiotic resistance of A. hydrophila has been reported by several researchers throughout the world (De Vicente et al., 1990; Davies, 1992; Qureshi and Qureshi, 1992; Vivekanandhan et al., 2002; Radu
et al., 2003; Thayumanavan et al., 2003; Hatha et al., 2005; Lakshmanaperumalsamy et al., 2005; Subashkumar et al., 2006a). The release of such type of organisms through faeces may ultimately pave the way for the contamination of aquatic environments (Thangavel, 2004). In addition, antibiogram is considered as one of the useful techniques in the characterization of pathogens (Obi et al., 2004).

The exchange of multidrug resistant genes through horizontal transfer (HGT) within bacterial genera is frequently occurred in natural environments. Moreover, the presence / persistence of antibiotic residue in environment might be the chance for the multidrug resistance among pathogens and pose a serious problem. Hence, elimination of these organisms could be added to the gravity of the problem. To solve the problems, the indiscriminate use of antibiotics should be controlled.

**Haemolysin production of *A. hydrophila***

*Aeromonas* is known to produce a variety of virulence factors (Holmberg and Farmer, 1984; Sha et al., 2002). Among them, haemolysin is the important one, also considered as the primary toxin, produced by most of the pathogenic strains of *A. hydrophila* (Janda, 1991; Howard et al., 1996). Attention has been given on haemolytic *A. hydrophila* strains because the production of haemolytic toxins has been regarded as the indication of pathogenic potential, though non-haemolytic aeromonads also being implicated as human pathogens (Namdari and Battone, 1990). Aerolysin (Howard et al., 1987) is the best-studied haemolysins, but *Aeromonas* strains can produce more than one haemolytic toxin with higher virulence properties (Chopra et al., 1991; Wong et al., 1998). Wong et al. (1998) demonstrated the presence of two unrelated haemolytic genes in one strain of *A. hydrophila*, both involved in virulence, but not the only gene responsible for pathogenesis.

Allen and Stevenson (1981) reported that hemolytic activity appeared extracellularly during the early stages of growth, reaching a peak just before an increase
in the proteolytic activity. A study conducted by Burke et al. (1981) in Western Australia, it was found that almost all the enterotoxigenic strains of *A. hydrophila* produced haemolysin. Occurrence of haemolysin production of *A. hydrophila* reported worldwide by several authors (Callister and Agger, 1987; Chopra et al., 1991; Krovacek et al., 1994; Pin et al., 1995; Gosling, 1996; Wong et al., 1996; Wong et al., 1998; Chopra and Houston, 1999; Santos et al., 1999; Castro-Escarpulli et al., 2003; Lakshmanaperumalsamy et al., 2005)

In this study, it was observed that 97.5% of the isolates have been found positive for haemolysin production. The isolates varied in their ability to lyse red blood cells of human origin. In a study conducted by Abdullah et al. (2003), it was seen that all the *A. hydrophila* strains isolated from children diarrhoeal samples produced β-haemolysins, while, Aslani and Hamzeh (2004) reported 82% of the strains isolated from children diarrhoeal samples producing β-haemolysin. In a study, Wong et al. (1996) reported that all *A. hydrophila* isolates with haemolysin-positive genotype were virulent in the suckling mouse model assay. They also observed that after 24 h at 37 °C, the production of haemolysin was found high, whereas Wretlind et al. (1973) and Riddle et al. (1981) observed the haemolytic activity during the exponential growth phase, reaching a maximum before maximal growth, and then falling on prolonged incubation.

Burke et al. (1986) also reported that 78% of the *A. hydrophila* strains were positive for haemolysin and they also found an overall correlation between the production of haemolysins, cytotoxins and enterotoxins and they concluded that haemolysins, cytotoxins and enterotoxins were not identical. In contrast to this, Ljungh et al. (1981) considered that haemolysins and enterotoxins were distinct, and interpreted the minimal effect of β-haemolysin in the rabbit ileal loop (RIL) test as negative. In a recent report, 95% of the *Aeromonas* isolates from children diarrhoea were capable of exhibiting haemolysin activity, but varying in their ability to lyse the RBCs of human origin (Subashkumar et al., 2006a).
Based on the available reports and the present investigation, the diarrhoeal isolates of *A. hydrophila* with higher degree of haemolysin production would be one among the several virulence factors that determining pathogenicity.

**Protease activity of *A. hydrophila***

Proteases are thought to contribute to virulence of aeromoands for fish and other hosts (Gonzalez-Serrano *et al.*, 2002; Savithamani, 2002), however, their contribution to human pathogenicity still needs to be determined. Gonzalez-Serrano *et al.* (2002) studied that *A. hydrophila* and *A. veronii* biovar *sobria* isolates produced variable amounts of proteases at 28, 37 and 4 °C. Considerable differences between the number, types and quantities of protease produced by *Aeromonas* have been reported and attributed to own strain variation, origin, incubation temperature or culture media (Cahill, 1990; Mateos *et al.*, 1993; Santos *et al.*, 1996). Few reports have described the kinetics of haemolysis and protease production by *A. hydrophila* grown in culture media (Wretlind *et al.*, 1973; Riddle *et al.*, 1981). In the present investigation, all the strains of *A. hydrophila* have produced protease enzyme after 24 h, but the amount of production varied depending on different strains, which is in agreement with the findings of Abdullah *et al.* (2003). This might be because of the species diversity among the *A. hydrophila* strains isolated from various area, and it was further supported by the findings of Wakabayashi *et al.* (1981), Nieto and Ellis (1991) and McMahon (2000). Among two different broths used for the production of protease, Esteve and Birbeck (2004) observed that Tryptic Soy Yeast Extract (TSYE) broth supported the enhanced production than the BHIB broth. Vivas *et al.* (2004) suggested that nutrient rich culture media helped to produce protease in higher level by the *A. hydrophila* strains.

Howard and Buckly (1985) reported that both the proteolytic and haemolytic activity are interrelated, while Gonzalez-Serrano *et al.* (2002) observed that there is no relation between these two activities. But in our study, we have observed that a
non-haemolytic strain (Ah-D29) has produced considerable amount of protease (105.78 µg mL\(^{-1}\)). This clearly revealed that both the proteolytic and haemolytic activities are interrelated. This also clearly indicated that there are two separate genes which are responsible for the production of haemolysin as well as protease enzyme. In the present investigation, it was also observed that the non-haemolytic strains of *A. hydrophila* have also produced protease enzyme, whereas all the haemolytic isolates were rich in production of protease enzyme. This obviously indicates that both the haemolysin and protease enzyme production are inter-related and independent.

Proteolytic activity was found in the culture supernatant but not in the cells and the increase in activity did not directly parallel cell growth (O’Rielly and Day, 1983; Perez *et al*., 2002). Protease was mainly released during stationary phase (Esteve and Birkbeck, 2004). In the present investigation, the culture supernatant (crude enzyme) digested the protein substrate at 30 °C by 30 min. Castro – Escarpulli *et al.* (2003) reported that 61 % of the *Aeromonas* isolates produced protease and strongly suggested the involvement of protease in pathogenicity. However, it has not been thoroughly investigated (O’Rielly and Day, 1983). The protease cleaves the prohaemolysin (activated aerolysin-protoxin) resulting in the removal of a 42 amino acid peptides, which converts the prohaemolysin into haemolysin (Nomura *et al*., 1999). The haemolysin and protease production were found more frequently in the clinical samples, which may be important in colonization through the disruption of the intestinal barrier (Sechi *et al*., 2002) and further it promotes their pathogenicity.

**DNase activity of *A. hydrophila***

DNase is one of the putative virulence factors, which plays a vital role in causing disease in human by inducing gastroenteritis (Wong *et al*., 1997; Soler *et al*., 2002; Castro-Escarpulli *et al*., 2003). In the present study, 80 % of the *A. hydrophila* isolates produced DNase enzyme. All the clinical and environmental strains of *A. hydrophila*
found to produce DNase enzyme (Sharma et al., 2005). Wong et al. (1997) reported that all the strains were positive for DNase activity on DNase agar medium. Weckman and Catlin (1957) also reported that *A. hydrophila* strains could produce the DNase enzyme even in BHIB medium. Castro-Escarpulli et al. (2003) reported that the gene which is responsible for the production of DNase enzyme is conserved in most of the clinical as well as environmental strains of *A. hydrophila*. Wong et al. (1997) also reported that the non polar insertional / inactivation of *flaA* gene had no effect on the activity of DNase enzyme.

**Slime activity of *A. hydrophila***

Slime is the another type of virulence factor, which is a viscous glycoconjugate material, produced by most of the Gram negative bacteria. It is also helpful in the formation of biofilm. The slime is highly significant to the pathogenesis; it appears to inhibit the neutrophil, chemotaxis, phagocytosis and antimicrobial drugs.

In the present investigation, it was recorded that 71.5 % of the strains were positive for slime production. In a study, Sechi et al. (2002) observed 50 % and 35.3 % of clinical and environmental strains respectively producing slime. In conclusion, strains having the capacity to produce slime should be given much more attention in connection with the pathogenesis. *A. hydrophila* can easily colonize and produce the antigenic slime production in human.

**Serum resistance of *A. hydrophila***

Several genospecies of *A. hydrophila* produce enterotoxin and haemolysin, they also show resistance to normal human serum (Janda et al., 1984; Rolston, 1988; Singh and Sanyal, 1993). Serum resistance is an important virulence factor for clinical (Janda et al., 1994) and wild *A. hydrophila* strains (Leung and Stevenson, 1988; Leung et al., 1994) and may be modulated by environmental condition. These are prominent antigens and play important role in the pathogenicity, which protecting from complement mediated serum killing. *In vitro* demonstration of serum resistance of all *A. hydrophila*
strains isolated from diarrhoeal samples showed serum resistance. Both the rough and smooth strains of \textit{A. hydrophila} also play a major role in the serum resistance. Merino \textit{et al.} (1998) reported that smooth strains of \textit{A. hydrophila} are resistant to serum, while rough types are sensitive to serum. As all the isolates were positive for serum resistance, we came to a conclusion that all the strains used in this study might be belonged to resistance form.

All the isolates showed variable resistance to NHS. It significantly induces the virulence mechanisms. In this present study, it was observed that two strains exhibited higher degree of resistance. The observation that isolates of \textit{A. hydrophila} may account for even higher pathogenic potential in extraintestinal infection and also versatility in various infections (Roantree and Rantz, 1960). When compared with these two isolates, other isolates may contribute serum resistance at moderate level. All of them have ability to pose infection at lower level.

Microbiological studies on serum resistance often focus on bacterial components that confer resistance, such as surface proteins. Such types of proteins are inhibiting the binding or activation of complement (Jarva \textit{et al.}, 2003). These same mechanisms have been related to the serum resistance of several fish and human bacterial pathogens, including Gram negative bacteria (Trust \textit{et al.}, 1981; Davies, 1992; Merino \textit{et al.}, 1994) and it is public threat to health hazard. So it is needed for characterization of the putative virulence mechanisms of \textit{A. hydrophila}.

\textbf{Cytotoxicity of \textit{A. hydrophila}}

Generally, a microorganism must colonize before causing infection. The first step in pathogenesisis the adherence of eukaryotic cells, followed by colonizations and toxin production, which leads to the development of diseases. In the present study, we have observed that 88.8 \% of \textit{A. hydrophila} exhibited cytotoxic activity. Reports of Cumberbatch \textit{et al.} (1979), Pollard \textit{et al.} (1990), Majeed and MacRae (1994) and
Castro-Escarpulli et al. (2003) showed that all the strains of *A. hydrophila* showed cytopathic effect on HEp-2 cells, and Aslani and Hamzeh (2004) reported 69 %, 63 % and 68 % of *A. hydrophila* strains producing cytotoxin respectively.

The cytopathic effect induced on Vero and HEp-2 cells infected with by *A. hydrophila* was more evident when the cells were infected with the strains which produced cytotoxin (Sechi et al., 2002). Cytotoxic effects of *Aeromonas* enterotoxins have been extensively studied by several authors by exposing various tissue culture cell lines (Vero, Int 407, Chinese hamster, ovary, HEp-2 or He La cells) with cell free supernatant (Cahill, 1990; Carrello et al., 1998). *A. hydrophila* could produce two types of enterotoxins, cytotoxic (Ast) (cholera like) enterotoxin (Chakraborthy et al., 1984) and cytotoxic enterotoxin (Act) (Asao et al., 1984; Albert et al., 2000). Cytotoxicity was found as the major toxigenic activity among the diarrhoeal isolates of *A. hydrophila*.

Among the seven diarrhoeal isolates of *A. hydrophila*, six strains have been found to produce cytotoxic activity. The virulence gene amplification study also showed that all the six cytotoxic positive strains exhibited *act* gene, which is responsible for the cytotoxic activity (Heuzenroeder et al., 1999; Albert et al., 2000; Sen and Rodgers, 2004), while the cytotoxic negative strains (*Ah-D12*) did not have the *act* gene. In conclusion, the organisms having *act* gene, only may be able to produce the cytopathic effects.

**Distribution of *act* gene in *A. hydrophila***

The Gram-negative cell wall also contains surface proteins that function as adhesions, allowing the bacterium to adhere intimately to host cells and other surfaces in order to colonize and resist flushing. Some Gram-negative bacteria also produce invasiveness, allowing some bacteria to penetrate host cells. Pili, flagella, capsules, and exotoxins also play a role in the virulence of some *Enterobacteriaceae*

In this study, PCR technique was used to detect cytolytic enterotoxin gene (*act*) among the selected strains of pathogenic *A. hydrophila*. Janda (2001) reported that *act* gene
is responsible for the expression of cytotoxin, which is one of the major virulence factors of *A. hydrophila*. As virulence in *Aeromonas* is said to be multifactorial, the PCR approach developed in this study has value in characterizing *A. hydrophila* (Sen and Rodgers, 2004) from diarrhoeal, fish and milk isolates.

The cytotoxic virulence gene (*act*) encodes the cytotoxic enterotoxin to host cells and leads to diarrhoeal infections (Albert *et al.*, 2000). Three enterotoxin genes were selected as targets because the cytotoxic enterotoxin, *act* / *hly* / *aerA* and cytotonic enterotoxins, such as *alt* and *ast* have been implicated as important virulence factors in diarrhoeal diseases (Sha *et al.*, 2002). In the present study, except *Ah*-D12, all the strains of *A. hydrophila* (*Ah*-D5, *Ah*-D6, *Ah*-D15 and *Ah*-D24) were positive for the presence of *act* gene. This was confirmed by the cell line study. Except *Ah*-D12, all the strains showed cytopathic effects on Vero and HEp-2 cells. Absence of cytopathic effect by *Ah*-D12 might be due to the absence of *act* gene. But the strains have been observed positive for the production of haemolysin, protease and DNase enzyme.

Albert *et al.* (2000) observed a significant association between the diarrhoeal isolates and *act* genes. The presence of putative virulence genes provides evidence that each subpopulations could use a different mechanisms to colonize or infect their specific host (Bin-Kingombe *et al.*, 1999; Albert *et al.*, 2000; Aguilera-Arreola *et al.*, 2005). In a study conducted by Bin-Kingombe *et al.* (1999), it was found that among 350 clinical and environmental isolates, 65% of the *Aeromonas* strains were positive for *act* / *hly* / *aerA*. Albert *et al.* (2000) also reported a significant correlation between *Aeromonas* isolates having both the *act* and *alt* genes and diarrhoea in children harbouring such strains. They observed that 54% and 15% *A. hydrophila* strains of diarrhoeal and environmental origin respectively, had both *act* and *alt* genes. Type III secretion system associated with cytotoxic enterotoxin have altered the virulence of *A. hydrophila* and seems to be an important factor related to their pathogenicity (Vilches *et al.*, 2004).
The act gene is widely distributed among the clinical and environmental isolates. The natural environment is said to be the best media where the horizontal gene transfer (HGT) occurs frequently. In this condition, if any strains of A. hydrophila possess/received the act gene and that gene may get transferred from one clone to another.

**Profiling of outer membrane proteins (OMP) of A. hydrophila**

Outer membrane proteins (OMP) are the major and important virulence factors produced by most of the virulence strains of A. hydrophila. Few studies (Quinn et al., 1994; Merino et al., 1998; Kirov et al., 1999) indicated that some filamentous structures. LPSs and OMPs could be useful in intestinal adhesions, leading to cause disease. Several species of Aeromonas exhibited characteristic proteins that may be a useful marker for the identification of Aeromonas at species level (Delamare et al., 2002; Kresters and De Ley (1990); Muller and Millership (1993).

Since the sarkosyl method of extraction of OMP is much superior than the Triton X 100 (Amaro et al. 1989; Maruvada et al., 1992), we adopted the Sarkosyl method in the present study. The OMP profiles of strains of A. hydrophila showed clear protein bands with a molecular weight range of 12.5 to 98.5 kDa. The comparison of OMP profiles on 12.5 % SDS–PAGE showed identical and clear profiles with 12.5, 32.5 and 46 kDa as common amongst most of the strains, and a predominance of the OMP in the 45 to 56 kDa regions. It has been shown that a 43 kDa OMP produced by A. hydrophila binds to H antigen expressed on the surface of most human erythrocytes (Quinn et al., 1994). Gosling et al. (1993) observed 45 kDa protoxin and both the 43 and 45 kDa were reported as cytotoxic enterotoxins. This toxin is widely distributed among motile Aeromonas strains isolated from clinical, fish and environmental sources (Rodriguez et al., 1993; Perez et al., 1998).

It was observed that all the strains have produced bands, that too mainly with in the toxin range, i.e., 43 – 57 kDa, which was similar to the findings of Maruvada et al.
(1992). Dooley and Trust (1988) also observed that most of the *A. hydrophila* strains have produced OMP with a molecular weight of 52 kDa. In the present study, few common bands have been observed between 43 and 57 kDa, which was similar to the findings of Maruvada *et al.* (1992), who reported that most of the *Aeromonas* shared an immunodominant polypeptide at the 52 kDa position. It was also observed that all the strains shared a common band with a molecular weight of 46 kDa, which falls within the toxin range, previously reported. Lakshmanaperumalsamy *et al.* (2005) also reported that *A. hydrophila* strains shared a common band with 52 kDa of protein. The presence of species specific OMP indicated that these immunogenic polypeptides could be exploited to develop antibody-based diagnosis and the identification of *A. hydrophila* (Maruvada *et al.*, 1992).

Two major protein molecular masses with 45 and 56 kDa were common among all strains used in this study. The protein was poorly dissolved in 12.5 % acrylamide gel and appeared as a large smear. This effect could be due to the presence of LPS, which also migrated to the same area of the gel as the smeared protein. Esteve *et al.* (1994) found that all the Spanish isolates have commonly produced two major bands with 45 and 50 kDa, while the Japanese isolates and other isolates have produced different bands with varying molecular weights.

Millership and Want (1993) confirmed high reproducibility of protein fingerprinting analysis. Among fifteen strains of *Aeromonas* isolated from various sources tested for OMP profiling, Delamare *et al.* (2002) demonstrated that all the isolates yielded similar protein electrophoretic patterns with several bands (>50) and found many strains exhibiting characteristic patterns that may be useful markers for the identification at species level. In our study, the protein profiles were highly reproducible.

According to the previous reports whole cell protein fingerprinting could be used as a standard marker in the differentiation of *A. hydrophila*, isolated from various sources, while Alavandi *et al.* (2001) reported that whole cell protein
fingerprinting could neither differentiate the isolates from clinical and domestic water sources nor phenospecies of the genus *Aeromonas*. They also found that *A. hydrophila* strains formed small separate clusters at 5–10% hierarchical levels and this indicate that the strains among same phenospecies could produce different SDS–PAGE fingerprintings; hence, whole cell protein fingerprinting could not distinguish the epidemiologically unrelated phenospecies of the genus *Aeromonas*. Millership and Want (1993) also reported that the whole cell protein fingerprints did not correlate with phenospecies of *Aeromonas*.

DNA–DNA hybridization along with cellular protein profiles studied by SDS–PAGE indicated that most *Aeromonas* strains recovered from clinical material belonged to the DNA hybridization group 1 (HG1), which was not found in freshwater and drinking water samples (Hanninen, 1994). Kirov *et al.* (1994) also reported the predominance of HG1 in clinical samples, while the environmental isolates belonged to HG3. In a recent report, Szczuka and Kaznowski (2004) found that all the clinical isolates tested belonged to HG1. On the basis of previous reports, it may be considered strongly that the clinical strains used in the study belong to HG1 and the remaining might belongs to other HGs. The cell wall protein is associated with the DNA-DNA HG classification. As one of the aims of the present work is to characterize the OMP of *A. hydrophila* isolated from diarrhoeal samples, the HG grouping study was not carried out.

**Profiling of lipopolysaccharides (LPS) of *A. hydrophila***

The properties of LPSs from mesophilic *Aeromonas* spp. follow the general characteristics of the endotoxin from Gram negative bacteria. Secretion of some endotoxins are dependant on the presence of O-antigen LPS. The rough strains (lacking O-antigen LPS) secrete less amount of toxin than smooth strains (rich O-antigen LPS). This fact correlates with some descriptions in *Enterobacteriaceae* (Merino *et al.*, 1992). The endotoxin is much more heat stable than protein toxins and consists of
polysaccharide side chains, core polysaccharide and lipid A, which is the component that confers toxicity to the complex (Blair et al., 1999). The homogenous O-polysaccharide of the lipopolysaccharides (LPS) and the S-layer protein have been reported by Dooley et al. (1985). Three major antigens in Gram-negative bacteria responsible for serotyping are the outer membrane proteins, lipopolysaccharides and phospholipids, which normally thought to be constant for a given species (Aoki and Holland, 1985). Serotyping in case of Aeromonas has been reported to be polymorphous (Leblanc et al., 1981).

The SDS-PAGE analysis of LPS showed existence of heterogeneity among the A. hydrophila strains isolated from various area. In the present investigation, LPS band was seen in all the strains of A. hydrophila which were used in OMP profiling. It ranged between 9.5 and 32.5 kDa. Strains Ah-D3, Ah-D12, Ah-D15, Ah-D24 and Ah-D29 produced identical profiles with an unique molecular weight of 20 kDa. In a work conducted by Maruvada et al. (1992) on outer membrane proteins against whole cell antisera blots with three species of Aeromonas, (A. hydrophila, A. caviae and A. sobria), A. sobria exhibited predominant regarding reactivity to LPS, showing LPS antigenicity mostly between 20 and 65 kDa range, and it seems to provide evidence in support of previous report by Smith (1977). Liberation of O-antigen LPSs in the A. hydrophila strains with the molecular weight of 31 – 38 kDa have been highly antigenic / pathogenic to human (Subashkumar et al., 2005). Thayumanavan (2005) has also observed the identical and apparent profile of LPS with higher heterogeneity amongst environmental and diarrhoeal strains of A. hydrophila. The antigenic LPS patterns displaying O-polysaccharide of homogenous side chain length was characterized by three or four bands (Esteve et al., 1994). A report of Kokka and Janda (1990) showed that LPS profiles mainly consisted of core polysaccharide antigens, which showed clear bands. The miniature ladders like bands elucidate the side chain of the polysaccharides. Our data relating to the banding pattern of all the
strains showed both the core and side chain polysaccharides, and also observed the
diffused fast migrating bands, which carry a lipid A core oligosaccharide fractions
(Merino et al., 1996).

Santos et al. (1996) reported the heterogeneity in the LPS profiles of motile
Aeromonas strains not only among the different serogroups, but also within the same
serotypes. The existence of variability in profiles of LPS and membrane proteins have been
previously described in Aeromonas isolated from salmonid fish (Aoki and Holland, 1985;
Dooley et al., 1985; Nomura and Aoki, 1985; Esteve et al., 1994). Santos et al. (1996) also
observed that the strains of Aeromonas grouped in the same serotype exhibited differences
in their membrane protein patterns irrespective of the species and the degree of virulence,
and these results were confirmed by immunoblot.

Merino et al. (1992) and Aguliar et al. (1997) reported that growth
temperature and medium osmolarity changed the degree of substitution of R and S
forms of LPSs among mesophilic A. hydrophila of serogroup O: 34. They also found
that strains from serogroup O: 34 showed smooth LPS (S and R forms of LPSs) when
they grew at 20 °C in low osmolarity or at 37 °C in high osmolarity medium. But
serogroup O: 34 showed rough LPS (only the R-form LPS), when they grew at 37 °C
in low osmolarity medium. This phenomenon changed some pathogenic factors and
the virulence of these strains grown under different conditions, i.e., temperature and
osmolarity. Merino et al. (1992) also investigated this phenomenon with O: 1 to O: 44
serogroups of Aeromonas sp. and found that strains from serogroups O: 13, O: 33 and
O: 44 showed a LPS that was influenced by temperature and osmolarity of growth
medium. In the present work, all strains were grown at 37 °C and in low osmolarity
medium. According to the previous reports (Merino et al., 1992; Aguliar et al., 1997;
Merino et al., 1998), it was concluded that the LPS produced by the strains used in
this study might be rough form (R-form of LPS). Merino et al. (1992) stated that
mesophilic Aeromonas sp. expressed smooth LPS and it was very similar to that
obtained for strains from serogroup O: 34. They also suggested that the method used for obtaining specific LPS antisera for serogrouping may be an important factor for finding sera rich in antibodies against O-antigen LPS.

**Molecular typing of *A. hydrophila* using RAPD and ERIC-PCRs**

Taxonomic identification with in the genus *Aeromonas* has been subject to considerable debate and differentiation at species level is somewhat confused (Blair et al., 1999). The need of a good system for the identification and classification of *Aeromonas* is justified by their ecological and clinical importance. Different methods such as biotyping (Popoff and Veron, 1976), isozyme electrophoretic analysis (Picard and Goullet, 1987), DNA hybridization (Popoff and Veron, 1976), lipopolysaccharide analysis (Shaw and Hodder, 1978), serotyping (Havelaar et al., 1992), rDNA typing (Altwegg et al., 1988), outer membrane proteins (Millership and Want, 1993; Muller and Millership, 1993), RAPD–PCR (Miyata et al., 1995; Oakey et al., 1996), ERIC-PCR (Davin-Regli et al., 1998), AFLP fingerprinting (Huys et al., 1995) and PCR (Oakey et al., 1996) have been used to type the isolates for epidemiological investigations. Genotyping methods have been applied to these species; plasmid analysis is unhelpful because, plasmid carriage is infrequent (20 to 58 %) in *A. hydrophila* (Janda, 1991). Although there are some arguments against the reproducibility of PCR–based typing methods (van Belkum et al., 1994; Perez et al., 1998), in the present study, these methodologies were highly reproducible in overall banding patterns and band repetition. These results could be agreed with excellent correlation, not statistically validated, between RAPD and ERIC methods reported recently (Davin-Regli et al., 1998; Szczuka and Kaznowski, 2004). Previous investigations on the reproducibility of RAPD allowed the standardization of parameters for routine use (Abed et al., 1995; Davin-Regli et al., 1995; Sinha et al., 2004).

However, all the above techniques are time-consuming, expensive and laborious and are not able to provide reliable and rapid results on a routine basis. Rapid, simple
and sensitive methods are desirable for hospital and food industries based laboratories. Polymerase chain reaction (PCR) based techniques have been applied successfully to the epidemiological typing of various bacterial species. It is one of the molecular technique, which relies on the \textit{in vitro} amplification of a DNA fragment and the result is obtained with in a short period of time after receiving the samples. Random amplification of polymorphic DNA (RAPD) is based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequence (Williams \textit{et al.}, 1990). RAPD has been used widely for epidemiological investigation of numerous bacterial species (van Belkum \textit{et al.}, 1994; Millemann \textit{et al.}, 1996). Because of the low stringency inherent in this procedure, the patterns generated by RAPD may be affected by experimental parameters and standardization is crucial. RAPD analysis was chosen because it is reliable and can be use to differentiate between strain with in the species (Austin and Adams, 1996).

In recent reports, Davin-Regli \textit{et al.} (1998), Aguilera-Arreola \textit{et al.} (2005), Thayumanavan (2005), Subashkumar \textit{et al.} (2006b) proved that both RAPD and ERIC–PCRs are powerful tools for differentiating the strains of \textit{A. hydrophila}, while Szczuka and Kaznowski (2004) reported that both RAPD and ERIC–PCRs have the same discriminatory power of the species of \textit{Aeromonas}, and found that the repetitive extragenic palindromic (REP) PCR is less effective for differentiating \textit{Aeromonas} isolates into species, since the REP sequence may not be widely distributed in \textit{Aeromonas} sp. genome. In contrast, Alavandi \textit{et al.} (2001) used whole cell proteins and RAPD–PCR to distinguish the clinical strains of \textit{Aeromonas} spp. and concluded that both RAPD and whole cell protein finger printing techniques could not differentiate the phenospecies of the genus \textit{Aeromonas}.

The combination of RAPD and ERIC–PCRs for the epidemiological typing of environmental strains of \textit{A. hydrophila} was first reported by Davin-Regli \textit{et al.} (1998) and latter by Szczuka and Kaznowski (2004) and Aguilera-Arreola \textit{et al.} (2005). The aim of the
present study was to distinguish the \textit{A. hydrophila} strains isolated from children diarrhoeal samples. Two molecular typing methods based on PCR, RAPD and ERIC–PCRs were used to discriminate the isolates of children diarrhoea, milk and fish.

In the present investigation, we have observed a perfect correlation between RAPD and ERIC–PCR profiles. This is strongly supported by the findings of Davin-Regli \textit{et al.} (1998) who also observed similar type of correlation between RAPD and ERIC–PCR profiles for the isolates of \textit{A. hydrophila} isolated from hospital patients and tap water in France. Szczuka and Kaznowski (2004) also reported the same, and it was between clinical and environmental strains of \textit{Aeromonas} spp.

A greater heterogeneity among within the strains of \textit{A. hydrophila} has been demonstrated by RAPD and ERIC–PCRs. Eventhough, all the isolates are of clinical origin, it was observed that none of the \textit{A. hydrophila} isolates produced identical profiles. This clearly reflects the genetic diversity of the strains tested. This was well reputable with RAPD and ERIC-PCRs. In their study, Davin-Regli \textit{et al.} (1998) found 6 different patterns among 10 clinical isolates of \textit{A. hydrophila} tested. They also found that all the 13 water isolates of \textit{A. hydrophila} used as control gave different profiles. Moyer \textit{et al.} (1992) and Davin-Regli \textit{et al.} (1998) also found no fingerprints were common among environmental and clinical isolates of \textit{A. hydrophila}. Recently, Thayumanavan (2005) reported a wide spread variation in clones of \textit{A. hydrophila} isolated from freshly caught and marketed seafood. Work on \textit{A. hydrophila} isolated from river water (Sharma \textit{et al.}, 2005) and raw milk (Subashkumar \textit{et al.}, 2006b) have also supported the findings of present investigation.

Both the RAPD and ERIC-PCRs could be highly reproducible, reliable and exactitude. These techniques could be used in the epidemiological exploration of bacterial diseases. All the above reports strongly support the wide genetic diversity of
A. hydrophila strains of diverse origin. On the basis of the previous reports and the present investigation, the coexistence of genetic diversified strains of A. hydrophila among the diarrhoeal samples collected from different areas was well established.

Coefficient simple matching dendrogram for RAPD and ERIC profiles of A. hydrophila

The UPGMA cluster analysis on the basis of RAPD and ERIC profile of Aeromonas depicted all the strains having the several clusters and it suggests that RAPD and ERIC profiles are species specific. The dendrogram has been developed for the RAPD–PCR fingerprinting and 12 clusters have been observed. The RAPD profiles of A. hydrophila isolated from the milk and fish formed a separate cluster.

Visible observation on RAPD profiles showed that none of the A. hydrophila strains tested produced identical RAPD profiles and this was further confirmed by the dendrogram generated for RAPD profiles and revealed the substantial wide genetic diversity among the strains tested. Strains Ah–T2, Ah–D3, Ah–D6, Ah–D12, Ah–D15, Ah–D19 and Ah–D24 formed a minor cluster (cluster 7) while Ah–D5 and Ah–D26 formed separate minor cluster against the cluster 10. This indicates the genetic relationship among the strains within the clusters. With the help of the dendrogram, it is clear that one can easily understand the percentage of genetical similarities between the strains.

Similarly, none of the ERIC profiles have been found identical, but greater homogeneity (0.87) was observed for the strain Ah–D3 from the other strains of A. hydrophila. It formed a separate cluster showing a wide genetic diversity from other strains. A total of 12 clusters were observed with a maximum number of 8 strains grouped into major cluster 8. However, all of them have been isolated from diarrhoeal sources and one from fish source. But the type strains formed a separate cluster (5) and this formed a new cluster (9) along with the milk isolate (Ah-M1). Here it is clear that all diarrhoeal isolates have been arranged in a big cluster (cluster 9). The dendrogram clearly exhibited that clinical and environmental isolates of A. hydrophila are not genetically similar. Moyer
et al. (1992), Szczuka and Kaznowski (2004) and Davin-Regli et al. (1998) also reported that none of the *Aeromonas* species are genetically similar. And it was further confirmed by the dendrogram. The combined RAPD and ERIC profiles also supported the co-existence of genetically varied *A. hydrophila* strains among the samples. Here also none of the strains have been observed to produce identical profile, similar to the findings of Davin-Regli *et al.* (1998) and Bauab *et al.* (2003).

The rationale for performing molecular typing was to understand whether any particular clone of *Aeromonas* species was more often associated with diarrhoea. Both RAPD and ERIC profiles clearly revealed that all strains tested in this study are having high heterogeneity.

**Coefficient simple matching dendrogram for combined RAPD and ERIC profiles of *A. hydrophila***

The most consistent dendrogram was obtained when the RAPD and ERIC data were combined. Twelve clusters have been observed in the dendrogram. Here both the type strains formed a separate cluster and the milk and fish isolates have formed another separate cluster. It is a visible spectacle that most of the diarrhoeal isolates were grouped under a major cluster. Even though they were genetically diverse, because of many characteristic band (s), they fell under this major cluster. This could be one of the reasons for the type strains, fish and milk isolates also. Interestingly, the dendrogram reflects the major cluster of diarrhoeal strains and showed the significant variation among them. Whereas the type strains and environmental strains did not follow any such kind of specificity. This strongly supports the previous reports that both the clinical and environmental strains of *A. hydrophila* varied and this variation (s) are because of the genetic structure of the clones.

Haemolysin was considered as the major criteria for the selection of the strains for the molecular typing study and it was a random selection. On this basis, we came to a conclusion that all the isolates might be genetically heterogenous and the *A. hydrophila* isolated from all the sampling areas might be genetically diverse.
SUMMARY

The incidence of *A. hydrophila* in children diarrhoeal samples collected from four places of Tamil Nadu state, India has been studied for a period of one year (January 2003 to December 2003). All the isolates were subjected to multiple antibiotic resistance assay and production of haemolysin, protease and DNase. Also, their ability to produce slime and serum resistance capacities have been observed. In addition to this, the cytopathic effects and detection of *act* gene, which is responsible for the production of cytolytic enterotoxin have also been tested. To accentuate the present investigation, the genetic diversity of the strains have also been observed by means of RAPD and ERIC-PCRs and profiling of OMPs and LPSs.

Among the frequency of diarrhoeagenic bacteria, isolated from all the sampling areas, *E. coli* was recorded as the predominant bacterium with 58.57 %, followed by *Vibrio, Salmonella, Aeromonas, Shigella, Pseudomonas, Streptococcus, Proteus* and *Staphylococcus* with 40.58 %, 33.47 %, 30.54 %, 10.46 %, 10.04 %, 10.04 %, 7.11 % and 5.43 % respectively. This clearly suggests the significant place of *Aeromonas* as one of the diarrhoeagenic genera.

Since the main work is focussed on the incidence of *A. hydrophila* among the diarrhoeal samples of children, it was further concentrated only on this organism and the percentage of incidence of *A. hydrophila* was recorded as 16.7 %. The maximum percentage incidence of this organism has been observed in Coimbatore, with 17.6 %, followed by Kovilpalayam, Pattukottai and Tirupur with 16.6 %, 15.1 % and 14.3 % respectively. It is clear that the frequency of incidence was seasonally dependent.

The multiple antibiotic resistance (MAR) study revealed that all the strains of *A. hydrophila* isolated from children diarrhoeal samples showed resistance to bacitracin and cephalexin and sensitive to netillin. It is quite interesting that chloramphenicol resistance (12.5 %) strains have also been isolated, which is one of the important features of this present investigation.
It was observed that 97.5 % of *A. hydrophila* strains have been identified as haemolysin producers. Similarly, 100 %, 80 % and 71.5 % of the strains have been observed to produce protease, DNase and slime respectively. Also, all the strains of *A. hydrophila* have been observed to have serum resistance.

Among the selected strains of *A. hydrophila* subjected to cytopathic study and amplification of *act* gene, all the strains, except one strain (Ah-D12) have been observed to cause damage in Vero and HEp-2 cell lines. Since the strain Ah-D12 did not have the *act* gene, the cytopathic effect could not be established.

Both the random amplification of polymorphic DNA (RAPD) PCR and enterobacterial repetitive intergenic consensus sequence (ERIC) PCR profiles reveal the coexistence and wide-genetic diversity among the *A. hydrophila* strains. Both the environmental and type strains used in this study also showed significant variations. It was confirmed by the dendrogram.

It was observed that two molecular masses of outer membrane proteins (OMP) with 43 and 57 kDa were found common among all the strains used in this study. The lipopolysaccharide (LPS) profiles showed the existence of heterogeneity among the strains, and ranged between 9.5 and 32.5 kDa.

In conclusion, the present study reveals the occurrence of toxigenic and heterogeneous strains of *A. hydrophila* among children diarrhoeal samples collected from various parts of the state, which is an important threat to the public health point of view. The percentage of incidence of this organism along with the other important diarrhoeagenic pathogens is one of the serious problems to be concentrated in, since *A. hydrophila* has been recognized as a re-emerging pathogen. Studies on various virulence factors add the magnitude to the present investigation. The molecular typing techniques have once again proved their powerful discriminating capacity in differentiating *A. hydrophila* strains. Hence, it is strongly recommended that these techniques could be useful in the epidemiological investigations.