Review of Literature
REVIEW OF LITERATURE

Diarrhoeal illness is well recognized as a major cause of morbidity and mortality in young children in many developing countries (UNICEF, 1992; WHO, 1995; Urbino et al., 2003). About 24.1% of all infant deaths and 40% of all deaths in the first two years of life is due to diarrhoeal disease (Simanjuntak et al., 1998). WHO predicts that there will be about 5 million deaths in children younger than five years by 2025, of which 97% will be in the developing countries and mostly caused by infectious diseases, within which diarrhoea will continue to play a prominent role (WHO, 1998). It is reported that about 2.5 million children die from the diarrhoeal illness in every year (Taper and Sanderson, 2004). One of the major challenges in the gastrointestinal diseases is the recent increase in the number of probable aetiological agents. In developing countries, pathogens were identified in 65% of stool samples from children with acute diarrhoea (Kang et al., 2001). In India, one third of the total paediatric admissions in hospitals are due to diarrhoeal diseases and 17% of all deaths in indoor paediatric patients are diarrhoea related (Park, 1998). The main causes of diarrhoea are poor personal and food hygiene and lack of clean drinking water (Sanderson and Walker, 1993).

Bacteria associated diarrhoeal infections

The members of Enterobacteriaceae are the most important aetiologic agent of childhood diarrhoea and represent major public health problems in the world (Taneja et al., 2003). In recent years, a significant proportion of human infectious diarrhoea are associated with enterotoxigenic bacteria. The best known of these are V. cholerae and enterotoxigenic E. coli (ETEC). These organisms produce intestinal fluid loss by elaborating enterotoxins which interfere with net trans-mucosal fluid and electrolyte transport. These and other enterotoxigenic bacteria produce either a heat labile toxin (LT), which resembles cholera toxin (CT), or a heat stable toxin (ST) or both. The
extent of enterotoxigenicity amongst human intestinal bacteria is still undefined; it is undoubtedly much more extensive than realized only a few years ago (Robinson et al., 1983). Epidemics of cholera have been reported from various parts of India (Taneja et al., 2003). It is caused by Vibrio cholera and other species belonging to the genus Vibrio are well known as causative agent of gastrointestinal, extraintestinal and wound infections with a fatal outcome in some cases (Dumontet et al., 2000). Escherichia coli is the most important aetiological agent of childhood diarrhoea and represent a major public health problem in developing countries (Nataro and Kaper, 1998). Other bacteria which are reported to cause gastroenteritis include Salmonella, Shigella, Campylobacter, Aeromonas and Yersinia species (Collee et al., 1997). Robinson et al. (1983) isolated Aeromonas, Salmonella, E. coli, Shigella and Vibrio from 500 children with gastroenteritis.

*Aeromonas* infections

Zimmermann (1890) was the first one who described a member of the present genus *Aeromonas*. The history of the genus *Aeromonas* reflects the confusing history of microbiology in the last 100 years. The first isolation and description of *Aeromonas* in connection with human and disease was done by Aitken et al. (1936). Stainer (1943) reclassified the organisms based on the fundamental taxonomic studies of the genus *Aeromonas* (the seventh edition of *Bergey's Manual*). Further the organism was named as *Aeromonas hydrophila* (Snieszko, 1959).

Members of the genus *Aeromonas* are gram negative, rod-shaped, 1 X 3.5μ, aerobic and facultative anaerobic. They are motile by a single polar flagellum with the exception of *A. salmonicida* and *A. sobria* which are non motile and were assigned to the family *Aeromonadaceae* (Colwell et al., 1986). The genus is characterized by broad phenotype variations (Bottarelli and Ossipandi, 1999). Thirteen species of the 15 included in the genus have been reported from human infections.
The mesophilic *Aeromonas* species are primarily organisms of aquatic environment and are present in fresh water, estuarine and coastal water bodies. They also occur in untreated and chlorinated water, ground beef, pork, fish, shell fish, poultry products and raw milk. The bacteria are distributed world wide in the primary habitats *i.e.*, surface water and soil (Lakshamanperumalsamy *et al.*, 2005). The global distribution of *Aeromonas* species in many aquatic ecosystems indicates the successful adaptation of the bacterium to such environments (Hazen *et al.*, 1978; Williams and LaRock, 1985).

The microbial flora of aquatic animals like fish, snakes and leeches include *Aeromonas* species (Okafor and Nzeako, 1985). *A. hydrophila* was first implicated as a causative organisms of gastroenteritis more than 20 years ago and the recognition of its enteropathogenicity (Sanyal *et al.*, 1975) suggests that these organisms may cause intestinal fluid loss and watery diarrhoea by the secretory effects of toxins (s) on the intestinal mucosa, analogous to the mechanism involved with *V. cholerae* and enterotoxigenic *E. coli*. *A. hydrophila* has been recognized as an opportunistic pathogen in person with impaired local or systemic immunity in which it can cause severe infections in humans, such as gastroenteritis, bacteraemia, cellulitis, meningitis, endocarditis, peritonitis, endophthalmitis, corneal ulcer, septic arthritis, wound infections, osteomyelitis, suppurative arthritis, intraabnormal abscess, urinary tract infections, evolving into pneumonia, lung abscess, colicititis and soft tissue infections (Janda and Abbott, 1998; Khardori and Fainstein, 1988).

The occurrence of a haemolytic-uremic syndrome following an *Aeromonas* gastric diseases has been described and it is very similar one by *E. coli* O157:H7. It is caused by an *Aeromonas* cytotoxin, which is genetically and antigenically different from the *E. coli* cytotoxin. *A. hydrophila* can also be responsible for localized infections on the skin and soft tissues. The condition is characterized by myositis or necrotizing myositis.
and it is often associated with wounds contaminated by water or soil; usually it involves the lower limbs (Khardori and Fainstein, 1988).

Majority of the serious infections caused by this organism occur in individuals with certain predisposing conditions; the most relevant examples being that of *Aeromonas* septicaemia. Individuals with hepatic disorders, malignancies and biliary obstructions leads a greater risk in contracting septicaemia, although it can occur in patients with no apparent immunological and physiological deficits (Janda *et al.*, 1994).

*Aeromonas* meningitis may involve all age groups and can be either community or nosocomial acquired (Paras *et al.*, 1993; Collee *et al.*, 1996). The species may attribute with that acquisition of certain pathogenic determinants that confer it with greater invasive capabilities (Paras *et al.*, 1993). Other infections are found in the post surgical leach application on damaged tissue with questionable arterial perfusion (Lineaweaver, 1991). Spontaneous bacterial peritonitis (SBP) presents in 15% of the patients with cirrhosis and ascitis (Gilbert, 1995). Spontaneous bacterial empyema currently exists and caused by *A. hydrophila* (Steeiw and Colodny, 1994).

Over the past ten years, *A. hydrophila* has been documented as respiratory pathogen. The clinical features have ranged from pneumonia empyema and formation of fatal lung abscesses. In Cuba Bravo *et al.* (2003) have described the association of *A. hydrophila* with immunocompromised patient suffering from the lung cancer.

The role of aeromonads, and particularly of *A. hydrophila*, as human pathogen and their transmission have been revised during the past few years. According to some authors, the isolation of highly virulent strains is increasing; thus these bacteria can no longer be classified among the "opportunistic" agents, as they were in the past. Moreover, the epidemiological and ecological relationships between *Aeromonas*, man, environment and animal have aroused increasing interest in this bacterial genus, in the human field (Bottarelli and Ossiprandi, 1999).
**Aeromonas associated diarrhoea**

Over the last few years, increasing the interest in *Aeromonas* species has gone beyond the boundaries of fish pathology; this is due to the increase of disease in man which is caused by these agents, as they can often act as opportunistic agents in ipoergic individuals, or in patients with chronic and weakening disease (Janda, 1991). Among bacterial etiological agents of diarrhoea, *Aeromonas* is increasingly recognized as an enteric pathogen (von Gravenitz and Mensch, 1968). In addition to role of “secondary pathogens” aeromonads also play a role as primary pathogens (Janda, 1991; Palumbo et al., 1992) being responsible for enteric or, less often, extra-enteric infections (Janda and Duffey, 1998). Due to increasing reports of acute diarrhoea caused by these bacteria, *A. hydrophila* can now be considered a relatively common enteropathogen. Most of the studies on the causes of human gastroenteritis cases have focused on the transmission by contaminated food and water. *Aeromonas* associated diarrhoea is similar to that of the infections caused by different *V. cholera* – O1 and non-O 139 serogroups (Sinha et al., 2004). The most common syndrome is confined to the gastro-enteric tract (Varone et al., 1998); sometimes it runs a course identical to the condition known as ‘traveller’s diarrhoea’ (Yamada et al., 1997). The clinical findings are similar, although less dramatic, to the ones of cholera; watery diarrhoea, fever and vomit; occasionally the disease can be more severe, with mucus and/or blood in the faeces. In compromised patients, the gastro-enteric form can develop to a severe abdominal or septicaemic infection (Saito and Schick, 1973).

A greater risk of diarrhoeal infection was reported in young children, elderly people and immunocompromised patients (Freij, 1987; Janda, 1991; Anandhan et al., 1998). At least three distinctive gastroenteritis syndromes following gastroenteritis caused by *A. hydrophila* have been described (a) acute, watery diarrhoea; (b) dysentery and (c) sub-acute chronic diarrhoea (Janda and Duffey, 1988). It invades the blood stream through defective intestinal mucosa (Altwegg and Geiss, 1989). The virulence factors
enable the bacterium to colonize the host and to obtain nutrients and growth factors in vivo (Allen and Stevenson, 1981; Santos et al., 1988; Paniagua et al., 1990). Diarrhoeal infections account for most illness, with pathogens employing ingenious mechanisms to establish disease. In the developed countries, an upsurge in immune-mediated gut disorders might have resulted from a disruption of normal bacterial-epithelial cross-talk and impaired maturation of the gut’s immune system (Thapar and Sanderson, 2004). A study in southern India has revealed that *A. hydrophila* is the predominant species in diarrhoeal sources (Komathi et al., 1998).

*Aeromonas* associated diarrhoea has a distinct seasonal pattern with a sharp summer peak (von Gravenitz and Mensch, 1968). This may be related to the prevalence of the organism in environmental sources, such as water during the summer months, particularly as it has been proposed that the portal entry of the gastrointestinal tract (Trust et al., 1979).

Acute diarrhoea with self limiting and dysentery like illness with bloody and mucous diarrhoea, mimicking childhood inflammatory bowel disease was seen occasionally (Janda and Duffey, 1988). A wide differences in the frequency of isolation of *Aeromonas* spp. from stool has been observed, but it is more common in tropical countries like Peru and Tropical Australia (Gracey et al., 1982; Nishikawa and Kishi, 1988; Pazzaglia et al., 1991). In Kuala Lumpur, children with acute diarrhoea attending outpatient clinic, were reported of *Aeromonas* infection, the isolation rate being 5.2 % (Koe et al., 1991). In India, *Aeromonas* associated diarrhoea has been reported from Bombay (Deodhar et al., 1991), Calcutta (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) and Coimbatore (Subashkumar et al., 2006a) and the incidence in these areas was reported between less than 1 % and 13 %.
Aeromonas have been isolated from 10% of 500 children with diarrhoea in western Australia but from less than 0.4% of an age matched group of 500 children without diarrhoea (Robinson et al., 1983). Lee and Puthucheary (2001) reported that Aeromonas was a rare cause of gastroenteritis in urban Malaysian children. It was isolated almost exclusively from gastrointestinal tract, caused mostly by mild gastroenteritis with no serious complications. Asymptomatic stool carriage among newborns admitted to special care nursery and older children with no diarrhoea were also observed.

Antibiotic resistances of A. hydrophila

The emergence of resistance to antibiotics in several pathogenic bacteria in the past two decades has gradually rendered traditional antimicrobial treatment less effective. The horizontal transfer of resistance genes to other bacteria, even across various species, rapidly creates bacterial populations with one or more of the following antimicrobial properties; (a) an increased ability to degrade antimicrobial compounds, (b) decreased permeability, (c) decreased affinity for the antibiotic and (d) increased efflux of many different antibiotics (Sritharan and Sritharan, 2004). The use of antibiotics is limited because bacteria have evolved defenses against certain antibiotics. One of the main mechanisms of defense is inactivation of the antibiotic. Another form of defense involves a mutation that changes the bacterial enzyme affected by the drug in such a way that the antibiotic can no longer inhibit it. This is the main mechanism of resistance to the compounds that inhibit protein synthesis, such as the tetracyclines. All these forms of resistance are transmitted genetically by the bacterium to its progeny. Genes that carry resistance can also be transmitted from one bacterium to another by means of plasmids, chromosomal fragments that contain only a few genes, including the resistance gene.

The infective risk for humans related to potential virulence of A. hydrophila appears too improved by the ability to acquire plasmids carrying antibiotic resistance and/or antibacterial activity. The strains of A. hydrophila isolated from Infectious Disease
Hospital (IDH), Kolkata exhibited resistance to commonly employed antibiotics (Kannan et al., 2001). In addition, many pathogens are becoming increasingly resistant to standard antimicrobial drugs; making treatment difficult and in some cases impossible (Gomez-Lus et al., 2000). Sader and Jones et al. (2005) evaluated variable significance of antimicrobial susceptibilities between species/genera and the geographical regions. Radu et al. (2003) reported that all *A. hydrophila* strains isolated from retail seafood outlets were resistant to three or more than three antibiotics tested.

Ramteke et al. (1993) reported that one fourth of water isolates of *Aeromonas* spp. were resistant to chloramphenicol, streptomycin while almost all the strains were sensitive to gentamicin, kanamycin and polymyxin–B. Jesudasan and Koshy (1990) observed that an increasing trend in the resistant pattern among *A. hydrophila* isolated from faeces of patients closely associated with the cause of gastroenteritis, which was caused by consumption of contaminated drinking water. Almost all the *Aeromonas* spp. are resistant to penicillin and carbenicillin. The acylureidopenicillins showed a varying degree of growth inhibition, with pipercillin was the most active agent. *In vitro* tests of imipenem indicated good action against *Aeromonas* group (Altwegg and Geiss, 1989). Aminoglycosides exhibited good activity against *Aeromonas* strains, except for streptomycin, which has proved to be less active (Altwegg and Geiss, 1989). Sinha et al. (2004) reported that majority of the *Aeromonas* strains exhibited multidrug resistance and exposed a significant threat to management of *Aeromonas*-mediated diarrhoea because of the progressive development of new drugs.

**Multiple antibiotic resistances among *A. hydrophila***

Today, a global concern has emerged that we are entering into a post-antibiotic era with a reduced capability to combat microbes. Hence, the development of novel therapeutic approaches to the treatment of bacterial infection has become a global emergency in the management of infectious diseases (Sritharan and Sritharan, 2004).
The first systematic tests of antibiotic activity against *Aeromonas* strains were performed by Schaperclaus, Wlf and Havelka (cited in Caselitz, 1966). Growing incidence of multiple antibiotic resistance (MAR) among *A. hydrophila* strains isolated from various sources has been reported from many parts of the world (Chaudhary *et al*., 1996; Pettibone, *et al*., 1996; Radu *et al*., 1997; Ko *et al*., 1998; Shome and Shome, 1999; Vivekanandhan *et al*., 2002; Radu *et al*., 2003; Thayumanavan *et al*., 2003). Pathak *et al*. (1993) reported that strains of *A. hydrophila* exhibited a frequency of resistance against ampicillin followed by streptomycin, chloramphenicol and nalidixic acid. But all the isolates exhibited susceptibility to tetracycline. Under these circumstances, it will be worthwhile to find out the prevalence of multiple antibiotic resistance of *A. hydrophila* strains that may be considered as an emerging pathogen and to identify the high-risk source.

Further, Ko and Chung (1995) observed increased levels of drug resistance among clinical isolates of *A. hydrophila* to commonly used antibiotics. Like Gram-negative bacilli, the emergence of resistance among aeromonads will be accelerated by the extensive clinical use of antibiotics (Chaudhary *et al*., 1996). Such high level of multiple drug resistance may arise from selective pressure due to the indiscriminate use of antibiotics in human therapy and animal husbandry. The variation in the drug resistance may be related to the source of *A. hydrophila* isolated and the frequency was prescribed for treating *Aeromonas* infections in geographical area (Radu *et al*., 1997). Although antibiotic resistance is common, antibiotics are still indicated in the management of diarrhoea. Antibiotics shorten the duration of diarrhoea, decrease stool output and may mitigate complication (Black, 1993). These reports revealed that geographical, socioeconomical parameters and local selective pressures could influence antibiotic resistance among *Aeromonas* spp.

Multidrug resistance among *A. hydrophila* has been reported by several researchers worldwide (Radu *et al*., 2000; Vivekanandhan, 2000; Savithamani,
The problem is more serious in developing countries, where antibiotics are used widely. In India, antibiotics are extensively applied in animal husbandry (Wegner and Frimodt-Moller, 2000) and aquaculture (Vivekanandhan et al., 2002). The use of antibiotics is the most important factor in amplifying the level of resistance in a given reservoir (Wegner and Frimodt-Moller, 2000).

The susceptibility patterns of the strains to certain common antibiotics differed from those determined by some other workers (Fainstein et al., 1981), who observed that the strains of *A. hydrophila* were highly susceptible to tetracycline and trimethoprim-sulfamethoxazole. The occurrence of resistance plasmids in *Aeromonas* has been described by Aoki et al. (1986). Plasmids harbouring multiple antimicrobial resistance determinants (R-plasmids) were transferred in simulated natural microenvironments from various bacterial pathogens of human and animal origin. R plasmids seem not to be the major mechanism for the resistance in the genus *Aeromonas* (Altwegg and Geiss, 1989).

**Pathogenicity of *A. hydrophila***

Attributes of the motile aeromonads which have potential to contribute to their pathogenicity include the production of endotoxin, extracellular enterotoxin, haemolysins, nuclease, cytotoxins and proteases; the ability to adhere to cells; and the possession of certain surface proteins. The ability of *A. hydrophila* to produce exotoxins (haemolysins, enterotoxins and cytotoxins) and enterotoxins (protease and lipase) is associated with its capacity to cause disease (Janda and Duffey, 1988; Janda, 1991). These extracellular products have been reported to be useful indicators of pathogenicity (Santos et al., 1988; Cahill, 1990). There is substantial evidence that some strains (particularly of the species of *A. hydrophila* HG1, *A. caviae* HG4 and *A. veronii* biovar *sobria* HG8/10) are primary gastrointestinal pathogens (Kirov, 1997). However it is still
not possible to identify these virulent strains definitively as *Aeromonas* since pathogenic mechanisms are not well understood (Kirov *et al.*, 2002).

**Haemolysin production**

Species of *Aeromonas* are capable of expressing a number of extracellular toxins and enzymes (Gosling, 1996; Howard *et al.*, 1996). Among the various virulence factors reported in *Aeromonas* spp., haemolytic molecule seems to be related to enterotoxigenicity. *Aeromonas* spp. produces cytolytic as well as cytotoxic enterotoxin (Deodhar *et al.*, 1991; Kirov, 1997). The primary toxins produced are haemolysins, of which, the most significant is aerolysins, expressed by many strains of *A. hydrophila* and *A. sobria* (Janda, 1991; Howard *et al.*, 1996; Xu *et al.*, 1998; Buckley and Howard, 1999). The haemolytic enterotoxins have been reported by some authors (Chopra *et al.*, 1991; Gosling, 1996). Limited reports are available in the distribution of the main virulence factor, aerolysin, and cytotoxicity of environmental strains (Wong *et al.*, 1998).

A number of studies have shown that haemolytic factors are involved in the virulence of aeromonads. Two haemolytic toxins have been described in *A. hydrophila*: the AHH1 haemolysin (Hirono and Aoki, 1991) and aerolysin (Howard *et al.*, 1987). These haemolytic toxins have only 18 % homology and are distinct (Hirono and Aoki, 1991). Aerolysin (Howard *et al.*, 1987) is the best studied haemolysins, but *Aeromonas* strains can produce more than one haemolytic toxin with virulence properties (Chopra *et al.*, 1991; Wong *et al.*, 1998). Kirov (1997) reported that 50 % of the *Aeromonas* spp. was the carriers of aerolysin. 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.

Much attention has been given on the haemolysin of motile *Aeromonas* spp. because the production of haemolytic toxins has been regarded as indication of pathogenic potential, though non-haemolytic aeromonads also being implicated as human
pathogens (Namdari and Battone, 1990). Wong et al. (1996) found that all Aeromonas isolates with haemolysin positive genotype were virulent in suckling mouse assay model. Haemolysin producing A. hydrophila strains are considered to be enteric pathogens and this suggestion is supported by the discovery of various toxins, including enterotoxin, haemolysin and cytotoxic proteins. Cumberbatch et al. (1979), Asao et al. (1984) and Janda (1985) have proved that enterotoxin production is significantly correlated with haemolysin production. Radu et al. (2003) reported that more than 90% of the Aeromonas strains isolated from retail fish in Malaysia were haemolysin producers, while Callister and Agger (1987) observed that all A. hydrophila isolates from retail grocery store products were highly cytotoxic at 35 °C, but not at 37 °C.

Two patterns of haemolysin accumulation in culture supernatants have been described for A. hydrophila cells. In the first, attributed to the α-haemolysin was released into the culture fluid during stationary phase (Ljungh et al., 1981). In the second, attributed in the extracellular fluid during the active growth phase and decreasing quickly thereafter (Ljungh et al., 1981; Asao et al., 1986).

Kirov et al. (1994) considered that Aeromonas strains are potential food borne bacteria possessing at least two of the following characteristics; hemolytic activity against rabbit erythrocytes, cytotoxic activity against Vero cells and enterotoxigenicity in the suckling mouse test. Direct detection of the hemolytic genes aer A and hly A has been suggested as a reliable approach for identifying potentially pathogenic Aeromonas strains (Heuzenroeder et al., 1999).

**Protease enzyme production**

Protease enzyme produced by bacteria have been shown to cause tissue damage, aid invasiveness and establishment of infection by overcoming host defenses and provide nutrients for bacterial proliferation (Shimada et al., 1996; Rao et al., 1998). Studies on the protease of Aeromonas have mainly been performed with A. hydrophila (Rivero et al.,
The protease composed of 624 amino acid residues with molecular weight of 66 and 73 kDa. The amino acid sequence showed the characteristics features of a bacterial serine protease (Okamato et al., 2002). Castro-Escarpulli et al. (2003) reported that 61% of the *Aeromonas* isolates produced protease.

The virulence of *A. hydrophila* is multifactorial and includes wide variety of surface characteristics and extracellular products (ECPs) (Cahill, 1990; Paniagua et al., 1990; Cascon et al., 2000; Gonzalez-Serrano et al., 2002). The proteolytic activity of some extracellular enzymes of *A. hydrophila* was recognized early (Dahle, 1971) and is considered to play a major role in the virulence and pathogenicity of the bacterium and the role of this type of enzyme is to provide nutrients by breaking down host proteins into small molecules capable of entering the bacterial cell (Sakai and Kimura, 1985). These activities are often implicated in the pathogenesis of many bacterial infections (Goguen et al., 1995; Beith, 2001).

Proteases are thought to contribute the virulence of aeromonads, however, their contribution to pathogenicity still needs to be determined. Gonzalez-Rodriguez et al. (2004) reported that direct tissue damage enhanced invasiveness or provision of nutrients by the protease activity. Another protease activation of proaerolysin (Howard and Buckley, 1986) has relationship between proteolytic and haemolytic activities (Gonzalez-Rodriguez et al., 2004).

Temperature, pH and aeration are influencing the production of maximum protease enzymes (O’Rielly and Day, 1983). These protease enzymes would participate in the virulence mechanisms for homeothermic animals and it has been proved that it is one of the important factors in the development of infections in human by *A. hydrophila* (Allen and Stevenson, 1981; Ellis et al., 1981). Even though protease activity of the human strains decreased at 37 °C, unlike the haemolytic, cytolytic activities, some of them are higher at 37 °C than at 28 °C, which would indicate the relevance of these
extracellular virulence factors in the pathogenicity mechanisms for homeothermic animals. Several serine and metalloprotease have been purified and characterized from extracellular products of *A. hydrophila* (Leung and Stevenson, 1988; Rivero *et al*., 1990, 1991; Rodriguez *et al*., 1992; Loewy *et al*., 1993) which degrades casein but not elsatlin. The *A. hydrophila* genes *Ahp* A and *Ahp* B, which encodes a 68 kDa thermolabile serine protease and 38 kDa thermostable protease, respectively, have also been cloned and efficiently expressed in different bacteria (Rivero *et al*., 1990, 1991). 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).

*Aeromonas* protease enzyme is mainly involved in the formation of induction and lesion in normal human. It was strongly suggested the involvement of protease in pathogenicity and the regulation of protease production by *A. hydrophila*. 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 aminoacid peptides, which converts the prohaemolysin into haemolysin (Nomura *et al*., 1999). The haemolysin and protease production were found more frequently in the clinical strains of *A. hydrophila*, which is important in colonization through the disruption of the intestinal barrier (Sechi *et al*., 2002).

Protease are thought to contribute the virulence of aeromonads for fish and other hosts. However, their contribution to human pathogenicity still needs to be determined (Gonzalez-Serrano *et al*., 2002). Considerable differences between the number, types and quantities of protease produced by aeromoands 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). Gonzalez-Serrano *et al*. (2002) reported that there was no interrelationship between proteolytic and haemolytic activities.
The extracellular protease and other components of those extracellular products of *A. hydrophila* are major antigenic components of a vaccine against haemorrhagic septicaemia (Allen and Stevenson, 1981). The *in vivo* expression of these antigens is recognized as an important parameter not only for understanding pathogenesis but also for the development of effective vaccines. The expression of the protease enzyme in the live attenuated vaccine may contribute to the superior protection afforded by these kind of vaccines (Vivas *et al.*, 2004).

**DNase enzyme production**

From numerous biological materials it has been detected that the DNase (deoxyribonucleases) enzymes have the capacity to degrade highly polymerized deoxyribonucleic acids (Weckman and Catlin, 1957). Most of the bacterial DNases resemble pancreatic DNase in the cation requirement and optimum pH level and it is distinguished on the basis of antigenic differences or differences in responses to specific DNase inhibitors (Catlin, 1956). DNase digests the collagen of subcutaneous tissue and muscle (Brooks *et al.*, 2001).

Production of DNase enzyme by cells may be constructive tools for taxonomic characterization of bacteria (Weckman and Catlin, 1957). DNase genes are common in most of the *Aeromonas* spp., and are responsible for encoding putative virulence factors to humans (Castro-Escarpulli *et al.*, 2003). Weckman and Catlin (1957) reported that high level of DNase activity was found in brain heart infusion cultures incubated with continuous shaking at room temperature for 40-72 h.

**Slime production**

A slime layer is a zone of diffusible unorganized material that can be removed easily from the surface of bacteria. A glycolgalyx is a network of polysaccharide extending from the surface of bacteria and could encompass both capsules and slime layers. Capsules and/or slime layers usually are composed of polysaccharides, but they
may be constructed of other materials. Slime is a viscous extracellular glycoconjugate that allows these bacteria to adhere to smooth surfaces, such as prosthetic medical devices and catheters. Extensive microbial growth, accompanied by excretion of copious amounts of extracellular organic polymers, thus leads to the formation of visible slimy layers (biofilms) on solid surfaces (Brooks et al., 2001).

Most of the human gastrointestinal tract is colonized by specific groups of microorganisms that give rise to natural biofilms. These biofilms provide protections from other pathogenic microorganisms, antibiotics and body's normal defense mechanisms. Thus the biofilm also provides a source of infection for other parts of the body as bacteria detaching during biofilm sloughing. Slime also appears to inhibit neutrophil, chemotaxis, phagocytosis and antimicrobial agents (Ananthanarayan and Panicker, 1999).

Bacteria secreting large amounts of slime produce mucoid growth on agar, with string consistency when touched with loop. It has the little affinity for basic dyes and is not visible in gram stained smears. Most of the diarrhoeal strains of *A. hydrophila* produced higher slime than the environmental strains (Sechi et al., 2002).

**Endotoxin production**

Endotoxin is the lipopolysaccharide component of outer membrane of the cell, which is toxic to man and animals. Endotoxin is much more heat stable than protein toxins, and consists of polysaccharide side chains, core polysaccharide and lipid A, which is the component confers toxicity to the complex (Altwegg and Geiss, 1989).

**Enterotoxin production**

The extracellular products may act on intestinal epithelium. The enterotoxins have been classified into two types.

*Cytotonic enterotoxin:* Cytotonic enterotoxins stimulate cyclic adenosine 3',5'-monophosphate (cyclic AMP) – mediated sequence of events in cells. Activation of
adenylate cyclase in the intestine stimulates salt and water secretion which leads to diarrhoea (Jiwa, 1983). *Aeromonas* adheres to the intestinal mucosa and produces a cytotoxic enterotoxin but does not invade intestinal cells. The profuse of watery diarrhoea may leads to death in severe condition.

*Cytotoxic enterotoxin*: It gives rise to cell damage or cell death, and produce dysentery like symptoms with the species of *Aeromonas* and other bacterial pathogens, ability to invade the cells is essential for pathogenesis, and diarrhoea associated with these infections usually contains blood and mucus (Cahill, 1990). In about 20% of infections with enteropathogenic strains of *Aeromonas* are dysenteric symptoms (Gracey *et al.*, 1982). These cytotoxic effects of *Aeromonas* enterotoxins have been studied by exposing various tissue culture cell lines to a cell free supernatant fluids of *Aeromonas* cultures (Kindschuh *et al.*, 1987)

*Cytotoxicity of A. hydrophila on cell lines*

Unlike most of other gram negative organisms, aeromonads produce a wide range of extracellular toxins and enzymes. The multiplicity of extracellular products of motile *Aeromonas* have led to difficulty in characterizing these factors and to disagreement about their properties, as well as about their direct involvement in the enteropathogenicity of *Aeromonas* spp. This has resulted in a great deal of controversy and confusion. However, such extracellular products are biologically active compounds such as enterotoxins, cytotoxins, haemolysins and proteases that have been reported to be produced by motile aeromonads and their pathogenicity have been extensively studied (Donta and Haddow, 1978; Cumberbatch *et al.*, 1979; Allan and Stevenson 1981; Johnson and Lior, 1981; Stelma *et al.*, 1986). However, relatively little is known about the relationship between enterotoxin, haemolysin and cytotoxin of motile *Aeromonas* isolates. The parallel activity of these three factors has been reported by Donta and Haddow (1978) and Cumberbatch *et al.* (1979).
For many of the enteropathogenic bacteria, the ability to adhere the intestinal mucosa is a first step in the colonization and development of diseases. It has been reported that the *Aeromonas* strains usually adhere to the intestinal mucosa, followed by colonization and production of cytotoxins (Krovacek *et al.*, 1991) which results in cell damage and cause dysentery like illness. Gracey *et al.* (1982) reported that 20% of the gastrointestinal infections due to *A. hydrophila* are of the dysenteric type. Furthermore, it has been widely reported that the majority of clinical isolates of *Aeromonas* produce cytotoxins (Johnson and Lior, 1981; Janda *et al.*, 1983; Gosling, 1996). Motile *Aeromonas* spp. has other properties that have been reported to be associated with virulence, such as adherence and invasiveness. The attachment of enteric pathogens to the intestinal mucosa is an essential step in the pathogenesis of gastrointestinal infection.

Vero cells are fibroblast like cells. Their source is the kidney of a normal adult African green monkey. The cytopathic effects (CPE) starts to shatter the rounding cell and aggregation of cell monolayer due to infection (toxicity) of *Aeromonas*. Infected cell monolayer gradually developed histological evidence of cell damage, as newly formed virion spread to involve more and more cells in culture. After 24 hours of infection, cells were gradually started to change in shape to produce CPE. CPE was characterized by granularity in cytoplasm, rounding of infected cells, development of micro plaque, clustering of infected cells, intracytoplasmic bridge connecting those clusters, vacuolization in the cell system and the formation of syncytia, an irregular shaped eosinophilic intracytoplasmic inoculation. A large number of clear syncytia were observed after about 30 to 40 hours of infection which were followed by the formation of multinucleated giant cells (Holmeberg and Farmer, 1984).

The important virulence factors of the bacteria are the enterotoxin which are responsible for gastroenteritis, the major enterotoxin is either cytolytic beta-haemolysin or aerolysin. It has been shown to cause fluid accumulation in infant mice and rabbit ileal loops (Sanyal *et al.*, 1975). It has been demonstrated that elaboration of
enterotoxins by different strains of *Aeromonas* may cause disease through different mechanisms (Holmeberg and Farmer, 1984; Sha *et al.*, 2002). Schiavano *et al.* (1998) reported that cytotoxin production was the most common virulence factor compared to adhesive and invasive ability.

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

Colonization and persistence of live bacterial pathogens in host tissues can be related to the interactions between different host factors and bacterial virulence factors (Casadevall and Pirofski, 1999, 2003). Serum resistance is considered to be bacterial virulence factor, because it endows the bacteria with the ability to avoid the bactericidal activity of host specific blood serum components, resulting in severe infections (Roantree and Rantz, 1960). It is known that the culture conditions (e.g., culture medium, incubation temperature) used to grow bacterial strains can modulate the expression of multiple virulence factors including serum resistance (Griffiths and Lynch, 1989), as demonstrated for *A. hydrophila* and *A. salmonicida* (O’Rielly and Day, 1983; Merino *et al.*, 1994; Garduno and Kay, 1995). Several genospecies such as *A. hydrophila* (HG1), *A. veronii* biovar *sobria* (HG3), but rarely *A. caviae* (HG4) are resistant to normal human serum (NHS) (Janda *et al.*, 1984; Rolston, 1988; Singh and Sanyal, 1993). Modification of culture conditions can also increase the immunogenicity of killed and live vaccine (Nitzan *et al.*, 2004).

Perhaps more appropriate terms for resistance could be bactericidal activity of the serum. Immunological research on such bactericidal serum activity often focuses on the components that can affect the viability of the pathogens inside the host, such as antiproteases, lysozyme, antibodies and complements (Bayne and Gerwick, 2001). Microbiological studies on serum resistance often focus on bacterial components that confer resistance, such as surface proteins. Such type of proteins inhibit the binding or activation of complement (Kubens *et al.*, 1998; Cirillo *et al.*, 1996; Jarva *et al.*, 2003).
These same mechanisms have been related to the serum resistance of several fish and human bacterial pathogens, including gram negative (Trust et al., 1981; Davies, 1992; Merino et al., 1994) and gram positive bacteria (Barnes et al., 2004).

Serum resistance is an important 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. It is demonstrated that in vitro resistance of A. hydrophila mutant to serum can be modulated by the growth in TSB and BHIB medium, it favored as indicated by a decrease in complement consumption and an increase in survival in non immune serum showing only basal titers of agglutinating antibodies (Vivas et al., 2005).

**Molecular characterization of A. hydrophila**

Epidemiological studies of Aeromonas infections require the use of different molecular markers to trace precisely the diffusion of strains. Phenotypic and genotypic techniques have been used for typing of Aeromonas, which includes serotype, phage and bacteriocin typing, protein profile, lipopolysaccharide profile, plasmid profile analysis, restriction fragment length polymorphisms (RFLP) PCR, ribotyping, pulsed field gel electrophoresis and random amplification of polymorphic DNA (RAPD) PCR (recently called arbitrary primed polymerase chain reaction) and enterobacterial repetitive intergenic consensus sequence (ERIC) PCR (Millemann et al., 1996). When clinical strains are identified by molecular methods, A. caviae and A. veronii are more common than A. hydrophila (Abbott et al., 1998). Eventhough biochemical tests have been proved to be less accurate for the identification of Aeromonas (Abbott et al., 1998), still they are broadly used. Recently, Abbott et al. (1998) reported several biochemical schemes that can be useful for the phenotypic identification of Aeromonas spp. However, the use of these procedures in the clinical setting is difficult.
Knowledge of the distribution of *Aeromonas* genomospecies among environmental and clinical sources is essential for establishing the epidemiological pattern involved in human infection. DNA-DNA hybridization techniques is used as the proper method for identifying isolates at the genomospecies level. Therefore several approaches for the phenotypic characterization of the different genomospecies have been described (Abbot *et al.*, 1992; Oakey *et al.*, 1996). At least 15 genospecies or HGs related to 14 phenospecies have been validated (Carnahan and Altwegg, 1995). Presently 17 hybridization groups have been assigned (Minana-Galbis *et al.*, 2002). Of several scheme proposed by Abbott *et al.* (1992), hybridization technique seems to be very useful identifying *Aeromonas* strains (Janda *et al.*, 1996). Recently, biochemical methods are not reliable for identifying the *A. hydrophila* (Borrel *et al.*, 1997; Figueras *et al.*, 2000; Kozinska *et al.*, 2002; Castro-Escarpulli *et al.*, 2003).

**Outer membrane proteins (OMP) of *A. hydrophila***

Generally, Gram negative bacteria are bounded by two membranes. The outer membrane consists of phospholipids, lipopolysaccharides and lipoproteins and integral outer membrane proteins. Recently much progress has been made in the elucidation of the mechanisms of outer membranes. Proteins present in the outer membrane are of either two classes; lipoprotein, which are anchored to the outer membrane with a N-terminal lipid tail, and integral proteins will be further referred to as outer membrane proteins (OMPs). It is a highly specialized structure that lies outside the cytoplasmic membrane and peptidoglycon layer forming the inter-surface between the cell and its external environment. Protein fingerprinting has been reported to correlate with phenospecies (Kresters and De Ley, 1990). Adherence and invasion to host cells have been shown to be important steps in the pathogenesis of enteropathogenic bacteria, including *Aeromonas* (Rocha-de-Souza *et al.*, 2001). Little is known about cellular and molecular mechanisms of *Aeromonas* spp. adherence and invasion in to host cells. Recent studies indicated that some filamentous structures,
LPS and OMP of pathogenic bacteria could be helpful in intestinal adhesions (Quinn et al., 1994; Merino et al., 1998; Kirov et al., 1999).

Esteve et al. (1994) reported that O-serotyping could be used as a marker in the epidemiological investigation of *A. hydrophila*. In a study, they observed that all the epizootic *A. hydrophila* strains (O: 19, recovered from eels, respective of their geographical origins) have produced the protein with the molecular weight range between 36 and 45 kDa. Delamare et al. (2002) also reported that several *Aeromonas* spp. have exhibited characteristic proteins which may be useful markers for the identification of *Aeromonas* at species level. The presence of species–specific OMP indicated that these immunogenic polypeptides could be exploited to develop antibody–based diagnostics for the identification of *A. hydrophila* (Maruvada et al., 1992). On contrary, a high variability in profiles of LPS and OMP have been described among *A. hydrophila* isolated from warm water fish (Aoki and Holland, 1985; Nomura and Aoki, 1985).

Gosling et al. (1993) recovered the cellular protein from *A. hydrophila*, in which both the 43 and 45 kDa of proteins responsible to produce cytotoxic enterotoxins were identified. This toxin is widely distributed among motile *Aeromonas* strains isolated from clinical, fish and environmental sources (Rodriguez et al., 1993; Perez et al., 1994). Maruvada et al. (1992) reported that *Aeromonas*, in general shared an immunodominant polypeptide at the 52 kDa position.

Millership and Want (1993) and Muller and Millership (1993) suggested that SDS-PAGE analysis of cell proteins could be a useful method for the identification of *Aeromonas* species. DNA-DNA hybridization along with whole cell 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 *A. hydrophila* from freshwater and drinking water samples (Hanninen, 1994). Kirov et al.
(1994) reported the predominance of HG1 in clinical samples, whereas the environmental isolates belonged to HG3. In a recent study, Szczuka and Kaznowski (2004) reported that all the clinical isolates tested were belonged to HG1.

In contrast, Alavandi et al. (2001) reported that whole cell fingerprinting and cluster analysis could neither differentiate the isolates from clinical and domestic water sources nor phenospecies of the genus Aeromonas. They 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 fingerprinting. Hence, whole cell fingerprintings 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.

**Lipopolysaccharide (LPS) of A. hydrophila**

Lipopolysaccharides (LPS) are essential components of bacterial endotoxin, which define many of the properties of host parasites interactions, and they are the immunodominant antigens of the most Gram negative bacteria (Altwegg and Geiss, 1989). Surface polysaccharides such as O antigen and capsule are considered as important cell surface components. The O-antigen polysaccharide is covalently ligated the lipid A core complex and extends outward from the cell surface. The capsule is an extracellular polysaccharide unit (Reeves et al., 1996). They act as prominent antigens and play important roles in the pathogenicity of many bacterial pathogens, such as protecting bacterial cells from complement mediated serum killing (Joiner, 1988; Merino et al., 1992) acting as adhesion factors (Merino et al., 1996) protecting the bacteria from the effects of desiccation and aiding survival in phagocytes (Smith et al., 1999).

The serogrouping of bacterial strains within a genus is determined by the structural variability of surface polysaccharides. Aeromonas strains have been serotyped on the basis of the O–antigen lipopolysaccharide (LPS) (Sakazaki and Shimada, 1984).
Serotyping in case of *Aeromonas* has been reported to be polymorphous (Leblanc et al., 1981). *A. sobria* was found predominant regarding reactivity to LPS, exhibiting LPS antigenicity mostly in the 65 to 20 kDa range, and it seems to provide evidence in support of previous report by Smith (1977). From an epizoological point of view, a serological study helps in the identification of disease-associated antigens. Homogenous O-polysaccharide of the lipopolysaccharides (LPS) and the S-layer protein have been considered and well elaborated (Dooley et al., 1985).

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 also exhibited differences in their LPS and membrane protein patterns irrespective of the species and the degree of virulence, and these results were confirmed by immunoblot. Esteve et al. (1994) reported that the LPS patterns were different depending on the serotype. Aoki and Holland (1985) and Nomuru and Aoki (1985) observed high variations in profiles of LPS and OMP among the water isolates of *A. hydrophila*.

The genus *Aeromonas* has been classified into 96 serogroups (Sakazaki and Shimada, 1984), and a role for surface polysaccharides in the pathogenicity of certain *A. hydrophila* strains has been proposed, for example the O-antigen lipopolysaccharide (LPS) of *A. hydrophila* O: 34 strains has been found to play an important role in adhesion to HEp-2 cells (Merino et al., 1996). The genetic and genomic organization of surface polysaccharides of *A. hydrophila* have not been studied well (Zang et al., 2002).
Temperature plays an important role in the production of two different forms of lipopolysaccharides (Rough and Smooth). 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 in strains of mesophilic A. hydrophila from 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 or high osmolarity medium and at 37 °C, only in high osmolarity medium, but 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 and this could be an useful method of identifying virulence markers of Aeromonas spp. Merino et al. (1992) also investigated on 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.

Santos et al. (1996) found that some virulent strains synthesized O-polysaccharides of heterogenous chain length and it was in contrast to the previous report by Dooley et al. (1985). Santos et al. (1996) also reported that the structural diversity among the LPSs belonged to the serogroups O:3 and O:19, while the immunoblot assay demonstrated that these serotypes were immunologically homogenous. On the basis of the results obtained by Leblanc et al. (1981) and Santos et al. (1996), they considered that neither serotyping nor LPS patterns could be used as criteria to differentiate virulent and avirulent strains of motile Aeromonas. Based on the presence of species-specific immunoresponsive polypeptides as defined by Maruvada et al. (1992), we can develop a method, which may be helpful in the detection and diagnosis of aeromonads of different origin.
DNA based analysis

The application of DNA primers/probes in pathogen detection and identification has made a significant impact on the development of such rapid diagnostic methods. Standardization and validation of these methods, however, has in most cases not been addressed (Hiney and Smith, 1998). Many bacterial pathogens can now be detected in samples of various kinds without the need to culture the organism. PCR methods are not only specific and quick, but they also can lead to the detection of 'nonculturable' bacteria (Brauns et al., 1991). PCR and in situ hybridization methods are currently being developed for the detection of pathogens. These include tests to identify pathogens such as *Renibacterium salmoninarum* (Brown et al., 1994), *A. hydrophila* (Cascon et al., 1996), *A. salmonicida* (Hiney et al., 1992), *Vibrio anguillarum* (Hirono et al., 1996), *Photobacterium damselae* subsp. *piscicida* (Aoki et al., 1995, 1996) and many other species are under development.

PCR is used for the identification of pathogens in tissue, blood, water and sediment samples due to its high sensitivity and specificity. PCR can be performed as a single or nested assay and these products can be identified using a variety of methods. Each assay has been optimized and the methods have been selected according to the type of pathogen and kinds of samples. Quantification is also desired so that upward trends in pathogen titer can be detected.

Since *A. hydrophila* has been recognized as a significant opportunistic pathogen for humans, many efforts were made to find out suitable methods for a good molecular typing system. In most of the cases, the origin of the infection is suspected to be environmental, but the causative strain is only rarely isolated, despite intensive screening and the utilization of highly discriminatory typing methods. Phenotypic methods used to characterize *Aeromonas* spp. include biotyping, serotyping, esterase electrophoresis, radiolabelled cell proteins and outer membrane proteins (Stephenson et al., 1987; Kuijper
et al., 1989; Carnhan et al., 1990; Kuhn et al., 1992). Genotypic typing methods have been applied to these species; plasmid analysis is not helpful because plasmid carriage is infrequent (20 – 58 %) in A. hydrophila (Janda, 1991). However, all the above techniques are time consuming, expensive and labour intensive and are not able to provide reliable and rapid results on the routine basis. Rapid, simple and cheap methods are desirable for hospital based laboratories and PCR techniques have been applied successfully to the epidemiological typing of various bacterial species. Random amplification of polymorphic DNA (RAPD) is based on the amplification of random DNA segments with a single primer of an arbitrary nucleotide sequence to amplify genomic DNA in a low stringency inherent, the patterns generated by RAPD may be affected by experimental parameters and standardization is crucial (Williams et al., 1990). RAPD has been used widely for epidemiological investigations of numerous bacterial species (van Belkum et al., 1994). Previous investigations on the reproducibility of RAPD allowed the standardization of parameters for routine use (Abed et al., 1995; Davin-Regli et al., 1995). An alternative approach based on families of short and repetitive sequences, such as enterobacterial repetitive intergenic consensus sequence (ERIC), has been used by Versalovic et al., (1991) in a genomic fingerprinting method called ERIC-PCR. ERIC sequences represent an intergenic, highly conserved and dispersed DNA sequence that has been observed in many bacterial species. Consensus primers complementary to each of the repeated sequence are oriented such that PCR amplification of DNA sequences proceeds between adjacent repeated ERIC elements.

**RAPD and ERIC PCRs**

DNA polymorphism in genomic fingerprinting generated by RAPD markers can distinguish between strains of all bacterium. RAPD analysis has been applied previously for the typing of A. salmonicida subsp. salmonicida and a few strains of A. hydrophila by Miyata et al. (1995). Although this technique is highly reproducible and discriminatory, they are not widely used as they are time consuming, tedious and
technically complicated (Liu et al., 1999). The combined RAPD and ERIC PCRs on *A. hydrophila* was first reported by Davin-Regli et al. (1998) and demonstrated great heterogeneity among the clinical and environmental strains of *A. hydrophila*. They also reported both the RAPD and ERIC PCRs are the most powerful tools in the epidemiological and pathological investigation of *A. hydrophila*. Also, it is reliable for the strain and species differentiation. But Alavandi et al. (2001) reported that RAPD–PCR could not be used in the identification of *Aeromonas* of clinical origin. In recent reports, Szczuka and Kaznowski (2004) and Aguilera-Areola et al. (2005) also confirmed the same. The report of Oakey et al. (1999), RAPD PCR band identity would be the hybridization experiments or direct sequence comparisons of cloned fragments from single RAPD – PCR bands and helps to find out the genetic relationships and to be useful for classifying a number of bacterial species (Medico et al., 1996).

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

PCR based method is rapid, sensitive and specific for the detection of virulence factors of *Aeromonas* spp. It overcomes the time consuming biochemical and other DNA based methods (Bin-Kingombe et al., 1999). As virulence in *Aeromonas* is certain to be multifactorial, the PCR approach developed in these studies has value in characterizing *A. hydrophila* (Sen and Rodgers, 2004). A similar approach has been used by other workers to detect one or more virulence gene in *Aeromonas* (Gustafson et al., 1992; Shibata et al., 1996; Wang et al., 1996; Khan et al., 1999; Bin-Kingombe et al., 1999; Heuzenroeder et al., 1999; Biscardi et al., 2002; Gonzalez-Serrano et al., 2002; Soler et al., 2002; Sechi et al., 2002; Sen and Rodgers, 2004). However, a complex virulence mechanism has been involved in the pathogenicity (Aguilera-Areola et al., 2005), among them, the expression of the *act* gene was responsible for cytotoxic enterotoxin (Janda, 2001)
The cytolytic virulence gene *act* encodes the cytotoxic enterotoxin (Act) to hosts cells and it is one of the main factors for causing diarrhoeal infections (Albert et al., 2000). Sen and Rodgers (2004) reported that three duplex PCR assays were used to analyse for cytotoxic enterotoxin (Act) potential virulence gene and also identified the cytotoxic enterotoxin (*act*) gene in the diarrhoeal, fish, milk isolates. Studies of Sha et al. (2002) revealed that three enterotoxin genes were selected as targets because the cytotoxic enterotoxin, act / hlyA / aerA and the cytotoxic enterotoxins, alt and ast have all been implicated as important virulence factors in diarrhoeal disease.

The distribution of the *act* gene was wide amongst the diarrhoeal and environmental isolates, but an amplified product from extra intestinal subpopulation could not be obtained. Similarly, other virulence-associated *aexT*, *ascV* and *alt* genes do not seem to have a relevant role during an extraintestinal infection, other virulence determinants are probably involved in specific adherence to other kinds of epithelial host cells (Sha et al., 2002).

The overall interest of this study is to determine the prevalence, multidrug resistance and pathogenicity of *A. hydrophila* isolated from diarrhoeal stool samples, since it causes gastroenteritis to children. Reports on the occurrence of gastroenteritis in Tamil Nadu are high among children and the incidence due to *A. hydrophila* is not known except at Chennai and Vellore. Clinical and environmental *A. hydrophila* isolates possess many virulence factors, which are responsible for the pathogenicity in human and animals. The virulence within the genus *Aeromonas* might be a clonal and only few clones may be responsible for progressive disease. However, there have been no reports determining clonal structure within *Aeromonas* and the spread of specific clones in human population and in the environments. The diversity of virulence factors, lack of suitable technique with their detection, and their uncertainty of their clinical relevance have so far precluded the definition of a virulence factor-associated disease spectrum. These findings need to seek the identification of virulence genes in
A. hydrophila. The literature survey delineate the taxonomy of the genus Aeromonas is still confused. So there is need for the rapid identification and classification of Aeromonas is justified by their ecological and clinical importance. The molecular characterization assists to find out the clonal relatedness and genetic similarities of strains derived from diarrhoeal stool samples and environmental strains.