6. DISCUSSION
Diseases are caused only by a very small minority of bacteria. The host-pathogen interactions are very complex, involving combat between host defenses and the disease-causing ability of the invading organisms. *Aeromonas* is a genus of growing interest due to its pathogenicity for aquatic organisms, its potentially pathogenic effects in humans and its spoilage action in food (Janda and Abbott, 2010). *Aeromonas* was isolated in human feces for the first time in 1937 and only in 1980 it was recognized as pathogenic bacteria causing gastrointestinal diseases in human beings. The principal aim of investigating bacterial pathogenesis is to understand the way that pathogens interact with the host to cause disease. As there is no well-defined animal model for human-specific pathogens, it is difficult and impossible to conduct gene expression studies during real infections. Fish are considered to be an important model in comparative immunology studies, representing a unique link in the study of the immune system, which was chosen for our study. The outcome of host-pathogen interactions is dependent on the properties of microbial pathogen to establish infection and the ability of the host to control infection. It is necessary to understand the host factors that function to protect the host from infection or that are required by the bacterial pathogen for efficient infection. This current research was carried out to understand the *Aeromonas* spp. interaction with the common carp. From the results it is evident that *Aeromonas* establishes its pathogenesis in common carp, where it interferes with the immune gene regulation. Based on the findings of this current research, the results were compared and discussed as follows.

6.1 Identification of *Aeromonas* spp.

It is well known that aeromonads are ubiquitous and most of the species among them are opportunistic pathogens to cold and warm blooded animals. Their occurrence has been reported in numerous sources so far by many researchers. Based on this, sources like fish, soil and included in this study. Basic
enrichment, enumeration and preliminary identification methods were followed for the identification of *Aeromonas* spp., such as selecting yellow to honey coloured colonies on SAA and screening of catalase, oxidase positive isolates, before going for genotypic identification schemes. In order to avoid the false positive results by completely depending on the phenotypic tests, all the *Aeromonas* isolates used in this study were confirmed on the basis of amplification of housekeeping genes such as 16S rRNA and *rnpB* using specific primers and the expected amplicon size of 1050 bp and 400 bp were observed in all the isolates that showed positive for *Aeromonas* spp. in the presumptive identification tests. The conventional microbiological procedures for isolation and identifying *Aeromonas* spp. may be laborious and time consuming (ICMSF, 1996).

Conventional biochemical characterization may lead to misidentification of *Aeromonas* isolates due to lack of uniformity of some biochemical characteristics and atypical reactions. Although other conventional identification techniques such as serotyping (Janda, 2001), phage typing (Altweeg *et al*., 1988), and whole-cell protein electrophoresis (Stephenson *et al*., 1987) have been used, a lack of sensitivity to identify species exactly has also been reported. Similarly, a number of phenotypical identification methods such as multilocus enzyme electrophoresis, cellular fatty acid analysis and DNA-based methods such as ribotyping, amplified fragment length polymorphism study, randomly amplified polymorphic DNA analysis have all been reported to have some handicaps to discriminate all *Aeromonas* species (Ghatak *et al*., 2007). Martinez-Murcia and co-workers (1992) have reported that rDNA signatures might be useful to differentiate most *Aeromonas* species. In another study the *gyrB* gene has been considered to be useful as a target to identify species and strains simultaneously (Yanez *et al*., 2003). Among the DNA-based approaches for identification of *Aeromonas* species, the 16S rRNA gene RFLP in which different digestion combinations with restriction endonucleases were used has been shown to
be able to discriminate most Aeromonas species (Borrell et al., 1997; Figueras et al., 2000). The method performed by Borrell et al. (1997) has enabled identification of most Aeromonas spp.

As already discussed, although several conventional identification methods are available for Aeromonas, further confirmation of these organisms on the basis of 16S rRNA gene or screening of some other conserved regions such as rnpB, gyrB, rpoD rpoB etc., is necessary as Aeromonas are often misidentified as members of enterobacteriaceae. So, 16S rRNA and rnpB gene based identification/ribotyping can be a suitable method to characterize Aeromonas isolates which was adapted in this study. Kumar et al. (2009) isolated Aeromonas sp. from water samples and performed a 16S rRNA PCR for the bacterial identification and resulted in a 1453 bp of 16S rRNA product. In contrast the 16S rRNA analysis of the Aeromonas sp. resulted in the product of about 1050 bp. This variation is due to the conserved region for which the primer designed and set to amplify the expected product of 1050 bp.

In a study, PCR identification scheme was used to identify numerous clinical isolates Borrell et al., 1997. This suggests that 16S rRNA gene can be used as tool to aid in initial Aeromonas species identification. Sharma and Kumar (2011) used a total of 332 animal food samples (fish, poultry meat, pork and chevon) of which 11% isolates were identified as Aeromonas spp. by using the 16S rRNA gene based PCR.

Due to some false positive results while using 16S rRNA based identification, nowadays more specific signature region of Aeromonas species namely rnpB gene is used. Rubin et al. (2005) further stated that the 16S rRNA gene is quite large about 1500 bp and therefore may be laborious to sequence when full-length sequencing is necessary. Ribonuclease P (rnpB) gene was one among the housekeeping gene that is conserved region of any genome and specific for a particular organism. RNase P is present and essential in all cells and subcellular compartments that synthesize tRNA, but catalytic proficiency by the RNA alone has been demonstrated only for the
bacterial RNAs (and in all such RNAs tested). The structure of bacterial RNase P RNA has been studied in detail, primarily using comparative methods (Pace and Brown, 1995; James et al., 1988). Although recognizable RNase P RNAs are present in all organisms, they are more variable in both sequence and secondary structure than are the ribosomal RNAs and transfer RNAs. Bacterial and archaeal RNase P RNAs are more similar in sequence and structure than either is to the eukaryal RNAs (Brown and Haas, 1996; Haas et al., 1996; Chen and Pace, 1997). In the present investigation all the 16S rRNA positive isolates were subjected to \textit{rnpB} gene based identification, thinking that the 16S rRNA results may be false positive, which would have occurred due to some evolutionary changes. But contrast to that, all the 16S rRNA positive \textit{Aeromonas} isolates showed the amplification of \textit{rnpB} gene. These results made the current research even narrow.

Rapid and reliable identification of pathogenic microorganisms, including \textit{Aeromonas}, is important for surveillance, prevention, and control of food-borne diseases. The established methods for bacterial identification in clinical microbiology are often time-consuming and laborious. Time is required for purification and the identification of pathogenic bacteria subspecies by biochemical typing procedures also requires long incubation times, and therefore there is a delay in final identification. These procedures require experience in interpretation and can be limited by subjectivity and low specificity. There is an increasing need for alternative procedures that allow rapid and reliable identification of microorganisms (Dieckmann et al., 2008). The presence of species-related disease syndromes, coupled with differences in antimicrobial susceptibilities among the species, strongly suggests that conventional identification and antimicrobial susceptibilities should be determined for clinical aeromonad isolates (Perez et al., 2003). In this regard Akyar and Can (2013) developed a method, where a combination of CHROM agar \textit{Salmonella} Plus and
MALDI–TOF was used which helped to detect *Aeromonas* species in 24 h in a cost effective, practical and reliable manner.

### 6.2 Incidence of *Aeromonas* isolated from various sources

In the present investigation the prevalence of *Aeromonas* spp. was recorded in marine fish intestine, soil and clinical (blood, pus, urine and diarrhoeal) sources. A total of 212 samples were processed and about 42% showed positive for *Aeromonas*. Of the positive *Aeromonas* isolates, 74% were from fish, 51% from soil and 11% isolates were of clinical origin. Higher prevalence of *Aeromonas* spp. was observed in fish samples when compared to other samples used in this current research, which indicates the opportunistic nature of *Aeromonas* spp. and it was also a normal flora of fish intestine. It was reported that fish may also be a vehicle for pathogenic bacteria naturally occurring in aquatic environments referred to as indigenous or derived from polluted waters and or from postcapture contamination, storage and handling (Huss *et al*., 2003).

The incidence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture (Post, 1989; Zorrilla *et al*., 2003). Vivekanandhan *et al*. (2005) examined 536 samples of fishes and 278 prawn samples from the major fish market of Coimbatore, South India, over a period of 2 years for the presence of aeromonads (reported as *A. hydrophila*) and the results were varied from 18% in prawns to 33.58% in fishes. Radu *et al*. (2003) isolated 69% of *Aeromonas* spp. from market fish samples. Oliveira *et al*. (2012) obtained 114 *A. hydrophila* isolates from fish. Ottaviani *et al*. (2006) isolated 32 *Aeromonas* strains from 144 mussel samples used in their study and 12 of them showed virulence and enterophatogenicity on mice. Erdem *et al*. (2010) isolated 65% of *Aeromonas* spp. from fish samples. These reports confirm the presence aeromonads among fish, soil and clinical sources which justifies the sources preferred in isolating the aeromonads in present study. In the present study about 74% of *Aeromonas* spp. was isolated from fish.
A report given by WHO in 2002 stated that *Aeromonas* spp. are microbial etiological agents of diarrhoea particularly in developing countries, where diarrhoeal diseases constitute a very important cause of morbidity and mortality among children and young adults. In the current research clinical samples such as diarrhoeal, blood, urine and pus were used for recording the incidence of *Aeromonas* spp., in which 11% of the samples were found to be contaminated with aeromonads. The prevalence of *Aeromonas* spp. among clinical isolates were found to less when compared with other sources. This was supported by Oberhelman and Taylor (2000), who reported that the isolation rate of *Aeromonas* in many developing countries may range from 5 to 28% in clinical isolates.

Ramalivhana *et al.* (2010) obtained 17% of *Aeromonas hydrophila* isolates from stool samples and water samples collected in different municipalities in Limpopo province, South Africa. Similarly, the occurrence of *A. hydrophila* in acute gastroenteritis among children was reported in the Coimbatore region, Tamil Nadu by Subashkumar *et al.* (2004), where the clinical isolates were collected in the present study. Of the 216 samples they collected, (10%) were positive for *A. hydrophila*.

A recent study conducted by John and Hatha (2013) revealed the presence of motile *Aeromonas* spp. in ornamental fish samples and associated carriage water samples. One hundred and seventy five isolates from the fish samples and 182 isolates from the water samples were to species level, *Aeromonas sobria* predominated in both cases - 41% in fish samples and 35% in water samples. *A. caviae* was the second dominant spp. in both samples but its percentage of occurrence was much higher in fish samples, (31%), when compared to water samples (17%). *A. hydrophila*, *A. jandaei*, *A. schuberti* and *A. veronii* predominated in water samples when compared to fish samples. This variation is may be due to the host specificity and sensitivity.

According to our present research, a total of 97 clinical samples were collected and screened for the presence of *Aeromonas* from February, 2011 to March, 2012, in
which we could record 11% occurrence of *Aeromonas* spp. Various clinical sources such as urine, pus, blood and diarrhoeal samples were screened in the present study. The prevalence of *Aeromonas* was recorded high among the diarrhoeal samples.

Aeromonads are primarily aquatic organisms occurring naturally in different freshwater bodies that include rivers, water streams and lakes. They are predominant in estuarine waters and easily isolated from seashores but not from deep sea. They also occur in raw sewage, treated sewage and activated sludge (Neilson, 1978). Pablos *et al.* (2009) screened for the presence of motile mesophilic *Aeromonas* in drinking water samples and they recorded 27% of incidence.

Joseph *et al.* (2013) screened for the occurrence of *Aeromonas* spp. in tropical seafood, aquafarms and mangroves of Cochin coast in South India and they recovered 11% of *Aeromonas* spp. by 16S rDNA sequence analysis. In the present study about 18% of *Aeromonas* spp. was recorded in soil samples (mangroves of Muthupettai region), which indicates aeromonads are ubiquitous in occurrence. The variability in the prevalence among West coast and east coast regions of South India may be due to many reasons like river flow and anthropogenic activities.

### 6.3 Distribution of virulence genes

The pathogenesis of *Aeromonas* infections is complex and multifactorial. Because no single virulence factor or defined combinations of these factors has been found to be clearly correlated to virulence among *Aeromonas* spp., the presence of certain accepted virulence factors should be monitored to investigate the pathogenesis and epidemiology of *Aeromonas* infections (Aguilera-Arreola *et al.*, 2007). To determine the distribution of virulent genes in *Aeromonas* spp., several PCR protocols have been used as speed and sensitive tool (Chuang *et al.*, 1997; Yu *et al.*, 2005). Here, the presence of four virulence genes (*aexT, ascU, hcp2* and *hyl*) among *Aeromonas* spp. was studied for using specific primers using PCR.
Aeromonas species produce an array of virulence factors including enterotoxins, haemolysin, exoenzymes, siderophores, flagella and secretion mechanisms (Castilho et al., 2009).

For the majority of diseases caused by bacterial pathogens, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor. However, there are correlations between strains isolated from particular diseases and expression of particular virulence determinants, which suggests their role in particular diseases (Shokrollah et al., 2009). The production of a wide array of virulence factors by Aeromonas sp. is indicative of their potential to cause diseases in humans.

Bacterial secretion systems are macromolecular complexes that release virulence factors into the medium or translocate them into the target host cell. These systems are widespread in bacteria allowing them to infect eukaryotic cells and survive or replicate within them. Oliveira et al. (2012) analyzed 114 A. hydrophila isolates and reported that, 100 isolates (87.72%) represented at least one of the virulence factors screened in their study. The virulence factors were widely distributed among the A. hydrophila isolates. Aerolysin was the most frequent virulence factor present in the isolates analyzed.

Several pathogenic bacteria use ADP ribosylation as a key mechanism to modify the properties of host cell proteins and thus to modulate their function and induce disease. Hence ADP ribosylation of eukaryotic regulatory proteins is the underlying pathogenic mechanism of a heterogeneous family of bacterial protein toxins. According to Braun et al. (2002), aexT gene, was shown to be important for A. salmonicida virulence in fish. The type three secretion system (T3SS) is considered to be a virulence trait that correlates with bacterial pathogenicity, and its presence can be used as a general indicator of virulence. A study conducted by Chacon et al. (2004) revealed that out of 25 clinical isolates, 80% was found to be conserved with aexT gene. In the present investigation, of the 88 isolates about 53% showed the presence
of aexT gene. About 47% is conserved in fish isolates, 100% in soil isolates and 9% in clinical isolates. Higher prevalence of aexT was recorded in soil isolates, which indicates the presence of virulent isolates in the environment. A very low number of aexT genes was found to be conserved in clinical isolates, when compared with the other isolates, which indicates the incidence of virulent Aeromonas spp. in the soil and fish samples was high among the study area than clinical sources.

Sierra et al. (2007) identified a novel T3SS effector, AexU, from a diarrhoeal isolate of Aeromonas hydrophila SSU, and demonstrated that mice infected with the ΔaexU mutant were significantly protected from mortality. Braun et al. (2002) detected the aexT gene in all of the twelve A. salmonicida subsp. salmonicida strains tested in their study. Yu et al. (2005) performed a study in which an ascU homologue, near one end of the T3SS, was identified in A. hydrophila PPD134/91 by PCR. The present investigation revealed the presence of 320 bp ascU gene (T3SS) in 26% of the total isolates screened. Among which fish, soil and clinical isolates were conserved with 17%, 61% and 9% of ascU gene, respectively.

Ebanks et al. (2006) revealed the temperature-induced up-regulation of the T3SS in A. salmonicida A449 which occurs within 30 min of a growth temperature increase from 16 to 28 °C. They moreover shown that growth at 28 °C followed by exposure to low calcium results in the secretion of one of the T3SS effector proteins. The Aeromonas isolates possessing both effector (aexT gene) and regulatory (ascU) genes of about 8%, 61% and 9% of respective fish, soil and clinical isolates could be pathogenic and they may be capable of using T3SS machinery to deliver the virulence proteins and initiate damage in the host. Even if aexT gene is present without ascU gene, it cannot trigger the pathogenecity in the absence of ascU gene so it will remain non functional.

Type VI secretion systems (T6SSs) are macromolecular machines spanning the cell envelope in a large number of Gram-negative bacteria. Type VI secretion
system has been implicated in the virulence of several human, animal and plant pathogens. The *hcp* and *vgrG* genes are also often located within T6SS loci and encode proteins that can be exported extracellularly (Pukatzki *et al.* 2006).

Cascales (2008) observed that the deletion of *hcp* or *vgrG* prevents secretion of the other proteins. Importantly, both Hcp and VgrG proteins represent a hallmark of the T6SS secreted proteins in all of the bacteria that possess this system (Bingle *et al.*, 2008). There are several pieces of experimental evidences which suggest that Hcp and VgrG have dual functions, they are not only secreted proteins but also might act as machine components.

In the present study it was found that the *hcp2* gene of about 519 bp belonging to T6SS apparatus was found to be conserved in 19% of the isolates. Among which fish, soil and clinical isolates were conserved with 7%, 67% and 9%, respectively.

Further the isolates were also found to be conserved with *hyl*, the gene responsible for haemolytic activity. Screening of virulence gene *hyl* (haemolysin encoding) in *Aeromonas* isolated from fish, soil and clinical isolates made us to know about the pathogenic ability of this bacterium. Among 88 isolates about 70% showed the presence of the *hyl* gene with clear band at 550 bp in which 56%, 100% and 100% from fish, soil and clinical samples, respectively. The β-haemolytic gene has been detected in pathogenic strains of *A. hydrophila* using PCR. The research conducted by Xia *et al.* (2004) indicated that β-haemolysin-specific PCR might be useful to detect the virulent or pathogenic *A. hydrophila* in a limited condition.

Based on the virulence gene profile, all the four virulence genes (*aexT*, *ascU*, *hcp2* and *hyl*) were found to be conserved in 2%, 50% and 9% of the respective fish, soil and clinical isolates which helps us in identifying the more virulent isolates. It is evident that virulence factors are highly conserved and virulent isolates were found in the soil isolates. The absence of virulence factors in some of the isolates means, it was
not necessary for the gene to be functional in all the environmental conditions as regulation of all these genes are completely based on the availability of appropriate substrate. The results also reveal that the isolates posses the virulent protein gene, but the machinery for host infection was lacking in most of the isolates, which makes them less virulent. In the current research, the virulence factor-encoding genes might be differentially expressed in *Aeromonas* species depending on the environmental conditions such as water, soil, fish or the human host.

6.4 Haemolytic activity of *Aeromonas* isolates

Haemolysins are important virulence factors for several Gram-negative species, and detection of haemolytic activity in *Aeromonas* isolates from stool specimens may have clinical implications. β-haemolysin has been reported as a virulence factor in motile aeromonads (Majeed and MacRae, 1993; 1994). The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads (Turnbull *et al*., 1984).

Orozova *et al*. (2010) isolated *Aeromonas* spp. from drinking water samples and they reported that 60% of water isolates and 90% of clinical isolates possess haemolytic activity. Suhet *et al*. (2011) reported 57% hemolytic activity in the *Aeromonas* spp. isolates recovered from nile tilapia (*Oreochromis niloticus*). Pollard *et al*. (1990) and Mateos *et al*. (1993) reported that the virulence of *A. hydrophila* is closely related to β-haemolysin produced, and the β-haemolysin gene exists in all pathogenic strains isolated from humans.

The current investigation reveals the existence of haemolytic activity in 6% of the isolates with α-haemolysis, 52% showed β-haemolysis and 42% showed γ- or no haemolysis. Among that α-haemolysis was exhibited by 8% of the fish isolates and β-haemolysis by 44%, 100% and 18% of the fish, soil and clinical isolates, respectively. Whereas, 48% and 82% of fish and clinical isolates showed γ-
haemolysis and none of the soil isolates exhibited α- and γ-haemolysis, which indicates that the soil isolates obtained in this study are highly virulent. Since most *Aeromonas* haemolysins can be detected by using rabbit erythrocytes, we used only these RBCs. Haemolysis of the isolates could be a strong indicator of enterotoxigenicity by *Aeromonas* spp. in humans.

This work is further supported by Rahman *et al.* (2002) where they used 14 *Aeromonas* isolates obtained from fish, 26 soil isolates and 12 human diarrhoeal isolates and observed the haemolytic activity of about 100%, 69% and 50%, respectively.

The isolates of *A. hydrophila* and *A. jandaei* screened by John and Hatha (2013) were found to be capable of producing β-haemolysins. The results have variations with regard to the earlier reports, where equal distribution of α- and β-haemolytic activity among the *A. hydrophila* isolates from fish samples in India, has been reported (Illanchezian *et al.*, 2010). This reveals that the fish isolates of present research were highly virulent due to the higher prevalence of β-haemolytic activity.

### 6.5 Proteolytic activity

Proteases are a group of enzymes that hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. Sources of proteases include all forms of life, plants, animals and microorganisms. There are many reports describing the numbers and different nature of proteases found in the culture supernatants of *A. hydrophila*. Proteases are important factors in the spoilage of foods, and the presence of proteases and haemolysins are used as an indicator of potential pathogenicity (McMahon, 2000).

In the present study protease activity was found in 61% of the *Aeromonas* isolates which constitutes about 51% of the fish isolates, 100% of soil and 54% of the clinical isolates. As the presence of proteolytic activity is associated with the
pathogenesis of *Aeromonas* spp., from the results it is clear that virulent *Aeromonas* isolates are widely distributed in most of the samples used in this study, with high prevalence among the soil isolates. The presence of proteolytic activity among the isolates makes them highly virulent, which could exhibit a strong pathogenesis inside the host.

Austin and Adams (1996) suggested that the proteolytic activity of *A. hydrophila* can be correlated with its ability to induce pathogenesis in fish. Proteases are thought to contribute to the virulence of aeromonads for fish and other hosts. Hsu *et al.* (1981) observed that *A. hydrophila* produces a wide range of proteases, which play an important role in the invasiveness and establishment of infections, by overcoming the initial host defenses, and by providing nutrients for cell proliferation. Cho *et al.* (2003) purified extracellular protease, from *Aeromonas hydrophila* Ni 39, with an overall yield of 20%.

Pandey *et al.* (2010) reported that *Aeromonas hydrophila* strain An4 exhibited significant protease production in the form of clearance zone and liquefaction. This pathogenic isolate revealed the commencement of protease production after 18 h and with maximum level of production around 22 h of incubation. Presence of proteolytic activity is a part of pathogenesis in most of the pathogens; in this regard a future research is needed to be carried out in this field to detect the nature and type of proteases and to determine its actual role in pathogenesis.

### 6.6 Antibiotic sensitivity

In recent years development of resistant or multidrug resistant pathogens has become a major problem in India and many countries (WHO, 2013). Bacterial resistance is closely associated with the use of antimicrobial agents in clinical practice. Prolonged therapy with antibiotics can lead to the development of resistance.
in a microorganism which was initially sensitive to antibiotics. The aeromonads have been regarded as universally resistant to penicillins (penicillin, ampicillin, carbenecillin and ticarcillin) for quite a long time. Calomiris et al. (1984) and Poiata et al. (2000) isolated microorganisms that are resistant to both antibiotics and metals from nosocomial and burn wound infection.

The multiple drug resistance mechanism is attributed to several local selective stresses such as temperature, pH, habitat and frequent exposure to the antibiotic residues. Moreover, horizontal gene transfer of R-Plasmids between the species has been identified as one of the key factors for the spread of drug resistance.

In the present study all the Aeromonas isolates were tested against a panel of 10 antibiotics. The utmost resistance was found to be developed against cephalothin by the isolates and least resistance was shown towards aztreonam. All the isolates were found to be sensitive to chloramphenicol and gentamicin. The isolates exhibited 29 different types of multiple antibiotic resistance patterns. The ASR55 isolate obtained from fish showed resistance towards a maximum of seven antibiotics.

Multidrug resistance was shown by most of the isolates. With regard to amoxyclav, cefpodoxime, cephalothin and vancomycin, higher frequency of antibiotic resistance was recorded among the clinical isolates. All the isolates were found to be sensitive to chloramphenicol and gentamicin.

MAR index value higher than 0.2 is considered to have originated from high risk sources of contamination like human, commercial poultry farms, swine and dairy cattle where antibiotics are very often used. MAR index value of less than or equal to 0.2 considered as the origination of strain from animals in which antibiotics are seldom or never used (Krumpelman, 1983).

The MAR index in the range of 0.2 to 0.3 was shown by 41%, 28% and 37% of the fish, soil and clinical isolates, respectively. About 46%, 67% and 45% of the
respective fish, soil and clinical isolates showed the MAR index ranging from 0.4 to 0.5. Very few isolates showed the MAR index ranging from 0.6 to 0.7, which includes 3%, 6% and 18% of the fish, soil and clinical isolates, respectively. In the present study the MAR index value of *Aeromonas* isolated from various sources were mostly > 0.2, which suggests that the isolates have originated from high-risk source of contamination. About 10% of the sensitive isolates were recorded among fish samples. None of the soil and clinical isolates showed MAR index of < 0.2.

In the present investigation 77% of the *Aeromonas* isolates showed resistance towards amoxyclav, 4% towards aztreonam, 67% towards cefpodoxime, 79% towards cephalothin, 6.8% against rifampicin, 10% towards streptomycin, 31% towards tetracycline and 77% towards vancomycin.

Rogo *et al.*, (2009) recorded 100% resistance to ampicillin, cephalothin, streptomycin and tetracycline and 50% resistance to gentamicin in the isolates obtained from stool sample. Petersen and Dalsgaard (2003) found that most of their *Aeromonas* strains were resistant to commonly used antibiotics such as chloramphenicol, tetracycline and trimethoprim. In contrast to their reports, all the isolates of clinical as well as fish and soil sources used in the current research were found to be sensitive to gentamicin and chloramphenicol. This variation is may be due to less exposure of the isolates to these antibiotics.

Allen *et al.* (2010) stated that bacterial pathogens that are capable of infecting both humans and animals are considered as major offender for the origin of antibiotic resistance in the environment. Previous researchers have also documented, multiple resistance in aeromonads (Vivekanandhan *et al.*, 2002). More than 50% of the strains of *A. hydrophila* and *A. caviae* isolated from freshwater fishes of Kolkata, India exhibited MAR (Abraham, 2011).
The antimicrobial resistance test conducted by Suhet et al. (2011) showed that all *Aeromonas* spp. used in their study were resistant to amoxicillin and offered variable resistance to erythromycin.

Singer et al. (2006) documented that the antibiotic resistance research has typically been very disease focused. O’Brien (2002) reported that the resistance can either arise from mutations in genes native to the chromosome of the bacterial species or by transferable genetic elements such as plasmids. Guz and kozinska (2004) in their study reported that all the *Aeromonas* strains used in their study were resistant to ampicillin and penicillin and 43% of the strains were susceptible to cephalothin.

Vivekanandhan et al. (2002) reported only 3% of chloramphenicol resistant *A. hydrophila* in marketed sea foods. This evidence supports the current research results, in which all the *Aeromonas* isolates were susceptible to chloramphenicol. This shows that the use of chloramphenicol was found to be less among the study areas.

The *Aeromonas* isolates screened by Joseph et al. (2013) exhibited varying degrees of resistance to vancomycin (87%), ampicillin (47%), nalidixic acid (20%), tetracycline (7%), co-trimaxozole (7%) and rifampicin (7%) and were susceptible to antibiotics like gentamicin, streptomycin, trimethoprim, azithromycin, cefixime and chloramphenicol. And about 20% of their *Aeromonas* sp. showed MAR index > 0.2 indicative of the high risk environment.

The *Aeromonas* isolates tested by John and Hatha (2013) were found to be resistant to amoxycillin and sensitive to ciprofloxacin, chloramphenicol and gentamicin regardless of their source. While all the *Aeromonas* isolates from water were sensitive to nitrofurantoin, 1% of isolates from fish samples was resistant to nitrofurantoin. Resistance to ceftazidime was found in 13% of *A. veronii* and 6% of *A. sobria* isolates from fish samples and 8% of *A. veronii* isolates from water samples.

6.7 Minimum Inhibitory Concentration of metals
Although some heavy metals are important and essential trace elements, at high concentrations most of them can be toxic to microbes. Silver et al. (1989) reported that most of the bacteria developed resistance mechanisms in order to survive the high concentrations of metals in the environment. Miranda and Castillo (1998) isolated antibiotic and metal resistant Aeromonas isolates from polluted and unpolluted waters.

Odeyemi and Ahmad (2013) reported that the Aeromonas isolates obtained from marine sources in Kuala Sepetang region, Malaysia showed high rate of resistance against lead, chromium, copper and manganese with minimum inhibitory concentration (MIC) ranging from 2560 μg/mL to 5120 μg/mL. Paniagua et al. (2006) isolated A. hydrophila strains from the gastrointestinal tract of the charal (Chirostoma humboldtianum), an autochthonous Mexican fish, in which all the strains were resistant to lead (MIC=800-3200 μg/mL) and susceptible to chromate (MIC≤ 375 μg/mL), silver (MIC≤ 2.5 μg/mL), mercury (MIC=20 μg/mL) and zinc (MIC≤ 42.5 μg/mL).

Matyar et al. (2010) isolated Aeromonas spp. and Pseudomonas spp. from the water samples of Iskenderun Bay, Turkey (northeast Mediterranean Sea) and studied the antibiotic and heavy metal resistance species. The MICs of metals for the isolates ranged from 25 to >3,200 μg/mL. The Aeromonas isolates showed a higher resistance to copper and chromium than did the Pseudomonas isolates, and the Pseudomonas isolates showed a higher resistance to cadmium, lead, manganese and zinc than did the Aeromonas isolates. Tolerance to the maximum MIC (>3,200 μg/mL) for copper was 2.1% for Aeromonas and 1.6% for Pseudomonas isolates.

In the present study, the MIC of silver nitrate was observed at concentrations 0 μM, 10 μM, 30 μM, 50 μM, 100 μM, 150 μM, 200 μM and. At 250 μM concentration of silver nitrate, none of the isolates showed resistance. At 200 μM concentration of silver nitrate 14% of the isolates were found to be resistant, in which
11%, 18% and 15% from fish, soil and clinical isolates, respectively. The resistant isolates observed at 150 µM concentration of silver nitrate are of 49%, which was contributed by 52%, 50% and 33% of fish, soil and clinical isolates, respectively. About 90%, 95% and 100% of isolates from fish, soil and clinical isolates showed resistance at 100 µM concentrations, respectively. At 50 µM concentration, growth was observed in 98% isolates, 92% in 100 µM, 49% in 150 µM and 14% in 200 µM of silver nitrate concentration. At 10 µM and 30 µM silver nitrate concentrations, 100% of the isolates obtained from diverse sources used in this study were found to be resistant.

This analysis reveals that the minimum inhibitory concentration of silver nitrate towards the *Aeromonas* isolates used in this study is 250 µM. Among the isolates screened the clinical isolates have shown constant resistance till 150 µM, which indicates the prevalence of high silver resistance isolates from clinical source.

The reason may be due to the fact that no periodical monitoring of environmental quality testing set for drug residues like the permissible standards prescribed by the national environmental quality assessment system.

Minimum inhibitory concentration for copper sulphate was evaluated using LB agar medium supplemented with 0 mM to 7 mM of copper sulphate concentrations. None of the isolates exhibited resistance to 7 mM concentration of copper sulphate. At 6 mM concentration 25% of the isolates were found to be resistant, in which 26%, 23% and 21% from fish, soil and clinical isolates, respectively. The resistant isolates observed at 5 mM concentration of copper sulphate are of 63%, which was contributed by 63% of fish and soil isolates, where clinical isolates is of 70%. At 4 mM concentrations of copper 98%, 97% and 94% growth was observed in fish, soil and clinical isolates, respectively. At 1 mM, 2 mM and 3 mM copper sulphate concentrations 100% of the isolates obtained from diverse sources used in this study were found to be resistant.
The present study indicates that the minimum inhibitory concentration of copper sulphate towards the *Aeromonas* isolates used in this study is 7 mM. From the results it is evident that high copper resistant isolates were predominant among the clinical and fish isolates.

Miranda and Castillo (1998) in their study found about 29-40% of the aeromonads were mercury-resistant. They also observed that 3.6-8.3% of the isolates were resistance to chromium and 41-62% of them were resistance to copper (Babich and Stotzky, 1979). Several of the silver-resistant strains have been collected from silver-treated patients at burn centres, where these strains have sometimes caused outbreaks (Pirnay et al., 2003). Pike et al. (2002) screened for silver resistance on MHA containing 50 µM, 200 µM, 300 µM and 500 µM AgNO₃ and their results correlate with the results of present study.

In the present investigation further silver (*silP*) and copper (*copA*) resistance genes which are of 1200 bp and 1300 bp, respectively were screened in all the isolates. The *silP* gene was conserved in 70% isolates and *copA* gene in 43% of the isolates. About 81%, 61% and 27% of fish, soil and clinical isolates was found to be conserved with *silP*. The *silP* gene was highly conserved among the fish isolates when compared with soil and clinical isolates. However the presence of *silP* gene do not have complete role in existence of silver resistance among the isolates screened for varying concentrations of silver nitrate except for some isolates. The 43% existence of *copA*, was found to be distributed in 51%, 17% and 45% of fish, soil and clinical isolates, respectively. In both the cases of *silP* and *copA* resistance genes, higher prevalence was noticed in fish isolates when compared with the other isolates.

### 6.8 Gene expression profiling

A highly virulent strain is needed for the current research to carry out the host-pathogen interactions study. Hence, a fish isolate ASR30 was selected and was
confirmed by sequencing 16S rRNA and rnpB genes and it was found to be *A. hydrophila*, which was conserved with virulence factors such as *aexT*, *ascU*, *hcp2* and *hyl* as well as exhibiting β-haemolytic and proteolytic activity, also possess multiple antibiotic resistance. Isolates with high virulence can infect healthy fish; however, the stress coming from intensive fish farming can also contribute and triggers outbreaks (Suomalainen et al. 2005). The species *A. hydrophila* is the most common within the *Aeromonas* genus (Cipriano, 2001; Miranda and Zemelman, 2002; Santos, 2010; Silva, 2011). This species is considered to be the most virulent within the *Aeromonas* complex (Cyrino et al., 2004).

Chiang et al. (1999) performed the in vivo gene expression studies and revealed the importance of novel virulence factors in the pathogenesis of several microbial infections. Heithoff et al. (1997) reported that various genes involved in bacterial metabolism were up-regulated during in vivo growth, which might be necessary for organisms to better survive in the host.

The production of a wide array of virulence factors by *Aeromonas* species is indicative of their potential to cause severe diseases in humans. These virulence factor-encoding genes might be differentially expressed in *Aeromonas* species depending on the environmental conditions, such as water or the human host (Khajanchi et al., 2010). In addition to physical barriers such as the intestinal epithelium, an important biological barrier to disease-causing microbial infection is the defense of the host. The evolution of the T3SS is associated almost exclusively with pathogenic organisms, and it is not surprising that T3SSs are involved in interactions with host immune responses. T3SSs have been adapted by pathogens to provoke or evade host innate immunity in a variety of ways, including the direct activation of host signalling cascades, triggering of host pattern recognition through extracellular and intracellular pattern recognition receptors (PRRs), and the suppression or evasion of innate and adaptive defenses (Coburn et al., 2007).
Aeromonas are among the most common disease causing agent in fish. In order to evaluate the influence of T3SS in host-pathogen interactions, pathogenic Aeromonas hydrophila strain (ASR 30) with T3SS gene aexT and ascU and T6SS gene hcp2 were intraperitoneally injected into carp and the expression of virulence genes were recorded at various time intervals in selected organs.

The effector protein aexT gene was highly expressed in liver and kidney after 12 h and in spleen after 24 h of post infection (PI). The transport apparatus of T3SS ascU was highly expressed in liver after 12 h and in spleen and kidney after 24 h of PI. The T6SS gene hcp2 was expressed in liver, spleen and kidney after 12 h of PI. The expression hcp2 gene found to be lowered when compared to the T3SS genes at the same time point.

The virulence genes were expressed differently at various time intervals, which may be due to effective immune responses produced by the host. With the start of the infection or as soon as the pathogen enters the host, the immune system of the host was triggered on and it somehow compete with the pathogen to neutralize or eliminate the pathogen. This elimination process of the pathogen by the host varies depending on the nature of the pathogen, its surface virulence factors or antigenic determinants, infection dose, type and nature of the host. One of the main criteria regarding this is whether the host is exposed to the same pathogen or it was a second attack. If it was the first attack by the pathogen, the immune system of the host is triggered slowly termed primary immune response, where it can control the pathogen in a slow rate when compared to secondary immune response.

This is clear by amplifying the virulence gene expression in immune related organs of the host such as liver, spleen and kidney at various time intervals. In all the organs, the virulence genes showed increased expression till 12 or 24 h and later its expression was decreased due to the heightened immune response by the host. This
can be very well justified by observing the expression of immune related genes simultaneously in the host.

6.9 Host-pathogen interactions

Host-pathogen interactions can be interpreted as the battle of two systems. The main aim of investigating bacterial pathogenesis is to understand the way that pathogens interact with the host to cause disease. For many human-specific pathogens there is no well-defined animal model and it is difficult as well as impossible to conduct gene expression studies during in vivo infections. As the fish occurs at the basal position in vertebrate phylogeny, it gets more importance for genomic and practical functional comparative studies; especially of the immune system.

Galan and Cossart (2005) elucidated that the interface between pathogens and the innate immune system has attracted and will continue to attract a lot of attention from researchers, not only to understand non-specific mechanisms to control bacterial infections but also to unravel principles that direct the mounting of specific immune responses.

An immunoeccological study conducted by Rohlenova et al. (2011) in common carp (Cyprinus carpio) suggested that the seasonal variability affects host immunity and physiology through energy allocation in a trade-off between life important functions, especially reproduction and fish condition as well as different parasite life-strategies influence different aspects of host physiology and activate the different immunity pathways.

The ultimate goal for host-pathogen systems biology is not only the discovery and comprehension of underlying biology, but also the establishment of a robust framework for more efficient drug development and therapeutic intervention.

The present study helps us to elucidate the host-pathogen interactions between common carp and pathogenic Aeromonas hydrophila (ASR30). The expression of
virulence genes as well as immune gene profile in the host immune related organs gave insight into the infection progression inside the host and also helps us in understanding the underlying mechanisms behind the host defense.

6.10 Immune gene regulation

Lieschke and Trede (2009) reported that the lack of sufficient knowledge in fish immunity limits the investigation of immune system evolution and the development of vaccines. The fish immune system has great impact to understand the evolutionary history of immune system. Zhang et al. (2011) preferred the common carp as a candidate model system for immunology research.

The regulation of several immune-response genes (IL1, IL8, IL10, IL11, IL12, TNFα, TNFβ, TNFγ, CXC chemokine, CC chemokine, MHC class I, MHC class IIα, MHC class IIβ, TCRα and TCRβ) were studied using the spleen from the infected host. The immune gene expression of saline or control group and infected group were compared at various time intervals such as 0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h. In all the cases of saline group the immune genes were expressed throughout. In case of infected group there was down-regulation of IL1 at 12 h of PI and after 12 h it gets up-regulated. While in case of IL8 up-regulation was noticed at 12 h of PI and further it was down-regulated. This up-regulation and down-regulation of immune genes in the host infers that the virulent Aeromonas interferes with the normal gene expression due to its pathogenic nature. Whereas the expression of IL10, CXC chemokine and MHC class IIα, were constant throughout the course of the experiment in both the infected and saline group. This shows that the expressions of IL10, CXC chemokine and MHC class IIα were not altered by the pathogen, which means they are turned on constantly to somehow to compete with the pathogen until other immune factors come into action.
In the infected group, at 12 h of PI the immune genes such as IL12, TNFα, MHC class I, TCRα and at 3 h TNFβ, TCRβ, were up-regulated, and further it gets down-regulated due to the severity of infection. This down-regulation occurred because these immune factors of host were not able to compete with the pathogenic *Aeromonas* strain. In case of IL11, it was up-regulated after 3 h and 12 h and down-regulated at 6 h and 24 h; again it gets up-regulated after 72 h. This shows the host immune mechanism keep on regulating the immunity against the pathogen and its exoenzymes.

The gene TNFγ was down-regulated after 3 h and later it gets up-regulated, which indicates that the entry of pathogen triggered the immune system of host, which shows a primary immune response. The expression of CC chemokine was up-regulated at 6 h of PI, down-regulated after 12 h and again the gene gets up-regulated. The MHC class IIβ was down-regulated when the infection time proceeds. The results reveal that some of the immune genes were up or down-regulated as the infection progress in infected group. The immune gene expression of the saline group remained constant due to the absence of pathogen. Only if a pathogen enters the host it could provoke the immune system of the host and alters the immune gene expression, either it is up-regulated or down-regulated. The findings may be useful for developing a marker of early detection of pro-inflammatory response or acute phase immune response in carp upon infection with pathogenic *Aeromonas*.

Bird *et al.* (2002) stated that the discovery of IL-1β genes in mammals, amphibians, bony fish and cartilaginous fish has major implications for investigation into the immune systems of vertebrates.

Li *et al.* (2013) investigated the transcriptional effects of virulent *Aeromonas hydrophila* infection in channel catfish skin, *Ictalurus punctatus*. They observed dysregulation of genes involved in antioxidant, cytoskeletal, immune, junctional and nervous system pathways. In particular they have noticed that the *A. hydrophila*
infection rapidly altered a number of potentially critical lectins, chemokines, interleukins, and other mucosal factors in a manner predicted to enhance its ability to adhere to and invade the catfish host.

Classical immunoregulatory tissues and organs such as spleen, liver and lymphoid follicles often control the nature and scope of the secondary, systemic response, the local immune actors in the gastrointestinal, respiratory, and genitourinary tracts determine the success of critical early steps in pathogenesis including adhesion, entry, and replication. Similarly, interest in understanding components of the mucosal immune response in fish (gills, skin, gastrointestinal tract) is growing (Rombout et al., 2011; Rajan et al., 2011).

The virulent *Aeromonas* isolates are capable of producing mass mortality <24 h after exposure, mimicking the epidemiology of natural outbreaks on catfish farms. Mu et al. (2011) examined the transcriptional levels of several key genes in the head kidney of channel catfish exposed to attenuated and virulent *A. hydrophila* by intraperitoneal injection.

Similarly, Chu and Lu (2008) found that gills and damaged skin (wounded or mucus removed) were routes of invasion in crucian carp using GFP-labeled *A. hydrophila*. They concluded that while intact skin was not a primary portal, even minor disturbances of the mucosal layer allowed invasion. Studies in zebrafish with a virulent *A. hydrophila* isolate also revealed that initiating a slight tail cut was necessary to initiate infection using a bath challenge (Rodriquez et al., 2008).

Recent study of several *Aeromonas* species has highlighted that their infection pattern is characterized by stimulation of robust host production of reactive oxygen species (ROS) and nitrite oxide radical (NO), leading to loss of mitochondrial membrane potential and apoptosis (Krzyminska et al., 2011). The most potent virulence factor of *A. hydrophila* strains infecting mammalian species, cytotoxic
enterotoxin Act, has been shown, upon binding, to stimulate monocyte/macrophage infiltration and to induce release of ROS (Chopra et al., 2000; Sha et al., 2002).

Although different bacterial pathogens tend to exploit similar pathway components in the host, the way in which they 'hijack' host cells usually differs. Studying how these bacterial pathogens subvert host-cell pathways is central to understanding infectious disease. Host-pathogen interactions result in the production and delivery of specific virulence factors that manipulate host cellular processes. Studying the virulence of bacterial pathogens will help control disease and develop new strategies to prevent bacterial infection.

The virulence factor concept has been a powerful engine in driving research and the intellectual flow in the fields of microbial pathogenesis and infectious diseases. At a practical level the finding that effective immune responses often target virulence factors provides a roadmap for future vaccine design.