Chapter IV

DISCUSSION
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The diversification and intensification of aquaculture activities at national and global level has led to an increased focus on studies pertaining to microbial and parasitic diseases of finfish and shellfish. In India, the state of Andhra Pradesh has established a strong hold on commercial and semi-intensive farming of two major carps, *L. rohita* and *C. catla*, with major culture activity taking place in and around Kolleru Lake located at West Godavari and Krishna districts. Unfortunately, for the past few years, the indiscriminate farming practices adopted by fish farmers for increasing production invited many problems leading to outbreak of diseases, fish mortalities and the consequent economic loss. However no serious attempt has been made to undertake in-depth studies on fish diseases from the Indian region. Thus, investigations involving various epidemiological aspects of disease, and its impact on fish health is pertinent and demands due attention from the scientific community.

During the present investigation, studies undertaken on parasitic and microbial diseases of carps *L. rohita* and *C. catla* revealed some important attributes of the host, pathogen and disease. An overall analysis of the data indicated infections with three varieties of parasitic and four varieties of bacterial diseases in major carps *L. rohita* and *C. catla* collected from culture ponds of Kolleru Lake. Among the parasitic infections, ectoparasitic diseases like dactylogyrosis caused by monogenoidean parasites (*Dactylogyrus catlaius* and *D. labei*) and Argulosis caused by copepod parasites (*Argulus siamensis*) were found to be common.

Heavy infections with Dactylogyrid species were noticed quite frequently on the gills of *L. rohita* and *C. catla*, however, no mortalities were
reported so far (Ramakrishna, 2005). During the present study excessive mucus secretion and gill hyperplasia were commonly noticed in carps heavily infected with dactylogyrid species.

But the crustacean parasite *Argulus siamensis* has emerged as a significant problem and is considered as a notorious pathogen in semi-intensive and intensive aquaculture systems (Mishra, 1991; Saurabh and Sahoo, 2010; Saurabh *et al.*, 2011). One of the major clinical symptoms of the Argulosis disease is extensive damage to gills or skin, resulting in puncture of the skin, followed by development of small eruptions, which will be inhabited by bacteria in due course of terms, it left untreated. These parasites feed on blood, skin, mucus and epithelial cells (La Marre and Cochran, 1992). Besides direct loss due to mortality, the parasite retards the growth of fish, brings behavioural changes and reduces their market value. Several instances of Argulosis associated with fish mortality have been reported from fish culture ponds (Singhal *et al.*, 1990; Sheela *et al.*, 2002). During the present investigation, mortalities due to Argulosis was reported only once by farmers during summer months.

Bacterial pathogens are a major cause of infectious diseases, in wild fish stocks and in fish reared in culture conditions. Concurrent with the rapid growth and intensification of aquaculture and the associated activities, the list of new pathogenic bacterial species isolated from fish has increased steadily (Harvell *et al.*, 1999). Besides the virulence and the host range of existing pathogens has also been increasing, posing considerable challenge to fish health researchers, who are aiming at developing more efficient vaccines and therapeautic drugs to combat fish bacterial diseases.

Bacterial diseases observed in *L. rohita* and *C. catla*, during the course of present investigation fall under two major categories i.e. those caused by
Aeromonas- Pseudomonas group and those caused by Flexibacter group of bacteria. Aeromonas - Pseudomonas group of bacteria were found to be responsible for causing infections like Haemorrhagic Septicaemia and Abdominal Dropsy, where as Flexibacter bacteria including F. branchiophilum and F. columnaris were found to be responsible for Bacterial Gill Disease and Columnaris Disease. Among these four bacterial infections HS was found to be most dominant and highly prevalent disease, followed by BGD, AD and CD.

The major pathogen involved in causing HS in L. rohita and C. catla was identified as A. hydrophila, which was always found in association with P. fluorescence. Abdominal Dropsy (AD) has been considered as a latent stage of HS, as the same pathogens (may be different strain) are reported to be involved in causing the disease (Gopalakrishnan, 1966, Karunasagar, 1986). The ulcerative, septicaemic, ascetic diseased condition of fish caused by A. hydrophila in association with P. fluorescence has been reported from several South East Asian and also from other countries (Angka et al., 1988; Roberts et al., 1992; Shome et al., 1996; Yesmin et al., 2004; Hossaen et al., 2006; Abowei and Briyani, 2011).

The septicaemic condition noticed during the present study in L. rohita and C. catla is mostly characterized by internal haemorrhage, putrification of all internal organs, enlargement of spleen and gall bladder and haemorrhages on the external body surface, often resulting in ulcer formation.

Gopalakrishnan (1961) was the first to observe the disease in Indian major carps, where he reported C. catla to be the most affected host, followed by C. mrigala and L. rohita. He reported ascetic and ulcerative form of the disease in these fishes. Karunasagar, (1986), made a similar observation in
C. catla and reported A. hydrophila to be the causative agent responsible for causing white cutaneous lesions on the snout, loose scales and fin rot. Karunasagar et al., (1989) reported an acute septicaemic condition in L. rohita, C. catla collected from culture ponds of West Godavari districts. A severe outbreak of infectious Abdominal Dropsy followed by heavy mortalities was reported by Shome et al., (1986) in C. mrigala from South Andaman.


During the present study, septicaemic and dropsy condition was observed both in L. rohita and C. catla collected from culture ponds of Kolleru Lake. However, prevalence was high in the case of HS, when compared to dropsy, which was regarded as one of the diseased condition caused by A. hydrophila (Snieszko and Axelrod, 1971). Many others have attributed that variations in clinical symptoms of the disease caused by A. hydrophila is due to strains having different virulent properties (Torres et al., 1990; Lallier and Daigneault, 1984; Santos, 1987). Karunasagar et al., (2003) reviewed characteristics of virulent strains of A. hydrophila.

A. hydrophila is referred as a facultative pathogen which causes infection under stressed conditions, when the resistance capacity of the host is low (Snieszko, 1974; Mazeaud et al., 1977; Sinderman, 1979).
Raghavendrapal et al., (1995) noticed mortalities in the fingerlings of *C. carpio* when exposed to *A. hydrophila* in stressed condition.

Though *P. fluorescence* was always found to be associated with *A. hydrophila*, in infections showing clinical symptoms of Haemorrhagic Septicaemia, it is mostly considered as an opportunistic pathogen inhabiting the tissues already infested by *A. hydrophila*.

Flavobacteria are another important group of bacteria found to be responsible for causing disease in fishes from culture ponds. They are ubiquitous as they are found in the soil, freshwater and marine environments and are noted for their noval gliding motility (Leahy and Colwell, 1990). The two important diseases caused by these bacteria are Bacterial Gill Disease and Columnaris Disease.

*F. branchiophilum* is the causative agent of Bacterial Gill Disease in several parts of the world (Heo et al., 1990). It is a serious fish pathogen causing substantial economic loss and rearing difficulties to commercial and conservation aquaculture (Bullock, 1990). The disease appears to be characterized by high to moderate morbidity and mortality rates in fishes due to massive bacterial colonization of gill lamellar surfaces and progressive branchial pathology as a result of lamellar epithelial necrosis (Speare et al., 1991; Ostland et al., 1994). A similar situation was noticed during the course of present investigation, where gills of both *L. rohita* and *C. catla* heavily infected with *F. branchiophilum*, showed characteristic clinical signs of BGD like clubbing, distal necrosis and excessive mucous secretion followed by massive colonization of gill lamellar surfaces during severe conditions.

BDG was regarded as the 2nd most important bacterial infection, next to HS, in carps from culture ponds of Andhra Pradesh especially in Kolleru Lake area. But so far no serious studies were undertaken, except for the report
on an epizootic caused by BGD in carps by Ramakrishna, (2005) in his thesis work and a report by Swain et al., (2007). The present study constitutes a detailed study on Bacterial Gill Disease of carps from the Indian region.

Columnaris Disease, another one important bacterial disease has been found occurring in carps from culture ponds of Kolleru Lake. The disease, though less prevalent is significant due to its serious pathological manifestation. It is reported to be caused by *F. columnare* (formerly *Cytophaga columnaris; Flexibacter columnaris*) in wild fish populations and ornamental fish industry worldwide (Bernardet, 1989). Classically during outbreaks, its morbidity and mortality rates escalate more gradually than BGD. Besides, unlike the pattern necrosis in BGD, fish with Columnaris will show severe necrosis of entire gill region and external surfaces of body as the bacterium invades inwardly (Speare and Fergusen, 1989).

It is reported to be mostly a stress oriented disease, appearing suddenly in fishes subjected to handling or transportation stress. Kumar et al., (1986) reported a handling stress to be a factor responsible for causing the disease in carps. Other stress factors like netting and transferring of fish during early morning hours when the dissolved oxygen is low, keeping fish in crowded condition for a long time, transferring them in closed bags without aeration are some of the factors to be avoided to prevent disease out breaks.

The data on various diseases during different seasons indicated high prevalence during winter (38.56%), followed by summer (34.29%) and rainy season (16.48%) for bacterial infections. Among the various bacterial infections HS appears to be dominant with relatively high prevalence during all the three seasons. Whereas infections with BGD was most common during winter when the temperatures were comparatively low. It has been reported in many cases that decrease in temperature reduces the disease resistance
capacity of the fish and increases environmental stress. Borg (1960) and Holt (1988) reported high prevalence of cold water disease caused by *F. psychrophilum* during winter and spring when low temperature generally prevails. Bly *et al.*, (1993) reported winter kill disease in channel catfish caused by saprolegniacea fungi, when the temperatures drop suddenly. It was noted that low temperatures favour this fungus to produce enormous number of spores and thus increase their dispersal. The observations made during the present study lend support to the view that low temperatures enhance disease prevalence and sometime sudden disease outbreaks under abnormal conditions.

Differences in the prevalence of infections were noticed in different species of carps. Of the two species of carps studied during the present study, *L. rohita* appears to be more vulnerable to infections with high rate of prevalence when compared to *C. catla*. The prevalence of HS is almost same in both species of carps, but BGD appears to be more specific to *L. rohita* with high prevalence. In *C. catla* it is mostly noticed along with dactylogyrosis infection. It is probable that BGD gets manifested on the gills of *C. catla* when the fish is under stress due to parasitic infections or environmental stress.

Bacteriological studies undertaken during the present investigation included isolation and identification of bacterial pathogens viz. *A. hydrophila*, *P. flourescence* and *F. branchiophilum* from diseased fish showing clinical symptoms of HS and BGD respectively. The results of all the culture and biochemical tests with standard test results given in the Bergey’s manual of Bacteriology (1984) and Medical Bacteria 3rd edition by G.I. Barrow and R.K.A. Feltham (1993).
The pathogenicity tests conducted to determine the lethal dose of the three bacterial pathogens in the fingerlings of *L. rohita* and *C. catla* revealed significant variation in the LD$_{50}$ value of *A. hydrophila*, *P. fluorescence* and *F. branchiophilum*. The estimated LD$_{50}$ value for *A. hydrophila* was $1 \times 10^{6.3}$ cfu/ml in *L. rohita* and $1 \times 10^{6.15}$ in *C. catla*, for *P. fluorescence*, it was $1 \times 10^{7.15}$ in *L. rohita* and $1 \times 10^{7.43}$ in *C. catla*, whereas the LD$_{50}$ value for *F. branchiophilum* was recorded as $1 \times 10^{6.08}$ in *L. rohita* and $1 \times 10^{5.6}$ in *C. catla*.

Based on the LD$_{50}$ values, Lallier and Dargneault (1984) grouped bacterial isolates as virulent when the LD$_{50}$ value lies between $10^{4.5} - 10^{5.5}$, weakly virulent when the value is between $10^{5.5} - 10^{6.5}$ and as avirulent when it is more than $10^7$ cfu/ml. Following the above categorization, the bacterial isolates of *A. hydrophila* can be defined as weakly virulent, *P. fluorescence* as avirulent and *F. branchiophilum* also as weakly virulent as their LD$_{50}$ values falls in the range determined for each type.

*A. hydrophila* was found to be pathogenic to several species of fish fingerlings, showing variable LD$_{50}$ doses. It is found to be pathogenic to *Clarias batrachus* when infected intraperitonially (Supriyadi., 1986), to rainbow trout (Krovacek., 1989); to walking catfish when an intraperitonial infection of $10^7$ cfu/ml. is given (Angka., 1990); to fingerlings of catfish at a dose of $1.5 \times 10^9$ cfu/ml. (Shome et al., 1996); to *C. carpio* (Harikrishnan et al., 2003). Santos et al., (1991) determined LD$_{50}$ of *A. hydrophila* during seven day experimental set up for several species of fish viz. *Salmo trutta* ($2 \times 10^5$ cells/mg); *Anguilla japonica* ($7 \times 10^8$ cells/ml) *Plecoglossus altivelis* ($8.6 \times 10^4$ cell/ml); and *Lepomis macrochirus* (> $10^8$ cells/ml). Karunasagar et al., (1989) reported that the LD$_{50}$ of *A. hydrophila* isolates to carp fingerlings ranged from $10^5$-$10^6$ cfu/ml.
In the present study, the LD$_{50}$ value recorded for *A. hydrophila* in *L. rohita* and *C. catla* falls in line with those reported for other species of fish. The present study shows that *P. fluorescence* which often occurs in association with *A. hydrophila* can be regarded as avirulent strain with low LD$_{50}$ values. At various times, the species *P. fluorescence* has been found to be associated with fish spoilage (Gillespic, 1981), as an opportunistic pathogen (Estive *et al.*, 1993) or as secondary invader that take advantage of the damaged tissue. Omprakash and Manohar (1991) studied in detail the pathogenicity of *P. fluorescence* in natural and experimental infections and reported its association with septicaemic condition. However, during the present study, *P. fluorescence* was always found to be associated with infections showing clinical symptoms of HS disease caused by *A. hydrophila*.

Information on pathogenecity studies with respect to *F. branchiophilum* is quite scanty. Virulence properties of various strains of *F. branchiophilum* in experimentally infected salmonid fish were studied by Ostland *et al.*, (2006). Lamsden *et al.*, (1994) studied challenge doses of *F. branchiphilum* in rainbow trout for causing mortality. He noted that very high doses of $8 \times 10^{10}$ cfu /ml of bacteria are required for causing mortality, whereas a lower dose of $2 \times 10^9$ cfu /ml decreased the cumulative percent mortality in fishes.

There are several studies dealing with fish bacterial identification, experimental infection or disease resistance (Azad *et al.*, 2001; Al-Harbi and Uddin, 2004; Cai *et al.*, 2004) but little relates, changes in haematological response to bacterial infections. The haematological parameters are considered as important tools of diagnosis that reveal the health status of fish (Rehulka, 2002; Martins *et al.*, 2004 a). Knowledge of haematology is very important since it deals with the morphology, physiology and the
biochemistry of blood. Analysis of blood cell characteristics help to identify disease status in fish (Anderson, 2003). In fisheries, it is important to have early diagnosis of illness or disease status to undertake or to develop control and preventive measures. Hesser (1960) while recognizing the use and reliability of haematological analysis in human medicine adopted these techniques in fish haematology as an aid in the diagnosis of fish diseases. Studies during the past few decades have unequivocally established applicability of haematological and biochemical parameters in not only assessing fish health but also in understanding the nature of the physical and chemical condition of their external milieu (Secombes and Fletcher, 1991; Anderson et al., 1997).

The present investigation dealing with the effect of three different doses of bacterial pathogens A. hydrophila, P. fluorescence and F. branchiophilum on haematological and serum biochemical parameters of L. rohita and C. catla clearly fall in line with similar works carried out by others on different species of fish. Variations were revealed by reduction in the values of haematological parameters like total RBC, WBC, Hb% and PCV, and biochemical parameters like total protein, albumin, globulin, glucose levels, SGOT, SGPT and BUN is exposed fishes when compared to unexposed, control fish.

Among the various haematological parameters considerable reduction was noticed in total RBC count, Hb percentage and PCV values. A dose response relationship was noticed in the pattern of reduction of values for these parameters.

The results presented in this study have revealed an interesting pattern showing that the level of total RBC count, Hb% and PCV values were significantly decreased in fish inoculated with the three lethal doses of
A. hydrophila, P. fluorescence and F. branchiophilum when compared to control fish. A dose response relationship was noticed in the reduction of these values with an increase in the concentration of pathogen dose. A similar situation is reported in other species of fish infected with EUS with different isolates of A. hydrophila (Pathiratne and Rajapakshe, 1998; Rehulka, 2002; Martins et al., 2004; Haniffa et al., 2011). Similarly decreased RBC and PCV values due to infections with bacterial agents was reported for different species fish like Coho salmon (Oncorhynchus kisutch) infected with Vibrio anguillarum (Harbell et al., 1979), Asia Cichlid fish (Etroplus suratensis) infected with EUS (Pathiratne and Rajapakshe, 1998), rainbow trout with ulcerative dermatitis (Rehulka., 1998) rainbow trout infected with A. caviae and A. sobria (Rehulka, 2002), and C. carpio infected with A. hydrophila (Harikrishnan et al., 2003).

The decreased Hb% trend may be the result of swelling of RBC as well as poor mobilization of Hb% from spleen to other haematopoietic organs (Scott et al., 1981) or it can be due to hypochromic microcytic anaemia caused by a bacterial pathogen like A. hydrophila (Haniffa et al., 2011).

According to Haney et al., (1992), a decrease in the erythrocyte number and Hb concentration may be caused by bacterial agent and suggested destruction of erythrocytes as the possible reason. In the present study a decreasing trend was noticed in Hb% of fingerlings of L. rohita and C. catla exposed to different lethal doses of A. hydrophila, P. fluorescence and F. branchiophilum. Since haemorrhage and gill tissue damage is a conspicuous feature of A. hydrophila and F. branchiophilum infections, the haemolysis of blood cells by these bacteria would be the possible reason for these changes and could contribute to the decreased levels of RBC, Hb% and PCV.
It is a well known fact leukocytes which are normally lower in healthy fishes, could be used as a significant indicator for infectious diseases. In the present study the total WBC count was comparatively high in fishes exposed to all the three bacterial pathogen over the control fish. Increase in total WBC is mainly considered as a response of the fish immune system against the bacterial invasion (Ikeda et al., 1976). An increase in the number of WBC with induction of inflammatory processes during acute intestinal coccudiosis of carp was reported from differential WBC counts of parasitized carp (Steinhagen, et al., 1997). Martins et al., (2008) reported increased WBC and lymphocyte number in Nile tilapia infected with Enterococcus species at a concentration of $10^6$ cfu/ml. Manoj et al., (2010) reported increase in the number of WBC in *L. rohita* experimentally infected with *A. hydrophila* by immersion challenge. Increase in the number of leucocytes was also reported by Harikrishnan et al., (2003) in common carp *C. carpio*, by Bailone et al., (2010) in Nile tilapia (*Oreochromis neiloticus*) by Haniffa et al., (2011) in *Channa striatus* experimentally infected with *A. hydrophila*.

Compared to the work carried out on alterations in serum biochemical parameters of fishes in relation to toxicants, the work available on these lines in response to infection due to bacterial or viral pathogens is relatively less. With the advent of aquaculture as an industry, studies pertaining to fish health management have gained lot of significance. One important component of these studies dealing with serum biochemical responses of normal and diseased fish is that they can be used as markers for early disease diagnosis. In the present study, significant variations were noticed in several biochemical parameters like total proteins, albumin and globulin levels, glucose levels, in SGOT, SGPT enzyme activities and BUN levels in fishes exposed to different doses of bacterial pathogens.
Alterations in serum proteins and glucose levels have been suggested as a useful indicator of stress or an early warning signal of disease. In the present study, increase in total proteins, albumin and globulin levels was noted in fishes *L. rohita* and *C. catla* exposed to different doses of *A. hydrophila*, followed by *F. branchiophilum*. Whereas fishes exposed to different doses of *P. fluorescence* showed either no variation or slightly reduced level of these parameters over controls. Increase in the serum total protein level, especially globulin content was considered as an immunological response of fish, to overcome the effect of diseases by Wiegertjes *et al.*, (1996). Rehulka, (1998) while working with Aeromonas infections in rainbow trout reported that skin lesions caused by bacteria increased the total proteins and other enzyme activities like ALT and AST. A similar view was expressed by Zhou *et al.*, (2009), while working on the haematology and serum biochemistry of cultured and wild Dojo loach. Zorriehzahra *et al.*, (2010) noted marked increment in the mean level of blood serum total proteins in three experimental farms, in companion to control group from raceway culture.

In accordance with the opinion put forward by others (Rehulka *et al.*, 2005), the present study also indicates the fact that, such increase in total protein levels may be an indication of immunological response by way of increased antibody productivity during infectious diseases.

Glucose is a key product of cellular respiration. Since glucose is an essential fuel for the body tissues, the glucose level was maintained throughout the exposure period. In the present study, the concentration of serum glucose was increased in both *L. rohita* and *C. catla* exposed to different doses of bacterial pathogens, though it was more pronounced at LD$_{50}$ dose. Generally, glucose concentrations were selected as a stress
indicator, and increased glucose levels were mostly reported from infected or stressed fishes (Yoganandan et al., 2003; David et al., 2005). Increased glucose levels were also reported in tilapia infected with *Saprolegnia parastica* (Zaki et al., 2008), and also in common carp following infections with a monogenetic parasite (Ali and Ansari, 2012).

The hyperglycemic condition in natural as well as experimentally stressed fish, can be attributed to a number of factors, viz. the activation of glycogen stress (Nakano and Tomlinson, 1967) or increasing demand for carbohydrate reserves by fish in response to infection (Colombo et al., 1990), or may be due to the induced activation of adrenal pituitary glucocorticoid hormones (Chezthian et al., 1998) or impairment of hormone level in the blood involved in carbohydrate metabolism (Srivastava et al., 2004).

All these studies indicate the fact that an infection with any bacterial pathogen was considered as a stressor condition, which was indirectly confirmed by the enhanced glucose levels during that period. A similar explanation can be attributed to increased glucose levels noticed during the present study.

Enzymes play a significant role in the metabolic activity of any animal and thus important in maintaining their health status. In the present study the activity of transaminase enzymes though fluctuated at times, followed almost a similar trend in *L. rohita* and *C. catla*, exposed to different doses of the three pathogens. An increasing trend was noticed in the case of SGOT enzymes with more significant values at LD$_{50}$ dose, whereas the activity of SGPT exhibited a decreasing trend from lower dose to higher dose. The elevated or decreasing levels of serum transaminases (SGOT and SGPT) as observed during the present study are generally used as markers of liver function and can be related to disturbance of normal metabolism which may
be due to extensive alternations in the liver histology (hepato
cellular necrosis), indicating dysfunction of liver. Vasudevan and Sreekumar
(1998), Ali and Ansari (2012) noted a somewhat similar situation while
dealing with monogenean infected common carp, *C. carpio*.

Fluctuation in serum urea indicates declining liver function or a failing
of gill osmoregulatory capability in conjunction with creatinine (Walsh *et al*.,
2003; Allen *et al*., 2005). The present study indicated elevated levels of
Blood Urea Nitrogen in *L. rohita* and *C. catla* inoculated with different dose
of pathogens. The hypoxic conditions developed due to alternations in
haematological parameters may cause an alternative osmotic pressure (Shen
*et al*., 1991) and could be responsible for higher values of urea nitrogen
(Zhou *et al*., 2009).

Data analysed for various haematological and serum biochemical
parameters of *L. rohita* and *C. catla*, indicated significant interspecific
variations, especially in biochemical parameters like enzyme activity of
transaminases and BUN. Such inter specific variations were mostly studied in
healthy fish (Larsson *et al*., 1976; Rambhaskar and Srinivas Rao, 1987,
1989). Comparative studies on diseased fishes will help in understanding the
physiological responses and disease resistance capacity of fish to various
pathogenic bacteria. Similarly variations in physiological responses of fish
towards different bacterial pathogens will reveal the virulence or pathogenic
nature of the pathogen involved in causing the disease.

The variations in haematological and serum biochemical parameters
obtained during the present study emphasizes the need to undertake more
such studies involving longer exposure periods, different fish size groups, sex
and doses, so that the data generated can be effectively utilized as diagnostic
tools or as early warning signals of disease in fishes.