9. EFFECT OF ARGULUS SPP.ECTOPARASITES AND THE SECONDARY MICROBIAL INFECTION ON FISHES

9.1. Introduction

Vertebrates such as fish generally have two lines of immunological defense: innate, unspecific immunity, which attacks all invading pathogens and acquired, specific immunity, which is activated after the first encounter with a particular pathogen (Manning, 1994). Modern fish farming conditions with high fish densities and intensive production units provide ideal conditions for the invasion and persistence of a range of pathogens and parasites (bacteria, viruses, protozoan and metazoan parasites). Infections by these disease-causing agents reduce the condition and survival of fish causing economical losses to farmers (Rintamaki-Kinnunen and Valtonen, 1997).

Bacterial diseases are responsible for heavy mortality in both wild and cultured fish. The real role of these microorganisms may vary from that of a primary pathogen to that of an opportunist invader of a host rendered moribund by some other disease process (Richards and Roberts, 1978). The non-specific nature of diseases induced by non-fastidious and opportunistic bacterial organisms makes them unpredictable and complicates their differential diagnosis. Many of these organisms are a usual component of the bacterial flora in aquatic habitats particularly eutrophic systems. Stressors often inevitable in most culture systems predispose fish to bacterial borne sicknesses (Snieszko, 1974).

Argulus species are detrimental ectoparasites of fish and several epizootics have occurred throughout the world (Menezes et al., 1990; Hakalahti et al., 2004). Argulids fix to fish skin by means of suckers and spines and move on the skin destruction the mucus layer. The parasite repeatedly penetrates the skin of the fish to
feed on blood which leads to wounds and eventually to ulcers and opens a potential gateway for the secondary microbial invasions (Singhal et al., 1990).

Moreover, argulids frequently attach and detach from their hosts and potentially act as vectors spreading pathogens between fish (Cusack and Cone, 1986). Except at very high infection levels argulids alone rarely lead to mortality among adult rainbow trout (Lester and Roubal, 1995) but they may considerably reduce the survival of small sized fish (Poulin and FitzGerald, 1987). It has been shown however that ectoparasitic infections may induce multiple changes in fish physiology leading to decreased disease resistance (Tully and Nolan, 2002). Also extended *A. foliaceus* infections (6 parasites/fish) led to reduced immune response among infected rainbow trout when subsequent confinement stress was applied but not when the parasite was acting as the single stress factor (Ruane et al., 1999). This indicates that the negative influence of *A. coregoni* on fish may appear when a secondary stressor is present.

It is known that *Argulus* infestations lead to secondary parasitic infestation of the skin (Bauer et al., 1991; Soulsby, 1986). Some authors reported that *Costia necatrix* accompanied by *A. foliaceus* in infected fish, and *Trichodina* sp., *Trichodinella* sp. and *Apiosoma* sp. were observed in skin and gills preparation (Bauer et al., 1991; Burgu and Oguz, 1984). In this study no other parasites were observed on the body surface and gill.

Many studies have emphasised the possible role of parasites in enhancing infections of fish with secondary pathogens such as bacteria (e.g. Cusack and Cone, 1986; Busch et al., 2003; Pylkko et al., 2006). The pathways that can lead to such increased susceptibility might be direct for example when an entrance route for bacteria is created due to epidermal injuries induced by a parasite (Kanno et al., 1990;
Buchmann and Bresciani, 1997) or when a parasite acts as a vector for a disease (Cusack and Cone, 1986). Also, a parasite may enhance bacterial infections indirectly via decreased host immunocompetence (Bowers et al., 2000). Studies aiming to test hypotheses concerning interactions between parasites and pathogenic bacteria in fish are rare (Busch et al., 2003; Suomalainen et al., 2005; Pylkko et al., 2006) and we know of only one experimental study that has demonstrated enhanced bacterial invasion due to a parasitic infection (Pylkko et al., 2006).

9.2. MATERIALS AND METHODS

9.2.1. Collection and Conditioning of Fishes

The brackish water Mugilidae fish species were collected from the Vellar estuary, Parangipettai. They had a mean fork length of 10 cm ± 2 cm. The wild caught fishes were at first treated with fresh water with diluted formalin and commercial antibiotic for removing the external parasites attaching on the body surface, caudal fin, gill inside, outer areas and avoiding the wound healings, the secondary microbial infections. The wild caught fishes were maintained in UV treated sterilized marine water containing fiber tanks for seven days (28±2 ppt maintained). Then the fishes were maintained on a 12:12 h light and dark cycle with the continuous aeration. The oyster muscles were well boiled and gave for feed three times per day. The fecal discharges of fishes were siphon out by using tubes.

9.2.2. Argulus spp. Infection to Fishes

Before the experimental work, two 250 litter capacity of fiber tanks were taken and they were coated bleaching powder then washed with freshwater thoroughly. These tanks were dry and sterilized under direct sunlight. Four days after that two tanks were washed with freshwater then they filled with UV treated and oxygenated marine water 28±2 ppt. Then the tanks were stocked with the fishes in
each tank containing 10 fishes with 50 parasites ($\delta=25$, $\varphi=25=50$ parasites). One tank was control tank without parasite infection; remaining four tanks were introduced of identified Argulus spp. parasites to the host fishes (A. foliaceus, A. indicus, A. japonicus and A. siamensis).

9.2.3. Isolation of Bacterial Pathogens from Argulus spp. Infected Fishes

The live fishes in tanks were monitored constantly for Argulus parasites attachments. After 24 hours, the argulus parasites attach on host fishes, they start their counter attacks. They make the ulcer likes wounds on the fish body. The infected body regions were scraped using sterilized inoculation loop and streaked in Zobell Marine Agar (HIMEDIA, Mumbai) slants.

9.2.4. Identification of Pathogenic Bacteria

The morphological and biochemical characterization were carried out for the isolated bacterial cultures from diseased ornamental fishes and the results were confirmed by using standard Bergey's manual of Systematic Bacteriology. Following tests viz., Gram staining, motility test, IMViC, Catalase, oxidase, TSI, urease and Nitrate reduction tests were carried out to identification of pathogenic bacterial strains. Then genomic DNA from particular bacterial isolates was extracted and the sequencing analysis was performed.

9.3. Molecular Characterization of Bacterial Species

9.3.1. PCR amplification of 16S rRNA

The morphologically isolated individual bacterial colonies were used for direct PCR amplification. Bacterial colony was prepared as follows: to 50µl of sterile double distilled water a loop full of bacterial culture was dispensed and incubated in a dry bath maintained at 62°C for 30 minutes or loop full of culture in 50µl sterile water vigorously vortex (10x speed) and incubated at -20°C overnight. Following
incubation 2 µl bacterial culture was used as template for Polymerase chain reaction (PCR) and the primer pairs 8F(5’- AGAGTTTGATCATGGCTAG-3’)) and 1492 R (5’- CGGTTACCTTGTTACGACTTT-3’) (Lane,1991) were used for 16SrRNA gene amplification, using thermal cycler model TC-3000 (GeNei, India).

Amplification was carried out in a 50µl mixture containing 3mM concentration of MgCl₂, 2.5 mM of each deoxynucleotide triphosphate (dNTP), 10pM of each primer, 3U/µl of taq polymerase in 1x taq buffer (Bangalore Genei, India) and 2 µl of bacterial isolates. The PCR conditions were: initial denaturation at 94°C for 5 minutes, 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 90 seconds and final extensions at 72°C for five minutes. Two reactions, appositive control with DNA template with known to be successfully amplified and a negative control lacking template DNA was setup in each PCR.

9.3.2. DNA Sequencing of Editing and BLAST Analysis

The sequence were compared with other sequence with other sequence in National centre for Biotechnology information (NCBI) database through Basic Local Alignment searching tool (BLAST) analysis (Zhang et al., 2000). The 16S rRNA gene similarity (Visible in BLAST search) equal to or more than 98% was considered to be the same species (Stackebrand and Goebel, 1994).

9.3.3. Phylogenetic Analysis

All compiled full length 16S rRNA gene sequence were multiple aligned with Clustal X version 2.06 (Larkin, 2007). The 16S rRNA phylogram was constructed in Molecular Evolutionary Genetic Analysis (MEGA version 6.06) software (Tamura et al., 2013) using Kimura 2 parametric distance model. Pair wise distance data were
generated in MEGA using Kimura-2-parametric formulae and converted to percentage value (multiplied by 100) for better understanding

9.4. RESULTS

Maintaining the disease free fishes at 28±2ppt in water, they are active and adapt to captive conditions. When introducing the *Argulus* spp. parasites for making infection on its host fishes, the activities of parasites were noted, a) Parasites active swim was slowly reduced, b) They controlled their motion activities for few minutes’ bottom side of the tank for finding their host, c) When the parasites find host fishes they attached, after 24 hours monitoring the host fishes body only 12 parasites were survived. Highest numbers of eight parasites were noted in only one fish. Those mass attachment *Argulus* spp. parasites killed the fish and they transform to next targeted fishes. During feeding on the host fish’s theses parasites’ sting apparatus was virtually at right angles to the body. The epithelial layer was destroyed with the insertion of poison by the help of sting apparatus and inflammation; hemorrhage symptoms were fashioned on the host fish body (Plate.2, 3).

On the basis of morphological, biochemical characterization *Aeromonas salmonicida*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas fluorescence*, *Vibrio harveyi* were identified and the genetic analysis the bacterial species like *Aeromonas hydrophila* (KP676002), *Pseudomonas aeruginosa* (KP676004) and *Vibrio alginolyticus* (KP676006), *Vibrio parahaemolyticus* (KP676006) were isolated from the injured areas of parasite infected fishes (Plate.4, 5, Table.5, Figure.26).
Plate 2. Ventral view of Argulus sp. parasite

- Ventral surfaces of lateral lobes covered by pectin scales
- Pre oral sting with poison gland
- Basal palate
- Proboscis
- Sucker
- Respiratory areas
- Second maxillipede
Plate. 3. Effect of *Argulus* spp. and the Bacterial infection on Brackish water *Mugilidae* fishes
Plate. 4. Sample Collection and Identification of Bacterial spp.

- Sample collection
- Spread plate
- Streak plate

- **Aeromonas** species.
- **Bacillus cereus**
- **Escherichia coli**

- **Pseudomonas aeruginosa**
- **Pseudomonas fluorescence**
- **Vibrio parahaemolyticus**
Plate 5. Biochemical Tests for Bacterial spp. conformation
Table 5. Biochemical identification of bacterial pathogens

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<th>S.NO</th>
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<th>A. hydrophilia</th>
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<th>B. cereus</th>
<th>E. coli</th>
<th>V. alginolyticus</th>
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Figure 26. Phylogenetic tree drawn using 16S rRNA gene sequences of culturable isolates. Genbank having close similarity (indicated with accession numbers and species name) with the sequences obtained from the present study were used for phylogenetic tree construction. A- *A. hydrophilia*, B- *P. aeruginosa*, C- *V. parahaemolyticus*, D- *V. alginolyticus*. 
9.5. DISCUSSION

Bacteria disease is an illness of fish body caused by bacteria organisms creating infection or internal disorder. It is an expression of a complex interaction between a susceptible host, a pathogen and the environment. In the presence of an infective agent in an effective number, a susceptible host suffers an infection in adverse conditions. Bacteria diseases manifest in various ways for the impairment of the normal physiology in the host (Bassey, 2011).

Infections of bacterial constitute the most important source of disease problems in all the various types of production. Gram-negative bacteria cause epizootics in nearly all cultured species (Meyer, 1991). In this present study, infection by the *Argulus* spp. of ectoparasites on Mullet fishes under the captive condition, these ectoparasites can tolerate in the highest salinity 28±2 ppt and give opportunity to the bacterial infection. Most of the isolated and identified bacteria were Gram negative in nature.

The bacteria isolated from small mouth bass in the Potomac River have been shown to be enhanced by parasite infections in other fish species. Increased mortality due to *F. columnare* occurred when fish were infected with the ectoparasites *A. coregoni* (Bandilla *et al.*, 2006).

However, the magnitude of this sort of artificial injury is probably severe compared with injuries caused by the argulids and it does not exclude increased stress (van der Salm *et al.*, 2000) and consequently decreased immunocompetence (Salonius and Iwama, 1993; Engelsma *et al.*, 2003) as mechanisms through which *A. coregoni* could have enhanced the columnaris disease. In future work it would be worthwhile to determine if *A. coregoni* can deliver *F. columnare* to the host tissues acting as a vector.
In this present study, in captive condition of Mullet fishes infected with Argulus spp. seven Gram negative bacterial strains like Aeromonas hydrophila, A. salmonicida, E. coli, Vibrio alginolyticus, V. harveyi, V. parahaemolyticus, P. aeruginosa and P. florescence and one Gram positive bacterial strain like Bacillus cereus were isolated. Mortality of mullet fish during in the experimental work due to the reasons of secondary pathogenic life of above said bacterial species.

Aeromonad, Pseudomonad, Vibrio and Edwardsiella tarda are the major bacterial fish pathogens which are widely distributed in aquatic fauna (Banu, 1996; Islam, 1996). In coastal regions, fishes have been suffering from Vibriosis which severely affects the fish production (Rahman, 2005).

The first time experimental support for the hypothesis that ectoparasites can increase susceptibility of fish to a serious bacterial diseases. Similar combined effects of several stress factors on fish condition have been reported earlier but in different systems. For example, signs of immunosupression caused by extended A. foliaceus infection on rainbow trout only became evident in the presence of additional confinement stress (Ruane et al., 1999). Also, mixed infections of two ectoparasites, Trichodina murmanica (Protozoa) and Gyrodactylus pleuronecti (Monogenea) in winter flounder led to a decrease in fish growth compared with single infections (Barker et al., 2002). Surprisingly few studies have addressed links between parasitic and bacterial infections in fish although the co-occurrence of these diseases at fish farms is the rule rather than the exception (Buchmann and Bresciani, 1997).

A variety of ectoparasites were noted on the small mouth bass that may enhance infections with secondary or opportunistic bacterial pathogens. These included leeches, trematode metacercariae, myxozoans, and monogenes. Parasites, both protozoan and metazoan, can cause damage to the epithelial layer of skin, gill, or
intestine and create a route of entry for bacterial or viral pathogens (Thoney and Hargis, 1991; Bandilla et al., 2006; Pylkko et al., 2006).

An infection experiment revealed that the level of secondary bacterial infection increased after parasitization by the copepod A. coregoni. Fifty juvenile masou trout (Onchorhynchus masou) (30-50 g/fish) and 500 A. coregoni were kept in a container supplied with running water at 13° C -15° C contaminated with viable cells of A. salmonicida, the "furunculosis" agent. The other 50 O. masou free from the parasite were used as a control. More fish infected by A. coregoni died than the control fish. PM examination revealed that external lesions of furunculosis occurred in all parts of the body and were slightly more abundant in the posterior half of the body of both infected and uninfected fishes; the infected fish had more extensive lesions than the uninfected. However, no definite correlation was detected between the site of the external lesions and that of attachment of A. coregoni (Shimura et al., 1983).

The fatty acid sensitivities between Gram-positive and Gram-negative bacteria may result from the impermeability of the outer membrane of Gram-negative bacteria since the outer membrane of Gram-negative bacteria is an effective barrier against hydrophobic substances (Galbraith and Miller, 1973; Sheu and Freese, 1973). In fact, Gram-negative bacteria are more resistant to inactivation by medium and long chain fatty acids than Gram-positive bacteria (Kabara, 1981).

In this present study concluded that captive condition of Mugilidae fishes, infected with the Argulus spp. ectoparasites in 28±2 ppt, these parasites adopted to survive in that higher salinity nature. This adoptive nature instinct to live long period on host fishes in any extreme environmental conditions. Their survival skill highly harmful not only to Mugilidae fishes but also to other fish species in the tanks as well.
as natural environment. The parasites give a way to opportunistic secondary bacterial pathogens.