MICROBIOLOGY AND HISTOPATHOLOGY
Chapter VI – MICROBIOLOGY AND HISTOPATHOLOGY

6.1. Introduction

Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers a favourable culture environment for the microorganisms. The importance of intestinal bacteria in the nutrition and well being of their hosts has been established for homeothermic species, such as birds and mammals (Floch et al., 1970). Though the digestive tract of endotherm that is mainly colonized by obligate anaerobes (Finegold et al., 1983) the predominant bacterial species isolated from digestive tracts most of the fish have been reported to be aerobes or facultative anaerobes (Trust and Sparrow, 1974; Bairagi et al., 2002 and Saha et al., 2006).

Gut microflora plays an important role in the digestive process, growth and disease susceptibility (Fenchel and Kofoes 1976 and Yingst, 1976). The intestinal lumen contains numerous and many species of bacteria. This THBP population preserves the integrity of the body from the continuous threat of infection by the intestinal bacteria, the non – specific protection strategies of the gut are augmented by the presence of immune system. The mucous membranes lining the digestive, respiratory, and urogenital systems have a combined surface area which acts as the major site for the entry of most
pathogens. This mucosal immune system is formed by IgA secreting plasma cells located in the Lamina propria beneath the epithelium.

These IgA secreting cells in the gut wall are clearly the result of B cell stimulation induced by microbial and food antigens in the gut lumen since such antibody producing cells are virtually absent in the gut tissue. More commensal bacteria live in the gut and which enhances the digestive ability, these commensal bacteria live in the gut and which enhances the digestive ability both to promote development of the gut-associated lymphoid tissues and to maintain its function. THBP count in the different regions of the gut included symbiotis as well as pathogenic microbes. Symbiotic microbes have probiotic role and promotive digestive ability by producing microbial enzymes (Van der Waaji, 1987).

The decrease in THBP in different regions of gut antigen treated fish indicated the decrease in probiotic microbes. The reduction of beneficial gut microflora had found to be interfered with the digestive ability, food consumption energetic and overall health of the fish. Fish diseases are one of the major problems in the fish farm industry. Even though vaccines are being developed and marketed, they cannot be used as a universal disease control measure in aquaculture. The use of antibiotics to cure bacterial infection and prevent fish mortality in aquaculture is becoming limited as pathogens develop resistance to the drugs (Gonzalez et al., 2000 and Gomez Gil et al., 2000). One of the major problems in aquaculture is fish diseases and serious infections have been reported in those countries in which aquaculture is reasonably developed like Nigeria, South Africa, Zamia and Zimbabwe (Toranzo et al., 2005 and Li et al., 2009).
Intensive aqua farming accompanies several disease problems often due to opportunistic pathogens as evident from general aquaculture. High stocking densities, high food inputs and other organic loads stimulate the selection and proliferation of opportunistic bacteria (Austin et al., 1995). Due to this negative balance of the microbial community in rearing water as well as in fish gut, the aquaculture is often faces mass mortality of their stocks. However, with changing scenario, farmers are emphasizing on diagnosis and prevention of infection to promote health and production efficient. Histopathological knowledge has been successfully used to diagnose diseases of aquatic organisms (Awal et al., 2001).

Animals in the aquatic environment carry bacterial flora in their intestine which is a reflection of the flora in the environment (Al- Harbi and Uddin, 2004). Gut microflora play an important role in the digestive process, growth and disease susceptibility of marine deposit feeders (Fenchel and Kofoes 1976 and Yingst, 1976). However, some bacteria which possess the ability to tolerate the low pH in gastric juices, resist the action of bile acids and lysozyme secreted in intestines and the immune responses, adhere to mucus and/or enteric wall surface, could persist for a relatively long time and eventually make intestinal microflora specific to each host animal (Olsson et al., 1992). Intestinal bacteria such as *Aeromonas* sp. and *Vibrio* sp. often cause opportunistic infections (Asfie et al., 2000).

The symptoms of the infected fishes are apparently not so visible in the external feature or free-living movement of fish. The examination begins with external observation of any fish suspected of having a disease. These pathogens obtain food by
breaking down body tissues or by absorbing digested food from the intestines. In the fills, hyperplasia takes place between the gill lamellae. The parasites feed on the newly produced cells and damage gill tissue (McKenzie and Hall 1976 and Hossain et al., 2007). Usually in histopathology, the test is carried out with tissue sections. However, the tissue is fixed for subsequent test. At the present time, hematological investigations have been ever increasing in importance in practical fish pathology. Usually any hematological study is based upon the examination of smear, although hemoglobin and total protein estimations, serum electrophoresis and other biochemical determinations are also carried out. The blood undergoes serious changes, especially in the case of bacterial infections, but feeding and starvation also affect its composition such as total protein level, hemoglobin, and number of erythrocytes (Tonguthai, 1993). Histopathology is an important disease diagnostic tool in affected fresh water fishes (Ahmed and Hoque 1999; Ahmed et al., 2000 and Hossain et al., 2007).

Melano macrophage centers (MMC) are physiological features in fish spleen and kidney (Agius and Roberts, 2003). They are believed to be functional equivalents of the germinae centres of spleen and lymph nodes in mammals (Ellis, 1980). MMC may contain four types of brown pigments; melanin, lipofuscin, ceroid and hemosiderin (Couillard et al., 1999), Wolke et al., (1985) found that stressful situations related to aquaculture practices have resulted in increased numbers of spleenic and kidney MMC. Clinical studies have shown an association of MMC with highly resistant intracellular bacteria such as *Mycobacteria* and parasite such as *Myxobolus* sp. (Roberts et al., 2001).
Histological analysis appears to be a very sensitive parameter and is crucial in determining cellular changes that may occur in organs such as the gills, liver and gonads (Dutta 1996). Histopathological investigation may therefore prove to be a cost effective tool to determine the health of fish populations, hence reflecting the health of an entire aquatic ecosystem in the bio-monitoring process.

Fish histopathology could therefore make a valuable contribution in the monitoring of aquatic ecosystems and forms an integral part of environmental impact assessments in the environmental management process.

6.2. Materials and Methods

6.2.1. Histopathology

6.2.1. a. Preparation of histological slides

After dissecting diseased fish tissues, the samples were quickly washed with physiological saline solution (0.75% NaCl) and immediately fixed in 10% formalin. The following protocol was maintained for preparing histological slides.

6.2.1. b. Dehydration

The fixed tissues were gradually dehydrated in upgrading concentration of alcohol with 50%, 70%, 90% and 100% in 15 min, 45 min, 15 min and 15 min respectively. After dehydration the tissues were cleared in xylene for 10 min.

6.2.1. c. Paraffin impregnation

The function of paraffin impregnation was to provide a hard supported block for sectioning. A paraffin bath with 60°C was used. The melting point of the paraffin was 54 – 58°C. The tissues were treated in molten paraffin for 30 min - 1 hr.
6.2.1. d. Paraffin embedding

Metallic moulds and various types of containers including ice-cube trays and watch glasses were used for this purpose.

6.2.1. e. Blocking out of impregnated tissues

Molten wax was poured into the mould for solidification. With heated forceps the tissue was transferred and oriented so that the face to be cut was firmly embedded in the solidifying layer. When a thin layer of wax was hardened on the outer surface the mould was quickly, submerged in cold water. After complete solidification the block was removed from the mould.

6.2.1. f. Trimming and Sectioning

The blocks were then trimmed properly to the size of the tissue by cutting off the extra paraffin around the tissue. After trimming, the blocks were kept in the ice chamber for a while before cutting the section. The block was fitted to the microtome for sectioning. Sections were cut on a microtome fitted with a sharpened microtome knife. The temperature of the water bath was maintained between 55 – 56°C for stretching the cut out ribbons. Blocks of fish tissues were cut at 6 – 8 µm in thickness.

6.2.1. g. Affixing and deparaffinization

The ribbons were attached on the glass slides by means of Mayer’s albumen. In 20cc distilled water, 3 – 5drops of Mayer’s albumen was added and Shaken well and then allowed to dry at 25°C – 34°C. The slides with ribbons containing tissues were kept at 60°C for 10 min. for melting, followed by immersing the slides in xylene I and xylene II for 10 min each to remove the paraffin.
6.2.1. h. Rehydration of the Tissues

The slides with tissues were then transferred to the following grades of alcohols: 100% for 10 min, 90% for 5 min, 70% for 5 min and 50% for 5 min. At the end of this process tap water was used to remove alcohol.

6.2.1. i. Staining

The slides were then dipped in haematoxyline for 5 min. Then they were washed properly with running water. They were then dipped in 0.5% alcohol for 30 sec and washed again with running water. Finally they were dipped in eosin for 1 – 2 min.

6.2.1. j. Dehydration and Cleaning

The slides were then dehydrated gradually keeping in alcohol in the following way 70% for 5 min, 90% for 3-5 min and 100% for 15 min. The stained tissues were finally cleaned in xylene for 10 min.

6.2.1. k. Mounting and labeling

The permanent mounting of the slides was made by DPX and were labeled according to samples.

6.2.2. Total heterotrophic bacterial population

The total heterotrophic bacterial population was enumerated by pour plate technique using nutrient agar medium. The stomach, intestine and rectum samples were homogenized individually using a known volume of sterilized distilled water to make serial dilutions. After serial dilution with precaution, one ml of aliquots of appropriate dilutions of the sample was pipetted out into sterile petridishes and 15 to 20 ml of sterile
nutrient agar medium were poured. The medium and the inoculums were thoroughly mixed using turn table and the medium was allowed to solidify. Duplicate plates were also maintained. The numbers of bacterial colonies were counted after 48 hrs of incubation. The bacterial populations were expressed as number of colony forming units (CFU) per gram samples analyzed.

6.2.3. Generic composition of gut micro flora

Representative of morphologically dissimilar well isolated colonies were selected at random from the nutrient agar plates of stomach, intestine and rectum samples. The selected colonies were sub cultured to check purity after noting morphology and pigmentation of colony. Then the pure bacterial strains were again sub cultured in nutrient agar slants. The slant cultures were stored at 4°C in refrigerator and periodical subculturing was done to maintain the viability of the bacterial strains. The bacterial cultures were identified up to generic level by employing the scheme Fig.6.1.

All the experiments were carried out in triplicates and the mean ± standard deviation were estimated.
Figure 6.1. Bacterial strains identification up to Generic level

Gram Staining

Positive

Cocci  Small rods  Rods

(Microccous sp.)

Pencillin sensitive test (2.5 IU/disc)

Spore Stain

Positive

(Bacillus sp.)

Negative

(Corynebacterium sp.)

Huge & Leifson medium

Fermentative  Non Fermentative

(Pseudomonas sp.)

Kovacs Oxidative Test

Positive

(Aeromonas sp.)

Negative

(Enterobacter sp.)

Non-Luminescent

(Vibrio sp.)

Luminescent

(Flavobacterium/ Cytphaga)

Acid & Gas

Positive

(Aeromonas sp.)

Acid & No Gas

Yellow/orange

Negative

(Achromobacter)

Pigmentation test

(Kings medium)

Flavobacterium/ Cytphaga
6.3. Results and Discussion

Fish maintained as control showed normal structural organization of cells with granulated cytoplasm and uniform nuclei in liver. Fish treated with pathogen showed total loss of hepatic architecture with abnormal hepatocyte nuclei, hepatic necrosis, cytoplasmic vacuolization and conjunction of sinusoids. The liver shows a perivascular round-cell infiltration.

In fish maintained as control the lymphoid areas of spleen were found to be normal. In fish treated with antigen congestion and free apoptotic debris were observed in spleen. Histopathological analysis of spleen using light microscope revealed the aggregation of Melanomacrophage Centre (MMC) and production of mast cells in heat killed and live *A. hydrophilia* induced experimental groups than that in control. The spleens show increased melanomacrophage activity and it is surrounded by a massive accumulation of round cells and fibroblasts. Small chains of coccoid bacteria are visible within this area of inflammatory cells.

In fish, treated with combination of antigen, adjuvant and in control fishes the gross muscle mass was found to be stable. In fish treated with bacterial antigen muscular atrophy and high inflammatory cellular infiltration were observed.

Several histopathological changes that were seen in *Catla catla* fishes treated with *A. hydrophila* heat killed antigen and Vaccine treated fishes were shown in **Plate 6.1**.
Plate 6.1. Histopathological changes in *Catla catla* treated with the heat killed antigen and vaccine

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Normal</th>
<th>Antigen treated</th>
<th>Vaccine treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td><img src="image" alt="Liver Normal" /></td>
<td><img src="image" alt="Liver Antigen treated" /></td>
<td><img src="image" alt="Liver Vaccine treated" /></td>
</tr>
<tr>
<td>Muscle</td>
<td><img src="image" alt="Muscle Normal" /></td>
<td><img src="image" alt="Muscle Antigen treated" /></td>
<td><img src="image" alt="Muscle Vaccine treated" /></td>
</tr>
<tr>
<td>Spleen</td>
<td><img src="image" alt="Spleen Normal" /></td>
<td><img src="image" alt="Spleen Antigen treated" /></td>
<td><img src="image" alt="Spleen Vaccine treated" /></td>
</tr>
</tbody>
</table>

MMC – Melanomacrophage centres
hn – hepatic necrosis
The THBP count in different regions of gut of control and antigen treated fish was analysed. In the present study, the reduction of Total heterotrophic population in the gut is due to the administration of antigens, which affected the gut associated epithelial cells, where lymphoid tissues were present.

A decrease in bacterial population occurred in the gills, intestine and muscles when compared to the control fishes. The antigenic stress inhibited the digestive enzymes, changes in gut epithelial cells and alterations in food and feeding reduce the bacterial load. The poor digested food failed to provide the suitable medium for the growth of microflora in the alimentary canal, which lead to reduction in total heterotrophic population.

Table. 6.1. Total heterotrophic bacterial population in the alimentary tract of fishes maintained as control fishes exposed to vaccine

<table>
<thead>
<tr>
<th></th>
<th>Vaccine concentration (µg/ml)</th>
<th>Bacterial density (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fore-gut</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Heat killed antigen treated fishes</td>
<td>75</td>
<td>40</td>
</tr>
<tr>
<td>Vaccine treated Fishes</td>
<td>75</td>
<td>42</td>
</tr>
</tbody>
</table>
The bacterial microflora of the gut is an extremely stable ecosystem, which prevents colonization of newly ingested bacteria, in conjugation with other non-specific defense mechanism to prevent infection by intestinal bacteria (Ranjithsingh et al., 2003).

The changes in the physiological mechanism of the body can reduce cytokines production. These cytokines play an important role in activating B cells, T cells, Macrophages and various other cells that participate in the immune response.

Comparison of THBP in different regions of gut of immunoprophylacted fish with that of control fish, revealed microbial population and was found to be increased in fish administered with adjuvant antigen.

Stress compromises the fish’s natural defenses so that it cannot effectively protect itself from invading pathogens. A disease treatment is an artificial way of slowing down the invading pathogen so that the fish has time to defend itself with an immune response. Any stress which adversely affects the ability of the fish to protect itself will result in an ongoing disease problem: as soon as the treatment wears off, the pathogen will build up its numbers and attack again (Butchiram et al., 2013).

Histopathological examination helps in confirmation and identification of primary factors that are responsible for disease establishment and the effect of vaccines in antigen treated fish.