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HISTOCHEMICAL STUDIES ON SKIN OF FRESHWATER FISH *CHANNA STRIATUS* INFECTED WITH BACTERIA AND FUNGI CAUSING EUS

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ABSTRACT

The harmful effect of the bacterial and fungal infection causes epizootic ulcerative syndrome (EUS). The present study focus on the histochemistry of skin of the freshwater edible fish *Channa striatus* infected with *Aeromonas hydrophila, Staphylococcus aureus, Aspergillus fumigatus* and *Aspergillus niger*. The histochemical observation revealed a marked reduction in the infected fishes. The glycogen content and total protein content of the skin cells was compared with the control fish. *C. striatus* control skin shows moderate presence of Glycogen (PAS) and infected skin shows strong the presence of Glycogen substances (PAS), *C. striatus* control skin shows pale blue presence of acid mucin (AB2.5pH) and infected skin shows light and dark blue presence of sulphated mucins (AB 2.5 pH), *C. striatus* control skin shows presence of mucin (AB 1.0 pH), infected skin shows presence of sulfated mucosubstances (ABl.0 pH) and control skin, displaying presence of Glycogen predominately (pink), less amount of mucin (blue) (AB2.5pH/PAS). *C. striatus* control skin, displaying presence of Glycogen predominately (pink), less amount of mucin (blue) (AB2.5pH/PAS) and infected skin displaying the presence of mucin predominately (AB 2.5 pH /PAS), control skin presented elastic fibers purple and collagen stained with light yellow (Aldehyde fuchsine) and infected skin shown elastin decreased (Aldehyde fuchsine). *C. striatus* control skin displaying presence of basic proteins (Bromophenol blue) and infected skin displaying depletion of basic proteins with (Bromophenol blue). The present investigation deals with the histochemical nature of the skin of *C. striatus* in order to discuss, the contrast among control and the effect of bacterial and fungal infected skin.

KEYWORDS: *Aeromonas hydrophila, Aspergillus niger,* histochemistry, EUS, Aldehyde fuchsine.

INTRODUCTION

The freshwater edible fish *Channa striatus* was sucpectiable to bacterial and fungal infection such as *Aeromonas hydrophila, Staphylococcus aureus, Aspergillus fumigatus* and *Aspergillus niger* which are pathogenic group of EUS charecterstics. These types of infections may affect fish even under the low temperature (20- 25^{o}C) and stressed environmental conditions (Podeti and Benarjee 2016). The skin is the primary barrier against the environment allowing normal internal physiological function Sarasquetel *et al.,* (1998). The skin mucus of fishes may be important in natural defense against parasites and pathogenic microorganism besides having a possible osmoregulatory and lubricate function (Fletcher 1978; Van Oosten 1957; Rosen; Conford 1971). The defense may be mechanical, due to continuous production of mucus secretion Pickering (1974). However, the presence of lysozymes, complement components of the IgM type, suggests that the mucus takes active role in the immunity (Hjelmeland *et al.,* 1983).

Most studies previously carried out in the field of histochemical studies on skin. Askawa (1970) investigated histochemical studies of the mucus of the eel *Anguilla japonica.* Mittal and Munshi (1971) investigated on a comparative study on the structure of skin of certain air breathing freshwater teleosts. Harris *et al.,* (1973) studied histochemical analysis of mucous cells in the epidermis of brown trout *Salmo trutta.* Banerjee and Mittal (1975) made investigations on histochemistry and the functional organization of the skin of a "livefish" *Clarias batrachus* made observations on histochemistry and the structure of skin. Mittal *et al.,* (1976) studied the protein and carbohydrate histochemistry in relation to the keratinization in the epidermis of *Barbus sophor* (Cyprinidae, Pisces). Mittal and Whitear (1979) reported keratinization of fish skin with special reference to the catfish *Bagarius bagarius.* Mittal *et al.,* (1980) defined the fine structure and histochemistry of the epidermis of fish. *Monopterus cuchia,* (Hjelmeland *et al.,* 1983) observed on skin mucus protease from rainbow trout, *Salmo gairdneri* and its biological significance. The skin of epidermis is sub-divided into three layers, an outermost layer, a middle layer and a basal layer. The outermost layer is made up of several layers of epithelial cells. At the surface, chloride cells, outermost cells, mucous cells, and sacciform granular cells are present. The middle layer is composed of

unicellular glands, mucous cells and sacciform granular cells. Epithelial cells fill the spaces between the gland cells (Sunita *et al.,* 2004).

Rai and Saikia (2008) made observations on effects of acid stress on the functional organization levels and distribution of Carbohydrate constituents of skin in *Heteropneustes fossilis.* Bonilla *et al.,* (2008) investigated skin histology and morphometry of the fish *Eremophilus mutisii.* Tarun *et al.,* (2010) described on histochemistry and functional organization of the dorsal skin of *Andstrus ddlichopterus.* Mohamed *et al.,* (2010) investigated experimental infection with *Gyrodactylus* species on the density of skin mucus in fries of catfish *(Clarias gariepinus)* with emphasis on the pathological changes observed on skin histochemistry of freshwater catfish *Clarias batrachus* (Laxma Reddy and Benarjee, 2011).

MATERIAL AND METHODS

The freshwater fish material for the present study was collected from different lakes of Hasanparthy, Bandham Lakes in Warangal district, Telangana, India. The *Channa striatus* EUS infected fish samples were collected alive by using fishing net, and they were brought immediately to the laboratory, in plastic containers with oxygen filled water. For histochemical studies the required tissues were removed and the blood vessels and mucous attached to them were scrapped off smoothly without damaging the original structure. The procedure followed for preparation of histological sections and for histochemical analysis of different substances is similar to the tissue. The tissues collected from control and infected fishes were immediately fixed and processed. The fixatives used in the present study were Alcoholic Bouin's, Zenker, Susa and Carnoy for mucopolysaccharides Bouin's Harris *et al.,* (1973) and cetyl pyridinium chloride (1% cetylpyridinium chloride in formalin) was used specially. Bouin’s and Susa comparatively gave good results. After fixation in Bouin’s for 18-24 hours material were washed in scots tap water. Susa fixed material had been treated with iodine alcohol. Depending upon the parts of the systems, the infiltration time varied from (1- 4) hours was minimized and then serial transverse or sagittal sections were cut 3-6mμ thickness for all the tissues. Excellent staining results for histological studies could be obtained by using Dalafields haematoxylin counterstained with eosin (Gurr 1962 and Heidenhain’s Azan Gurr, 1962).

The following various histoichemical methods have been employed to elucidat the chemical nature of carbohydrates and proteins in the skin of *Channa striatus*. The procedure as

outlined in Pearse (1968) for the different histochemical tests was adopted (Shyamasundari and Hanumantha Rao, 2007).

Identification of Carbohydrates

For testing carbohydrates, material fixed in Bouin's and Susa fixatives was used. Periodic acid/ Schiff (PAS) technique (Pearse 1968) was employed to detect the presence of carbohydrates and other groups. Periodic acid brings about oxidative cleavage of carbon to carbon bond in 1, 2 glycols to form dialdehydes, which are subsequently coloured by Schiff's reagent. This oxidant does not further oxidize the resulting aldehydes. As number of compounds give PAS positive reaction. The different PAS positive groups were further characterized by subjecting the sections to various procedures.

Polysaccharides

The conventional method for detecting polysaccharides is by the periodic acid Schiff (PAS) method. As varieties of substances were known to give a positive reaction with PAS technique. Suitable controls were employed to determine the actual compound responsible for the positive reaction. It was performed without prior oxidations with periodic acid to know whether the reaction was due to aldehydes to detect glycogen. Sections were subjected to PAS technique in conjunction with diastase digestion. Best's caramine method was also used to determine the presence of glycogen. The PAS reaction was conducted after acetylation (24 hours at room temperature in 16 ml of acetic acid and 24 ml of pyridine) and subsequent deacetylation (45 minutes in 0.1 N potassium hydroxide at room temperature or with 20% ammonia in 70% alcohol for 24 hours) to establish the presence of 1: 2 glycol groups. To determine whether the reactivity is due to lipid, sections were treated with various lipid solvents such as pyridine or methyl alcohol chloroform mixture prior to the application of PAS technique. The substance gives a positive PAS reaction but not extractable by diastase or lipid solvents and when it also gives positive reaction for protein tests, then it was considered as either a mucoprotein or glycoprotein. Finally identification was confirmed by employing methylene blue extinction technique which involves the staining of sections in methylene blue at different pH levels.

Mucosubstances

Tissue was fixed in either new Comer's fluid dioxane or 1% acetyl pyridinium chloride in 10% formaldehyde containing 2% calcium acetate modified Bouin's Harris *et al.*,(1973). Of all these acetyl pyridinimum chloride formalin was the best as judged by the integrity of cells

and by the intensity of staining especially for mucopolysaccharides. A variety of histochemical tests were employed to demonstrate different types of mucous cells and to characterize the mucosubstances elaborated by them in turn their vicinal hydroxyl groups their carboxyl or sulfate acid groups or both.

To differentiate mucosubstances, sections were subjected to the following testes PAS technique of McManus (1946). PAS technique with prior acetylation and deacetylation in digestion, PAS after diastase digestion. PAS after phenylhydrazine treatment (5% phenyl hydrazine for 1 hour at 25°C). (Spicer *et al*., 1967). These are all to demonstrate mucosubstances with vicinal hydroxyls.

Acid mucosubstances were detected by using following techniques; Alcian blue (AB) at pH 2.5 (1%AB 8GX in 3% acetic acid) for 30 minutes AB at pH 1.0 (1% AB in 0.1N HC1) for 30 minutes Mowry (1956); Lev and Spicer (1964); Mowry's (1963) modifications of Male's colloidal iron solution for 2 hours.

Acid mucosubstances were distinguished from the neutral mucosubstances by following tests. The combined technique with PAS and AB at pH 2.5 Mowry and Winkler (1956) at pH 1.0 (Spicer *et al.,* 1967).

The following procedures were adopted to distinguish sialomutins and sulfomucins. Aldehyde fuchsine (AF) of Halmi and Davis (1953), AF / AB (pH 2.5) (Spicer and Mayer 1960).

Most of the above techniques were done concomitantly with supplementary procedures and specific tests involving chemical blockage or enzyme removal of certain reactive groups in the mucosubstances. These include mild methylation (O.40 cc of concentrated HC1 in 50 cc of absolute alcohol for 4 hours at 37°C) followed by Alcian blue staining at pH 2.5 and active methylation (4 Hours at 60°C) followed by AB at pH 2.5 (Fisher and Lillie 1954; Spicer 1960). Mild methylation saponification (1% KOH in 70% alcohol for 20 minutes) followed by AB (pH.2.5) staining, active methylation soponification AB (Spicer and Lillie 1959). These methylation and demethylation treatments were performed in conjunction with Azure A, AB/PAS and AF/AB.

Proteins

Mercuric Bromophenol Blue

The presence of basic proteins in the tissues was demonstrated by using mercuric bromophenol blue. The method followed was that of Mazia *et al.*, (1953). Bromophenol blue is an anionic dye which binds with the cations of proteins in the presence of salts like mercuric chloride. It binds with both anionic and cationic groups of proteins and imparts blue color. This test was confirmed simultaneously by control slides in vanslike's reagents.

RESULTS

Comparative Histochemical Analysis of Control and Infected Skin

A series of histochemical techniques applied for revealing the chemical nature of control skin and infected skin of *C. striatus*. The skin tissue consists of flat cells, goblet cells or club cells, mucin producing cells, the dense collagen connective tissue or bundles of coarse collagenous fibers, melanin containing cells and free red blood cells which, usually respond in a similar way to all the histochemical reactions. The intensity of staining can be used for comparing the protein and glycogen contents present in the cells of the control fish with infected fishes at different concentrations. The reactions of the various cells to the histochemical tests employed and their nature was discussed in the present study.

Glycogen Content

Periodic Acid Schiff (PAS) Reaction

In the control skin, all cells in the epidermis were faintly stained with PAS. The goblet cells or club cells stained moderately. In the flat cells, the reaction was intense in comparison to the other cells. Where in the infected skin tissue, collagenous fiber was strongly positive to Periodic Schiff reaction, suggested the presence of glycogen substances.

Alcian Blue (AB) PH 2.5

This technique is used to detect the presence of acidic mucopolysaccharides. With AB at pH 2.5, the control skin tissue displayed pale blue, the presence of acid and the goblet cells showed a negative reaction. All the mucous gland cells and the flat cells had shown a positive reaction, suggesting the presence of weakly acidic mucopolysaccharides, hyaluronic acid and sialomucins. In infected skin tissue, at some areas light and dark blue bands were observed. It confirmes the presence of sulphated mucins.

Alcian Blue (AB) PH 1.0

With AB at pH 1.0, the control skin tissue cells have shown a moderate blue colour, indicating the presence of acidic sulfated mucosubstances in them. Negative reaction had been noticed in the flat cells and a few goblet cells. A majority of the mucous gland however, showed a faintly positive reaction.

When infected skin tissue is subjected with AB at pH 1.0. Cells in the superficial and outer middle layer, stained greenish blue. These reactions indicated the presence of glycoprotein's with carboxyl groups and or with sulphate esters in low concentrations at these sites. (Plate – I, Fig. 1 to 6).

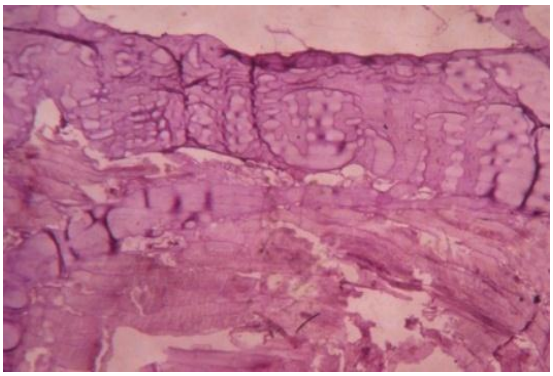
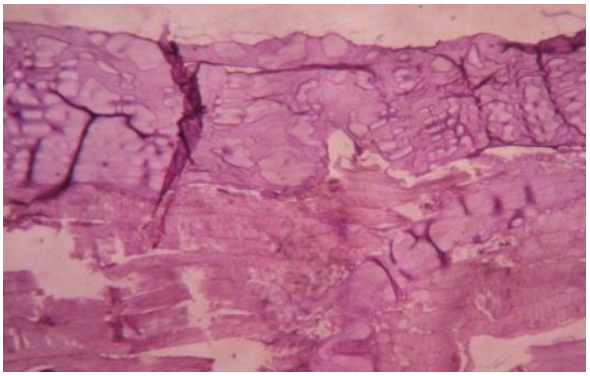


Fig.1



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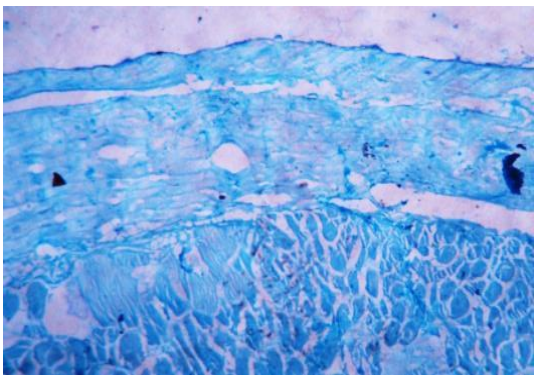

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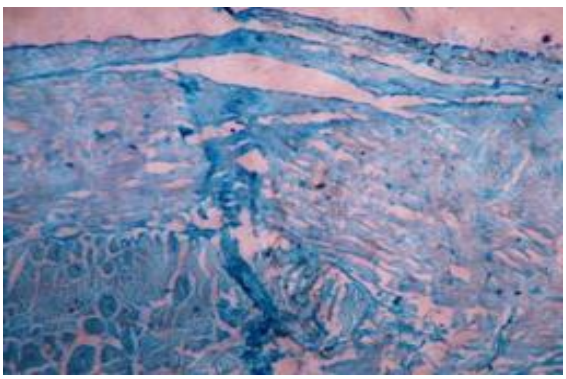
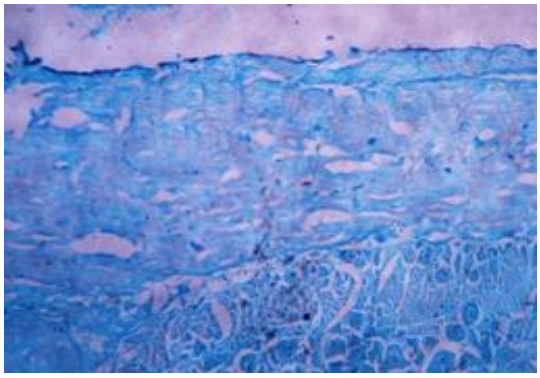


Fig.4



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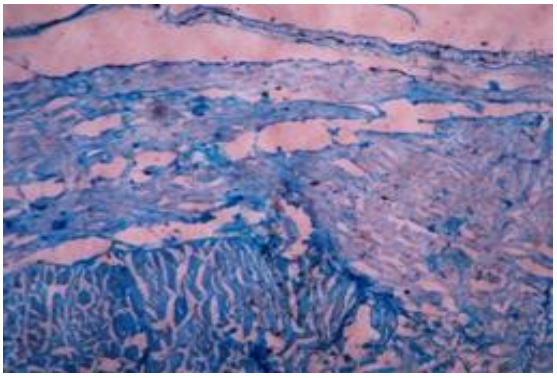

Fig.6

Fig. 1: T.S of *Channa straitus* Control skin shows moderate presence of Glycogen (PAS). Fig. 2: T.S of *Channa straitus* Infected skin shows strong the presence of Glycogen substances (PAS). Fig. 3: T.S of *Channa straitus* Control skin shows pale blue presence of acid mucin (AB2.5pH). Fig. 4: T.S of *Channa straitus* Infected skin shows light and dark blue presence of sulphated mucins (AB 2.5 pH). Fig. 5: T.S of *Channa straitus* Control skin shows presence of mucin (AB 1.0 pH). Fig. 6: T.S of *Channa straitus* Infected skin shows presence of sulfated mucosubstances (ABl.0 pH).

Alcian Blue PH 2.5/ PAS

With the combined technique of AB (2.5pH)/PAS an interesting picture emerges. The mucosubstances stained red with Alcian blue (2.5pH)/PAS technique. Neutral mucopolysaccharides stains blue are hyaluronic acid and sialomurins, those stains bluish purple include neutral mucopolysaccharides as well as the weak acidic mucopolysaccharides.

Control skin had shown pink colour, presence of predominately glycogen, and shown in faint blue colour, presence of less amount of mucin. The flat cells did not display any colour. The goblet cells appear blue indicating the presence of hyaluronic acid and sialomucins. It is to be remembered that these cells have also shown a positive reaction to PAS. Mucous gland cells seemes to contain carbohydrate protein complexes or mucoproteins. The dense collagen connective tissue however showed a bluish purple colour suggesting the simultaneous occurrence of neutral mucins and weakly acidic mucopolysaccharides. A few melanin cells however show a blue colour. Since no protein could be detected in these cells, as these cells contain only weakly acidic mucopolysaccharides.

Infected skin had shown increase in blue that indicates the presence of mucin, has increased proportionately a blue component.

Aldehyde Fuchsine

With the aldehyde fuchsine, control skin had shown elastic fibers purple and collagen stained with light yellow, the flat cells show negative reaction. Goblet cells appear as intense purple colour. The mucous cells were giving a red colour suggested that they were likely to be sulfomucins. The Infected skin tissues were strongly positive to Aldehyde fuchsine indicating the presence of sulfated mucosubstances. On the other hand the infected skin tissue showed that elastin was decreased. (Plate – II, Fig. 1 to 4).

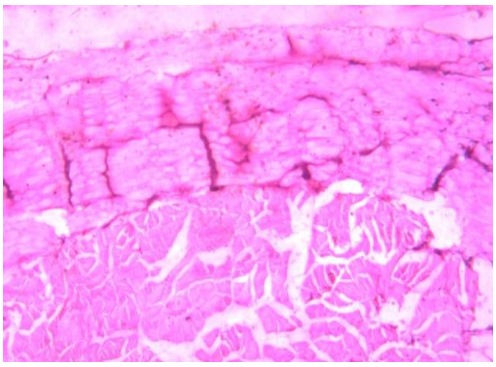

Fig.1

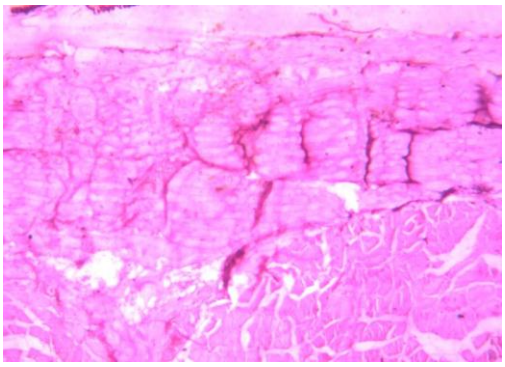

Fig.2

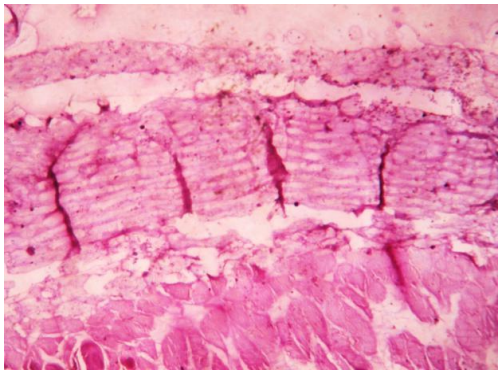

Fig.3

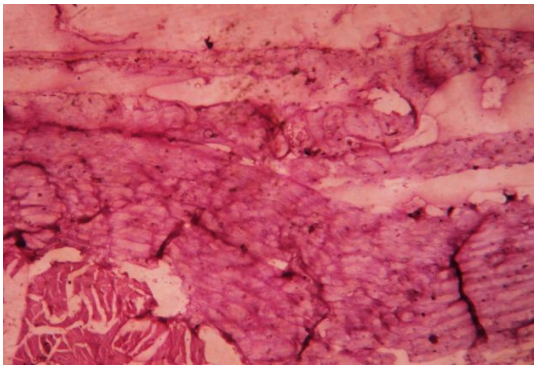

Fig.4

Fig. 1: T.S of *Channa straitus* Control skin, displaying presence of Glycogen predominately (pink), less amount of mucin (blue) (AB25pH/PAS). Fig. 2: TS of *Channa straitus* Infected skin displaying the presence of mucin predominately (AB 2.5 pH /PAS). Fig. 3: TS of *Channa straitus* Control skin presented elastic fibers purple and collagen stained with light yellow (Aldehyde fuchsine). Fig. 4: TS of *Channa straitus* Infected skin shown elastin decreased (Aldehyde fuchsine).

Proteins

Mercuric Bromophenol Blue

For testing protein in tissue sections, material fixed in Susa and Bouin's has been used. Large number of tests has been performed to study the presence of different types of proteins. The following tests were conducted. This test is applied to detect the presence of basic proteins. With Bromophenol blue the flat cells and goblet cells have shown positive reaction and the bundles of coarse collagenous fibers had stained blue shown positive reaction suggesting the presence of basic proteins. Whereas the infected skin tissue stained green, it denotes the depletion of basic proteins. Total protein was found to exhibit a noticeable decrease in flat cells, goblet cells and collagenous fibers. The histochemical procedures followed in the present study have clearly indicated that in the two species of fishes. Skin contains fairly good amount of proteinaceous material. This was confirmed, when the sections of skin were subjected to other specific stains for the detection various proteins. (Plate – III, Fig. 1 to2).

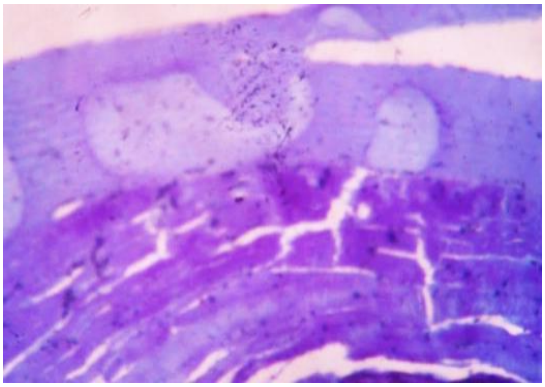

Fig.1

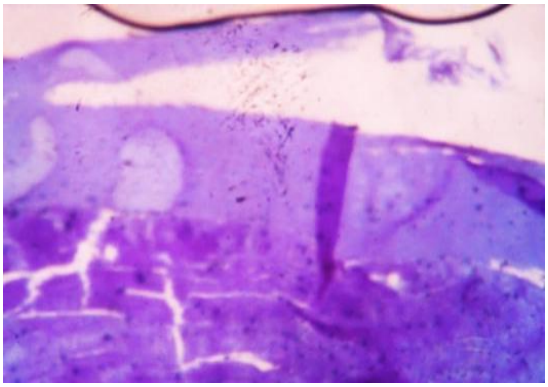

Fig.2

Fig. 1: T.S of *Channa straitus* Control skin displaying presence of basic proteins (Bromophenol blue). Fig. 2: T.S of *Channa straitus* Infected skin displaying depletion of basic proteins (Bromophenol blue).

DISCUSSION

In the present study it is possible to make the following histochemical observations. However, the control *C. striatus* fish epidermal cells were changed with large quantities of glycoprotein, the mucous cells also had large quantities of glycoprotein, as revealed by Bromophenolblue. Besides glycoprotein, the cytoplasm of these cells also contained neutral polysaccharides and acid mucosubstances with both sulfated as well as carboxlylated groups. Sulfated mucins were the predominant components of these cells. These observations were co-rrelated to many authors. In the different species of fishes, the skin mucous is composed

of mucopolysaccharides that contain sulphated and carboxyl radicals (Roberts, 1978). The present observations point out to the similar conditions. As a result of bacterial and fungal infections in *C. striatus*. However the severity of damage caused was more with the increased in the period of seasonal infection.

CONCLUSION

The histochemical investigation concludes both bacterial and fungal species such as *Aeromonas hydrophila, Staphylococcus aureus, Aspergillus fumigatus* and *Aspergillus niger* were pathogenic fungi of *Channa striatus* causes development of EUS charesterstics. The *Channa striatus* skin showed focal sloughing of the epidermis with hyperplasia and mucous cells. The dead cells formed due to sarcolysis. The histochemical reactions were determined that the control skin tissues of this fish contained glycogen, weakly acidic sulfated mucosubstances, hyaluronicacid, sialomucins, carboxlylated mucosubstances, basic, acidic proteins, glycoproteins. In the EUS infected fishes skin the tissue had shown some variation with the control.

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