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Studies on the effects of bacterial diseases on skin and gill structure of *Clarias gariepinus* in Dakahlia Provinence, Egypt

Hassan I.El-Sayyad*▫, Viola H.Zaki., Abdallah M El-Shebly*** and Dina A.El-Badry***

**Zoology Department, Faculty of Science, Mansoura University, Mansoura , EGYPT*
*** Fish & Infective Disease, Faculty of Veterinary Medicine, Mansoura University, Mansoura, EGYPT.*
**** Fish culture Department, National Institute of Oceanography and Fisheries, EGYPT.*

ABSTRACT

The sharp tooth catfish (Clarias gariepinus) is one of the most important fresh water fishes in the River Nile in Egypt. Ninty-four samples of alive mature catfish, Clarias gariepinus. were collected from the tributaries of River Nile, Egypt during summer and winter seasons of 2008-2009. Both healthy and diseased fish were examined macroscopically for skin and gill disease. The skin and gill of both healthy and diseased fish were subjected for microbiological identification of pathogenic bacteria, SDS-PAGE and DNA fragmentation analysis and light, scanning and electron microscopic investigation. The infected fishes possess ulcerative and haemorrhagic skin patches more intensified in winter more than summer. The isolated bacteria from both gills and skin of diseased were Actinobacter lwoffii, Enterobacter amnigenus , Escherichia coli , Citrobacter amlonaticus , Serratia odorifera and Aeromonas jandaei (gram-negative), Staphylococcus epidermidis (Gram-Positive). SDS-PAGE revealed no variations between healthy and diseased skin and gills. However, DNA fragmentation was moderately detected in diseased skin more than of the gill. Scanning electron microscopic examination (SEM) revealed necrotic patches in both skin and gills. Light microscopic study possesses massive atrophy, thinning and degenerative changes of both skin and gill. Transmission electron microscopic (TEM) study exhibited abundant distribution of cytoplasmic vacuoles as well as alterations of cytoplasmic organelles including mitochondria, rough endoplasmic reticulum and lysosomes of club cells and epithelial cells. The nuclear envelope of both club and epithelial become convoluted. The mucous cells showed widespread of vacuoles. The macrophage cells were apparently become degenerated. The epidermal-dermal junction lacked irregular pattern and the dermis exhibit internal haemorrhage and hyaline necrosis. Finally, C. gariepinus is highly susceptible to bacterial infection inducing pathological alterations in skin and gills of fishes and may cause mortality of it.

INTRODUCTION

The sharp tooth catfish (*Clarias garipinus*) is one of the most important fresh water fishes in the River Nile in Egypt. It has a great economic importance, contributing about 17.5 % of the total country catch (Gafrd, 1996). Most fishes obtain oxygen from water using efficient gills. Some fishes swim to the surface and take in the oxygen-rich water at the interface, while others have evolved the ability to breathe atmospheric air (Ishimatsu *et al.* 1998; Park *et al.* 2000; Zhang *et al.* 2000; Park, 2002). The substantial surface area of the gills in fish serves as an interface between the environment and blood, notably for the continuous diffusion of oxygen and the maintenance of acid–base and ion balance (Randall *et al.* 1996 ; Claiborne *et al.* 2002).

Fish are susceptible to a wide variety of bacterial pathogens especially when the fishes are physiologically unbalanced or nutritionally deficient, or subjected to stressors, i.e., poor water quality, and overstocking. Infectious diseases are the main cause of economic losses in aquaculture industry which is negatively impacted by various pathogenic organisms (Plumb, 1997) such as *Edwardsiella tarda* (Gram negative enterobacterium) which is the causative agent of edwardsiellosis in freshwater(Abdel-Lah and Shamrukh ,2001). Edwardsiellosis is a septicemic disease characterized by extensive lesions in the skin, muscle and internal organs and infected commercially important fish including eels, channel catfish, mullet, Chinook salmon, flounder, carp, tilapia and striped bass (Thune *et al*., 1993).

On the other hand, the skin and gills constitute the boundary tissue of the fish, and, being continuously hydrated, non-keratinized, and covered by a layer of slimy coating, is more suitable to water-borne toxicants. The skin of *Clarias gariepinus* acts as accessory respiratory organs (Banerjee and Mittal, 1976) and supplements any deficiency in oxygen uptake through conventional respiratory organs (gill).

The present study aimed to illustrate the bacterial pathogen of infected skin and gills as well as clarifying the detailed light, scanning and transmission electron microscopic investigation. Proteomic analysis and DNA fragmentation were taken in consideration.

MATERIAL AND METHODS

Ninty-four samples of alive mature catfish, *Clarias garipinus* were collected from the tributaries of River Nile, Dakahlia Governorate, Egypt during summer and winter seasons of 2008-2009. The infected fishes were isolated, in a glass aquaria measuring 60x30x30 cm each containing 30 L of water and sacrificed immediately. However, the healthy fishes were separated, fed *ad libitum* and the water was changed daily to discard the metabolic wastes till subjected for experimentation.

Investigated parameters:

1. *Macroscopic examination:*

Skin, gills and fins were examined for lesions and recorded.

2. *Microbiological studies:*

The skin and gills of both control and diseased fish were scraping with sterile loop excrement. Each of the, skin and gill samples (1 g suspended into 3 g of 0·9% m/v NaCl solution) was added into a presterilized McCartney bottle containing nutrient broth (9 ml) and incubated for 24 h at 30°C followed by thereafter, aliquots (0·1 ml) of each of the serial dilutions were spread onto Maconkey plates found on health cut. The plates were incubated for 24 h at 30°C. Single

colonies isolated from these plates, were purified and subjected to Gram staining identifies by automated Microscan system for biochemical identifications and an assessment of catalase activity (Cruickshank *et al.*, 1975) and Austin and Austin ,1987) .

3. Sodium Dodecyl polyacrylamides gel electrophoresis (SDS-PAGE):
The skin of both control and diseased fish were incised and homogenized and processed for SDS-PAGE according to Laemmli (1970). Electrophoresis was carried out with constant volt at 200v. The separated proteins on polyacrylamides gel stained with coomassie blue R-250. Coomassie blue staining requires an acidic medium for the generation an electrostatic attraction between the dye molecules and the amino groups of the proteins. Coomassie stain gives a linear response up to 20mg/cn.

4. DNA fragmentation:
DNA fragmentation (Ladder formation) was analyzed as described by Wyllie *et al.* (1980). Skin of both control and infected fishes were lysed with neutral lyses buffer. Genomic DNA was extracted by the phenol-chloroform method and analyzed by agarose gel electrophoresis at 46v for 2h on a 1.0 % agarose gel containing 0.1µg/ml ethidin bromide. DNA fragmentations were determined by inspection under Ultra-Violet light. The DNA bands were analyzed by DNA gel analyzer to determine the % of DNA fragmentation.

5. Scanning electron microscopic :
For scanning electron microscopic study, gill and skin samples were fixed in a mixture of paraformaldehyde 2.5 % and glutaraldehyde 2.5 % solution in 0.1 M phosphate buffer (Ph 7.4) for 4 hours at 4°C. After washing in the same buffer, the specimens were dehydrated in ascending grades of ethanol followed by critical point drying in carbon dioxide, then coating with sputter-coater and examined with Joel JSM 5300 scanning electron microscope, Faculty of Science, Alexandria University.

6. Light microscopic Investigation:
Both control and infected fishes were sacrificed and then the opercula cavity was opened. The gills were examined in situ and dissected. The skin and gill regions were separated and fixed in 10% phosphate buffered formalin for 18-24 hrs. After fixation, the samples were extensively washed in 70 % alcohol (3 x 24 hr) to get rid of the fixative before the subsequent step of tissue processing. The tissue samples were then dehydrated in ascending graded series of ethanol (80%, 95% and absolute), cleared in xylene and embedded in molten paraffin wax 58-62 C°. Paraffin sections (5µm) were cut and staining with haematoxylin and eosin according to Bancroft and Steven (1990).

7. Transmission Electron microscopy:
Biopsies from skin of both control and infected fishes were separated immediately, fixed in 2.5% glutaraldhyde and 2% paraformaldhyde in 0.1 M cacodylate buffer (pH 7.4), post fixed in 1% osmium tetra oxide at 4ºC for 1.5 hour. This was followed by dehydration in ascending grades of ethyl alcohol and embedded in epoxy-resin. Ultrathin sections were cut with a diamond knife on a LKB microtome and mounted on grids, stained with uranyl acetate and lead citrate and examined at Joel Transmission electron microscope.

RESULT

Macroscopically:
The examined fish exhibited discoloration of skin associated with the development of different patches of ulcerative and haemorrhagic skin. The apical caudal fin rays appeared necrotic. The lesions were invariably deep dermal ulcers with severe bacterial necrosis and an overlaying congery of necrotic tissue. In contrast, the gills appeared mucinous with widespread of pale yellow patches. The gills exhibited different swollen, often abrupt and protruding from the side of a gill filament. Other parts of the gill filaments took red colour as a result of pooled blood cells in ruptured or dilated capillaries. The gill filaments become comparatively shorter and wider with rounded or squared tips (Fig. 1 A-B).

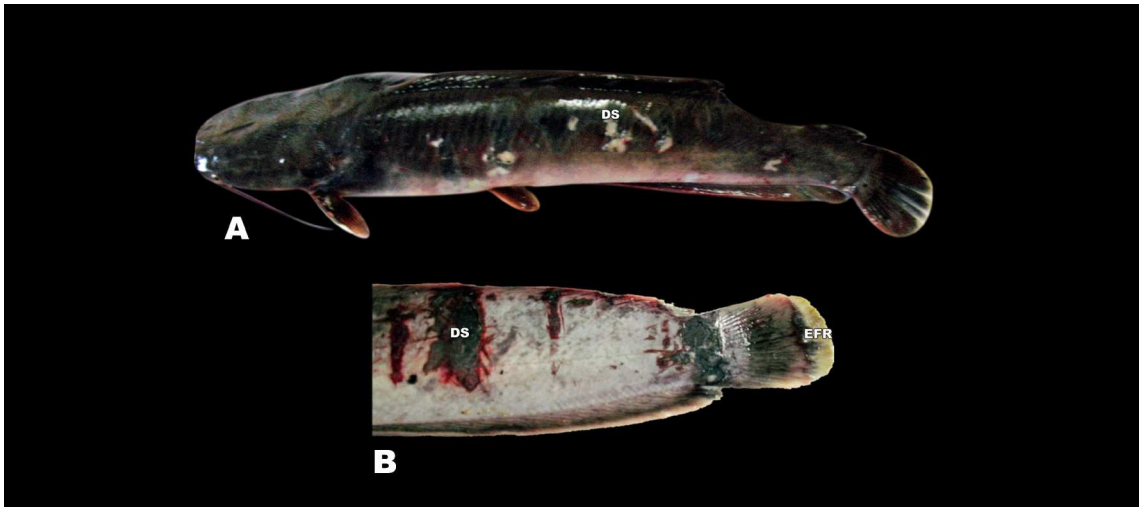
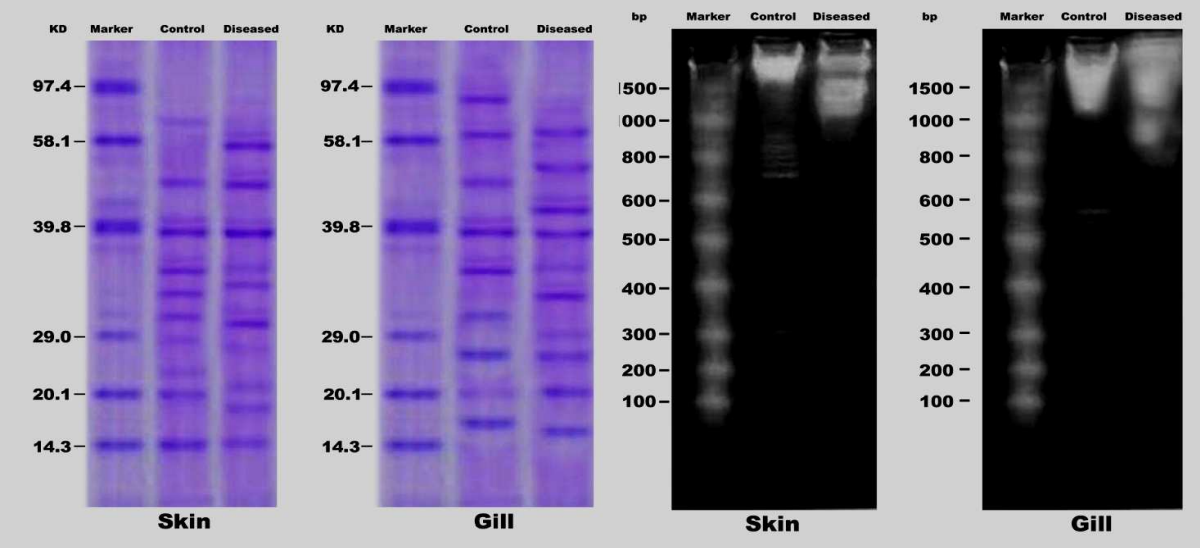


Fig.1 (A-B). Diseased *Clarias gariepinus* .

A. Showing different skin patches of superficial ulcers .
B. Showing necrotic skin with internal haemorrhage.

Microbiological isolation and identification of pathogenic bacteria:
Isolation and identification of significant bacteria from skin and gill lesions were carried out according to Cruickshank *et al.* (1975) and Austin and Austin (1999). The isolated bacteria from both gills and skin of diseased *Clarias gariepinus* were *Actinobacter lwoffii*. (Family, Neisseriaceae- Gram-Negative Aerobic Rods and Cocci), *Enterobacter amnigenus*, *Escherichia coli*, *Citrobacter amlonaticus* and *Serratia odorifera* (Family Enterobacteriaceae- Facultatively Anaerobic Gram-Negative Rods), *Aeromonas jandaei (*Vibrionaceae: facultatively anaerobic gram-negative rods) and *Staphylococcus epidermidis (*Family: *Micrococcaceae*, Gram-Positive Cocci (Aerobic, Catalase-Positive Genera). The intensity and spread of disease appeared more detected during winter season more than that of summer season.

SDS-PAGE of skin & gills:
Proteomic analysis of both skin biopsies revealed no variations of protein expression in skin; however, gills possessed a slight variation of lacked protein expression at protein bands 32.66, 29.31 and 22.96 KDa (Fig.2 A).



A **B**

Fig. 2 A. SDS-PAGE of healthy and diseased skin and gill of C. gariepinus.
Fig. 2B. DNA fragmentation of healthy and diseased skin and gill of C. gariepinus.

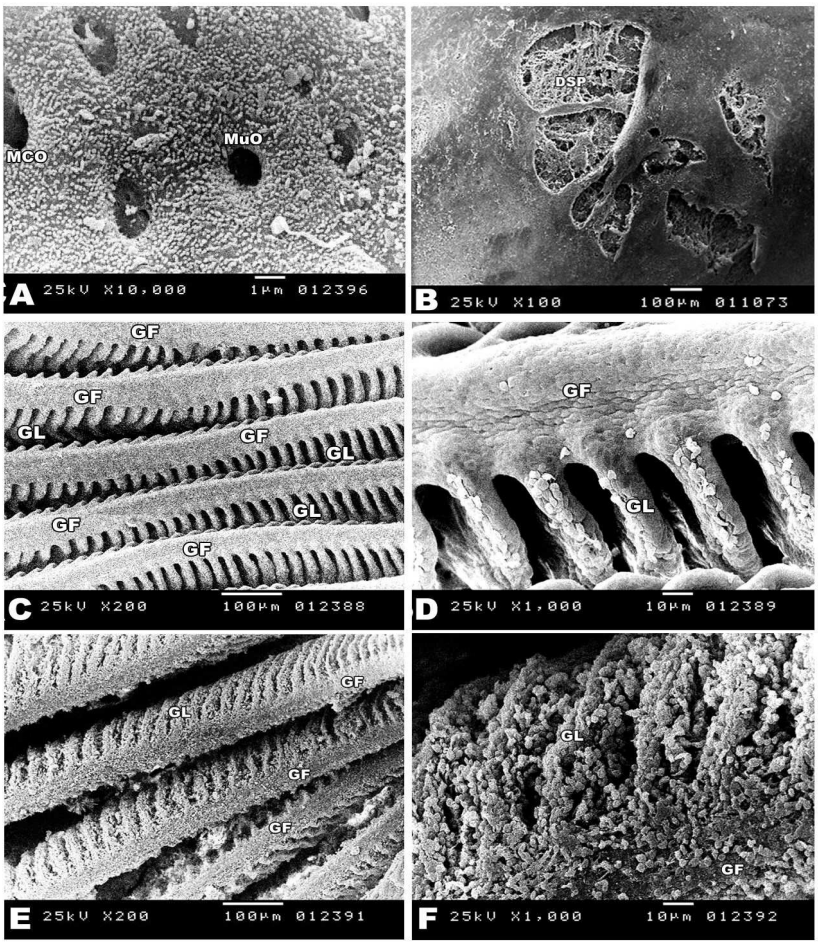


Fig.3 (A-F). Scanning electron microscopic photomicrograph of skin (Figs.A &B) and gills (Figs. C-F) of diseased *Clarias gariepinus*.

A. Healthy skin surface with detected mucous gland opening.
B. Diseased shin showing eroded skin surface.
C&D. Healthy gill showing gill filament carrying regularly arrangement gill lamellae bearing fine particles of mucous secretion. ; E&F. Diseased gill showing thinning and degeneration of gill filaments.

DNA fragmentation:

Comparing with healthy fish, skin of infected fish exhibited massive fragmentation of DNA. However diseased gills exhibited moderate damage of DNA (Fig. 2 B).
Scanning electron microscopy:

The healthy fish appears smooth with widespread of mucous gland opening. Fine accumulation of mucous secreting particles allover the skin surface (Fig.3 A). However, the infected one revealed massive erosion of skin surface associated with disruption of mucous gland opening (Fig. 3 B).
In addition, the healthy gill possesses regularly arranged gill filaments radiating from a gill arch. Leaf like lamellae branch of the filaments at regular intervals which are perpendicular to the filament's long axis. The lamellae are dramatically increasing the surface area of the gill filament epithelium and result in a small diffusion distance between the blood that perfuses each lamella and the respiratory water. It is easy to distinguish the pavement cells with their conspicuous microvilli and microridges, and largely infolding protrusions. The lamellar surface reveals outlines of the epithelial cells covered in places by scattered patches of mucus. Mucus cells occasionally open onto the lamellar surface and are often observed shortly after they had released mucus droplets (Fig. 3 C&D).

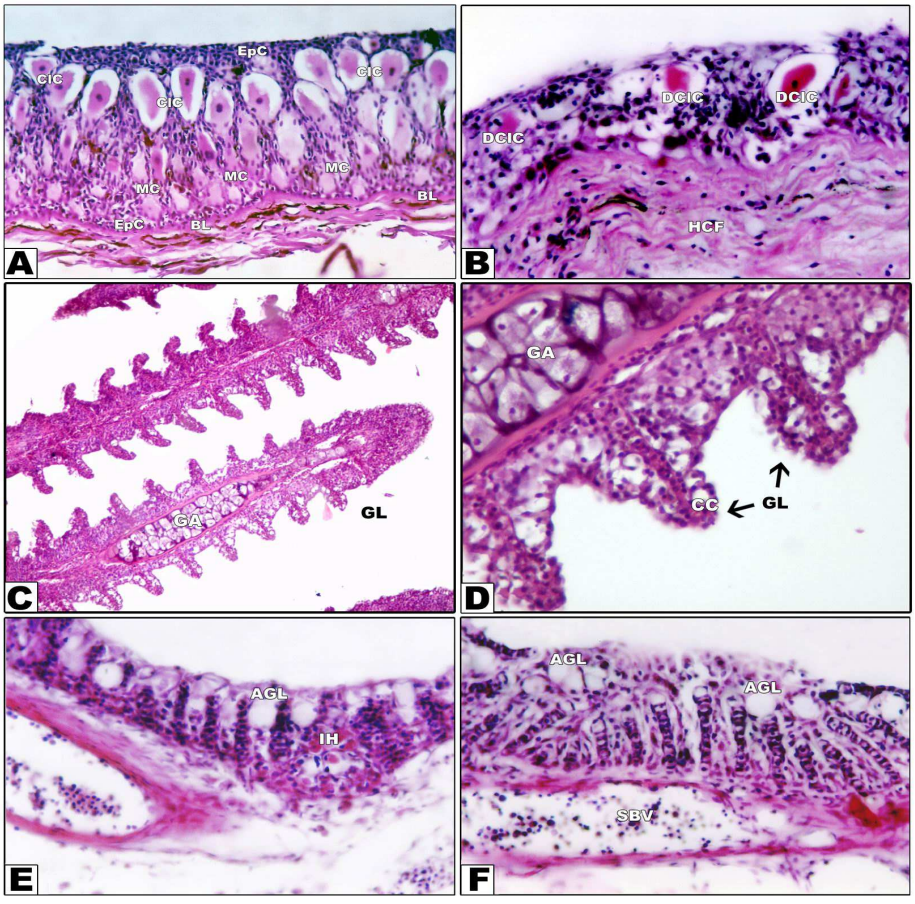


Fig.4(A-F). Photomicrograph of histological section of skin (V.S.) (Fig. A&B) and gills (Figs. C-F) of diseased *Clarias gariepinus*.

A. Healthy skin with detected epithelial, mucous and club cells. X250
B. Diseased shin showing massive skin atrophy. X250
C&D. Healthy gill showing gill filament carrying regularly arrangement gill lamellae having epithelial lining cells. DX250; E&F. Diseased gill showing thinning and degeneration of gill filaments, swollen blood vessels and internal haemorrhage. X250.

In infected fish, there was a considerable alterations of the gill filaments including extreme swelling of the gills caused by an abnormally large number of cells at the outer edge of the gill filaments. The gill lamellae exhibited massive thinning and distortion (Fig.3 E & F).
Light and transmission electron microscopy of Skin:

At light microscopy, the skin of *C. gariepinus* has no scales and is composed of two distinct layers: epidermis and dermis. The epidermis is a semi-like stratified epithelium with numerous mucous cells, club cells and epithelial cells. The epithelial cells scatter in between the other types of cells, especially in the basal layers. The dermis is composed of connective tissue, muscle fibers, and capillary blood vessels (Fig.4 A).

TEM observations of the healthy epidermis reveal the presence of three kind of cells; epithelial cells, club cells and mucous cells. Beside the mentioned major cells, clusters of macrophage cells appear in the middle layer. The macrophage cells possess enlarged nuclei with irregular nuclear envelope and thin coat of cytoplasm. The club cells are large with prominent nuclei rich in euchromatin and thin peripheral heterochromatin. The nuclei enclose by one or two nucleoli and their nuclear envelope appears irregular. The cytoplasm is rich in lysosomes, beside the presence of mitochondria and rough endoplasmic reticulum. Epithelial cells represent the major representing structure of the epidermis. It is more spread in the different layers of the epidermis. It is characterized by large centrally located nuclei and their cytoplasm is rich in mitochondria and rough endoplasmic reticulum. Few numbers of lysosomes are detected. The third type, mucous cells appeared pear-shaped with basal nuclei and very thin coat of cytoplasm. Their lumina enclosed by mucinous secretion. Few numbers of macrophage cells are detected in close contact with the mucous cells. The basal lamina of the epidermis appears enfolding with the underlying dermis. Hemidsmosomes are observed nearer the basal lamina in-between the basal epithelial cells. The epidermal-dermal junction appears folded. The dermis exhibits abundance of spindle-shaped fibroblast cells infiltrated the collagen fibrils and blood capillaries (Fig.5 A-H).

On the other hand, light microscopy of the infected fish exhibited massive thinning of epidermis .This was followed by sloughing of epithelial cells from the surface. Simultaneously the content of the club cells at the surface of the epidermis was squeezed out, leaving empty spaces behind. Many of the club cells showed extensive vacuolation, especially around their nuclei. The density of mucous cells was decreased (Fig. 4 B).

At TEM level, there was apparent damage of the macrophage cells. Most of them become pyknotic. The club cells possessed convoluted nuclei with electron-dense chromatin material. The cytoplasm exhibited massive degeneration of organelles and enclosed by numerous vesicles. The epithelial cells showed numerous nuclear disintegration with characteristic irregular nuclear envelope and their cytoplasm exhibited disintegration of cytoplasmic organelles. The mucous cells were distorted and lacked normal integrity and occupied the superficial layer. The mucoid secretion was enclosed by vacuoles and its upper surface possessed numerous small vacuoles. The basal lamina of the epidermal-dermal junction lacked normal irregularity and the adjacent basal cells showed disorganized hemidesmosomes. The underlying dermis exhibited abundant distribution of erythrocyte manifesting internal hemorrhage. Apoptic cell death was more recognized in the fibroblast cells (Fig. 6 A-H).

Gill:
Light microscopic observations of the healthy fish possess gill arches with normal arrangement pattern. From the gill filament, lamellae are originated which is covered by a delicate layer of a stratified squamous epithelium carrying the active exchange pillar cells. In the core of the gill

filament, a rigid mass of cartilaginous tissues are observed. Between the layers of flattened pavement cells, chloride cells can be seen in either filament or lamellar epithelia, however, most of them are observed in lamellae (Fig.4 C-D).

In infected fish, there was extensive dilation of blood capillaries and swollen lamellae packed with erythrocytes (i.e., aneurism). Necrotic changes in gill lamellae were commonly noted with rupture of lamellar epithelium. Heavy infected gills exhibited severe pathological changes, including lamellar fusion due to simple apposition as well as hypertrophy and hyperplasia of epithelial cells. The fusion commonly occurred along the entire length of secondary lamellae. In some incidences, there was respiratory epithelial necrosis. There was a massive loss of chloride cells (Fig. 4 E-F).

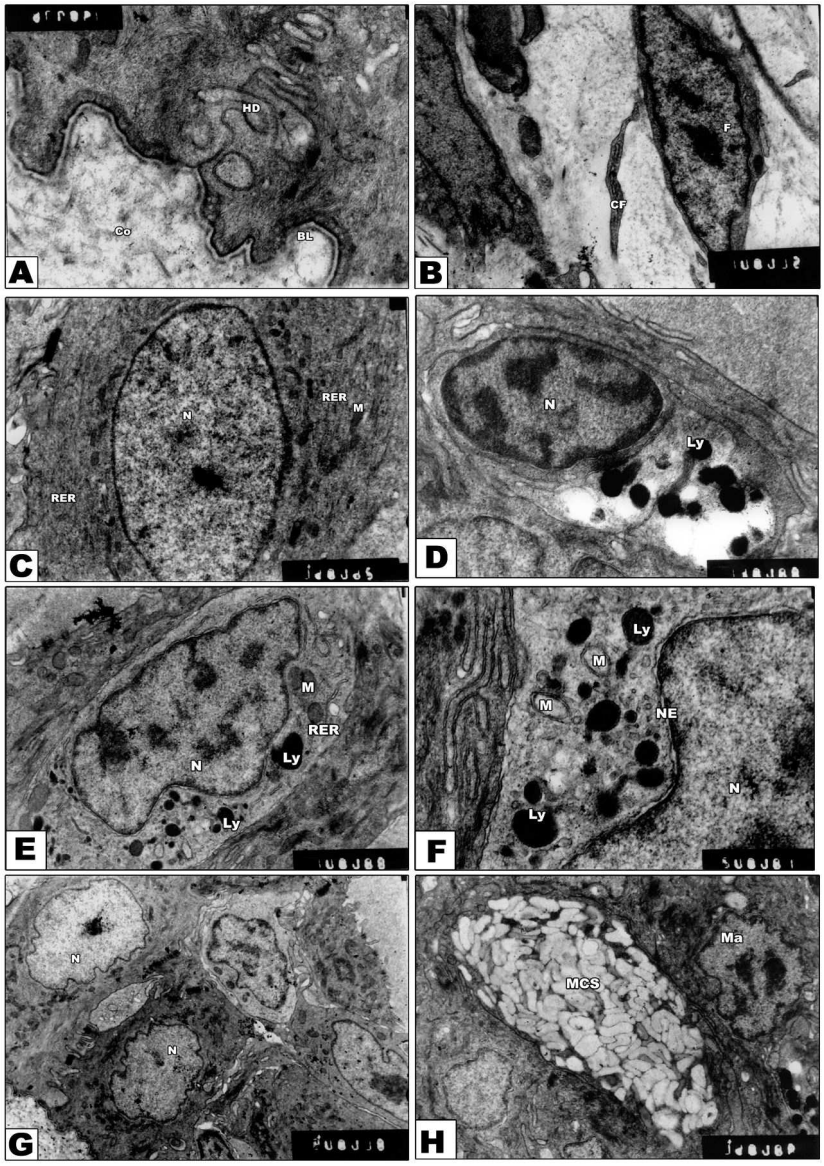


Fig.5(A-F). TEM micrographs of skin (V.S.) of healthy *Clarias gariepinus*.

A. Showing folded epidermal dermal junction and hemidesmosomes inbetween epithelial cells. X 15000
B. Showing dermis with regular arrangement of fibroblasts and ground collagen matrix. X10000
C. Epithelial cells with characteristic nuclei and cytoplasm rich in RER and mitochondria.X5000
D. Showing melanocytes with detected cytoplasmic melanocytes. X7500
E&F. Showing club cells with irregular nuclear envelope and cytoplasm rich in RER, mitochondria and lysosomes. EX15000
G. Showing macrophage cells. X5000; H. Showing mucous cells. X5000

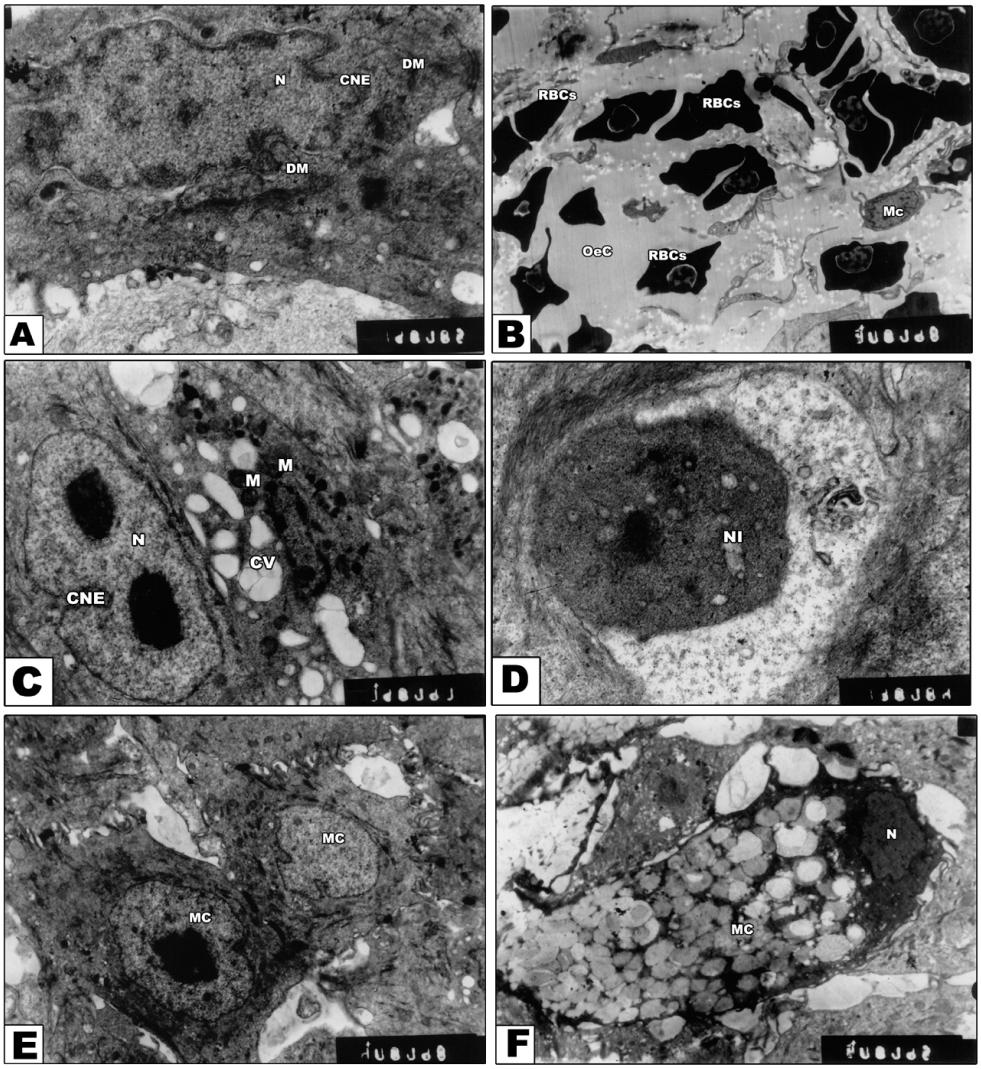


Fig.6(A-F). TEM of micrographs of skin of diseased *Clarias gariepinus*

A. Showing flat epidermal dermal junction and hemidesmosomes inbetween epithelial cells. The epithelial cell shows degeneratin nuclear chromatin. X 7500
B. Showing dermis with degenerated fibroblasts and internal haemorrhage. X7500
C. Club cells having vacuolated cytoplasm. with characteristic nuclei and cytoplasm rich in RER and mitochondria.X5000
D. Showing degenerated club cells. X7500
E. Showing degenerated macrophage cells. X5000
G. Showing macrophage cells. X5000
H. Showing mucous cells with vacuolated inner matrix. X5000

DISCUSSION

Clarias gariepinus, an omnivore freshwater fish, is a popular delicacy relished throughout tropical Africa. It is a prominent culture species because of its hardiness and fast growth rate. Fish are susceptible to a wide variety of bacterial pathogens causes large mortality in the aquaculture.

Macroscopically, the infected fishes exhibited ulcerative skin appearance of different patches. Many of these spots took reddish colour manifesting superficial skin hemorrhage. The gills were abnormally swollen and showed different white and yellow patches. The intensity and spread of disease appeared more detected during winter season more than that of summer season. The severity of fish disease is confirmed by massive degeneration of immune cells which detected in ultrastructural observation of skin of the studied *Clarias gariepinus*. The observed findings were confirmed by histopathological alterations in both skin and gills. The infected skin was abnormally thinning and loss of many of epidermal cells especially massive loss of epithelial, mucous and club cells. The epidermal-dermal interaction lacked normal integrity. Hyalinized degeneration and internal haemorrhage were detected in the dermal layer. Ultrastructurally, the epidermal cell exhibited massive cytological alterations. Although, fish skin is metabolically active, it responds to environmental stressors (Premdas *et al*., 1995; Austin 1999), and skin erosions are well accepted indicators of aeromonas bacterial infection.

The epidermis and its overlying mucous layer constitutes a major biological interface between a fish and its aqueous environment and forms a continuous layer over the fish (Whitear, 1986) playing great role in protection against injury, friction reduction (Rosen & Cornford 1971) and ion regulation (Handy *et al*., 1989 , Fouz *et al*., 2000). A number of chemical defenses in the epidermis and mucous, such as immunoglobulins, complement, lysozyme, natural antibiotics and agglutinins, may aid in protection (Ellis 2001), and the continuous shedding of mucous may prevent microbial colonization. However, exposure to environmental stressors may affect the epidermis and interfere with its protective role (Munro & Hastings 1993; Noga, 2000).

Abnormal structure of both the epidermis and dermis led to impairment of its functional activity as well as partially decrease the respiration activity of the integument which is the main source of transported oxygen to internal organs.

Stress from many different sources may induce a cellular response and cause skin damage (Udomkusonsri *et al.*, 2004). The effects of stress on fish may be severe, causing immunosuppression and reduced growth of the fish (Wendelaar Bonga 1997).

Consequently, the epithelium that covers the gill filaments and lamellae provides a distinct boundary between a fish's external environment and extracellular fluids and also plays a critical role in the physiological function of the fish gill. The gill epithelium of the fish is the major site of gas exchange, acid-base balance, ionic regulation, and excretion of nitrogenous waste (Thopon *et al.*, 2003).The presence of mucous-filled cavity (edema) observed in the gill filaments of *C. gariepinus* may be considered as an ion trap, in a way to concentrate free elements from surrounding water between the neighboring secondary lamellae.

The observed alterations of the infected gill possessed massive alteration of histological and scanning ultrastructures revealing different pattern of pathological alterations including disruption, degeneration and thinning of the lamellae and cellular hypertrophy structure led to a decrease in the respiratory capacity between the lamellae, and impairs the diffusion of oxygen across the gills due to the swollen condition of the epithelium and decrease in free gas exchange which in turn increase the mortality of the fish. These may lead to impairment of gill function and increased the incidence of fish mortality. DNA fragmentation confirmed the pathological alteration of both infected gill and skin.

The altered skin and gill were confirmed by the detection and identification of pathogenic bacteria including *Actinobacter lwoffii* , *Enterobacter amnigenus* , *Escherichia coli* , *Citrobacter*

amlonaticus and *Serratia odorifera*, *Aeromonas jandaei* (gram-negative) and *Staphylococcus epidermidis* (Gram-Positive Cocci) were detected in both affected gill and skin of *Clarias gariepinus*.

All bacterial infections were found as mixed infections. Mixed bacterial infections with Aeromonas & Pseudomonas sp. was recorded by Ahmed and Shoreit (2001). Thus, it could be concluded that both Aeromonas and Pseudomonas septicemias were more prevalent during winter season. This could be attributed to the suppressed immunity of the cultured fishes.

Concerning *Acinetobacter lwoffii* , Aucken and Malnick, (1999), Ku *et al*., (2000), Murray and Hospentahl, (2008) and Regalado *et al*., (2009) reported a normal flora of the oropharynx and skin in approximately 25% of the healthy human individuals. However, they caused respiratory failure and ventilator related pneumonia, bacteremia from indwelling catheters and wound infections. The bacterium colonizes the skin and mucous membranes in approximately 25% of patients.

Similar findings were achieved by many authors in different kinds of fish farms. Boonyaratpalin (1989) reported that the epizootic ulcerative syndrome, involving both cultured and wild fish in Burma, Indonesia, Lao People's Democratic Republic, Malaysia, Singapore, and Thailand, was associated with bacterial pathogens, primarily *Aeromonas hydrophila*, occasionally *Pseudomonas* sp. Subsequent skin erosion resulted in the formation of ulcerative lesions on the body.
Pal and Pradhan (1989) detected four types of bacteria, two fluorescent pseudomonads, one aeromones (*Aeromonas hydrophila anaerogens*) and one coccus (*Micrococcus variant*) were isolated from skin lesions of airbreathing fishes. The bacterial culture in mixed condition induced severe ulcers in healthy *Anabas testudineus*.

In vivo and *in vitro* in tilapia, *Oreochromis aureus* infected with *Staphylococcus epidermidis* was found to induce apoptosis in lymphocytes and macrophages in spleen and kidney of the fish. Apoptotic figures were observed occasionally in the brain, liver, gonad, mesentery, stomach, intestine and skeletal muscle of infected fish. Fragmented DNA was detected (Huang *et al*., 2000).

Fish diseases caused by aeromonas and pseudomonas considered to be the major bacterial problems facing the aquaculture development causing mass mortalities, reduced production and low quality of aquatic organisms. Both Aeromonas spp. (*A. hydrophila, A. sobria and A. caviae*) and Pseudomonas spp. (*P.fluorescens, P. putida and P. aeruginosa*) were incorporated in severe outbreaks among *O. niloticus* in fish hatcheries (Ahmed and Shoreit, 2001). *A. hydrophila* alone was isolated from gills of the naturally infected male monosex *O. niloticus* suffering from motile Aeromonas septicemia in floating cages (Gamal *et al*., 2002).
Proliferative gill inflammation was detected in farmed Atlantic salmon *Salmo salar L*. infected with chlamydia-like bacteria and Atlantic

salmon paramyxovirus (Kvellestad *et al*., 2005).
Kapetanoviæ *et al*.(2006) isolated *Stenotrophomonas maltophilia*, *Aeromonas salmonicida masoucida*/achromogenes and *Empedobacter brevis* from the gill of gilthead sea bream.
Finally, the authors concluded that *C. gariepinus* is highly susceptible to bacterial infection inducing pathological alterations in skin and gills of fishes and may cause mortality of it.

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