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Time and Dose Dependent Effect of *Pseudomonas aeruginosa* Infection on the Scales of *Channa punctata* (Bloch) Through Light and Electron Microscopy

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Abstract

Channa punctata (freshwater murrel) is an economically important fish species in Asian countries, including India. The present investigation demonstrates the pathogenic effects of *Pseudomonas aeruginosa* DJ1990 on the scales of fresh water *C. punctata* through optical and scanning electron microscopy. In order to determine the adverse effects of *P. aeruginosa* DJ1990, fish were exposed to different doses of bacterial load and sacrificed at 12 h interval up to 72 h of post-infection period. The light microscopic and electron microscopic examination of *C. punctata* scale clearly disclosed the bacterial invasion and concomitant destruction of the scale structure such as uprooted damaged lepidonts and dispersal of chromatophores. In the present study, it was observed that the advent of anomaly in scale structure (chromatophore dispersion, circuli damage, circuli disorganization, lepidontal breakage, lepidontal uprooting, lepidontal sockets exposing, lepidont displacement and scale loosing) was dependent on the pathogen loads and time of expose. Till date, several studies have been conducted in the field of fish pathology; however, scales are given less priority in comparison to hematopoietic organs. To the author's best knowledge, it is the first report describing the effect of bacterial pathogen on the scale of a fish species, *C. punctata*.

Keywords: *Channa punctata*, fish pathogen, scale structure, light microscopy, SEM

Introduction

Aquaculture in India has evolved as a viable commercial farming practice with considerable diversification in terms of species and systems, and is showing promising future with varied resources and lots of potentials. Intensive fish farming is only possible through the effective feeding and high fish densities, but it increases the risk of disease outbreaks (Nandi, Banerjee, Dan, Ghosh, & Ray, 2016). Aquatic animals including fish are always susceptible to a wide range of pathogenic bacterial strains (Banerjee, Nandi, & Ray, 2016). Bacterial diseases in fish are the major challenges that hamper the production and affect the country economy. The skin is reported to be the most common target for infectious agents in fish. Ferguson (1989) has recorded numerous disease symptoms in skin in different species. Fish epidermis plays an important role in maintaining homeostasis. There is a close relationship between skin damage and microbial colonization (Weber, Chen, & Milton, 2010; Lowrey, Woodhams, Tacchi, & Salinas, 2015).

The damage of epidermal layer provides access for infectious agents that enhance the osmotic stress and ultimately foster the mortality rate. There is evidence that very high acute mortality (nearly 50%) can occur even if the ulceration of body surface is as little as 10% and the degree of mortality is proportionate to loss of skin (Bouck & Smith, 1979). Thus, pathogens that are totally restricted to the epidermal layer (ectoparasites) are able to kill fish due to the result of osmotic shock related damage of the epidermal layer. Skin damage may affects fish in different ways such as increasing susceptibility to predation (Abbott & Dill, 1985; Lowrey et al., 2015) and reduces oxygen uptake from water (Whitear, 1986). Furthermore, epidermal damage and skin ulcers are also associated with the development of different diseases (Noga, 2000).

Several researches have been conducted regarding the fish pathogens and its associated effects on different organs (liver and kidney), blood parameters and immune system (Banerjee et al., 2016; Nandi et al., 2016). However, there are no such reports regarding the effect of bacterial pathogens on fish scale. The present communication demonstrates the physio-morphological changes occurred in the scale of a freshwater murrel (*Channa punctata*, Bloch) due to the pathogenic strain of *Pseudomonas aeruginosa*.

Materials and Methods

Experimental Fish

Healthy (no external disease symptom) *C. punctata* were collected from local market and acclimatize for 1 week in glass aquaria (size: 0.6m×0.3m×0.3m) under controlled laboratory conditions with continuous aeration following standard methods (APHA, 2005) at Department of Zoology, Gauhati University, Guwahati. During the acclimatization period, the fish were fed with *Tubifex*. The average Standard length and weight of the fishes were 15.67±0.29 cm and 22.5±1.5 g, respectively. Water temperature was maintained at 29± 2 °C during the whole experimental period.

Bacterial Culture and Maintenance

The fish pathogenic strain *Pseudomonas aeruginosa* DJ1990 (Acc No. KX709967) was used in this study. The strain was maintained [Pseudomonas selective agar base (SRL, India) medium supplemented with glycerol (2% v/v) and aztreonum (30 µg)] at Department of Zoology, Gauhati University, India. The pathogenicity of the strain was confirmed by *in vitro* (hemolytic activity) and *in vivo* studies (fish mortality test).

Artificial Infection Study

The strain was cultured in BHI broth (HiMedia, India) and incubated at 28 °C for 24 h. Harvesting of bacterial cells were done by centrifugation at 5000 × *g* for 10 min and washed in physiological saline (PS; 0.85% NaCl), and finally pelleted down in sterile PBS solution. The experimental fish were injected intra-peritoneally with 0.1 ml (1.5×10^4-10^8 CFU ml^{-1}) of bacterial suspension. A control group was also maintained which was injected with 0.1 ml PBS. The behavioral patterns as well as clinical signs of the fish were examined carefully. The control and infected

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fishes were anesthetized through chloroform (analytical grade) exposure and the scales (from the caudal and tail region) were removed immediately and processed for microscopy.

Behavior of the Fish

To monitor the disease progression, the swimming behavior, feeding efficiency and relative percent survival (RPS) was measured in regular basis. Relative percent survival was calculated following the equation of Amend (1981)

RPS = 1- (percentage of control fish mortality / percentage of infected fish mortality) × 100 %

Light Microscopy

Five scales from each of the fish were removed from the second row with the help of fine forceps. The scales were rinsed thoroughly with 70% ethanol to remove any extraneous materials and kept in paper envelope. It was then observed under sterio zoom microscope (Leica S8APO).

Scanning Electron Microscopy

Scales were carefully removed from both the control and diseased fish were first fixed in 2.5% glutaraldehyde prepared in 0.1M sodium cacodylate buffer (pH 7.2) for 3h at 4°C. Samples were then washed in 0.1M sodium cacodylate buffer, postfixed in 1% buffered osmium tetroxide for 1 h. After this, samples were washed twice in sodium cacodylate buffer, followed by washing in distilled water 30 minutes. Dehydration was done in ascending gradation of acetone and dried up by tetramethylsilane (TMS) drying technique through the replacement of acetone by TMS at 4°C for 10 min (Banerjee, Dan, Nandi, Ghosh, & Ray, 2015). The conductive coating was done by gold palladium to the samples with a JFC-100 Ion sputter (JEOL, Japan). For viewing, the JSM-6360 scanning electron microscope was used with a working distance of 8 mm and secondary electron emission mode was used with an accelerating voltage of 20 kV.

Measurements and Statistical Analysis

The morphological and functional changes in the scale were classified as per the method of Kar and Dua (2012). The percentage of occurrence of each anomaly in 100 scales (adjacent to caudal fin) of each fish was calculated by dividing the number of fish with a given anomaly by the total number of examined fish. Differences in percentage of anomaly for each treatment group were tested to compare the proportions for significant differences in changes among the different feeding habits using a binomial t-test for independent samples. The non-parametric Kruskall–Wallis test for independent samples with $P < 0.005$, followed by a multiple comparison of mean ranks for all groups was used to compare the bacterial dose dependent changes. All statistical tests were conducted using SPSS version 16.0 (SPSS Inc., Chicago).

Results and Discussion

Behavior and Relative Percent Survival of the Examined Fish

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We have examined the fish species (both control and infected) upto 96 h. A gradual ceased movements and less feeding efficiency were observed in infected fish compared to the control groups. Furthermore, at the end of 96h, most of the infected fish were seen floating dorsal side down at the water surface. The RPS was measured at three concentrations (1.5×10^4, 1.5×10^6 and 1.5×10^8 CFU ml^{-1}) of the pathogenic bacterium strain *P. aeruginosa* DJ1990, and the survival percentage was recorded to be 78.45%, 32.26% and 9.83%, respectively. Fishes are always surrounded by a wide range of bacteria. Monitoring of fish behavior is a very good technique which is commonly used in aquaculture sector to understand the fish health. Abnormal swimming, gasping at the surface of water, lethargic and low feeding efficiency are considered to be a maker to monitor disease condition or environmental stress in fish farming industries (Nandi et al., 2016).

Effect on Scale Structure Revealed Through Light Microscope

The scales from the control fish are cycloid, consisting of an anterior or rostral field where a number of radii are present, while the lateral fields and the posterior or caudal fins or lunula is not covered by adjacent scales. Circuli are present at the anterior and lateral regions of the scale (Fig. 1a). On the anterior side, the circuli are birfurcate, while the lateral side contains cooperatively thicker and widely spaced circuli. In the rostral field, the continuity of circuli is interrupted by the radii (R) (Fig. 1a) originating from the focus of the scale. Whereas, infected specimens (10^5 CFU/ mL for 3rd day p.i.) exhibited lepidontal damage, uprooting, exposed lepidontal sockets and damage disorganized circuli (Fig. 1b). Structural changes in the scales observed were breakage of lepidonts from the basal region and at the anterior one third portions. Individual uprooting of lepidonts and empty lepidontal sockets were also found (Fig. 1b). Fish are always susceptible to a wide range of pathogens that cause several types of diseases in skin, liver, kidney, and other organs (Banerjee et al., 2016; Nandi et al., 2016). In a study Khanna, Sarkar, Gautam, and Bhutiani (2007) have reported the relation between water pollution and scale morphology, that might be helpful in monitoring environmental pollution. In a similarly study, Dua and Gupta (2005) also have observed the toxic effects of mercury on fish scale and correlated the effect with the water pollution. In an investigation, Kar and Dua (2012) have stated the fish scale as a good indicator of water pollution, but till date no such report was published regarding the effect of pathogen on fish scale.

Effect on Scale Structure Revealed through Electron Microscope

In control fishes, the continuity of the circuli is interrupted by the radii originating from the focus of the scale in the rostral field (Fig. 2a). Denticles are also present in the older part of scale. In control fishes, sharply pointed lepidonts are developed well at the anterior part but disappeared as it progresses to the caudal part. Lepidonts are very sharp in appearance and are set in deep sockets, which has curve towards the interface (Fig. 2c). Whereas, in the infected fish (10^5 CFU ml^{-1} for 3rd day p.i.), damaged circuli, lepidontal uprooting and lepidontal breakage were observed in the scales (Fig. 2d). Individual displacements of the lepidont along with exposed lepidontal sockets were also found in the diseased fish (Fig. 2e). Individual displacement of lepidonts and exposed lepidontal sockets were also observed in infected specimens (Fig. 2f). There was no previous report found to demonstrate the effect of fish

pathogens imparted upon the scale. Though few reports have been published in this topic, however, these are related to effect of heavy metal toxicity on fish scale (Rishi & Jain, 1998; Yoshitomi, Koyama, Iida, & Ikeda, 1998; Kaur & Dua, 2012).

Effect of Pathogen on Scale Chromatophore

Scales collected from control specimens showed both dispersed and contracted chromatophores including black melanophores and yellow xanthophores (Fig. 3a). However, dispersed or reticulated chromatophores were observed in scales collected from fish infected with *P. aeruginosa* DJ1990 (10^4 CFU ml^{-1}) at 3rd day p.i. (Fig. 3b). Chromatophores are pigment containing or light reflecting cell. In this direction Kaleta (2009) stated that Chromatophores protect the fish from ultraviolet radiation. Chaplen, Upson, McFadden, and Kolodziej (2002) concluded that the mode of chromatophore responses (hyper dispersion of pigment granules and partial aggregation) may vary for different classes of agents.

Frequency of Disease Occurrence in Respect to Doses

Lepidontal damages were observed even at the lowest concentration of *P. aeruginosa* DJ1990 (10^4 CFU ml^{-1}). With an increased dose of *P. aeruginosa* DJ1990 (10^5 CFU ml^{-1}), uprooting of rows of lepidonts and sloughing of lepidonts from their original position were recorded (Table 1). Rows of lepidonts uprooted from their respective sockets and damaged disorganized circuli were observed at the highest concentration (10^6 CFU ml^{-1}) of the bacterial pathogen. Breakage and individual displacement of lepidonts from their point of anchorage was recorded at the lowest dose of *P. aeruginosa* DJ1990 (10^4 CFU ml^{-1}). At 10^5 CFU ml^{-1} dilution, increased frequency of lepidontal uprooting was observed (Table 1). Loosening of scales was also observed in all test specimens in a dose dependent manner when exposed to pathogen for duration of 7 day (6th day p.i.), as represented in the Table 1. Phenomenon of dispersion of chromatophores was more pronounced in specimens exposed to higher dose of pathogen (Chaplen et al., 2002). Ohta (1974) also has reported the super dispersion phenomenon in melanophores subjected to cytochaiasin B.

Frequency of Disease Occurrence in Respect to Time

Lepidontal uprooting, lepidontal breakage, and damaged circuli were observed in specimens subjected for a period of 3 days (2nd day p.i.). The frequency of breakage and uprooting of lepidonts and damage to the circuli was far prominent, when the exposure period was increased from 1 to 6 days (Table 2).Furthermore, the extreme and pronounced damage was recorded with an increase in exposure duration to 6th day p.i. Phenomenon of dispersion of chromatophores was more pronounced at 5th day p.i. (Table 2). Thereafter, the pigment granules in the centrospheres were markedly reduced. Kar and Dua (2012) have noted extremely active Brownian movement of pigment granules toward the branch extremities of the chromatophores with the long term exposure of polluted water.

Conclusion

In the present investigation, we have demonstrated the effects (dose dependent and time depended) of bacterial pathogen *P. aeruginosa* on the scale of *C. punctata*. Scales function as a physical barrier which protects the fish, and thus, damage of scale increases the susceptibility to infection. It also causes excessive uptake of water by freshwater fish or loss of water from marine species (osmotic stress). The observed alterations in architectural pattern of the scales (uprooted and damaged lepidonts, and the dispersal of chromatophores) strongly suggest that fish scales are also an indicator of pathogenic invasion or biological stress. To the author's knowledge, it is the first report describing the effect of fish pathogen on the scale.

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Table 1. Frequency of occurrence (in bold) of changes in scales in the three different dosage of *P. aeruginosa* DJ1990 at 6th day p.i. Number of fish with changes (number of examined fish)

Changes/dosage	10^4 CFU ml^{-1}		10^5 CFU ml^{-1}		10^6 CFU ml^{-1}	
Chromatophore disperson	5 (10)	50.0%[c]	8 (10)	80.0% [a]	10 (10)	100.0%
Circuli damage	6 (10)	60.0% [b]	7(10)	70.0% [a]	8 (10)	80.0% [a]
Circuli disorganization	6 (10)	60.0% [a]	8 (10)	80.0%[b]	9 (10)	90.0%
Lepidontal breakage	7 (10)	70.0% [b]	8 (10)	80.0%	9 (10)	90.0% [a]
Lepidontal uprooting	7 (10)	70.0%	7 (10)	70.0%	9 (10)	90.0%
Lepidontal socket exposing	6 (10)	60.0% [a]	8 (10)	80.0%[b]	9(10)	90.0%
Lepidont displacement	6 (10)	60.0% [b]	7 (10)	70.0%	8 (10)	80.0% [b\
Scale loosing	4(10)	40.0% [d]	6(10)	60.0%[a]	7 (10)	70.0% [a]

Superscripts letters indicate differences highly significant according to t-test for difference of proportion.

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Table 2. Frequency of occurrence (in bold) of changes in scale in the six different time point of post infection using sublethal dosage of *P. aeruginosa* DJ1990 (10^5 CFU ml^{-1})

Changes	1st day p.i.		2nd day p.i.		3rd day p.i.		4th day p.i.		5th day p.i.		6th day p.i.	
Chromatophore disperson	3 (10)	30.0% [c]	4 (10)	40.0%	4 (10)	40.0%	5 (10)	50.0% [b]	8 (10)	80.0% [a]	7 (10)	70.0%
Circuli damage	2 (10)	20.0%	3 (10)	30.0% [b]	3 (10)	30.0% [b]	5 (10)	50.0%	6 (10)	60.0%	9 (10)	90.0% [a]
Circuli disorganization	1 (10)	10.0%	3 (10)	30.0%	3 (10)	30.0%	4 (10)	40.0%	7 (10)	70.0%	8 (10)	80.0%
Lepidontal breakage	2 (10)	20.0%	4 (10)	40.0%	5 (10)	50.0%	6 (10)	60.0%	6 (10)	60.0%	8 (10)	80.0%
Lepidontal uprooting	0 (10)	0.0% [c]	3 (10)	30.0% [b]	5 (10)	50.0%	5 (10)	50.0%	7 (10)	70.0% [a]	7 (10)	70.0%
Lepidontal socket exposing	0 (10)	0.0%	0 (10)	0.0%	2 (10)	20.0%	4 (10)	40.0%	6 (10)	60.0%	8 (10)	80.0%
Lepidont displacement	0 (10)	0.0% [c]	0 (10)	0.0% [c]	2 (10)	20.0% [b]	3 (10)	30.0%	4 (10)	40.0% [b]	7 (10)	70.0% [a]
Scale loosing	0 (10)	0.0% [c]	0 (10)	0.0% [c]	1 (10)	10.0% [b]	2 (10)	20.0%	3 (10)	30.0%	3 (10)	30.0%

Superscripts letters indicate differences highly significant according to t-test for difference of proportion.

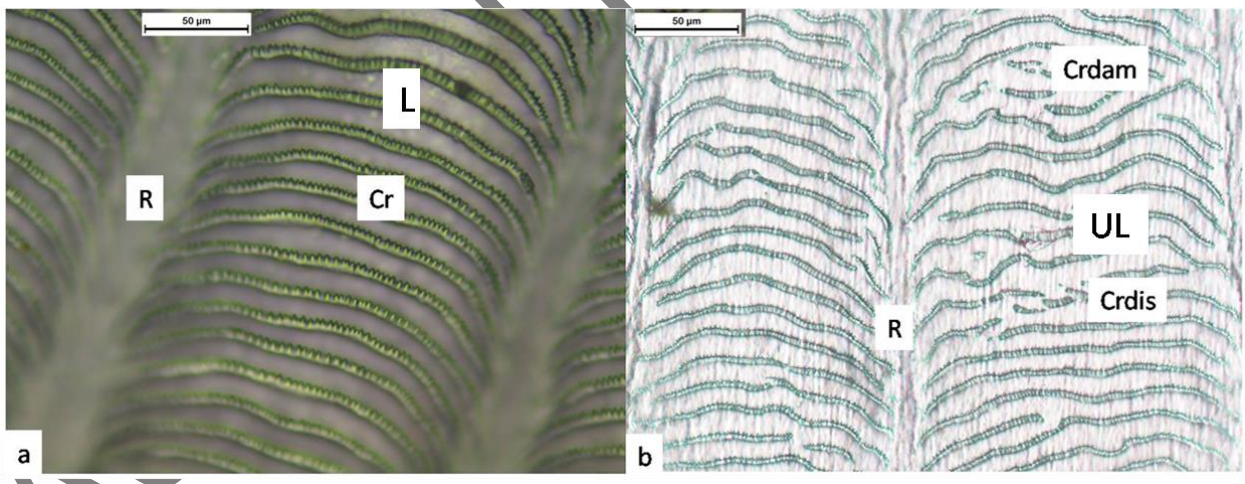

Figure 1. Light microscope photomicrographs of scale of *C. punctata* showing: a lepidont (L) and intact circuli (Cr) in control and b uprooting of lepidont (UL), damaged (Crdam) and disorganized (Crdis) circuli in the infected fish

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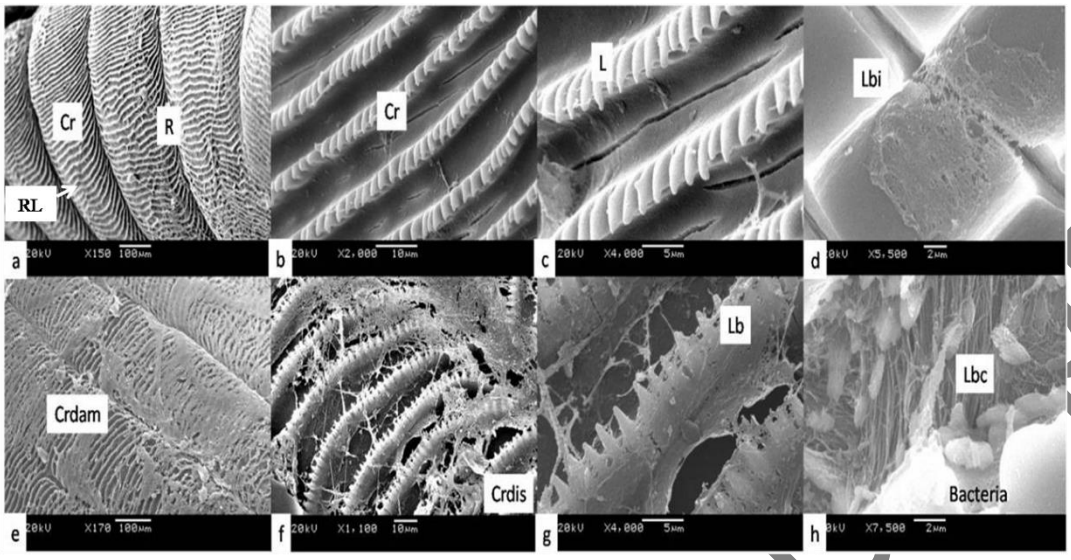

Figure 2. Electron micrograph of scale of *C. punctatus*(control) indicating the scale structure: a row of lepidont (RL), circuli (Cr) and ridges (R), b circuli (Cr), c lepidont (L), d intact lepidont base (Lbi); e-f Photomicrograph of infected *C. punctata* scales indicating the damaged scale structure: e circuli damage (Crdam), f circuli disorganization, g lepidontal breakage, h cracked lepidont base (Lbc) and bacteria

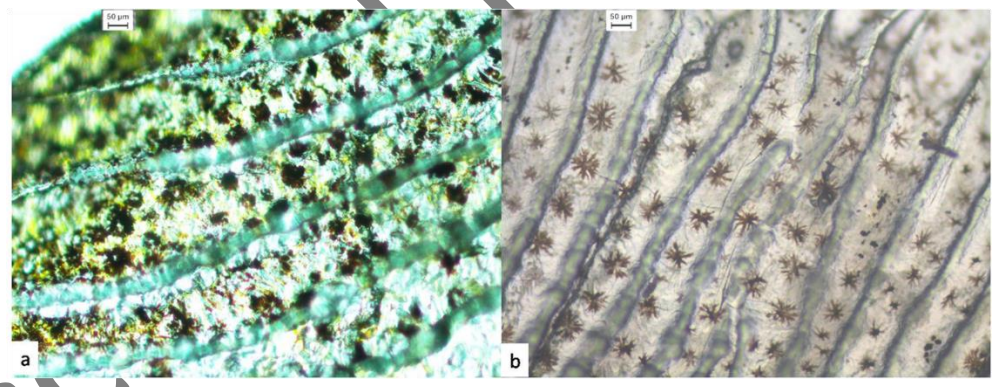

Figure 3. Light microscope photomicrographs of scale indicating pigment. a Reticulated and punctuated chromatophores along with yellow xanthophores in control fish (×40); b dispersed chromatophores in infected fish