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Mucus properties of Chinese carp and Indian carp: Physical barrier to pathogens

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Abstract

The study was aimed to investigate the probable role of mucus in reception and/or repulsion of *Lernaea* in Indian and Chinese major carps. *Ctenophryngodon idella*, *Hypophthalmichthys moiltrix*, *Labeo rohita*, *Cirrhinus mrigala*, *Catla catla* and *Cyprinus carpio* averaging 830±316 g each were collected and disinfected with $KMnO_4$ (8.0ppm) for collections of contamination free mucus. Total protein contents and size categorization were determined by Bradford Micro Assay and SDS-PAGE analysis. Lectin and alkaline phosphatase activities were also measured by hemagglutination (HA) titer and alkaline phosphatase test, respectively. Protein concentrations were the highest in *C. idella* and *C. catla* and the lowest in *C. carpio*. Considering protein profiles mucus samples from *C. catla* contained the highest molecular weight proteins while *C. carpio* has one unique protein band of 14.13 kDa the weight of which resembles the weight of lysozyme, a protective element of mucus. Lectin activity was highest in *C. idella* indicative of low resistance while it was the lowest in *H. molitrix.* Alkaline phosphatase level was the highest in *C. catla*, and was the lowest in *C. carpio*. Overall results indicted *C. carpio* as the most resistant species as it showed better values for the immune components. Higher values of protein contents and alkaline phosphatase for *C. catla* may be in favor of its higher susceptibility. These studies on mucus contents are good assessment indicators of the possibility of parasitic attacks in Chinese and Indian major carps.

Keywords: Fish, *Lernaea*, Pectin activity, Protein profile, Alkaline phosphatase

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Introduction

Fish depends upon its innate or non-specific immune mechanisms to fight against a variety of pathogens (Subramanian *et al.,* 2008). Fish mucus layer helps in osmoregulation, reduction of friction and disease resistance (Shephard, 1994). It provides mechanical, chemical and innate immunity against the pathogen invasion. It reduces pathogen access due to its constant downward movement along lateral sides of the fish and its various sprawling edges and sides. The presence of immunoglobulins, enzymes and lytic agents ensure the neutralization of microorganisms (Ellis, 1990; Palaksha *et al.,* 2008) when they invade. Number of experiments has been performed on fish skin immunity and skin bacterial infections (Lemaitre *et al.,* 1996; Aranishi *et al.,* 1998; Ebran *et al.,* 2000) however, little information is available on the role of skin mucus as the first line of defense against various pathogens.

Fish are vulnerable to many ectoparasites present in living aquatic environments but the family Lernaeidae has been and is a common family causing parasitic infestation in freshwater fish all over the world (Tasawar *et al.,* 2009). Members of Cyprinidae fish family have been observed to be the most common hosts of *Learnaea* -a persistent threat to fish and fish culture. Although considerable work has been done in various parts of the world and Pakistan (Bjorn *et al.,* 2001; Tasawar *et al.,* 2001; Boxaspen, 2006; Oktener *et al.,* 2007; Revie *et al.,* 2007; Tasawar *et al.,* 2007a; Tasawar *et al.,* 2007b) on level and intensity of its attack and its variability from species to species (Kabata, 1985; Tasawar *et al.,* 2007b) the causes of this selective attack have not yet been fully implicit. Earlier studies show that Leranaea remained ineffective till copepodid-I stage, and prior stages continue their further development off the fish host. This could be due to some degree of innate resistance or an immune mechanism in fishes against the parasitic copepodid-I, the basis for which may be morphological, biochemical or immunological or a combination of all. Identification and exploitation of this putative mechanism (s) can pave the way for its application to control its invasion and subsequent infections in susceptible species of fish. Jones (2001) opined that the physicochemical characteristics of the skin mucus, the presence of bio-reactive substances including lysozyme, complement, C-reactive protein, haemolysins and lectins and the epidermal migration of inflammatory cells and their secretions may affect the establishment and proliferation of ecto-parasitic copepods. Prem *et al.* (2017) compared immunological and biochemical properties of epidermal mucus of three brackishwater fishes, namely *Lates calcarifer*, *Chanos chanos* and *Mugil cephalus*. Ghafoori *et al.* (2014) observed significant changes in serum and mucus lysozyme in male and female Caspian kutum (*Rutilus frisii kutum*) under different seasonal temperatures, gonadal growth and reproductive migration. Irene *et al.*

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(2011), Nigam *et al.* (2012), Rombout *et al*. (2014) and Salinas (2015) investigated the adaptive immune system of teleost fish associated with each of their mucosal body surfaces. Evidence obtained from mucosal vaccination and mucosal infection studies reveal that adaptive immune responses take place at the different mucosal surfaces of teleost. The main mucosa-associated lymphoid tissues (MALT) of teleosts are the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT) and the recently discovered nasopharynx-associated lymphoid tissue. Similar studies related to comparative analysis of innate immune parameters of skin mucous secretions like alkaline phosphates, lysozyme total protein contents etc in different fish species were studied by a number of investigators like Loganatha *et al.* (2013) in *Channa striatus*, Bensussan *et al*. (2012), in *C. carpio)* Easy and Ross (2010) in Atlantic salmons and Palaksha *et al.,* (2008) in olive flounder. However an investigation of the possible causes for variability in the susceptibility to *Lernaea* in different carp species in the present experiments require further explorations to ensure that whether it is due to the biochemical, immunological, morphological and genetic characteristics of the host.

The present study was planned to explore the conceivable reasons and factors or bio-active molecules in the epidermal mucus of Indian and Chinese major carps which attract or repel this menace from attack. Mechanism of this host-parasite relationship and physiological role of mucus in this complex will also be explored.

Materials and methods

Experimental site

Studies were conducted in fish pond complex of Fisheries and Aquaculture Department at Ravi Campus Pattoki.

Experimental animal

Grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmicthys moiltrix*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), thaila (*Catla catla)* and common carp (*C. carpio*) were selected as experimental fish species for the planned studies.

Experimental procedure

A series of experimentation was conducted for the determination of possible causes of selective *Lernaea* attack in the Indian and Chinese carps. In the first trial Abbas *et al.* (2014) investigated *Lernaea s*usceptibility, infestation and treatment. After complete eradication of the parasite and on completion of the growth period, healthy fishes under similar environmental conditions were collected from commercial rearing ponds and transported alive in the same water to the fish hatchery for further commissioning the subsequent experimental steps. Each fish weighed 830±316 g on the average. Prior to initiation of mucus collection all the fishes were bathed in potassium permanganate ($KMnO_4$) solution (8.0 ppm) to remove any microbial or fungal

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infection/infestation to alleviate the possibility of any type of contamination in mucus samples. Then the fishes were acclimatized in cemented circular tanks in the hatchery building under a stress free environment for 24 hours for normalizing bodily functions of fishes. Five samples of each experimental fish species were collected and every sample was repeated three times.

Mucus collection

Fishes were anesthetized with MS-222 (100 mg L^{-1}) and laid on the smooth surface of a table with towel support for safe handling. A well immersed plastic spatula was gently wiped on the dorsoventral surface of fish which scraped off mucus from the skin surface. During the collection of mucus, maximum precautions were adopted to keep it free of common contaminants like blood, scales or intestinal fluids. Samples were centrifuged at 12000×g at 4 ˚C for 10 minutes and stored at -40 ˚C in a biomedical freezer. Prior to analysis, mucus samples were thawed at room temperature for smooth and hassle free operation of the equipment.

Total protein contents

Bradford Micro Assay technique was applied to determine protein contents using Bovine Serum Albumin (BSA) as standard. BSA solution and mucus dilutions were made in de-ionized water in flat-bottom microtiter plates. Bradford protein solution (50 µl) was added to each well and absorbance was recorded at 595nm. A standard curve was drawn from various but consecutive dilutions of BSA solution and protein concentrations in different samples were then calculated and determined by comparing their readings with the standard curve of BSA solution (Bradford, 1976; Zaika, 1988).

Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was carried out as described by Laemmli (1970) with slight modifications. 15% separating and 4% staking buffer were used to run the SDS- PAGE under constant voltage of 120. The FermentasPageRuler™ protein ladder was used as the standard marker for non-reducing protein. The gel was stained with PageBlue™ (Fermentas) stain for identification of protein bands for molecular weight determination.

Lectin activity

Lectin activity was determined according to the Ewart *et al.* (2001) method. 50 µl mucus was diluted in two-fold serial dilutions in 50µl phosphate buffer (PBS) (0.05 M, pH 6.2) in U bottom microtiter plates. 50µl rabbit RBCs solution (1%) was mixed in the dilutions and incubated at room temperature for one hour. RBCs in PBS were used as the control. The plate surface was read to determine hemagglutination (HA) titer. The HA titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was computed as one hemagglutination unit. (Specific activity is the number of heamagglutination unit's mg^{-1} lectin) (Wang *et al.,* 2001).

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Alkaline phosphatase test

Mucus was incubated with 4 mM p-nitrophenyl phosphate in ammonium bicarbonate buffer (100 mM) with 1 mM $MgC1_2$ (pH 7.8) at 30°C. The increase in optical density (OD) was measured continuously for 2 to 3 hours at 405 nm using a micro plate reader. The initial rate of the reaction was used to calculate the activity (One unit (U) of activity is defined as the amount of enzyme required to release 1µmol of p-nitrophenol product in 1 min) (Fletcher, 1986).

Statistical analysis

Statistical analysis was performed by using SAS software (9.1 Version). Data were analyzed using ANOVA statistical test. Significance level was fixed as $p<0.05$. Duncan's Multiple Range Test was applied to compare means from different treatment groups to identify group differences.

Results

Protein profile of mucus

The protein profile of mucus extracts of all the fish species was conducted on SDS-PAGE gel. PAGE showed molecular weight ranges of various proteins from 12.59 to 100 kDa. *L. rohita* has the most diverse group of proteins (12.59 to 89.13 kDa) whereas *C. mrigala* has the lowest number of bands (31.62 to 89.13kDa) marking less protein diversity than *L. rohita*. Proteins below 50 kDa were very common in all the fish species tested. High molecular proteins were more dominant in *C. catla* and were primarily of 100 kDa size (Table 1).

Table 1: Molecular weight of protein found in the skin mucus of experimental fish species (SDS-Page analysis).

Sr #	*Labeo rohita*	*Cirrhinus mrigala*	*Catla catla*	*Ctenophryngodon idella*	*Hypophthalmichthys Molitrix*	*Cyprinus carpio*
	M.Wt. (kDa)	M.Wt. (kDa)	M.Wt. (kDa)	M.Wt. (kDa)	M.Wt. (kDa)	M.Wt. (kDa)
1	89.13	89.13	100.00	89.13	84.14	89.13
2	84.14	57.54	84.14	74.13	74.13	74.13
3	74.13	39.81	74.13	58.88	58.88	58.88
4	58.88	31.62	58.88	50.12	43.65	39.81
5	50.12		50.12	29.51	31.62	15.85
6	31.62		25.12	25.12	16.60	14.13
7	26.92		15.85			
8	25.12					
9	23.44					
10	20.42					
11	17.38					
12	12.59					

Protein concentration

Protein concentration varied among different fish species and was highest in *C. idella* (3.29±0.13 mg ml^{-1}) and *C. catla* (3.02±0.57 mg ml^{-1}) which was significantly higher than their counterparts. Significantly ($p<0.05$) the lowest protein concentration was

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observed in *C. carpio* (Table 1). The protein concentrations in mucus of *C. mrigala, L. rohita* and *H. molitrix* fell in-between these two extremes and were quite similar to each other but different from other experimental fish species (Table 2).

Lectin activity

Lectin activity is the measure of the heamagglutination titer (HA). The *C. idella* showed maximum HA value (2^9) or the lectin activity hence showed the lowest resistance and the highest susceptibility to pathogen invasion. *H. molitrix* showed the lowest titer value (2^1) indicating fish was healthy with no or minimum pathogen infestation. The HA value for *C. mrigala* and *C. carpio* was recorded as 2^7. *L. rohita* showed the 2^8 HA value (Table 2). *C. mrigala* and *L. rohita* showed very little infestation by the pathogen however, *C. carpio* did not show any type of infestation throughout the whole study period.

Alkaline Phosphatase Activity

Alkaline phosphatase activity is another stress indicator and varies with the level of stress and type of fish species exposed. *C. catla* has the highest activity (184.6 U L^{-1}) and *C. carpio* the lowest (31.1±0.1) (Table 2). Alkaline phosphatase activity increased with intensity of *Lernaea* infestation. Totally free of parasite infestation *C. carpio* showed the minimum ALP value and *C. catla* with highest infestation displayed the highest value for ALP and proved to be the preferred host of *Lernaea.* ALP values (35.8±0.4) in the skin mucus of *H. molitrix* were very close to *C. carpio* (31.1±0.1) hence depicted no parasite infestation.

Table 2: Protein concentration, hemagglutination (HA) titer value and alkaline phosphatase (ALP) activity in mucus of experimental fish species.

Sr. NO.	Fish species	Protein Concentration (mg ml^{-1})	HA Titer value	ALP (U L^{-1})
1	*L. rohita*	2.63 ± 0.48^a	2^8	87.5 ± 0.7^a
2	*C. mrigala*	2.88 ± 0.07^a	2^7	61.3 ± 0.4^b
3	*C. catla*	3.02 ± 0.57^b	2^6	184.6 ± 0.1^c
4	*C. idella*	3.29 ± 0.13^b	2^9	152.5 ± 0.7^d
5	*H. molitrix*	2.87 ± 0.18^a	2^1	35.8 ± 0.4^e
6	*C, carpio*	1.80 ± 0.09^c	2^7	31.1 ± 0.1^f

Figures with different superscripts in column are significantly different (p<0.05)

Discussion

Living organisms have strong defense mechanisms against invading microorganisms as survival strategies. One of the defense mechanisms is the complement system, composed of more than 30 serum and cell surface components. This system collaborates in recognition and elimination of pathogens as a part of both the innate

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and acquired immune systems (Yano, 1996). Mucus of fish skin is a dynamic biological interface between fish and their environment and is composed of biochemically-diverse secretions from epidermal goblet and epithelial clavate (immune) cells (Pickering, 1974; Ellis, 2001; Powell, 2000). These cells secrete water and mucins like gel forming macromolecules (Negus, 1963; Shephard, 1994) ultimately transforming into mucus. This epithelial mucus has to face potential pathogen attacks and it retreats and deters possible parasitic attacks. It prevents colonization of various parasites.

Many members of the fish family Cyprinidae are the intermediate and definitive hosts of this bacteria and fungi (Ebran *et al.*, 2000) due to the inherent presence of certain enzymes, lytics and immunoglobin parasites. Baur (1962) and Hoffman, (1967) reported that *Lernaea cyprinacea* parasitizes *Carissus auratus*, *Anguilla japonica*, *Carassius vulgaris*, *Gobio fluviatilis* and *C. carpio.* They further claimed that parasite lacks host specificity to an extent that it can infect all freshwater fish and even frog tadpoles and salamanders. Our studies contradict the previous findings and confirm that this parasite does have host specificity during infestation. Probable reasons of this specific attack were explored on two Indian and three Chinese major carps. Primarily we used three indicators- an important factor in host defense mechanism. During the current studies, protein concentration was the highest in *C. idella* (3.29±0.13 mg ml^{-1}) and then came *C. catla* with a protein value of 3.02±0.57 mg ml^{-1}. The lowest protein value was observed in *C. carpio* (1.80±0.09 mg ml^{-1}) while values of other three species fell in between these extremes. Comparing the protein concentrations with level of *Lernaea* infestations it appears that a higher protein concentration might have attracted *Lernaea* because it was a convenient food and drastically proliferate its population. Arifa *et al.* (2011) reported protein contents in *L. rohita* (0.55 mg ml^{-1}) which is lower than the values observed in our studies. Stosik *et al*. (2001) observed protein concentration values (3.06 mg ml^{-1}) in *C. carpio* which are comparatively higher than ours which may be due to environmental differences and methods of collection of mucus. Contradictory to previous as well as our studies Mozumder (2005) observed quite higher values of mucus proteins in cod (38.28 mg ml^{-1}) and salmon (9.84 mg ml^{-1}). These broader variations can be due to species differences and treatment protocols. Ghafoori *et al.* (2014) also observed the variations in the protein contents of the male and female Caspian kutum (*R. frisii*) and reported that the values can be different due seasonal temperature, reproductive activity and migration. Nevertheless these observations can hardly be compared with our current species which are herbivorous while former are carnivorous in nature. Moreover carnivorous species do not face such parasitic challenges at least in our environment which demands further investigation to resolve these contradictory observations. Variations

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in the mucus proteins due to handling stress were also investigated by the Easy and Ross (2010) in the *Salmo salar* mucus. They suggested that there were correlations of some mucus enzyme/protein profiles with stress; however, the variability in *S. salar* mucus enzyme levels and actin fragmentation patterns suggested other triggers for inducing changes in mucus protein composition.

When various proteins were resolved on Polyacrylamide Gel Electrophoresis (SDS-PAGE), *C. catla* was the only fish species which showed the protein band with the highest molecular weight (100 kDa) (Table 2) which was not present in any other species. Contradictory to all other species *C. carpio* showed a distinct protein band of 14.13 kDa size which is quite close to lysozyme which has antibacterial property against pathogens (Fleming, 1922). It contributes to the innate immune system and is a natural form of protection from pathogens. The enzyme functions by attacking peptidoglycans (found in the cell walls of bacteria, especially Gram-positive bacteria) and hydrolyzing the glycosidic bond that connects N-acetylmuramic acid with the fourth carbon atom of N-acetylglucosamine. The catalytic role of lysozyme in various pathogens in fish is prominent except in bacteria. So it can be claimed that 100 kDa protein in *C. catla* attracted *Lernaea* while 14.13 kDa proteins in *C. carpio* completely deterred *Lernaea* attack. The catfish skin mucus also contained peptide of 13.3 kDa and 13.9 kDa band suggesting that it played a role in the innate immune system against bacteria because fish remained bacteria free during studies (Ramasamy *et al*., 2011). A band with a molecular weight of 31.62 kDa was present in the *L. rohita, C. mrigala* and *H. molitrix* whereas 29.51 kDa in *C. idella* may indicate the presence of carbonic anhydrase that has a molecular weight of 31.0 kDa (Broad Range BioRad SDS-PAGE Molecular Weight Standards, Catalog Number 161-0317).

Lectins are the proteins present in all living organisms. They recognize and bind to specific carbohydrates of the cell surface (glycoproteins and glycoconjugates) and agglutinate them (Lis and Sharon, 1998). Lectins play a variety of physiological roles especially in the innate immune system as a means of defense against invading pathogens (Hoffmann *et al*., 1999). Ng *et al.* (2003) observed lower lectin activity (2^7) in grass carp than we did (2^9). Sahoo *et al*. (2005) determined haemagglutination titer (lectin activity) in three Indian major carps to investigate normal physiological non-specific immune response under culture conditions. Haemagglutination titre values for *C. mrigala* (64 or 2^6), and *L. rohita* (512 or 2^9) are in line with the present study, but for *C. catla* (512 or 2^9) values are significantly higher than ours (2^6). Dash *et al*. (1993) also observed haemagglutination titre variation (32 to 512) in two catfish species, viz. *Heteropneustes fossilis* and *Clarias batrachus* in different seasons. Manihar *et al.* (1991) reported similar trends in HA activities in different species of the family *Channidae.*

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Higher values for agglutination titer can be used as an indicator of either a previous exposure to disease or contact with pathogen contaminated water (Troast, 1975). As the fish utilized in present study were from the same culture environment, the variation in agglutination titer observed during the current study may be due to variations in their immune responses.

Alkaline phosphatase (ALP) is a potential stress indicator in Atlantic salmon (*Salmo salar*) that plays a protective role in wound healing (Ross *et al*., 2000). Iger and Abraham (1990) were unable to know the cause of ALP activity differences in the mucus of rainbow trout however; they suggested that this variation may be related to stressors including acidity, thermal elevation, polluted water and the species response to these stressors. Omolbanin *et al.* (2012) analyzed mucus alkaline phosphatase in different weight groups of common carp and observed that the highest alkaline phosphatase activity (IU L^{-1}) were in older fishes however, concentrations increased in case of disease and wounding. Ross *et al*. (2000) reported that parasitic infestations initiate the secretions of sufficient alkaline phosphatase from Atlantic salmon skin which increased with increase in intensity of infestation. Other than fish, saliva of other arthropod parasites also exhibit phosphatase activity (Kerlin and Hughes, 1992) which might be an indicator of any sort and level of infestation. Sanchooli *et al*. (2012) and Kumar *et al*. (2010) reported ALP values in common carp blood serum (69.57±11.16 and 85±25.1 U L^{-1}, respectively) that are quite higher than that in the skin mucus of fish in the present study due to the difference of studied medium. However, ALP increased with the increase of infestation that is consistent with our studies. Thus our results support the phenomena that the different fish species have different resistances to the environmental stresses like temperature, growth periods and disease causing agents and were in comparison to the findings of Kumar *et al*. (2010) whose investigation indicated that mucus of *C. chanos* has stronger innate immune properties as compared to that of other two fishes and therefore, polyculture of this fish with other fish or shrimp species may have beneficial effects for disease prevention.

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