



Effect of Biofilm of *Aeromonas Hydrophila* Oral Vaccine on Growth Performance and Histopathological Changes in Various Tissues of Striped Catfish, *Pangasianodon Hypophthalmus* (Sauvage 1878)

M.A.A. Mamun, S. Nasren, P.B. Abhiman, S.S. Rathore,
N.S. Sowndarya, K.S. Ramesh, K.M. Shankar¹

10.18805/ijar.B-3814

ABSTRACT

Biofilm of *Aeromonas hydrophila* oral vaccine was evaluated on growth and histopathological features of striped catfish, *Pangasianodon hypophthalmus*. Fish were fed with feed incorporating biofilm (BF) or free cell (FC) of *A. hydrophila* vaccine at 10^{10} cells/fish/day for 20 days and basal feed for 60 days. From 30th day onwards, till end of the trial, the mean weight gain (g/fish) was significantly higher ($P < 0.05$) in BF treated group than both FC and control group. At the end of the study, histopathological changes in gills, liver and kidney were evaluated using haematoxylin eosin technique. The histopathological findings, in BF fed *P. hypophthalmus*, showed normal architecture of the gills, liver and kidney. However, fishes fed on FC and control group, resulted several histopathological abnormalities in all the organs.

Key words: *Aeromonas hydrophila*, Biofilm based oral vaccines, Growth, Histopathology, Striped catfish.

INTRODUCTION

Aquaculture is one of the fastest growing food production sectors in the world including India. World fish production has reached at about 171 million tonnes in 2016, where aquaculture contributing 47 per cent of the total fisheries production (FAO, 2018). There has been a phenomenal shift from extensive to intensive culture of carps and catfishes in the last three decades. Striped catfish, *Pangasianodon hypophthalmus* has quickly become the third most cultured freshwater fish in India (Kumar *et al.*, 2017). Intensive aquaculture offers an increased opportunity for spreading of infectious diseases at all stages of production. Pathogenic microorganisms (54.59% bacterial, 22.6% viruses, 3.1% mycotic) and parasitic agents (19.4%) are responsible for periodical disease outbreaks in aquaculture (Dhar *et al.*, 2014). Among the bacterial pathogens *P. hypophthalmus* is most susceptible to the *Aeromonas* spp. and *E. ictaluri* causing motile aeromonas septicemia (MAS) and bacillary necrosis (BNP) respectively (Subagja *et al.*, 1999; Ferguson *et al.*, 2001). Fish immune systems are weak, compare to the higher vertebrates which is the major factor for predisposing them to innumerable disease outbreaks. Over the last few decades, vaccination has become the integral part of modern intensive aquaculture. Norwegian salmon industry is the best example of benefit of vaccinations (Sommerset *et al.*, 2005).

With respect to vaccine studies, the injection of antigens provides for better immune protection than does the immersion protocol (Tatner *et al.*, 1986) and oral route (Ellis, 1988). However, injection vaccines are related to significant growth depression, internal adhesions and injection site

Laboratory of Aquatic Animal Health Management, Department of Aquaculture, College of Fisheries, Karnataka Veterinary Animal and Fisheries Science University, Mangalore-575 002, Karnataka, India.
¹Veterinary College, Karnataka Veterinary Animal and Fisheries Science University, Shivamogga-577 204, Karnataka, India.

Corresponding Author: M.A.A. Mamun, Laboratory of Aquatic Animal Health Management, Department of Aquaculture, College of Fisheries, Karnataka Veterinary Animal and Fisheries Science University, Mangalore-575 002, Karnataka, India.
Email: mamunff@gmail.com

How to cite this article: Mamun, M.A.A., Nasren, S., Abhiman, P.B., Rathore, S.S., Sowndarya, N.S., Ramesh, K.S. and Shankar, K.M. (2020). Effect of Biofilm of *Aeromonas Hydrophila* Oral Vaccine on Growth Performance and Histopathological Changes in Various Tissues of Striped Catfish, *Pangasianodon Hypophthalmus* (Sauvage 1878). Indian Journal of Animal Research. 54(5): 563-569.

Submitted: 27-02-2019 **Accepted:** 09-08-2019 **Published:** 09-11-2019

melanization resulting in carcass downgrading (Evensen, 2003). Oral vaccines are an attractive alternative to overcome such constraints. Oral vaccination is ideal for economically vaccinating large number of animals with least stress. Unfortunately, free cells (FC) or planktonic bacterial oral vaccine have given poor or uneven performance in terms of protection, due to digestive hydrolysis and low pH of gut. To protect oral antigens from the gastric destruction several strategies were evaluated, such as microspheres, bioencapsulation.

Our laboratory has conceptualized and developed a novel bacterial biofilm. Biofilm (BF) of *Aeromonas hydrophila*,

a fish pathogen developed on chitin flakes, inactivated and incorporated in fish feed (Fig 1). Bacterial biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix, glycocalyx. Within this glycocalyx, complex and differentiated associations can be formed, which facilitate nutrient uptake (Azad *et al.*, 2000; Hall-Stoodley *et al.*, 2004; Toutain *et al.*, 2004).

In the earlier biofilm based research has restricted on the measurements of antibody titre and protection upon challenge (Azad *et al.*, 1997, 1999; Nayak *et al.*, 2004; Sharma *et al.*, 2010; Siriappagounder *et al.*, 2014). Oral vaccines has great potential in Indian aquaculture. However, so far, there has been no published information on growth performance and histopathology of fishes fed on biofilm *A. hydrophila* as oral vaccine. Histological changes provide a rapid method to detect effects of irritants, especially chronic ones, in various tissues and organs (Bernet *et al.*, 1999; Wali *et al.*, 2018). This paper describes the effects of biofilm of *A. hydrophila* oral vaccines on growth and histopathological features of striped catfish, *P. hypophthalmus*.

MATERIALS AND METHODS

Bacterial isolate and maintenance of pure culture

A virulent *A. hydrophila* isolated from diseased gold fish was injected and re-isolated from common carp (*Cyprinus carpio*), using Rimler-Shotts (RS) selective medium for the pathogen (Himedia, Mumbai). One day old isolate grown in 1.5% (w/v) Tryptone Soya Broth (Himedia, Mumbai) was harvested by centrifugation at 10,000 rpm for 10 min and the cell pellet was re-suspended in sterile 1.5% TSB (w/v) with 15% glycerol. The cell suspension was aliquoted into 1.5 ml micro centrifuge tubes and stored at -20°C for further use.

Preparation of *A. hydrophila* biofilm cells

A. hydrophila biofilm was prepared according to Azad *et al.*

(1997). Conical flask containing TSB (0.225% w/v) and chitin flakes (0.3% w/v) (Himedia, Mumbai) was autoclaved at 121°C for 15 min and cooled to room temperature. The medium was inoculated with *A. hydrophila* culture and agitated for 6 h daily in an incubator mechanical shaker (120 strokes/min) at room temperature. On the fourth day, the supernatant was decanted and the chitin flakes were washed thrice in the same flask with sterile phosphate buffer saline (PBS, pH 7.2) to remove free cells. Biofilm cells on chitin were then heat inactivated at 100°C for 50 min before incorporating in the feed.

Preparation of *A. hydrophila* free cells

A. hydrophila free cells were prepared according to Azad *et al.* (1997). TSB (1.5% w/v) was sterilized at 121°C for 15 min and cooled to room temperature. The medium was inoculated with 18 h old *A. hydrophila* culture and the flasks incubated for 18 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min and further washed thrice using sterile PBS (pH 7.2). Finally the pellets were resuspended in PBS to 10¹⁰ cells/ml. The cells were heat inactivated at 90°C for 10 min before incorporating in the feed.

Incorporation of biofilm and free cells in feed

Feed ingredients, such as fish meal (24%), groundnut oil cake (24%), rice bran (17%), wheat flour (17%) tapioca flour (14%) were mixed together, cooked and cooled to room temperature. Cod liver oil (3%), vitamin and mineral mixture (1%) were added to the cooked ingredients separately followed by incorporation of heat inactivated biofilm cells on chitin (BF) and free cell (FC). Vaccine was incorporated in the feed according to Azad *et al.* (1999). Briefly, biofilm and free cells of *A. hydrophila* were grown to high density and quantified cells were incorporated at high density in feed, so as to achieve at the end 10¹⁰ cells/ fish/day. The feed paste was pelletized sun dried and dry pellets stored

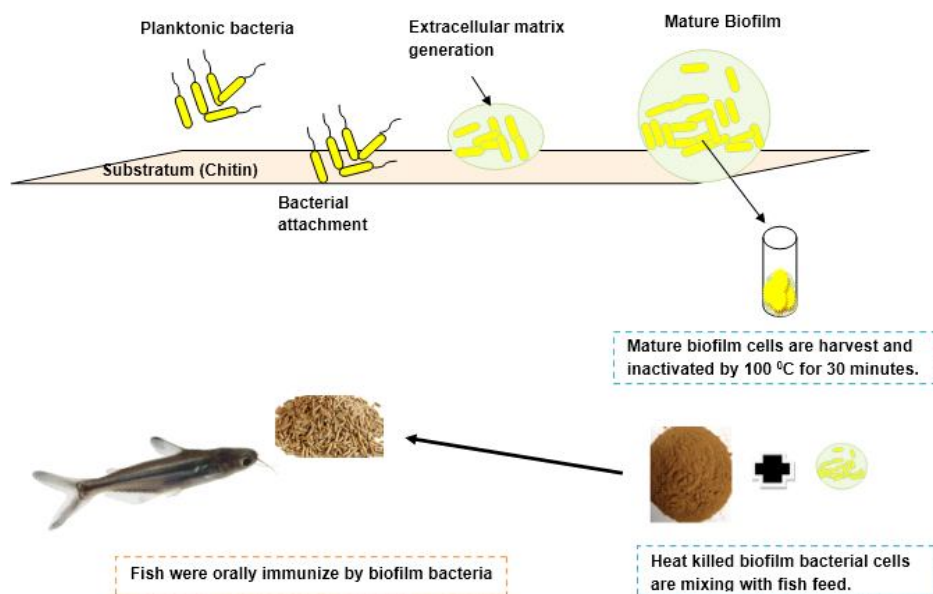


Fig 1: Attachment and growth of bacterial biofilm on the substrate and its application as oral vaccine for aquaculture species.

at 4°C in a refrigerator. A control diet (C) with above ingredients without *A. hydrophila* was prepared with sterile PBS (pH 7.2).

Fish stock maintenance

Healthy fry of *P. hypophthalmus* having mean weight (g) ranging from 0.524 ± 1.21 to 1.2 ± 1.13 and mean length (cm) 2.50 ± 0.94 to 4.50 ± 1.10 procured from Zonal Agricultural and Horticultural Research Station, Mudigere (13.1378°N 75.6060°E.), Chikkamagaluru, India. The fish was acclimatized in the Instructional Fish Farm, College of Fisheries, Mangalore and reared for 1 months to advanced fingerling. The fish were fed with dry pellets, prepared with feed ingredients such as fish meal 24%, groundnut oil cake 24%, rice bran 17%, wheat flour 17%, tapioca 14%, cod liver oil 3%, minerals and vitamins 1%.

Fish immunization

P. hypophthalmus (12.42 ± 0.14 g) were maintained in cemented rectangular tanks (950 l). Two treatments namely biofilm (BF) and free cell (FC) and one control (C) were setup with three replication in each. Each replicate tank was stocked with 30 fingerlings. Every alternate day, half of the tank water was siphoned off to remove the waste material to maintain better water quality. Fish were fed with biofilm cell incorporated feed at 10^{10} /fish/day and free cell feed at 10^{10} /fish/day. Fish in the control was fed with control feed mixed with PBS (Phosphate Buffered Saline). The vaccinated feed was given at 4% of the body weight for 20 days and complete acceptance of feed was ensured each day. First 20 days fishes were vaccinated and control feed was given at 2.5% body weight for next 60 days. At the end of experiments, growth performance was evaluated and fishes were collected for histopathological analysis.

Histopathology of the organs

Tissues for histopathological study, were aseptically collected. Healthy fishes were euthanized and tissue specimens of gills, liver and kidney were excised, rinsed in normal saline and fixed in 10% buffered formalin for 72 h.

After fixation, the tissues were dehydrated in an alcohol series of ascending concentration (70%, 80%, 90% and 100%, respectively), embedded in paraffin and sectioned at 5-6 μ m. The tissue sections were stained with haematoxylin-eosin (H&E) and were examined by light microscope. All the histological procedures were followed as detailed by Bullock (1989). The tissues on the glass slides were analysed through the light microscope (Olympus, BX3-25ND25, Japan) to check for any signs of tissue damage.

RESULTS AND DISCUSSION

Weight gain (g/fish)

Biofilms are gathering of microbial cells that is immutable associated with a surface material and enclosed in a matrix of primarily polysacchride material. Biofilm organism in natural environment are tiny and highly nutritious. Biofilm act as a bioactive compounds and dietary stimulants which can enhance growth performance of fishes. Several research conducted employing natural substrate based biofilm, revealed that higher biomass compare to control at the end of the experiments. Ramesh *et al.* (1999) have reported better growth of Rohu (*Labeo rohita*) fed on biofilm, settled on the substrates. Similar results found in shrimp (Thompson *et al.*, 2002), scampi (Abdullah *et al.*, 2012).

In the present study, biofilm oral vaccines enhanced the growth of pangasius catfish (Fig 2). From the initiation of the trial to 15 d, no significant difference ($P > 0.05$) was evident in mean weight gain (g/fish) in all the experimental diets. From 30th day onwards, till end of the trial, the mean weight gain (g/fish) was significantly higher ($P < 0.05$) in BF treated group that of FC and control group. The mean weight (g/fish) recorded after 80 days of feeding trial in BF treatment was 59.51 g, followed by 46.93 g in FC and 47.26 g in control. The average weight of fish in BF group was higher by 26.80, 25.92% than FC and control group respectively. Similar result was reported that in the presence of sugarcane bagasse, paddy straw and dried *Eichhornea* for settlement of biofilm, was higher the growth of rohu by 47.5, 29.1 and

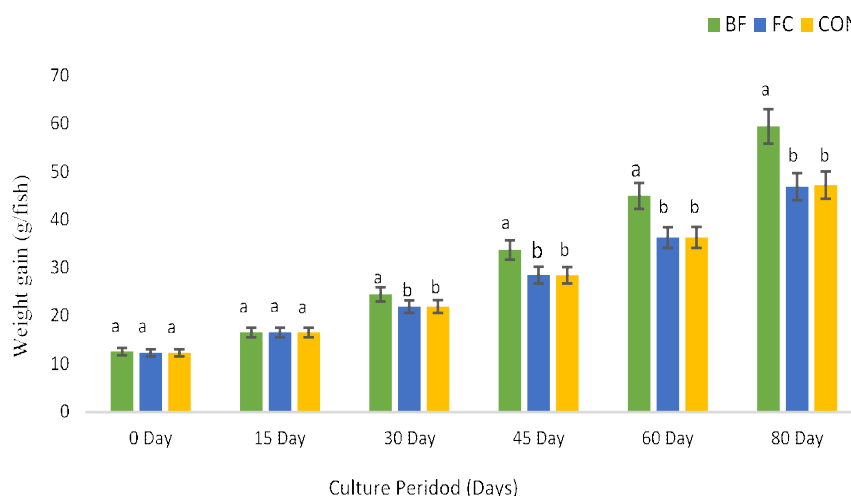


Fig 2: Weight gain (g/fish) of *P. hypophthalmus* fed different experimental diets for eighty (80) days.

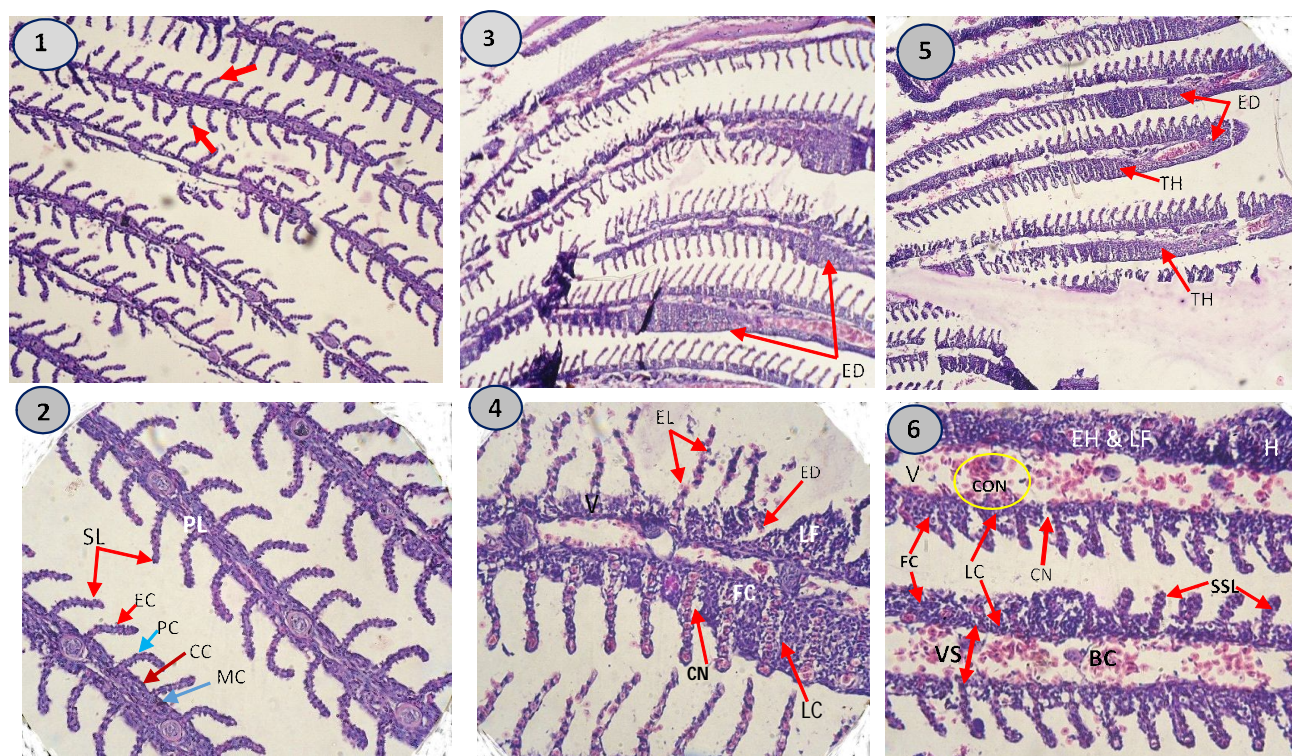


Fig 3: Photomicrographs of the gill of *P. hypophthalmus*. BF fed group, (1: 10X; 2: 40X) H & E showed normal architecture of the gill, showing primary lamellae (PL), secondary lamellae (SL) and arising from these filaments, parallel with them and perpendicular to the filament axis (arrow); mucous cell (MC), chloride cell (CC), pillar cell (PC) and epithelial cell (EC). Whereas FC group (3: 10X; 4: 40X) H& E showing lifting of Epithelial lining (EL) in the base and tips of secondary lamella, edematous separation of the epithelial layer (ED), lamellar fusion (LF), cellular necrosis (CN), infiltration of leukocytes (LC) and Control group (5: 10X, 6: 40X) H & E showing filamentous clubbing (FC), cellular necrosis (CN), blood cells (BC), vasodilation (VS), congestion (CON), hypertrophy (H), epithelial hyperplasia (EH) with lamellar fusion (LF), shortening of the secondary lamellae (SSL), showing irregular thickening (TH) of primary lamellar epithelium, infiltration of leukocytes (LC), vacuolation (V).

17.6%, respectively than the control (Ramesh *et al.*, 1999). Significant improvements of growth performance were noted in silver pomfret, *Pampus argenteus* diets supplemented with *Bacillus subtilis*, *Lactobacillus plantarum* and *Clostridium butyricum* (Gao *et al.*, 2017).

There is limited knowledge about the effects of oral vaccines on the growth of *P. hypophthalmus*, in particular with biofilm of *A. hydrophila* (Azad *et al.*, 1999, Nayak *et al.*, 2004; Sharma *et al.*, 2010; Siriappagounder *et al.*, 2014). This research demonstrates for the first time for growth-related effects in striped catfish, *P. hypophthalmus*, following the oral vaccination with the biofilm and free cell of *A. hydrophila* antigen. Biofilms are group or micro-organisms in which microbes produced an extracellular polymeric substances (EPS) such as proteins (<1-2%) including enzymes, DNA (<1%), polysaccharides (1-2%) and RNA (<1%) and in addition to these components, water (up to 97%) is the major part of biofilm which is responsible for the flow of nutrients inside biofilm matrix (Jamal *et al.*, 2015). The elevated growth in BF fed group fishes might be due to the extra protein substances generated by the biofilm of *A. hydrophila*. We assuming, glycocalyx matrix might protected

the *A. hydrophila* from the gastric digestion and fecalated to the hind gut enterocytes in a form which can be taken up by these cells and translocated in an immunogenic form to hind gut and provide the probiotic effect. Kahieshesfandari *et al.* (2019) and Azad *et al.* (2000) convincingly validated the availability of greater quantities of antigen and its prolonged retention and protection as demonstrated by GALT and MAb based immunofluorescence respectively. Studies revealed that *Bacillus subtilis* act as probiotics since they have promoted for growth (Hoa *et al.*, 2000). According to Balcázar *et al.* (2006) probiotic microorganisms are able to aggregate in the gastrointestinal tract when administered over a long period of time could generated their multiple benefits. The increased growth performance reported Gao *et al.* (2017) in silver pomfret fed with probiotics could be due to better utilization of the diets through enhancement of digestive enzyme activity.

Histopathological changes in the gill tissue

Gills are responsible for regulating the exchange of salt and water and play a major role in the excretion of the nitrogenous waste products. Histopathological changes in the gill tissues in various treatments have shown in Fig 3. In

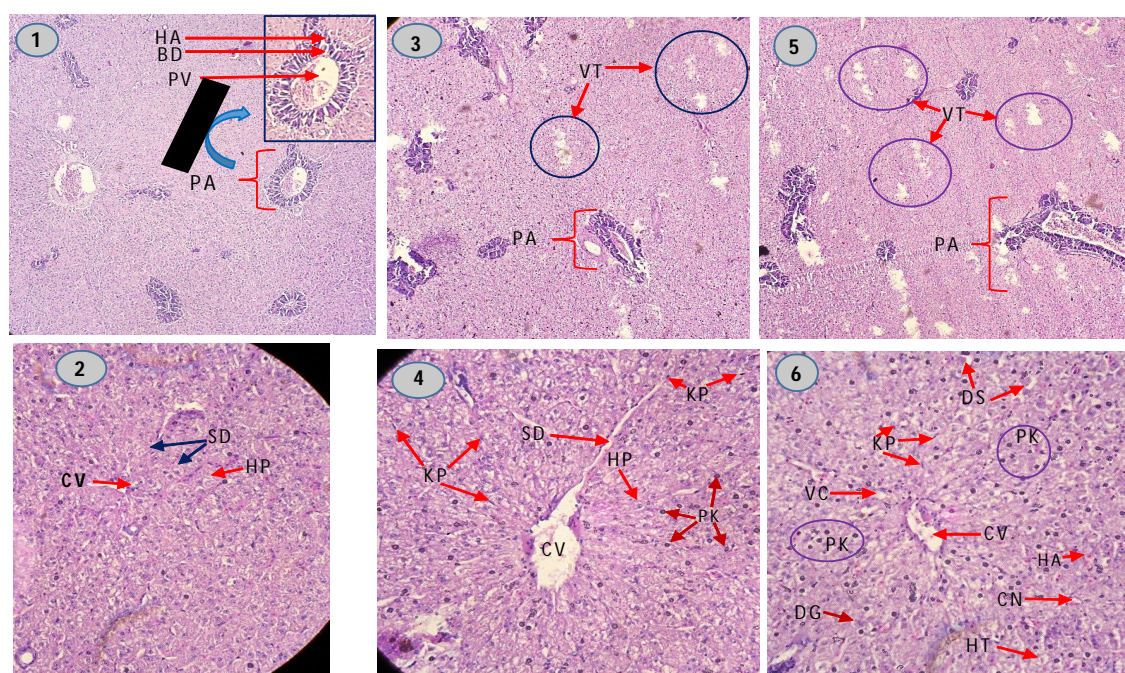


Fig 4: (1: 10X; 2: 40X) H & E, Liver section micrograph of *P. hypophthalmus* in the biofilm (BF) fed group, showing healthy liver tissue with quite regular liver cells, central vein (CV), hepatic cells (HP), sinusoids (SD), portal area (PA). Portal area mainly consist of hepatic artery (HA), bile duct (BD) and portal vein (PV) (3: 10X; 4: 40X) H & E, Free cell (FC) fed group, (5: 10X; 6: 40X) control group, showing vacuolization in the tissues (VT), dilated sinusoids (DS), vacuolization of cytoplasm (VC), cellular necrosis of hepatocytes (CN), increase in the number of Kupffer cells (KP), pyknotic nuclei (PK), degeneration of the nucleus (DG), hypertrophied hepatocyte (HT), haemorrhages (HA).

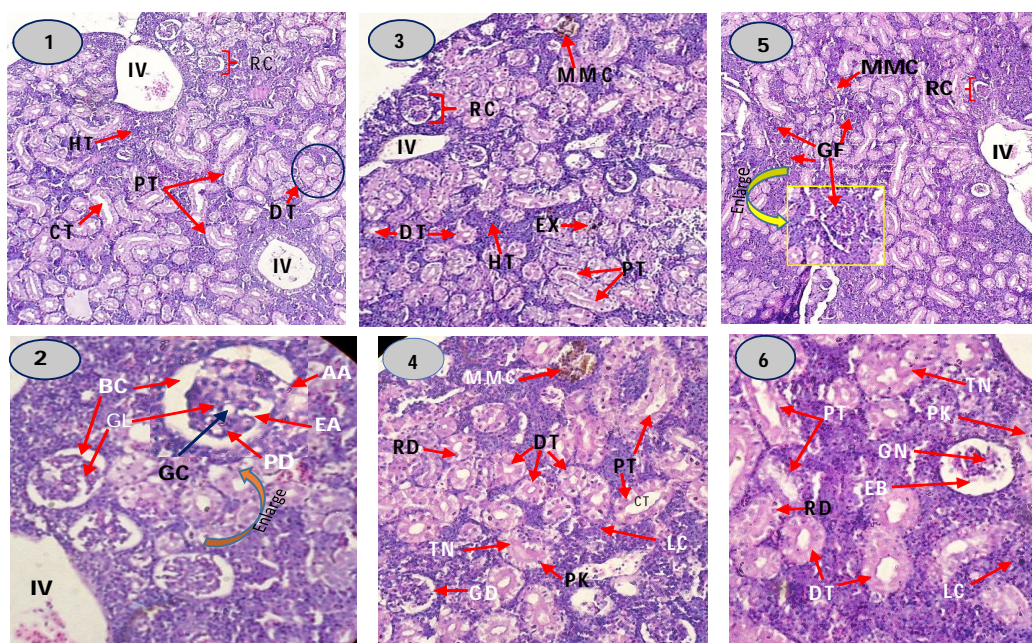


Fig 5: (1: 10X; 2: 40X) H & E, Kidney histopathological changes of *P. hypophthalmus* in the biofilm (BF) fed group, showing healthy kidney tissue with quite regular renal corpuscle {Glomerulus (GL) + Bowman's Capsule (BC)}, hematopoietic kidney tissues (HT), interlobular vein (IV), glomerular capillaries (GC) afferent arteriole (AA), efferent arteriole (EA), podocytes (PD) (3: 10X; 4: 40X) H & E, Free cell (FC) fed group, (5: 10X; 6: 40X) control group showing, inflammatory exudate (EX), glomerular distension (GN), tubular necrosis (TN), increase in the presence of leukocyte cells (LC), rodlet cells (RD), pyknotic cell (PK), glomerular fibrosis (GF), glomerulus necrosis (GN), extended bowmans capsule (EB).

biofilm fed fishes showed normal architecture of the gill. Similar findings of striped catfish *P. hypophthalmus* fed with dietary nucleotides has been reported (Pournori *et al.*, 2017). Nouh *et al.* (2009) has mentioned no remarkable pathological alterations were recognized in groups treated with single or mixed probiotic. Feeding with FC and control feed resulted several histopathological abnormalities in gill tissues, such as filamentous clubbing, cellular necrosis, blood cells, vasodilation, congestion, hypertrophy. Sharaf and Tag (2011), indicated that feeding of *Cyprinus carpio* with the higher dose of humic acid caused more potent destructive effect in the gill, liver and kidney tissues.

Histopathological changes in the liver tissue

Histopathological changes in the liver tissues in various treatments have shown in Fig 4. Liver tissue of biofilm fed group was natural and healthy with regular liver cells. This might be due to the capability of biofilm of *A. hydrophila* to reduce the effect of stressors. Proper nutrition is extremely important for maintaining sound health status of fish. Fish feeding on probiotics manipulate microbiota in the gastrointestinal tract, which produce exoenzymes that can increase nutrient digestibility and promote better health conditions (Zhang *et al.*, 2010). Babińska *et al.* (2005) reported no negative impact of *Lactobacillus acidophilus* on the morphology of the liver and gastrointestinal tract when given in piglets feed. In control and free cell treatments, vacuolization in the tissues, fibrosis of hepatocyte cells, dilated sinusoids, vacuolization of cytoplasm, cellular necrosis of hepatocytes, increase in the number of Kupffer cells, pyknotic nuclei were observed. The histopathological abnormalities observed in control and free cell vaccine either for nutrition imbalance or environmental irritants or presence of ubiquitous bacterial pathogen, *A. hydrophila* that are present in the same water environment.

Histopathological changes in kidney tissue

The kidneys are paired elongated structures placed above the alimentary canal close to the vertebral column. Histopathological changes in the kidney tissues of the fish in different treatments have shown in Fig 5. In the biofilm fed group, kidney was perfectly healthy consisting of glomeruli, tubules, hematopoietic tissue and a variable number of visible melanocytes. The fingerlings of *L. rohita* fingerlings fed with 10^6 CFU/g *L. rhamnosus* probiotic feed showed intact structure of gills, liver and kidney (Gobinath, 2014). Fishes in the free cells and control group showed histopathological alterations such destruction of Bowman's space, inflammatory exudate, glomerular necrosis, tubular necrosis, infiltration of leukocyte cells were detected. Most common alterations found in the kidney of fishes exposed to water contamination are tubule degeneration and changes in the corpuscle, such as dilation of capillaries in the glomerulus and reduction of Bowman's space (Takashima and Hibiya, 1995). Our results are in accordance with Moneim *et al.* (2019) reported disruption of kidney tissues in wild captured Nile tilapia, *Oreochromis niloticus*.

The induction of a local or systemic immune response after oral immunization is dependent on uptake of antigens from the gut lumen. Histological results revealed that biofilm fed fishes showed healthy structure of gill, liver and kidney tissues compare to free cell and control group. As there was no previous results we could assume that biofilm based oral vaccine might induced in the integumentary immune response in the gill, by providing the protection from ubiquitous bacterial pathogen, *A. hydrophila* that are present in the same water environment. Moreover, normal structural morphology of the biofilm fed group might be due to the probiotic effect. Furthermore, biofilm might reduce the stress and improved the health status of *P. hypophthalmus* fingerlings. Azad *et al.* (2000) found that quantity of BF antigen in the lumen of the gut, spleen and kidney were significantly higher than that of free cell with high retention time. It's because BF cells are encased in the glycocalyx material which renders protection against digestive hydrolysis.

CONCLUSION

Oral vaccines are ideal for aquaculture to vaccinate millions of fish of different size with least stress and cost. Till now, no known vaccines are being commercialized or marketed in India despite the fact that market attractiveness is very high. Against this, biofilm oral vaccine could be the ideal vaccine for Indian aquaculture. Biofilm fed fishes were revealed healthy status of gill, liver and kidney tissues compare to free cell and control group. It can be concluded that feeding with biofilm of *Aeromonas hydrophila* oral vaccines can provide the better growth and histological results in this experiment. Further studies should be carried out in field conditions to ascertain the benefit of biofilm based oral vaccine on growth and histopathology.

ACKNOWLEDGEMENT

First two authors gratefully acknowledge the funding received towards their Ph.D. from the Indian Council of Agricultural Research for awarded of Netaji Subhas- ICAR International fellowship 2015-16.

REFERENCES

- Abdullah-Al-Mamun, M., Al-Rashid, A.H., Mazumder, S.K., Shamsuzzaman, M.M., Hossain, M.M., Nasren, S. (2012). Improvement in the production phase of freshwater prawn, *Macrobrachium rosenbergii* in grow-out ponds through the use of substrates. J. Aquacult. Trop. 27(1-4): 53-64.
- Azad, I. S., Shankar, K. M., Mohan, C. V., Kalita, B. (1999). Biofilm vaccine of *Aeromonas hydrophila*—standardization of dose and duration for oral vaccination of carps. Fish Shellfish Immunol. 9(7): 519-528.
- Azad, I.S., Shankar, K.M., Mohan, C.V., Kalita, B. (2000). Uptake and processing of biofilm and free-cell vaccines of *Aeromonas hydrophila* in Indian major carps and common carp following oral vaccination antigen localization by a monoclonal antibody. Dis. Aquat. Org. 43(2): 103-108.

- Azad, I.S., Shankar, K.M., Mohan, C.V. (1997). Evaluation of an *Aeromonas hydrophila* biofilm oral vaccination of carps. IM; *Diseases in Asian aquaculture III* (T.W. Flegel and I.H. Macrae, eds.), Fish health section, AFS, Manila, pp. 181-186.
- Babińska, I., Rotkiewicz, T., Otrocka-Domagala, I. (2005). The effect of *Lactobacillus acidophilus* and *Bifidobacterium* spp. administration on the morphology of the gastro-intestinal tract, liver and pancreas in piglets. *Pol J Vet Sci.* 8(1): 29-35.
- Balcázar, J.L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J.L. (2006). The role of probiotics in aquaculture. *Vet. Microbiol.* 114(3-4): 173-186.
- Bernet, D., Schmidt, H., Meier, W., Burkhardt Holm, P., Wahli, T. (1999). Histopathology in fish: proposal for a protocol to assess aquatic pollution. *J. Fish Dis.* 22(1): 25-34.
- Bullock, A.M. (1989). Laboratory methods. In: *Fish Pathology*. [Edt. Roberts, R.J.] Bailliere Tindall, London, pp. 374-406.
- Dhar, A.K., Manna, S.K., Allnutt, F.T. (2014). Viral vaccines for farmed finfish. *Virusdisease.* 25(1): 1-17.
- Ellis, A.E. (1988). Optimizing factors for fish vaccination. In: *Fish Vaccination* (Ellis AE, editors): Academic Press, New York. pp. 32-46.
- Evensen, O. (2003). The vaccine formulation and its role in inflammatory processes in fish-effects and adverse effects. In: *3rd International Symposium on Fish Vaccinology*, April 9-11: 2003. Bergen Norway.
- FAO. (2018). The State of World Fisheries and Aquaculture 2018-Meeting the sustainable development goals. Rome. Licence: CC BY-NC-SA 3.0 IGO.
- Ferguson, H.W., Turnbull, J.F., Shinn, A., Thompson, K., Dung, T.T., Crumlish, M. (2001). Bacillary necrosis in farmed *Pangasius hypophthalmus* (Sauvage) from the Mekong Delta, Vietnam. *J. Fish Dis.* 24(9): 509-513.
- Gao, Q., Xiao, C., Min, M., Zhang, C., Peng, S., Shi, Z. (2017). Effects of probiotics dietary supplementation on growth performance, innate immunity and digestive enzymes of silver pomfret, *Pampus argenteus*. *Indian J Anim Res.* 50(6): 936-941.
- Gobinath, J. and Ramanibai, R. (2014). Histopathological Studies in the gill, liver and kidney of the freshwater fish *Labeo rohita* fingerlings. *Int. J. Innov. Res. Sci. Eng. Technol.* 3(3): 10296.
- Hall-Stoodley, L., Costerton, J. W., Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2(2): 95.
- Hoa, N.T., Baccigalupi, L., Huxham, A., Smertenko, A., Van, P.H., Ammendola, S., Cutting, S. M. (2000). Characterization of *Bacillus* species used for oral bacteriotherapy and bacteriophylaxis of gastrointestinal disorders. *Appl. Environ. Microbiol.* 66(12): 5241-5247.
- Kumar, S., Raman, R. P., Prasad, K. P., Srivastava, P. P., Kumar, S., Rajendran, K. V. (2017). Modulation of innate immune responses and induction of oxidative stress biomarkers in *Pangasianodon hypophthalmus* following an experimental infection with dactylogyrid monogeneans. *Fish Shellfish Immunol.* 63: 334-343.
- Moneim, A.A., Elmenshawy, O., Al-Kahtani, M., Sayed, A., Alfwuaires, M. (2019). Pattern of renal pathology in fish from Al-Hassa waterways, Saudi Arabia. *Indian J Anim Res.* 53(6): 751-755.
- Nayak, D.K., Asha, A., Shankar, K.M., Mohan, C.V. (2004). Evaluation of biofilm of *Aeromonas hydrophila* for oral vaccination of *Clarias batrachus*-a carnivore model. *Fish Shellfish Immunology.* 16(5): 613-619.
- Nouh, W.G., Mohamed, M.F., Aly, S.M. (2009). Pathological evaluation to the effect of some probiotics on the health and immune status of Nile Tilapia (*Oreochromis niloticus*). *Egyptain J Comp. Pathol. Clinical Pathol.* 22(2): 233-249.
- Pournori, B., Paykan Heyrati, F., Dorafshan, S. (2017). Histopathological changes in various tissues of striped catfish, *Pangasianodon hypophthalmus*, fed on dietary nucleotides and exposed to water-borne silver nanoparticles or silver nitrate. *J Aquat Anim Health.* 3(2): 36-52.
- Ramesh, M.R., Shankar, K.M., Mohan, C.V., Varghese, T.J. (1999). Comparison of three plant substrates for enhancing carp growth through bacterial biofilm. *Aquacult. Eng.* 19(2): 119-131.
- Sharaf, M.M. and Tag, H.M. (2011). Growth performance, gill, liver and kidney histomorphology of common carp (*Cyprinus carpio*) fingerlings fed humic acid supplemented diets. *Egypt J Exp Biol Zool.* 7(2): 285-294.
- Sharma, S.K., Shankar, K.M., Sathyanarayana, M.L., Sahoo, A.K., Patil, R., Narayanaswamy, H.D., Rao, S. (2010). Evaluation of immune response and resistance to diseases in tiger shrimp, *Penaeus monodon* fed with biofilm of *Vibrio alginolyticus*. *Fish Shellfish Immunol.* 29(5): 724-732.
- Siriappagounder, P., Shankar, K.M., Kumar, B.N., Patil, R., Byadgi, O.V. (2014). Evaluation of biofilm of *Aeromonas hydrophila* for oral vaccination of *Channa striatus*. *Fish Shellfish Immunol.* 41(2): 581-585.
- Sommerset, I., Krossøy, B., Biering, E., Frost, P. (2005). Vaccines for fish in aquaculture. *Expert Rev Vaccines.* 4(1): 89-101.
- Subagja, J., Slembrouck, J., Hung, L. T., Legendre, M. (1999). Larval rearing of an Asian catfish *Pangasius hypophthalmus* (Siluroidei, Pangasiidae): Analysis of precocious mortality and proposition of appropriate treatments. *Aquat. Living Resour.* 12(1): 37-44.
- Takashima, F. and Hibiya, T. (1995). An Atlas of Fish Histology: Normal and Pathological Features, 2nd Edition. Kodansha Ltd., Tokyo/ Fischer Verlag, Stuttgart/New York.
- Tatner, M.F. and Horne, M.T. (1986). Correlation of immune assays with protection in rainbow trout, *Salmo gairdneri*, immersed in *Vibrio bacteriens*. *J. Appl. Ichthyol.* 2: 130-139.
- Thompson, F.L., Abreu, P.C., Wasielesky, W. (2002). Importance of biofilm for water quality and nourishment in intensive shrimp culture. *Aquaculture.* 203(3-4): 263-278.
- Toutain, C.M., Caiazza, N.C., O'Toole, G.A. (2004). Molecular basis of biofilm development by pseudomonads. In *Microbial biofilms*. American Society of Microbiology. pp. 43-63.
- Wali, A., Balkhi, M.U.H., Maqbool, R., Darzi, M.M., Bhat, F.A., Shah, F.A. (2018). Seasonal histopathological alterations caused by *Adenoscolex oreini* in *Schizothorax niger* of Kashmir valley. *Indian J Anim Res.* 52(6): 911-913.
- Zhang Q, Ma H M, Mai K S, Zhang W B, Liufu Z G, Xu W. (2010). Interaction of dietary *Bacillus subtilis* and fructooligosaccharide on the growth performance, non-specific immunity of sea cucumber, *Apostichopus Japonicas*. *Fish Shellfish Immunol.* 29: 204-211.