Abstract

The aim of the presented study was testing the efficacy of an herbal concoction in goldfish (Carassius auratus) experimentally infected with Aeromonas hydrophila. After an intramuscular injection of the pathogen, the scales sloughed off on the site of the injection, with an appearance of a muscular haemorrhagic protuberance, which progressed into an extensive ulcerative dermatitis associated with focal haemorrhage, oedema, and dermal necrosis exposing the underlying muscle. The progression of the disease affected organs in the following order: muscle, gills, liver, and finally the heart. The dip treatment with the 1% herbal concoction for 5 min daily restored the macro- and microscopical structure of the altered primary gill lamellae, liver, heart, and muscles. The recovery changes were described.

Key words: goldfish, Aeromonas hydrophila, pathology, phytotherapy.

Aeromonas hydrophila is an opportunistic pathogen of a wide variety of hosts (30, 48). A. hydrophila infection causes a serious damage in pond and aquarium culture. The pathogenesis and histopathology of red-sore disease has been extensively studied in the common carp (Cyprinus carpio) (1, 53), and the Channel catfish (Ictalurus punctatus) (21). A. hydrophila has been considered to be responsible for red-fin disease in cultured eel (Anguilla japonica) (28), red disease in common carp (20), red-sore disease in largemouth bass (Micropterus salmoides) (30), and haemorrhagic septicaemia in several fish species (41). In Japan, A. hydrophila infection in cultured ayu (Plecoglossus altivelis), was linked to exophthalmia and cutaneous haemorrhage in the tail and anus (34, 39). Although the aetiology of epizootic ulcerative syndrome (EUS), another globally devastating fish disease is uncertain, it is believed that A. hydrophila contributes to the pathogenesis of the lesions (11). Histopathological changes in diseased animals provide a methodological platform to determine the causes of their mortality (32). For example, histopathological changes in A. hydrophila-infected catfish, Clarias batrachus, and Salmo gairdneri, have been documented (2, 7). The liver as the centre of metabolism is also involved in breaking down toxic substances. As a result, hepatic cells are subjected to more damage than cells of other organs (45). In largemouth bass (M. Salmooides), A. hydrophila infection leads to necrosis in the liver, kidney, and heart (30).

A. hydrophila outbreaks have a major impact in aquaculture (4) and such outbreaks can be controlled either by prophylactic measures, like vaccination or by antibiotic treatment. Most of the antibiotics used to control fish diseases today are of microbial origin. In medicine, problem with pathogenic microbes becoming increasingly resistant against most commonly used antibiotics and chemicals is being experienced (12, 38). Then, some derivatives of the antibiotics circumvent or even prevent rapid mutation of the pathogens into resistant forms (29). At present, there are no commercially available vaccines against A. hydrophila (52) and studies on aeromonad plasmids have assumed particular significance of the development of multidrug resistant strains. Nowadays, there is a growing interest in using medicinal herbs as immunostimulants in aquaculture. In the search for alternative, non-resistant, and cost-effective fish disease therapeutic agents, Indian medicinal plants are receiving an increasing attention (27). Harikrishnan and Balasundaram (26) examined the antibacterial activity of an aqueous herbal concoction in arresting the in vitro growth of A. hydrophila. The extract obtained from the rhizomes of Ulteria salicifolia has been shown to improve the healing of ulcer in
different animals (46), while Dombeya buettneri aqueous leaf extract has been shown to be beneficial to peptic ulcers (44). The objective of this study was to treat A. hydrophila infected goldfish with aqueous herbal concoction obtained from three plants (Neem, Tulsi, and Turmeric), and examine histopathological and recovery changes in lateral muscles, gills, liver, and heart.

Material and Methods

Goldfish (Carassius auratus) (weight 15±2 g; n=250) were collected from a local ornamental fish farm in Tiruchirappalli, India. The fish were acclimatised in 200 l tanks/50 fish (salinity 0.25±0.5 ppt, pH 8±0.5, temperature 24±2°C; dissolved oxygen 6.1±0.4 mg/L; photoperiod: light-dark cycle of 14:10 h) and provided with continuous aeration. During the holding period (15 d), the fish were fed a pelleted feedstuff (25% of crude protein) at 5% of their body weight, once a day at 10 am. Two thirds of water was replaced daily after siphoning out the unfed and faecal materials. After holding period, the fish were dipped into 25 ppm formalin for 15 s with no mortalities and released into freshly prepared experimental tanks. They were further acclimatised to these conditions for at least 15 d prior to commencement of experiments.

Aeromonas hydrophila (MTCC 646) was obtained from the Institute of Microbial Technology, Chandigarh, India, and maintained in the laboratory under standard conditions (27). Subcultures were preserved on tryptic soy agar (TSA: w/v; Himedia, Mumbai) in slopes at 5°C and routinely tested for pathogenicity (36) by inoculation (13) into goldfish. The stock culture was stored at -70°C in 0.85 % NaCl with 20% glycerol (v/v) in tryptic soy broth (TSB: w/v; Himedia, Mumbai) to provide stable inoculate throughout the experimental study (8, 55). Subcultured A. hydrophila was taken from TSA slope and harvested in TSB. The broth was incubated for 24 h in a shaker at 25°C and then centrifuged at 10,000 rpm for 20 min at 4°C (22). The supernatant was discarded and the bacterial pellet was washed three times with phosphate-buffered saline (PBS) at pH 7.2 (55).

Fresh leaves of neem (Azadirachta indica), tulsi (Ocimum sanctum), and turmeric (Curcuma longa) were collected in August 2003, from the Bharathidasan University campus, Tiruchirappalli, India. They were surface-sterilised separately with 0.1% mercuric chloride (w/v) solution for 10 min and washed thoroughly in running tap water for 10 min, followed by shade drying for about 10 d until weight constancy was achieved (27). Each sample was finely powdered in an electric blender. Ten grams each of C. longa, O. sanctum, and A. Indica, were evenly mixed manually in the ratio of 1:1:1. From this mixture, a sample of 10 g was weighed, suspended in 100 ml of sterile water, and left under standard conditions for one week (32). The mixture was then serially filtered through Whatman No. 1 and finally through 0.2 µm filters (10), and the obtained concoction was stored in a sterile bottle until use.

The goldfish were initially divided into control group (n=50) and two experimental groups consisting of 50 fish in each. Control fish were intramuscularly injected with 50 µl of PBS, while experimental fish were injected with 50 µl of PBS followed by 50 µl of A. hydrophila (10^7 colony-forming units/ml). After 3 d the same dose was repeated. On the day 12 post-infection, the fish were further divided into two groups: group I: infected un-treated (n=50) and group II: infected and subjected to 1% herbal concoction dip treatment (n=50) for 5 min/d at 11:00 am. Three fish were randomly chosen from each group on days 18, 24, 30, and 36, and were anaesthetised with MS-222 (Sigma, USA) to collect the samples of the lateral muscle from the site of injection, gill, liver, and heart for histopathological analysis. The samples were removed aseptically, processed through graded alcohol, and embedded in paraffin wax. Sections (5-6 µm) were stained with haematoxylin and eosin (H&E).

Results

Histological structure of lateral musculature of control goldfish comprises two compactly arranged distinct layers with an outer superficial red muscle layer and inner white muscle layer. Generally, the white muscle comprises a greater proportion of fibres than the red muscle (Fig. 1a). In the diseased fish, there was a gradual infiltration of macrophages on day 18 (Fig. 2a). In infected untreated group (Group I), the underlying lateral musculature contained many muscle fibres on day 24 post-infection, showing dermal lesions and subdermal necrosis that extended deeper into the underlying skeletal muscle accompanied with a moderate oedema and muscular necrosis (Fig. 2b). On day 30 post-infection, the fragmentation of muscle fibres was noticed along with the occurrence of sarcoplasmic debris, granulomatous inflammation in the epidermis, epidermal necrosis, and hyperplasia (Fig. 2c). After 36 d post-infection, the lateral musculature became markedly oedematous, characterised by cloudy or whitened muscle fibres (Fig. 2d). In infected treated fish (Group II), on the day 18 (6th d of treatment), regenerative process began with the appearance of macrophages (Fig. 2e), which was followed by the proliferation of interstitial cells on day 24 (Fig. 2f). Further regenerative response was observed on days 30 and 36, with the appearance of macrophages and fibrotic cells (Fig. 2g, 2h).

Histological structure of gills in control goldfish was characterised by the presence of primary and secondary lamellae, with mucus cells lying scattered on both sides. The primary lamellae were thickened; the secondary lamellae were uniform in length, straight, and evenly distributed. The primary lamellae and secondary lamellae were also covered by a single thin layer of epithelium that was closely opposed to the pillar cells (Fig. 1b). In infected fish, haemorrhagic necrosis
appeared by day 18 after the infection (Fig. 3a). On day 24 post-infection, histological analysis revealed irregular aggregates of macrophages in gill lamellae, the oedematous lamellae had a bacterial invasion into the capillaries, gill congestion, and hyperaemia (Fig. 3b). By day 30 after the infection, the lesions were found to be associated with thrombosis, oedema, haemorrhage, desquamation of the respiratory epithelium, and formation of haemorrhage globe (Fig. 3c). By day 36 after the infection, the disease had progressed to degeneration with necrosis of lamellar epithelial cells, diffuse infiltration of neutrophils and macrophages, formation of haemorrhagic necrosis and necrosis (Fig. 3d). In the infected treated group, histological analysis revealed the proliferation of granular cells, thickening of the secondary lamellae, and infiltration by leukocytes by day 18 of the treatment (Fig. 3e). By day 24, a moderate hypertrophy of lamellar epithelium and slight necrotic changes were visible (Fig. 3f). By day 30, infected gills exhibited fibrosis or fibrogranulation tissue and infiltration of neutrophils and monocytes (Fig. 3g). In contrast, by day 36 of the treatment, an aneurism was noted (Fig. 3h).

Histological analysis of the liver of control goldfish showed a continuous mass of hepatocytes arranged irregularly. The hepatic cells were large and polygonal in shape with almost centrally placed nuclei; blood sinusoids were also observed (Fig. 1c). By day 18 after the infection, multiple fibromas and macrophage granulomas were observed (Fig. 4a). By day 24 after the infection, the liver of infected fish showed deep vacuolisation and granulation in the cytoplasm, pyknosis of the hepatocyte nuclei, necrosis, granulomatous inflammation, and large number of macrophages and fibroblasts (Fig. 4b). By day 30 after the infection, the liver exhibited focal necrosis of the hepatocytes with tubular degeneration of the intestinal microvilli and hepato-cellular necrosis (Fig. 4c). By day 36 after the infection, liver tissues appeared oedematous and were congested with necrotic foci showing fibrin deposition or slight haemorrhage in the pulp, inflammation, free pre-granulomatous tissue, and mature granuloma (Fig. 4d). Treated fish exhibited initial regenerative response by day 18 (Fig. 4e). By day 24, the dilatation of sinusoids had ceased and formation of normal liver parenchyma occurred, indicating a regenerative response (Fig. 4f). Large-scale granulocytes were noticeable on the day 24 of the treatment (Fig. 4h); by day 36, the formation of normal liver parenchyma was in advanced stage (Fig. 4g).

Histological analysis of the heart in control fish did not show any pathological changes (Fig. 1d). Prominent pathological changes were observed in the heart of infected fish. By day 18 after the infection, marked haemorrhage and massive necrosis were evident (Fig. 5a). By day 24 after the infection, early signs of heart damage with spongiform and compact myocardium were noted. The sarcoplasma showed the separation of myofibrils, mild hyper eosinophilia with fragmentation of cardiac muscle fibres, and infiltration of macrophages and erythrocytes (Fig. 5b).

**Fig. 1.** Control (normal) muscle (a), gill (b), liver (c) and heart (d) of *C. auratus* (HE, 400x).
Fig. 2. Lateral musculature of *A. hydrophila* infected untreated (a, b, c, d) and herbal treated (e, f, g, h) goldfish, *C. auratus* (HE, 400x).
Fig. 3. Gills of *A. hydrophila* infected untreated (a, b, c, d) and herbal treated (e, f, g, h) goldfish, *C. auratus* (HE, 400x).
Fig. 4. Liver of *A. hydrophila* infected untreated (a, b, c, d) and herbal treated (e, f, g, h) goldfish, *C. auratus* (HE, 400x).
Extensive heart damage was evident by day 30 after the infection, with severe hyperplasia of the pericardium, leukocyte-like cells infiltration in the pericardial cavity, the presence of macrophages in loose irregular aggregates, and massive necrosis (Fig. 5c). By day 36 after the infection, affected pericardium exhibited slight to severe thickening due to hyperplasia, a variety of pleomorphic cells in the fibrous layer, fragmented myofibrils bundles; many degenerated mitochondria, granular degeneration, and focal haemorrhage (Fig. 5d). While there was no evidence of active bacterial cell multipication in necrotic lesions from day 18 to 24 of the treatment period, there was an initial regenerative response with dense infiltration of granulocytes (Fig. 5e, 5f). By day 30 of the treatment, marked regenerative responses were evident with dense infiltration of granulocytes (Fig. 5g) and advanced regenerative response on day 36 (Fig. 5h).
A. hydrophila, a Gram-negative rod shaped bacterium distributed widely in aquatic environments (23) is known to infect fish, amphibians, and reptiles (27, 52). In fish, A. hydrophila is widely believed to be the causative agent of ulcerative disease symptom (49) though the pathogenesis is complex and multifactorial (9). The virulence factors expressed by this bacterium include haemolysins, proteases, cholinesterases, enterotoxins, endotoxins, and adhesins (22, 50) all of which contribute to the overall development of the disease in fish (9). In this study, after a 24-day infection, goldfish exhibited fragmentation of muscle fibres with necrosis and sarcoplasmic debris. There were no external signs of infection, such as the appearance of skin ulcers in the early stages. The histopathological changes observed were similar to those in channel catfish (5), and rainbow trout (7) infected with A. hydrophila, and Cyprinus carpio infected with viruriaemia-associated ana-aki-byo (ulcer forming diseases) (44). We noticed signs of severe infection after day 30, with macrophages in lateral muscle and proliferation of interstitial cells. By day 36 of the infection, fibrotic and interstitial cells were easily identifiable. Largemouth bass infected with A. hydrophila also showed epidermal necrosis and extensive hyperplasia along with proliferation of fibrocytes and infiltration of inflammatory cells that extended upward from the oedematous tissues (30). In goldfish, A. hydrophila infection resulted in a higher cumulative mortality of 16.7% by day 24 of the infection, and 33.3% by day 36; there was no mortality in the control group. However, in infected fish subjected to herbal treatment, the mortality was only 3.3% by day 18 of the infection, and 6.7% by day 36. Similarly, the size of induced ulcers in infected fishes progressively increased while in treated group, the size of the lesions was 0.3 mm on day 30 post-infection and after 36 d they were completely healed (Harikrishnan et al., communicated). The present study indicates that the gill lamellae became oedematous due to bacterial invasion into the capillaries by day 24 of the infection. Other marked histopathological changes observed in the gills of C. carpio and I. punctatus infected with A. hydrophila are haemorrhagic hyperaemia, sloughing off, of the respiratory epithelium, and the formation of a haemorrhagic globe (24, 40).

In the present study, histological analysis of the gills after day 30 of herbal treatment showed the presence of oedematous spaces, proliferation of granular cells, and thickening of the secondary lamellae, fibrosis, and infiltration of leukocytes. Oedematous spaces are important in the activation of mucosal immune response in different fishes (15, 43). In contrast, histopathological changes in A. hydrophila-infected liver were characterised by developed granulomatous inflammation and necrosis of hepatocytes. A similar type of tissue destruction and the affinity of this bacterium to the liver were reported also by Miyazaki et al. (40), Grizzle and Kiryu (24), and Huizinga et al. (30). Ventura and Grizzle (51), Angka (2), and Candan (7) also suggest that Clarias bactrachus, S. gairdneri, and I. punctatus experimentally infected with A. hydrophila were affected with necrosis and haemorrhage in the kidney, liver, pancreas, and intestine. A Tiger Oscar (Astronotus ocellatus) infected with motile aeromonad septicaemia contained a large amount of red-ascitic fluid accumulated in the abdominal cavity along with haemorrhages in the liver and kidney (47). In this study, treated fish had faster regenerative responses such as diffusion of sinusoid dilation and formation of normal liver parenchyma by day 24, and multiple fibromas by day 36.

Infected fish exhibited fragmentation of cardiac muscle fibres, infiltrations with macrophages and erythrocytes, massive necrosis accompanied by haemorrhage in the myocardium by day 24 of the infection. In contrast, herbal treated fishes showed regenerative responses with a dense infiltration of granulocytes in the heart muscle between days 18 and 36. Recent in vitro and in vivo experiments in rats suggest that not only topical antimicrobials may be toxic to fibroblasts and keratinocytes, but also retard wound healing (35). In this regard, disease management in fish using phytotherapy is becoming an attractive alternative, mainly due to the biodegradability of raw materials and lack of side effects (27).

Extensive studies have documented the beneficial effects of herbal treatment in human diseases (6, 14, 19, 25, 37). Wu et al. (54), found that weight gain of eels (Anguilla anguilla) treated with traditional Chinese medicines (TCMs) increased significantly their resistance to common infectious diseases. Jian and Wu (33) observed that TCMs had a beneficial effect on the growth and on the prevention and treatment of common diseases in C. carpio. Neem leaves, garlic, and turmeric powder induced disease resistance in fry of Indian major carp (Catla catla) (16). Herbs have also been tried in other countries for the control of shrimp and fish diseases, and the successful results have been reported in Mexico, Thailand, Japan, and Turkey (3, 17, 18). These studies highlight the immense potential of herbal remedies and the need to extend the application of phytotherapy to fish disease management. In this study, we have shown that herbal application may also be of practical use in disease management strategy in fish. To the best of our knowledge, this is the first investigation to report on the restorative changes in the histoarchitexture of the gill, liver, heart, and muscles of gold fish infected with A. hydrophila.

Acknowledgments: The first author is grateful to the Council of Scientific and Industrial Research (CSIR), Government of India for the award of Research Associateship (RA), which made this work possible. The authors are grateful to the Department of Science and Technology for the award of FIST, and special assistance programme (SAP) granted by the University Grants Commission (UGC) for the facilities made available to the department. The first author would also like to acknowledge financial support through KOSEF Postdoctoral Fellowship, South Korea.
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