

## **The Pathology of the Walking Catfish, *Clarias batrachus* (L.), Infected Intraperitoneally with *Aeromonas hydrophila***

SRI LESTARI ANGKA

*Faculty of Fisheries  
Bogor Agricultural University  
Kampus Darmaga, Jalan Rasamala  
Bogor, Indonesia*

### **Abstract**

Selected organs from *Clarias batrachus* (L.) fingerlings that were experimentally infected with *Aeromonas hydrophila* were examined. Gross pathology, histopathology and the LD<sub>50</sub> are described. Histopathological changes, which included necrosis and hemorrhage in the kidney, liver, pancreas and intestine, were probably associated with the cytotoxin produced by the bacteria.

### **Introduction**

*Aeromonas hydrophila* is commonly isolated from fish affected by ulcerative disease outbreaks in Southeast Asia and some other countries (Ruangpan et al. 1986; Areerat 1987; Llobrera and Gacutan 1987; Angka et al. 1988). Although it is a common inhabitant of aquatic environments and is a constituent of the superficial and intestinal flora of fish (Lallier and Daigneault 1984; Dooley et al. 1986), this bacterium frequently behaves as a secondary invader (Roberts 1978; Tonguthai 1985). *Aeromonas hydrophila* is responsible for the disease known as motile aeromonad septicemia (see Lewis and Plumb 1979) and is considered one of the most important pathogens of freshwater fishes. Its pathogenic mechanisms are not known (Bullock and McLaughlin 1970), but are associated with toxins (Santos et al. 1987).

*Clarias batrachus* is intensively cultured in Indonesia and serious disease problems have occurred. In ulcerative disease

outbreaks in some parts of Java where 80-90% mortality has occurred *Aeromonas hydrophila* was the dominant pathogen isolated. Once an outbreak begins in wild or pond-raised fish, it is difficult to control, as rapid growth of the bacterium and elaboration of its toxic products may cause irreparable systemic damage which leads to death.

This study describes the pathological changes associated with *Aeromonas hydrophila* injected intraperitoneally.

### Materials and Methods

Fish used for this experiment, *Clarias batrachus* fingerlings about 5-8 cm long and weighing 3-4 g, were provided by the Fisheries Research Institute, Bogor. They were all derived from the same parent stock. Before the experiment the fish were first treated with NaCl at 30 ppt for 15 minutes to remove any ectoparasites. After two days they were exposed to 15 ppm tetracycline as a bath which was repeated daily for three days. Before stocking, the holding tanks were treated with potassium permanganate ( $\text{KMnO}_4$ ) at 20 ppm for 24 hours and drained and refilled with well water. Fish were acclimatized in the tanks for one week prior to the start of the experiment.

Water temperature was measured twice daily and water quality measurements were taken before the experiment started, and once a week until the end of the study. The experiment was carried out over a period of 30 days.

*Aeromonas hydrophila* was obtained from a natural epizootic in hatchery-reared fingerling *Clarias batrachus*. To determine its pathogenicity, the organism was injected into five fish intraperitoneally, all of which died two days after inoculation. It was then recovered from the mid-kidney region. The identity of the organism was confirmed using standard biochemical methods and subculture of the isolate was suspended in 0.85% saline. The number of bacteria was determined by pour plate colony counting as colony forming unit per ml (cfu/ml).

Forty-two walking catfish fingerlings per tank in each of six treatments were injected intraperitoneally with 0.1 ml of a suspension containing  $10^3$ ,  $10^4$ ,  $10^5$ ,  $10^6$  and  $10^7$  cfu/ml of *Aeromonas hydrophila*. A control group was injected with physiological saline. Susceptibility to experimental infection was expressed as  $\text{LD}_{50}$  calculated by the method of Reed and Muench (1938).

The behavior, clinical signs and appetite of the infected fish were noted daily and three from each group were killed by severing the dorsal aorta at days 2,3,4,7 and 14 post-infection. For evaluation of histological changes, liver, kidney, intestine, pancreas and dorsal musculature were removed and fixed in Bouin's fluid, embedded in paraffin and sectioned at 5-7  $\mu\text{m}$ . Tissue sections were stained with hematoxylin and eosin (see Humason 1972) and the Brown-Brenn modification of Gram's method (see Vacca 1985).

Bacterial reisolation was attempted from the injection site and the mid-kidney. The material was streak plated onto tryptic soy agar (TSA) using a flamed platinum loop. All cultures were incubated at 25°C for 72 hours. Representative colonies were restreaked until pure and maintained on agar slants. Purified isolates were identified by standard biochemical tests using the methods of McDaniel (1979) and Shotts and Bullock (1975).

## Results

### *Gross Pathologic Changes*

By day 3 post-injection (PI), petechiae and reddening of the abdomen were noticed in the fish injected with  $10^7$  cfu/ml of *Aeromonas hydrophila* and similar clinical signs were apparent in all groups the following day. Other signs were lethargy and the sick fish remained near the bottom of the tank or hung beneath the surface of the water.

By days 5 to 7 all fish had decreased appetites and had become sluggish. Fish injected with  $10^3$  and  $10^4$  cfu/ml had depressed appetite until day 14 when it returned to normal. In the other groups the appetite remained depressed and fish were sluggish until the experiment ended on day 21.

By day 14 PI the fish inoculated with  $10^6$  and  $10^7$  cfu/ml were covered with variable-sized pale foci of necrosis, which were accompanied by diffuse congestion of the abdominal skin in all fish.

During the first three days, one to two fish per day died in the  $10^7$  cfu/ml group. On day 14, six fish died and on day 15 eight fish died from this group (Fig. 1) and at the end of the experiment only one fish remained alive.

In the other infected groups, mortalities ranged from three to five fish per group up to the end of the experiment. Control fish died during the experiment due to causes other than *Aeromonas* infection.

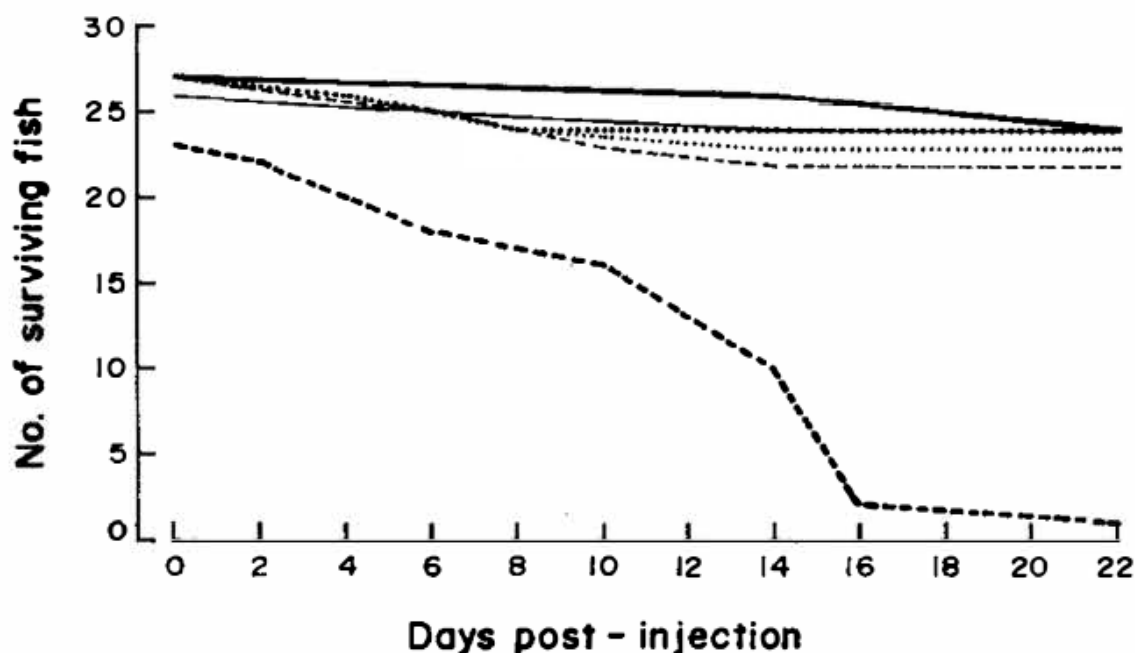


Fig. 1. Number of fish surviving after injection with *Aeromonas hydrophila*. (.....10<sup>3</sup> cfu/ml;.....10<sup>4</sup> cfu/ml;---10<sup>5</sup> cfu/ml;—10<sup>6</sup> cfu/ml;---10<sup>7</sup> cfu/ml;— Control).

From this data, the LD<sub>50</sub> of *Aeromonas hydrophila* to fingerling *Clarias batrachus* was calculated to be 10<sup>6.24</sup> cfu/ml (ranging between 10<sup>6</sup> and 10<sup>7</sup> cfu/ml).

### Bacteriological Findings

*Aeromonas hydrophila* biochemically indistinct from the isolate used to inject the fish was reisolated from 15 of 75 exposed fish but not from the controls (Table 1).

Table 1. *Aeromonas hydrophila* reisolated from the kidney and injection site.

No. bacteria injected	Organ <sup>1</sup> Days post-infection				
	2	3	4	7	14
10 <sup>3</sup> cfu/ml		I (1/3)	K (1/3)		
10 <sup>4</sup> cfu/ml		I (1/3)	K (1/3)		
10 <sup>5</sup> cfu/ml	K (2/3)		I (1/3)		
10 <sup>6</sup> cfu/ml			K (2/3)	I (1/3)	I(1/3)
10 <sup>7</sup> cfu/ml	K (2/3) I (1/3)				

<sup>1</sup> K = kidney; I = injection site; (no. infected/no. examined)

On day 2 bacteria were isolated from five fish, two from the  $10^5$  cfu/ml group with the organism isolated from the kidney and three fish were from  $10^7$  cfu/ml group in which the organism was isolated from the kidney and the injection site. On day 3, it was isolated from the injection site of the  $10^3$  and  $10^4$  cfu/ml injected fish. On day 4, bacteria were isolated from the kidney and the injection site of the  $10^3$ ,  $10^4$  and  $10^6$  cfu/ml groups, and from the injection site, of the  $10^5$  cfu/ml injected fish. At days 7 and 14 they were isolated from the injected area of the  $10^6$  cfu/ml injected fish.

### ***Microscopical Changes***

No gross internal lesions were seen during necropsies. The most prominent histopathological changes occurred in the kidney and liver of all experimental fish. In those injected with  $10^3$  cfu/ml, microscopic features were similar to the control fish, except on days 3 and 4 when slight inflammation occurred in the kidney. At day 3 there were focal tubular degeneration and slight interstitial infiltration consisting of neutrophils, small lymphocytes and a few macrophages. At day 4, dilated blood vessels and slight interstitial edema were detected. However, the degree of inflammation was similar to that observed on day 3.

Changes in the fish injected with  $10^4$  cfu/ml occurred at days 3 to 21 and consisted of focal necrosis in the liver. Multifocal tubular necrosis, moderate interstitial hemorrhage, congestion of the necrotic area and infiltration by neutrophils, macrophages and small lymphocytes were observed in the kidney (Fig. 2). Similar changes were also found in the dorsal musculature (Fig. 3).

For the  $10^5$  cfu/ml group, similar changes in the kidney occurred as for those injected with  $10^4$  cfu/ml. Obvious changes in the liver were slight focal necrosis and vacuolation of the hepatocytes; congestion and hemorrhages were also found from days 4 to 21. There were no prominent changes in the intestine, although hemorrhage and congestion in the submucosal layer were noted.

Similar changes occurred in the  $10^6$  and  $10^7$  cfu/ml groups, focal interstitial necrosis occurring in the kidney, the necrotic area gradually increasing from focal to intensive from day 7 onwards. Inflammatory cells in the necrotic area included lymphocytes, macrophages and a few neutrophils. Hemorrhage and congestion were also encountered.

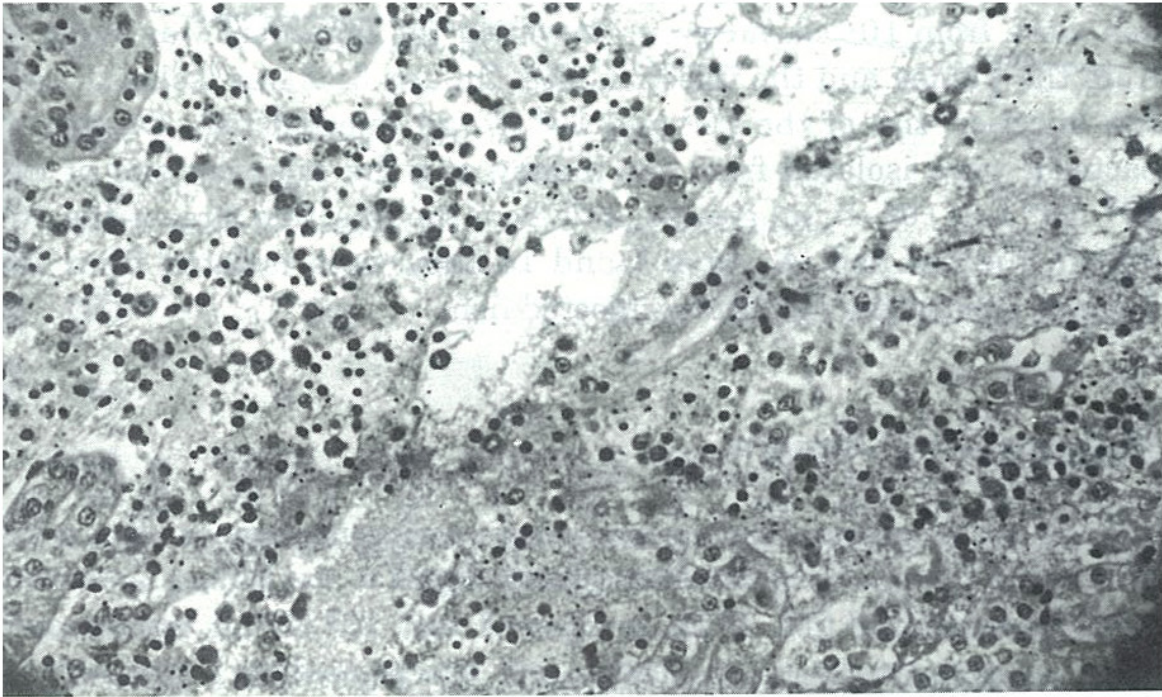


Fig. 2. Necrosis and hemorrhage in kidney. 400X magnification, hematoxylin and eosin.

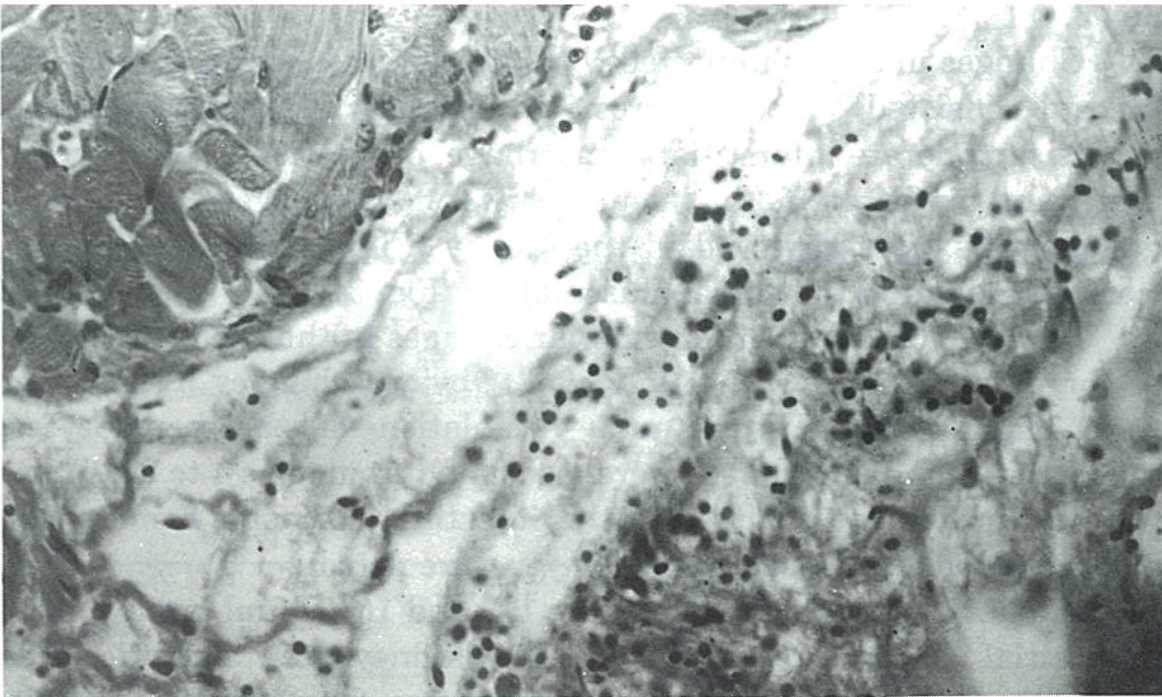


Fig. 3. Early degeneration and edema of dorsal muscle. 400X magnification, hematoxylin and eosin.

Similar changes were also detected in the liver. These included slight focal necrosis, hemorrhages and congestion. Moderate hemorrhage and slight inflammatory infiltration were detected in the submucosal layer of the intestine from day 4 until the end of the experiment (Fig. 4). Lymphoid cells and few neutrophils were found in this area.

One fish from the  $10^6$  cfu/ml group had villous atrophy of the intestine on day 7 which was not found in any other fish.

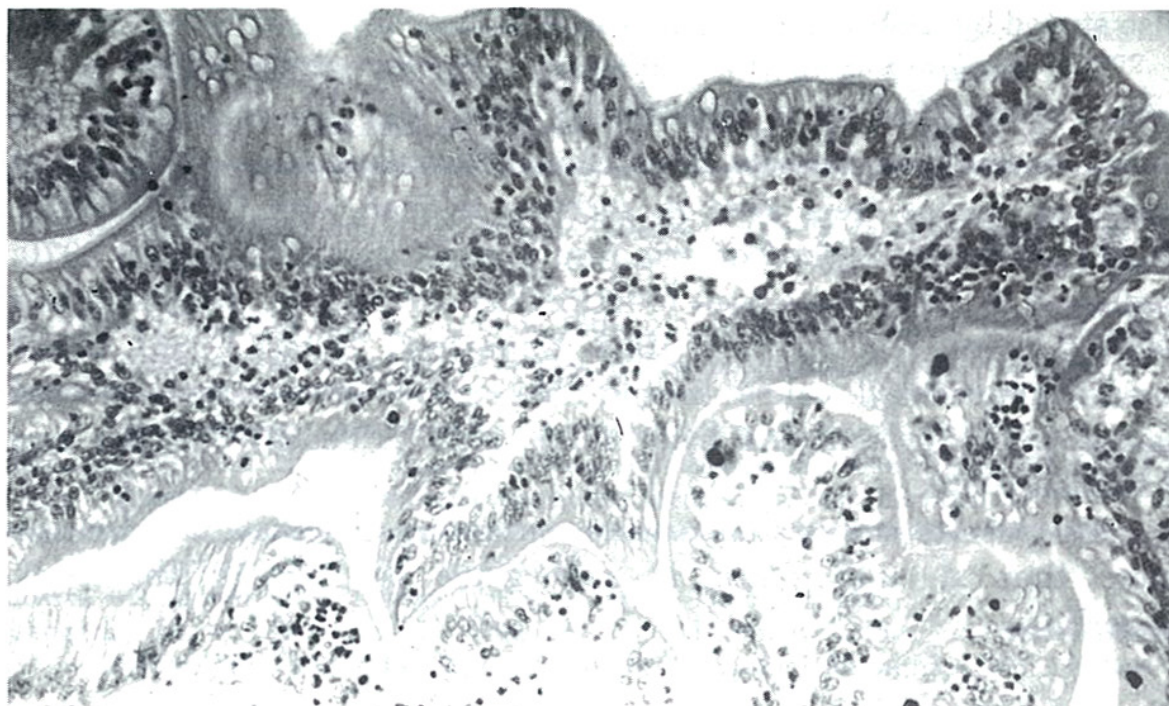


Fig. 4. Edema of intestinal submucosa. 400X magnification, hematoxylin and eosin.

### Discussion

In this study it was found that *Aeromonas hydrophila* injected intraperitoneally is pathogenic to *Clarias batrachus* fingerlings, causing 93% mortality in fish infected with  $10^7$  cfu/ml, with peak mortalities occurring on days 14 and 15. At lower dosage mortalities were significantly lower.

Intraperitoneal inoculation of  $10^3$  cfu/ml of bacteria did not cause significant disease in the fish. Histological examination showed only slight inflammation, focal renal tubular degeneration and mild interstitial infiltration of lymphoid cells. Organ damage and mortalities occurred in fish injected with  $10^4$ ,  $10^5$  and  $10^6$  cfu/ml bacteria, but in some fish recovery took place. From the histological view, it was shown that focal necrosis occurred in the liver and

kidney. Interstitial hemorrhage and inflammatory cells were found in the kidney, dorsal musculature and intestine. All this damage was reflected by loss of appetite and sluggishness of experimental fish, and subsequent mortalities. In fish injected with  $10^7$  cfu/ml fatal damage occurred and histologically, it was seen from day 2 onwards, that there was focal necrosis in the liver, kidney, intestine and dorsal musculature which gradually became extensive by day 7. Histological changes did occur in the injected fish, although *A. hydrophila* could not always be reisolated from the kidney or injection site. This was also stated by Llobrera and Gacutan (1987).

*Aeromonas hydrophila* possesses adhesin which allows it to attach to erythrocytes in fish (Munro 1982), and this may reduce the isolation rate from tissues. Even though tissue damage occurred, no bacterial clumps were seen in sections stained with Gram's stain. Toxins produced by the bacteria (see Bullock and McLaughlin 1970; Santos et al. 1978) have been identified as enterotoxin, proteases and hemolysin (see Laohaviranit 1983; Kanai and Takagi 1986). These toxins apparently cause irreparable systemic damage to the hematopoietic system and liver which leads to death (Brenden and Huizinga 1986).

The results of this study, while containing no toxicological information show that *A. hydrophila* is pathogenic to *Clarias batrachus* fingerlings.

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