Histopathological and Cytological Alterations of Leukocytes in Hybrid Catfish Infected with *Aeromonas* sp. Collected from the Market in Surin Province

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Abstract

The aim of this study was to isolate bacteria from hybrid catfish and then identify by morphological and biochemical characterization. The results indicated that bacteria isolated from hybrid catfish was *Aeromonas* sp. After histological study was performed, the alterations and damages of tissues were found in all organs studied including gill, liver, stomach, intestine and kidney. In gill, the alterations were damaged secondary lamellae. Moreover, vacuole degeneration and necrosis of hepatocytes were observed in the liver tissue. Necrosis of intestinal mucosa was found, and cell lesion occurred in intestine and stomach. Additionally, glomerulus was deformed and necrosis also occurred in the kidney. The results of cytological study in the leukocytes of infected hybrid catfish indicated that the leukocytes consisted of monocyte, and neutrophil which were higher than that of the control. In conclusion, *Aeromonas* sp. could be isolated from infected hybrid catfish. It was found that this bacteria caused the alteration in fish tissue and leukocytes.

Keywords: *Aeromonas* sp.; Hybrid Catfish; Histopathological Alteration; Leukocyte; Surin Province

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Introduction

In Thailand, fishery especially aquaculture in both seawater and freshwater have been extended in response to an increasing need of the population . At first, there were few species being cultured: common carp (*Cyprinus carpio*), snakeskin gourami/sepat siam (*Trichogaster pectoralis*), and sutchi catfish (*Pangasianodon hypophthalmus*). By now, there are at least 15 species of fish and invertebrates being cultured. For freshwater, Nile tilapia (*Oreochromis niloticus*), hybrid catfish (*Clarias gariepinus x C. macrocephalus*), Java barb (*Barbonymus gonionotus*), sepat siam, sutchi catfish, and snakehead murrel are the important species contributing nearly 86 percent in quantity and over 75 percent in value (Boonchuwong, P., 1995). FAO (FAO, 2011) reported that the hybrid catfish is a very important economic fish in Thailand. The domestic annual production was 146,000 tons worth about 300 million Thai baht. It is of higher value compared to the other economic freshwater fish. However, it frequently faces plague caused by pathogenic bacteria especially *Aeromona*s spp. This bacteria causes infection in many cultured fish: minnows, bait fish, carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), striped bass (*Morone saxatilis*), largemouth bass (*Micropterus salmoides*), and tilapia. *Aeromonas hydrophila* is indicated as "the most important bacteria" causing aeromonosis or motile Aeromonas septicemia and as the pathogen of haemorrhagic septicaemia (Austin, B. and Austin, D.A., 1999; Alagappan, K.M. et al., 2009). The production of infected catfish was decreased by nearly 10% every year. An example is motile aeromonas septicemia which is caused by *Aeromonas hydrophila*. In the case of infection in human, it may associate with gastroenteritis and localized wound infection. However, the most serious concern in public health issue focuses on individuals with immune deficiency (Alagappan, K.M. et al., 2009).

The aim of this study was to isolate and study the unique characteristic of pathogenic bacteria in hybrid catfish, then, the negative effect on the tissue and other immune response; type and amount of leukocytes in the infected catfish were also studied.

Methodology

Isolation and Characterization of Bacteria from Hybrid Catfish

Hybrid catfish was collected from the local market and then immediately transferred for culturing at the Department of Fisheries, Faculty of Agriculture and Technology, Rajamagala University of Technology Isan Surin Campus, Surin, Thailand.

The fish was grouped into 2; 1) non-infected (n = 10) and 2) infected group (bleeding and ulcers) (n = 10). Fish having symptom was observed for 24 hrs and bacteria around lesion were isolated by using aseptic technique. The isolated bacteria were cultured in tryptic soy agar (TSA) and then incubated at 37°C for 24 hrs. Single colony was selected and transferred into a new TSA plate for getting the unmixed strain. Bacteria strain was classified by using gram staining technique and biochemical testing; growth on SBA, growth on MCA, bile aesculin hydrolysis, % NaCl, oxidase test, triple sugar iron agar, motility, indole production, arginine dehydrolase, gas from glucose and acid from manitol by conventional microbiology procedure (Table 1).

Histopathological Alterations

After 24 hrs of culture and monitoring, the infected catfish was killed and the target organs: intestine, gills, liver, stomach, and kidney were collected for further histopathological study. The sectioned tissue was fixed in 10% neutral buffered formalin for 24h and then rinsed with tap water. After that, it was dehydrated by using a series of alcohols, then cleared in xylene and finally embedded in paraffin wax. A prepared tissue (6 μm thick) were cut and stained with hematoxylin and eosin (H and E) and then studied under light microscopy (Bancroft, D. et al., 1996).

Cytological Study on the Leukocytes of Hybrid Catfish

Ten hybrid catfishes (120 – 230 g weight and 25 – 20.5 cm length) were selected for cytological study. Caudal vein blood was collected by using syringes with 10% EDTA and then filled with benzocaine (1 g/10 l). For the morphological study, fish blood was smeared and stained with May Grünwald – Giemsa – Wright (MGGW). Next, the smear was fixed in methanol for 10 min and then washed in running distilled water. Then, it was immersed in toluidine blue solution (0.5 g of toluidine blue mixed with 1 g of sodium tetraborate in 100 ml of distilled water) for 1 – 2 min at room temperature. The smear was washed in running distilled water and air – dried (Tavares-Dias, M. and Moraes, F.R., 2006).

Statistical Analysis

The one – way analysis of variance procedure was performed to statistically analyze expression percentage of leukocytes comprising lymphocyte, monocyte and neutrophil in infected hybrid catfish compared to non-infected catfish by using SPSS 15 for window software (significant differences were considered at $p < 0.05$).

Results

1. Isolation and Characterization of Bacteria from Hybrid Catfish

After bacteria from infected hybrid catfish (n = 20) (Figure 1(b)) was collected, isolated, and studied, the results showed that it was round in shape, smooth, short straight rod, and white (Figure 2(a)). In addition, it was a negative gram bacteria and short straight rod (Figure 2(b)). The results of biochemical study indicated that it was *Aeromonas* sp. as shown in Table 1.

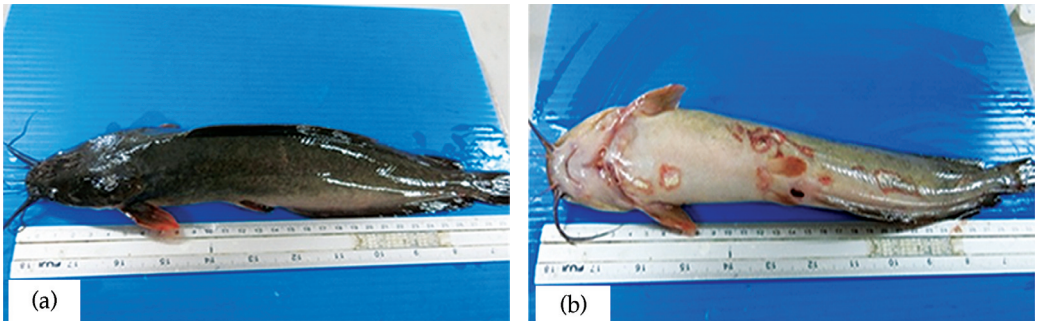

Figure 1 Hybrid catfish; (a) non-infected and (b) infected hybrid catfish (bleeding and ulcers)

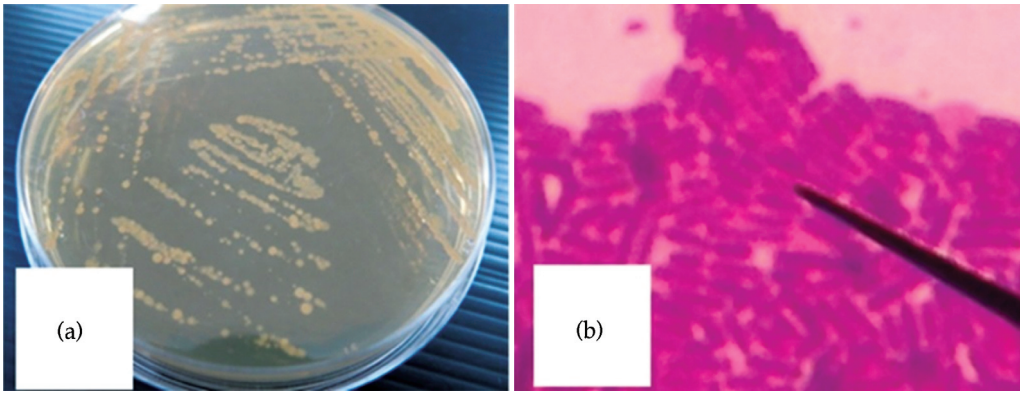

Figure 2 Morphological image of the bacteria isolated from infected hybrid catfish. (a) shows the characteristic of bacteria cultured on agar. Their colonies are round, smooth, and soft white, (b) morphological characteristic of the bacteria isolated from infected hybrid catfish are gram-negative, short straight rod, and stained red dyes of safanin o.

Table 1 Biochemical properties of the bacteria isolated from hybrid catfish

Biochemical Properties	Result
Acid from manitol	Positive
Gas from glucose	Positive
Arginine dehydrolase	Positive
Indole production	Positive
Motility	Positive
Triple sugar ion agar	Alkaline slant/Acid but with gas
Oxidase test	Positive
6% NaCl	Positive
3% NaCl	Positive
0% NaCl	Positive
Growth on MCA	Translucent (yellow)
Growth on SBA	2.5 - 4 mm, β hemolysis

2. Histological Alterations of Hybrid Catfish

After comparing histological alteration of the infected catfish and the non-infected, it was found that gill structure of the non-infected have gill filament, secondary lamellae, and nucleated erythrocyte (Figure 3(a)) while damaged secondary lamellae was found in the infected hybrid catfish (Figure 3(b)). In the liver cell of the infected hybrid catfish, vacuolation of hepatocyte and necrosis of liver cells were found (Figure 4(b)) compared to the non-infected hybrid catfish (Figure 4(a)). Moreover, we found necrosis of intestinal mucosa in the intestine of infected hybrid catfish (Figure 5(b)) but lesion of dead cell was instead found in the non-infected fish. In the non-infected fish, its intestine had four tissue layers (Figure 5(a)), and its stomach had mucous secreting cells with pink - violet coloration (Figure 6(a)). On the other hand, we found lesion of the mucous secreting cell in the infected fish (Figure 6(b)). Finally, glomerulus deformation and necrosis was found in the tubular in the kidney cell of the infected fish (Figure 7(b)) compared to the normal.

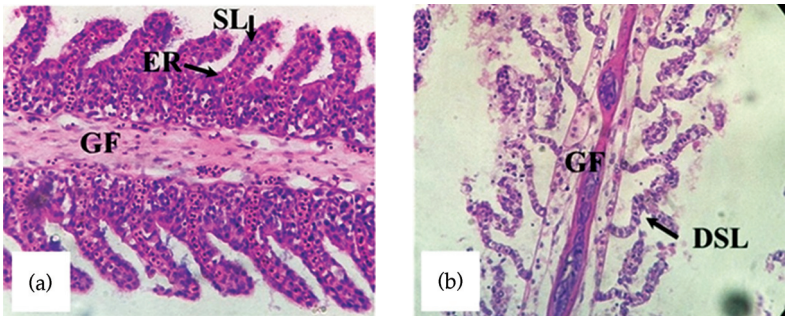

Figure 3 Histological alterations occurred in gill of non-infected (a) and infected hybrid catfish (b). Where GF: gill filament, ER: nucleated erythrocyte, SL: secondary lamellae, DSL: damaged secondary lamellae (Hematoxylin and Eosin, x40)

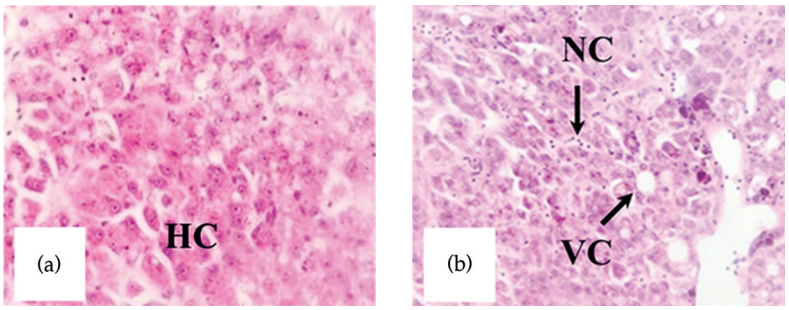

Figure 4 Histological alterations observed in liver of non-infected (a) and infected hybrid catfish (b). Where HC: hepatocyte cell, VC: vacuolation of hepatocyte, NC: necrosis of liver cells. (Hematoxylin and Eosin, x40)

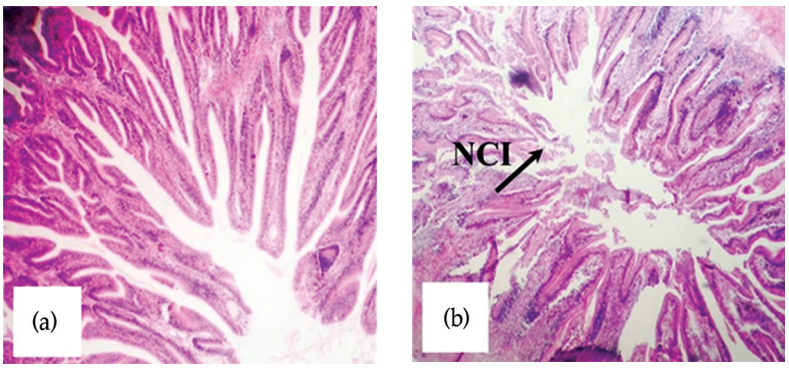

Figure 5 Histological alterations observed in intestine of non-infected (a) and infected hybrid catfish (b). Where NCI: necrosis of intestinal mucosa (Hematoxylin and Eosin, x10)

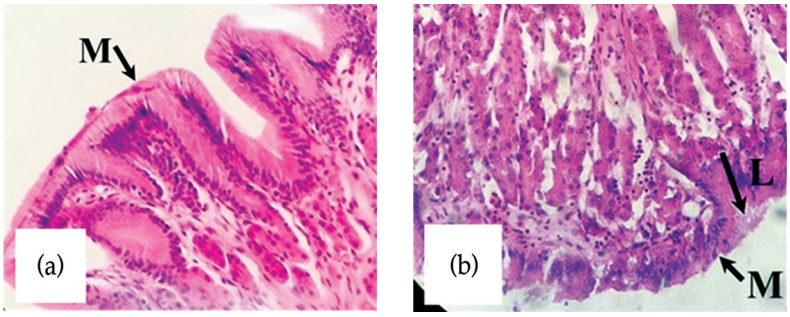

Figure 6 Histological alterations occurred in stomach of non-infected (a) and infected hybrid catfish (b), respectively. Where M: mucous secreting cell, L: lesion of mucous secreting cell. (Hematoxylin and Eosin, x40)

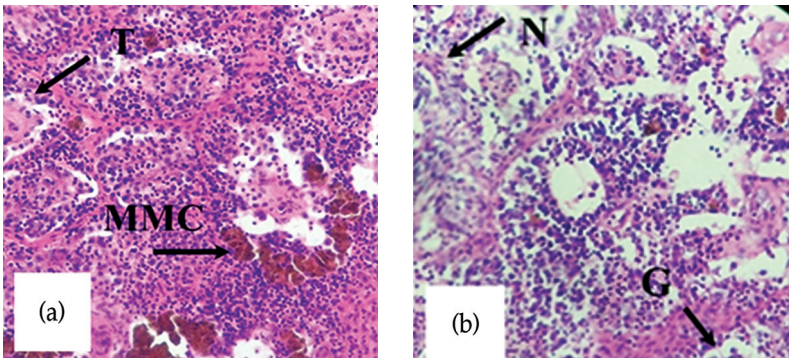

Figure 7 Histological alterations of kidney observed in non-infected (a) and infected hybrid catfish (b). Where T: tubular cell, MMC: melanomacrophage center, N: necrosis cell, G: glomerulus deformed (Hematoxylin and Eosin, x40)

3. Cytological Study on the Leukocytes of Hybrid Catfish

After the study on type and amount of leukocytes in infected hybrid catfish comparing with the non-infected catfish was performed, the results indicated that hybrid catfish infected with *Aeromonas* sp. had all three leukocytes: monocyte (Figure 8(a)), neutrophil (Figure 8(b)) and lymphocyte (Figure 8(c)). In addition, the amount of leukocytes comprising neutrophil and monocyte of infected hybrid catfish was significantly higher than that of the non-infected hybrid catfish ($p < 0.05$). For each type of leukocyte, the expression percentage of lymphocyte in the non-infected was 95±1.5% while it was 88±1.8% in the infected. The expression percentage of neutrophil in the infected was 15±1.8% while it was 4±1.5% in the non-infected. Finally, the expression percentage of monocytes of the infected was 7±0.8% while in was 1±0.4% in the non-infected (Figure 9).

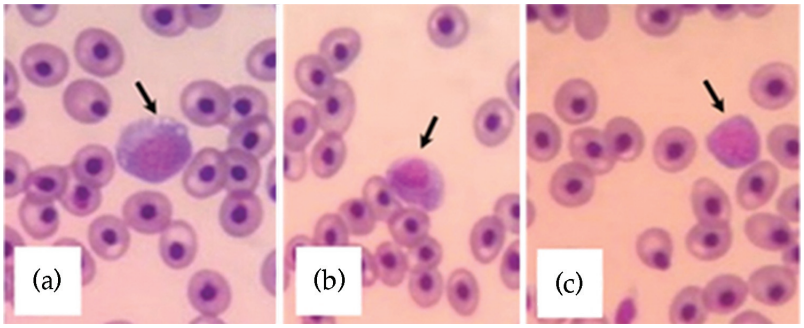

Figure 8 Three leukocytes; monocyte (a), neutrophil (b) and lymphocyte (c) in *Aeromonas* sp. infected in hybrid catfish

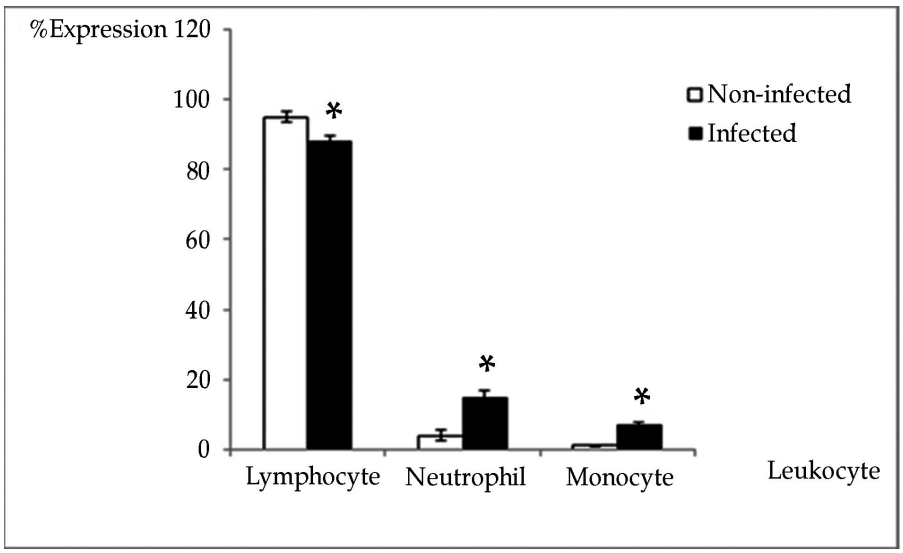

Figure 9 Expression percentages of leukocyte comprising lymphocyte, neutrophil and monocyte from hybrid catfish infected *Aeromonas* sp. compared with non-infected hybrid catfish.
Remark: * significantly different to control group ($p < 0.05$)

Discussions

The culture of hybrid catfish frequently faces many problems such as pathogenic bacteria especially *Aeromonas* sp. Aguilera-Arreola, M.G. et al. (Aguilera-Arreola, M.G. et al., 2005) reported that *Aeromonas hydrophila* was classified as negative-gram bacteria having short straight rod shape. Its colony was round, smooth, and white coloration which is to that observed in this study. The results of biochemical study indicated that it was *Aeromonas* sp. having the fundamental characters; growth on SBA, growth on MCA, bile aesculin

hydrolysis, % NaCl, oxidase production, triple sugar iron agar, motility, indole production, arginine dehydrolase production, gas produced from glucose and acid from manitol (Table 1). Our results is in agreement with the study of Awan, M.B. et al. (Awan, M.B. et al., 2005) who studied the infection of *Aeromonas hydrophila* in Tilapia. *Aeromonas* sp. could cause infection in many fish specie such as rainbow trout (*Oncorhynchus mykiss*) (Řehulka, J., 2002), estuarian catfish (Alagappan, K.M. et al., 2009) and *Oreochromis niloticus* (Noor El *Deen, A.I.* et al., 2014). Aguilera-Arreota, M.G. et al. (Aguilera-Arreota, M.G. et al., 2005) also reported that *Aeromonas hydrophila* was an opportunistic infection causing tremendous damage to fisheries around the world (Austin, B. and Austin, D.A., 1999). This bacterium could cause disease in catfish, called motile aeromonas disease. The symptoms were lesions on the body and hemorrhage. The infected fish shows severe acute symptom; abdominal swelling and lesion on the body. In this study, we found lesion on the fish and it continuously expanded. In order to study on the impact the catfish infected with *Aeromonas* sp., histological alterations was monitored and examined and the immune response system was also studied by identifying and counting leukocytes in infected catfish.

The organs examined in this study were gill, liver, intestine, stomach, and kidney. The role of teleost gills is directly linked with the respiratory system and the stability of the body (Genten, F. et al., 2009). Because gill is important, histological alteration in gills of infected fish, it can be used as bio – indicator for toxic chemicals exposure and water pollution. In this study, we found that *Aeromonas* sp. could damage secondary lamellae in the fish. Alagappan, K.M. et al. (Alagappan, K.M. et al., 2009) studied estuarian catfish (Alagappan, K.M. et al., 2009); The researcher infected *Aeromonas hydrophila* and found damaged secondary lamellae. After 24 hrs of infection, its filament was completly detached and dissociated from the secondary lamellae.

In addition, liver is another important organ playing role in assimilation of nutrient, production of bile, maintaining the body metabolic homeostasis, and detoxification (Genten, F. et al., 2009). After exposing to heavy metals, the fish liver was induced to function in reducing the toxicity. However, Noor El Deen, A.I. et al. (Noor El Deen, A.I. et al., 2014) found cell necrosis in the liver of *Oreochromis niloticus* after infected with *Aeromonas hydrophila*. In addition, there was vacuolation of hepatocytes in the liver of infected as well as observed in this study (Alagappan, K.M. et al., 2009).

The alteration of intestine and stomach occurred after the catfish was infected with *Aeromonas* sp. In this study, we found the congestion of submucosa, necrosis of intestinal mucosa, and cell lesion which is in agreement with the study of Noor El Deen, A.I. et al. (Noor El Deen, A.I. et al., 2014) who found the alteration in tilapia infected with *Aeromonas hydrophila*. The kidney of exposed fish plays an important role in excretory and

osmoregulatory mechanism (Genten, F. et al., 2009). In 2014, Alagappan, K.M. et al. (Alagappan, K.M. et al., 2009) found congestion of interstitial blood vessels and renal glomerular, focal interstitial hemorrhage with necrobiotic changes of the surrounding renal tubular epithelium, focal interstitial infiltration of mononuclear cells with degeneration of the surrounding renal tubular epithelium in the tissue sectioned from kidney of fish estuarian catfish infected *Aeromonas hydrophila*. For this studies, only glomerulus deformed and necrosis tubular was found.

As generally known, the leukocytes such as monocyte can destroy xenobiotic phagocytic ability and amoeboid movement or phagocytosis by neutrophil (Tripathi, A. 2014). Thus, we also studied the changes in type and amount of leukocytes comprising neutrophil, monocyte and lymphocyte occurred in infected fish. It was found that the amount of leukocytes comprising neutrophil and monocyte in infected fish was higher than that in non-infected fish; in contrast, lymphocyte was lower than non-infected fish. Thus, the amount of leukocytes can be used for monitoring *Aeromonas* sp. infection. Arnold, J.E. (Arnold, J.E., 2009) reported that the lymphocytes in infected fish were small round having a high N : C ratio and showed a rim of smooth light blue cytoplasm around the large oval condensed nucleus. The lymphocytes in some infected fish, especially elasmobranch species, frequently release out of cell membrane. Some studies separated its size as small and large while its functional difference requires more study. The morphology of fish monocytes is like those in mammals, birds, and reptiles. It is large and round cells with abundant blue cytoplasm containing in vacuoles. The nucleus is round and oval with loosely compact chromatin, and the neutrophils are large round cells having abundant clear cytoplasm, an eccentric and condensed nucleus (round or multi – lobed). The role of these two cells in fish also requires more study. Neutrophil in elasmobranchs often is large cell having colorless cytoplasm (containing round or multi-lobed condensed nucleus).

Conclusions

In this study, the bacteria that caused infection in hybrid catfish collected from local market in Surin Province was *Aeromonas* sp. The symptom was bleeding and ulcers. Alteration was found in the important organs: gill, liver, intestine, stomach, and kidney. The dominant alteration was damage of cell and necrosis. For immune response, *Aeromonas* spp. raises the amount of leukocytes, lymphocyte, momnocyte, and neutrophil in the infected catfish. Thus, we can monitor and identify the infection of *Aeromonas* sp. by histopathological examination and cytological study on the leukocytes.

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