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A model of rat's stomach orally infected by live *Anisakis typica* larvae : A histopatological study

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Abstract: This study was aimed at knowing the ability of *A typica* to damage the stomach tissues. Method used in the study was an observational design. Rats were divided into risk group and non-risk group (control). The former was orally infected with stadium-3 (L3) larvae of *A typica* and the latter was not infected. After infection of 2-8 hours, observations were carried out on both groups for larva occurrence in the stomach. The stomach tissues, where larvae were found, were preserved for histopathological analysis using Hematocylineosin (HE) staining. Stomach tissue defect analysis used 4 histopatholical lesions: epithelial exfoliation, hyperemia / hemorrhage, inflammation and amount of mucous cap cells. The histopathological analysis showed that there was no inflammation in control group but there were moderate to severe inflammation in the risk one. Acute inflammation is indicated with mucosal erosion and ulceration and the presence of polymorphonuclear cells (PMN) in the risk group.
Keywords: Histopathology, *A typica*, stomach, rat.

Introduction

Anisakid nematodes are known to be a zoonotic parasite that infects fisheries products[1,2] and cause anisakiasis. Anisakiasis (anisakidosis) refers to human infection by nematode larvae of family Anisakidae or Raphiscarididae[2,3]. Transmission to human is related to human tradition of consuming raw fish or medium cooked fish in several countries[4,5].

There are two major factors of risks resulting from larvae swallowing: first, accidental consumption of raw or half-cooked fish can cause live larvae infect stomach and intestine[5]. Second, dead nematode larvae could still bring about allergic reactions[2,6,7,8]. Eating up live larvae could result in acute and chronic stomach infection[9] and acute intestine infection as well[10].

Research development relating with serology and allergy in human and test animals has much been conducted. However, histopathological studies are mostly obtained from human cases, but less from test animals. Initiated of the histopathological studies on the test animal as model development for human. These were just done using *Anisakis simplex*, *Anisakis pegreffii* and *Anisakis* sp.[11,12,13] while the anisakis has approximately 9 species[14,15,16], and there is no report on the other 7 species. This study was aimed at understanding *A typica* infection using a histopathological study. It will add the histopathological evidences of *Anasakis* sp. infection using wistar rats.

Materials and Method

Collection of larvae

L3 *A typica* was collected from skipjack tuna (*Katsuwonus pelamis*) in traditiional market of Kupang Municipality. *K pelamis* was caught in Savu Sea, East Nusa Tenggara, Indonesia, using pole and Line. The nematode-infected fish were held in an intact form in a coolbox up to use in order to maintain the fish freshness so that the larvae remained healthy and alive.

Before use, the fish were dissected to gain the anisakid larvae dispersing in the flesh and stomach content. The larvae were then repeatedly washed in 0.9% NaCl solution up to clean that they were easily taken using feeding needle. Active-moving larvae were selected and collected in 0.9% NaCl-containing glass jar to be orally infected into the rat.

Ethical Clearing

Test animals were rats, *Rattus norvegicus* L, 150-250 grams, wistar strain obtained from the Integrated Research and Examination Laboratory of Gadjah Mada University, Jogjakarta, No/262/LP3HP/15/X/2012. The ethical clearing was tested by the Research Ethics Committee of Brawijya University validated through an Ethical Clearence Numbered 268-KEP-UB.

Experimental Method

This study used an observational design on two groups of rats, comprising the risk group and control group. The observation was done after the former group had been infected. Each rat of the risk group was orally given 10 individuals of selected anisakid larvae using a 7 cm-feeding needle. The observations were carried out after 2, 3, 4, 5, 6, 7, and 8 hours of infection preceeded with euthanasia and necropsy. Then, stomach was separated from other parts of digestive tract, dissected, and observed the larva presence. The stomach tissues suffering from larva invasion/penetration were then preserved in 10% formaldehyde for histopathological analysis using HE staining[17]. The stomach histopathological observation was focused on the relationship between post-infection time (risk factor) and defect level (effect).

Histopathological Analysis

Examination method used a stomach defect level scoring following modified method[18], in which the defect level of each sample was determined by totalling entire scores of 4 types of the histopathological lesions. The scores ranged from 0-13 with the following interpretation: (1) no inflammation appears (total score of 1-2), (2). Light inflammation (total score of 3-4), (3) moderate inflammation (total score of 5-8), and (4). Severe inflammation (total score of 9-13).

Data analysis was done descriptively through comparison between the histopathological image of exposed stomach part of the risk group and the same stomach part of the control group. The stomach image was obtained using common Nikon H600L light microscope facilitated with a 300 megapyxels-DS Fi2 300 digital camera and image processing software of Nikkon Image System.

Results and Discussion

Observation of larvae in the gastric tract

Larva movement in the gastric tract shows that 2-3 hours of post-infection, the larvae are in the stomach (50% in 2 hours, 10% in 3 hours and **jejenum** (50% in 2 hours, 90% in 3 hours). Four to six hours later, beside stomach (50% in 4 hours, 30% in 5 hours, 10% in 6 hours) and **jejenum** (30% in 4 hours, 40% in 5 hours, 50% in 6 hours), the larvae were also found in the ileum (10% in 5 hours, 20% in 6 hours). Seven to eight hours later no larva was found in the stomach and **jejenum**, but in the ileum (70% in 7 hours, 90% in 8 hours) and colon (20% in 7 hours, 60% in 9 hours). Four individuals of larvae, 2% (4/70), were recorded in the process of penetrating the stomach wall 4, 5 and 6 hours after infection.

Larva observations in the digestive tract revealed that (1) the penetration occurred on the stomach wall, but not on the other parts of the digestive tract. (2) Invasion of the larvae into the stomach wall occurred in 4, 5 and 6 hours after infection. (3) No larva was encountered penetrating other gastric tract parts. Penetration into

the stomach and intestine was very common from anisakidosis. Anisakiasis infection is in association with invasion into the stomach and intestine[10].

Invasion process occurs in the way that larvae embed the head into the stomach surface. When the head part penetrates the mucosal layer, the larva moves its body and tail to support the head penetrating deeper into the mucose. Sometimes the larva pushes its tail edge on the stomach to move its body and head parts. Fig. 1 a,b,c is the process where the head part has entered the mucosal layer of the stomach, while the body and tail parts are moving freely to help invasion process.

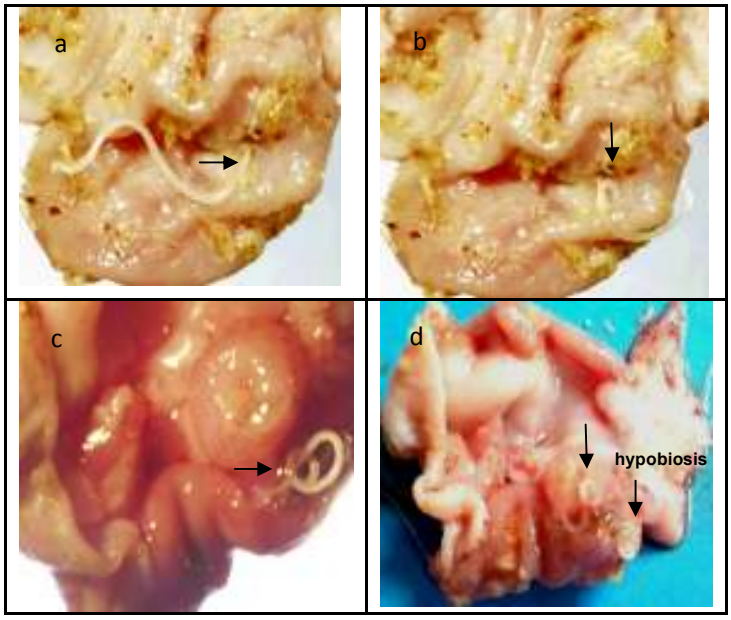

Figure 1. Larva invasion in the stomach. a, b, c: penetrating process into stomach mucosal layer. d: penetration followed with physiological adaptation (hypobiosis).

Fig. 1d is the process when the head part has entered the stomach mucosal layer, but the body and tail parts do not freely move and make a circle. In this position, the larvae are still alive but do not respond when touched. This is a form of physiological adaptation of the anabiosis atau hypobiosis[5,19,20]. The type of adaptation is similar to that in *K pelamis* as intermediate host. However, in this study the larvae have not encapsulated yet, while those in the intermediate host have encapsulated. This process is the "critical point" since the larvae can conduct the physiological adaptation. Parasitic worms can develop some mechanims to prevent and avoid the immune response of the host to maintain their survivorship[5]. When the adaptation succeeds, there will be an opportunity for the larvae to be longer in the host body followed with some possible illness risks, such as erosive lesion (4 hours-6 days after consumption), ulcerous lesion (7-14 days after consumption) and > 14 days, chronic ulceration[5].

Stomach defect level of the rat infected by live *A typica*

Defect level of the stomach mucose comprises 4 histopathological lesions, epithelial damage, hyperemia/haemorrhage, inflammation and number of mocous cap cells[18]. Histopathological observations on the rat's stomach are described as follows:

a. Epithelial defects

Stomach mucose consists of epithelial layer, lamina propia and muscularis mucosa. Epithelium is cellular layer covering the stomach surface. This layer in the mucose or inner part of the gastrointestinal (GI) tract and directly connected to the GI content[21,22]. Epithelial damages could be epithelial exfoliation, erosion, and ulceration. The image of epithelium is presented in Fig. 2.

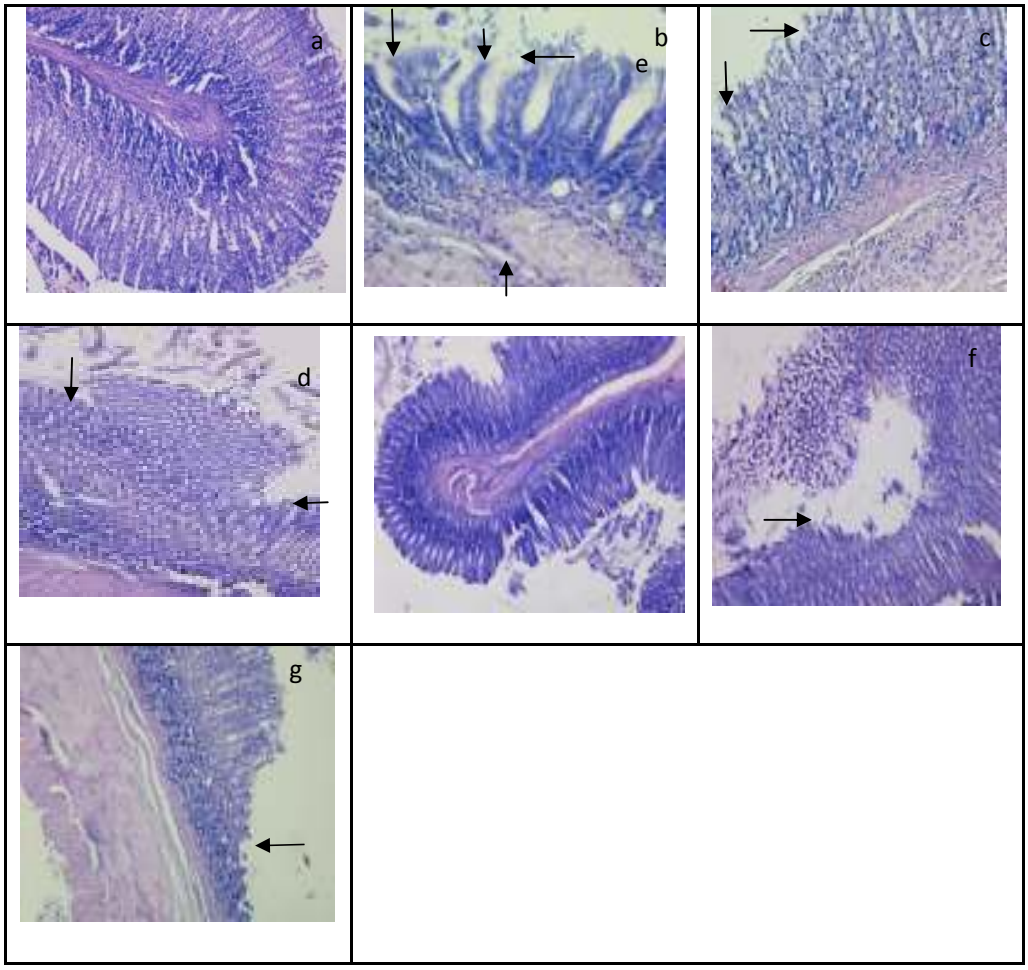

Figure 2. Histological images of stomach mucosa, epithelial damages. a: Normal structures, b and c: *Superficial erosion*, d, e and f: *deep erosion,* g: *acute ulceration*, (HE staining. 100x enlargement; Nikon H600L microscope; 300 megapyxels- DS Fi2 camera).

Stomach erosion is defined as a shallow injury or exfoliation of mucosal layer that does not penetrate the muscularis mucosa layer. Erosion of epithelial layer comprises superficial erosion and deep erosion, while that of lamina propia layer is *acute ulcer*. Erosion of muscularis mucosa layer is called chronic ulcer[23].

The rats of risk group suffer from mucosal destruction, such as epithelial damages, erosion, and ulceration, but no defect was recorded in the rats of control group. The histopathological lesion score of the epithelial defect in the rat control group was 0. The control group shows normal tissue structure of epithelial layer, lamina propia and muscularis mucosa (Figure 2: a). The epithelial damages of the risk group (Figure 2: b,c,d,e,f) cover epithelial exfoliation and erosion with number of cell necroses between 1-10. According to Toljamo[23] exfoliation occurs on the surface of epithelium (Figure 2: b,c) and beneath the epithelium (Figure 2: d,e,f). The epithelial exfoliation could reach lamina propia layer (Figure 2: g).

Romero[13], in their study, used live L3, *A simplex* and *A pregefii* on rats and found 16.3% (31/190) causing lesion on the digestive tract. In detail, 87.1% (27/31) causes lesion on the gut wall and antrum pilorus, and the rest 12.9% (4/31) in small intestine. The lesion size varies from <1 to 24 mm^2, comprising ulcus hermorrhage followed with edema and the larvae left scars the gut wall.

b. Hyperemia ot haemorrhage

Hyperemia is blood excess in certain body part or bleeding indicated with haemorrhage[21]. Histological imagess of tissues from *A typica* infection are given in Fig. 3.

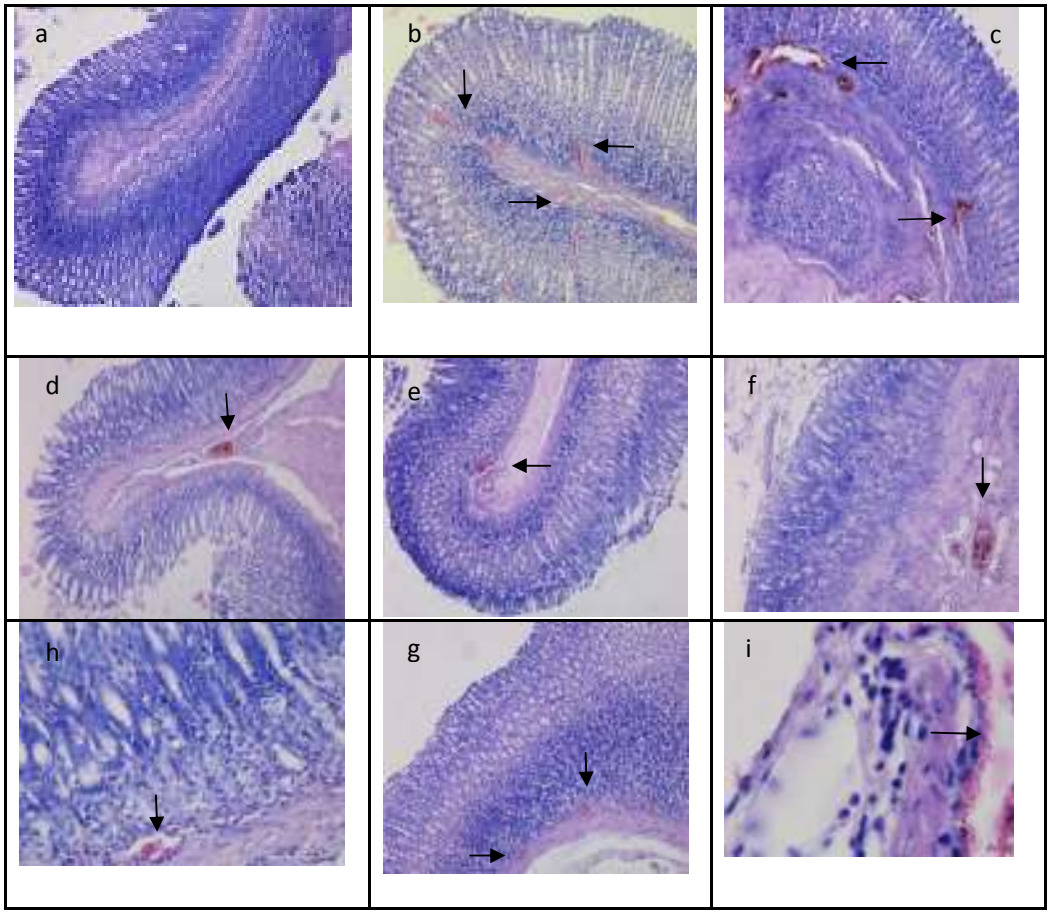

Figure 3. Histological images of hyperemia/haemorrhage. a: Normal structures, b,c,d,e,f,g,h: Bleeding on submucosal layer of the stomach (*hyperemia*) shown by the dilation of vascular blood vessel (*haemorrhage*). (HE Staining. 100x enlargement; Nikon H600L microscope; 300 megapyxels-DS Fi2 camera).

Figure 3 shows that the tissue hyperemia and haemorrhage of the control group occurred in less than 1/3 number of vascular vessels (Fig. 3: a), while the risk group sufferred from bleeding followed with vascular blood vessel expansion in the submucosal layer less than <1/3 and between 1/3 – 2/3 vascular vessels (Fig. 3: b,c,d.f.g.h.i). In spite of that, observations on several risk groups (6,7,8 hours after infection) show similar image to the control group, but it cannot be described yet in this study.

Bouree *et al*[10] who collected diagnostic reports on acute anisakidosis patients described the occurrence of hyperemia in stomach mucose and adhesion on mucose and submucose. Acute stomach anisakidosis was very active in penetration effort at the early stage and then getting weaker. Histological explanation of the acute phase is indicated with intact and obvious image of the larvae on the submucosal layer, stomach wall thickenning, edema and a great number of eosinophyls mixed with neutrophyls, macrophages and limphocyt infiltration.

c. Inflammation

Inflammation is a protective response of the tissue to the injury or damage that works to break or dissolve or hold the agent causing tissue damage**.** Inflammation is classified based on lesion duration and histological image.

Acute inflammation is an inflammation occurring in relatively short time, from few minutes, several hours to several days, indicated with pain, fever, red, and expansion. Chronic inflammation is an inflammation felt in a long period of time, particularly indicated with new connective tissue formation[21,24]. The histological image of the tissue inflammation is given in Fig. 4.

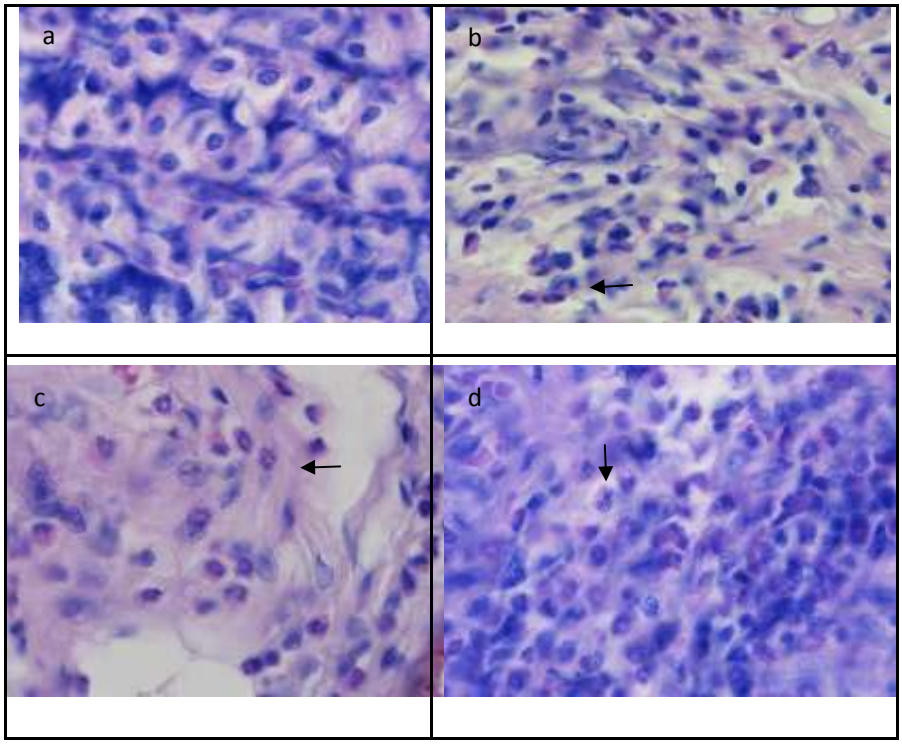

Figure 4. Histological images of inflammation, a: Normal structures, b,c,d: Infiltration of Polymorphonuclear (PMN) inflammation cells (arrows) on submucosal and mucosal layers. (HE Staining. 1000x Enlargement; Nikon H600L microscope; 300 megapyxels-DS Fi2 camera).

PMN or white blood cells are the first line of body immunal defense against disease. They are cells responsible for the damage of inflammatory tissue occurring during the acute infection[25]. Inflammatory image in control group is shown by number of PMN cells between 6-20 cells/field (Figure 4:a). In risk group (2,4,5,6,7 hours of post-infection), the number of PMN cells was between 61-100 cells/fields and >100 cells/fields with infiltration up to lamina propia (Figure 4: b,c,d), except in 8 hours of post-infection, it is the same as the control group.

Acute inflammatory reaction quickly activate the PMN cells and plasmic protein to the injured sites to eliminate the infection causing agent. Acute inflammation has two major components, **vascular and cellular alterations**. Changes inside the blood vessels cause increase in the blood flow (vasodilatation) and blood vessel wall alterations which enable the plasmic protein to leave the circulation. It could result from increase in blood vessel permeability. On the other hand, the adhesion and migration of leococyt through blood vessel wall occurs due to active **endothel** cells. This cellular event aims to diminish the inflammation causing agent[25].

The occurrence of PMN cells in the risk group indicates an acute inflammation. The symptom appears after 4-6 hours of consumption. From clinical point of view, the digestive tract infection is classified as acute and moderate conditions. Based upon its site, this disease is divided into stomach anisakidosis/anisakiasis and intestinal anisakidosis/anisakiasis[26,27]. The acute infection of live L3 *Anisakis* spp in rats was reported by Zuloaga *et al*[12] that live larvae were found in the stomach mucose (particularly in fundus part) and passed the gut wall after 20 hours of infection. *Anisakis* spp causes acute inflammation on the submucosal layer and its surroundings dominated by eosinophylic PMN cells and light inflammation.

The main cause of inflammation as purely caused by mechanical stress, including trauma from blunt object, any infection (bacteria, virus, fungi, parasite), and vibration[24,25,28]. In this case, the inflammation of stomach mucosal layer resulted from mechanical movement of *A typica*. The mechanical movement of Anisakis spp was also reported to bring about acute inflammation in wistar rats [12].

d. Mocous cap cells

Mucous cap cell is gland-containing mucus membrane layer covering the stomach surface, whose surface is fine and soft like velvet. It consists of epithelium, lamina propria, and muscularis mucosa[21]. Histological image of mucous cap cell is presented in Fig. 5.

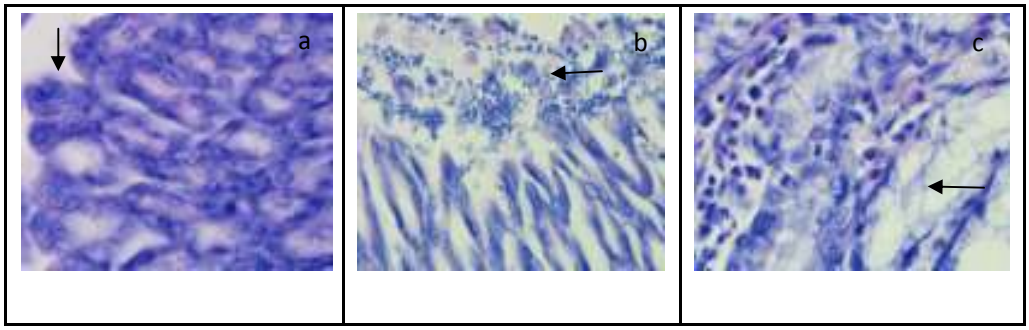

Figure 5. Histological images of mucous cap cells, a: Normal stomach epithel with image of *mucous cap cells* (arrows) close to more than 40 cells/viewing field, and b and c show that number of the cells declines from exfoliation (arrows). (*HE staining. 1000x enlargement; Nikon H600L microscope; 300 megapyxels-DS Fi2 camera*).

Rats of control group (Fig. 5:a) show stomach epithelium with number of mucous cap cells >40 cells/field, and similar condition is also demonstrated by the risk group (4,5,7 hours after infection). However, in other risk groups (2,3,7,8 hours after infection), number of mucous cap cells decreased between 24-39 cells/field and 12-23 cells/field (Fig. 5: b,c,d). Decline in cell numbers is related with the exfoliation of stomach epithelial layer.

Stomach infection from *A typica* starts when the larvae are swollen indeliberately in live condition. Reaching the stomach, the larvae attempt to do physiological adaptation. Before succeeding into hypobiotic phase, the larvae move a lot and try to penetrate the surface of the stomach mucosal layer. This mechanic movement of the larvae causes irritation on the stomach mucosal layer and results in tissue inflammation and expansion. Erosion also brings about reduction of number of mucous cap cells.

Referring to the histopathological lesion scoring of Bathel *et al*[18] total score of the control group lies between category 1-2, no inflammation appears, but that of the risk group at the observation 3,4,5,6,7,8 hours after infection ranges from category 5-8, moderate inflammation, and at the observation after 2 hours of infection it was in category 9-13, severe inflammation**.**

Estimates of prevalence and biological characterization of live L3 *A typica* danger

In general, *A typica* in this study posseses similar body shape and part to *Anisakis simplex*[29], particularly digestive tract, but both larvae are different in their size. Total body length of *A typica* ranged from 7.27 to 20.27 (12.07±6.85)[30] smaller than that of *A simplex*, 19.73~28.41(23.62±1.87)[31]. Different size also appears in other morphological characters.

Nematode life cycle requires a final host and one or two intermediate hosts. In this case, *K pelamis* is an intermediate host. Some nematodes use human as their final host when people consume infected fish and get infected. Study on test animals could help assessing the hazard potential of zoonoses. Hence, in relation with food security, the most important is to watch the larva distribution in the body part of the fish, particularly the edible one, flesh. Soewarlan *et al*[30] found that mean intensity of *A typica* infection in fish was 48.40±32.12 (4~123) to symbiosis 82.62±70.88(31~321). The highest distribution of *A typica* was recorded in the stomach content, 95.14% to 96.97% and the lowest in the flesh, 0.15% to 0.41%. In contrast, the prevalence of *A simplex* in the muscle reached 98%[31], and this becomes one of the factors making this spesies the most pathogenic.

Based on their distribution in the flesh, the presence of *A typica* is much lower than *A simplex*. This distribution is related with the opportunity to transmit when consumed. It means that the opportunity of *A typica* to distribute through food chain into human is lower than *A simplex*. Nevertheless, the pathogenity is not

determined by the extent of prevalence, but physiological adaptability and individual migration of the larvae in the host. When they succeed to adapt, there will be opportunity to get acute and chronic infection.

Prevalence, intensity and physiological adaptation are presumed to influence the pathogenic potential of *A typica* when they exist in the host (transmitted to the test animal or human). Studies of Suzuki *et al*[32] and Quiazon[33] found that *A simplex* ss had higher tendency than *A pegreffii* to migrate to the muscle parts, and their ability to penetrate the muscular tissue of the fish will affect their pathogenic potential. According to Soewarlan *et al*[30], the ability of *A typica* to penetrate the muscular tissue of the intermediate host is less than 0.5% compared with that of *A simplex,* 98%[31], meaning that *A typica* is capable of causing disease in human as accidental host with relatively lower risk, but hazard potential remains possible.

Conclusion

This study is 'the first report on histopathological study infected by *A typica* using test animals". The present study reflects that oral infection of live L3 *A. typica* for 2-8 hours could cause acute infection with moderate to severe rat stomach inflammation. In this case, the live L3 *A typica* can result in stomach anisakiasis in rat.

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