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NOTE

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Dynamics of liver enzymes in rabbits experimentally infected with *Fasciola* sp. (Intermediate form from Japan)

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ABSTRACT. Dynamics of serum liver enzymes in rabbits experimentally infected with metacercariae of *Fasciola* sp. (intermediate form between *Fasciola hepatica* and *F. gigantica*) were monitored. Gradual increase of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were observed from 3 weeks post-inoculation (WPI) and peaked at 6 WPI, which corresponded well to the period of migration and development of juvenile fluke in the liver parenchyma and the time when the young adult flukes migrated to the bile duct. However, no significant increase in serum gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) were observed. This could reflect reduced or minimal injury of bile ducts and biliary epithelia as the flukes had reached the adult stage. Alpha- fetoprotein (AFP) and carcinoembryonic antigen (CEA) were not detected in the infected rabbit during the course of the experiment. Serum liver enzymes monitoring might be useful for understanding the host-parasite relationship in fascioliasis.

KEY WORDS: *Fasciola* sp. intermediate form, fascioliasis, liver enzymes, rabbit

Fascioliasis is a hepatic parasitic infection in many mammalian species. The pathogenic effect on the definitive host begins with the ingestion of metacercariae, which become excysted and released the juvenile fluke in the intestinal lumen. The juvenile flukes then migrate through the intestinal wall into the peritoneal cavity and penetrate into the liver through the liver capsule. They then migrate through the liver parenchyma to find their way to the bile ducts and develop into adult flukes [11, 25]. Pathological changes mainly caused by migration of the juvenile flukes affect the complex vascular and biliary system in the liver. The complexity of vascular and biliary system examination has led to the use of a number of different laboratory tests based on biochemical analysis of serum parameters. These include the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) [7, 9, 29]. Two aminotransferase enzymes (ALT and AST) are localized mainly in the cytoplasm of hepatocytes and recognized as a marker of hepatocellular injury in rats, dogs and non-human primates [3, 8]. ALP and GGT are the two most commonly used enzymatic marker of cholestasis in the clinical practice of small animal [8]. The highest ALP activity is found on the border membranes of the bile duct whereas GGT is found in the hepatocytes and biliary epithelial cell [8, 12]. Increased level of these enzymes is an indicator of hepatobiliary injury, epithelial damage and stasis of the bile duct [8, 17, 27]. There are many previous studies on serum liver enzymes of sheep, cattle and monkey infected with *F. hepatica* and *F. gigantica,* but few on dynamics of serum liver enzymes in rabbit fascioliasis [5–7, 9, 17, 24, 25, 29]. Shoriki *et al* [18] had reported the existence of an intermediate form of *Fasciola* in Japan, which is characterized as aspermic, and having genotypic characters of both *F. hepatica* and *F. gigantica*. The clinical symptoms and enzyme profiles of the rabbit host infected by this *Fasciola* type has not yet been reported. The objectives of this study are to monitor the change of serum liver enzymes that characterize liver damage caused by *Fasciola* sp. (intermediate form) in rabbits during the invasive acute phase and to correlate the kinetics of these enzymes as a reference for interpretation of liver biochemical profiles in animal fascioliasis, especially during the migration of the larva to the bile duct.

The *Fasciola* fluke used in our study has been identified as *Fasciola* sp. (intermediate form between *F. hepatica* and *F. gigantica*) following the PCR- RFLP and multiplex PCR method designated intermediate form described by Shoriki *et al.* [18]. Metacercariae of *Fasciola* sp. were collected from laboratory-bred and artificially infected snail by encystation on the wall of the polyethylene bag of emerged cercariae following the protocol described by Taira *et al.* [23]. Briefly, infected lymnaeid snails

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Table 1. Eggs per gram of feces (EPG) of rabbits experimentally inoculated with metacercariae of *Fasciola* sp. (intermediate form)

WPI [a]	EPG		
	Rabbit No.1 Inoculated 20 MC [b]	Rabbit No.2 Inoculated 20 MC	Rabbit No.3 Inoculated 12 MC
6	0	0	0
7	0	0	0
8	22	8	0
9	108	103	0
10	138	222	0 [c]
11	219	171	-
12	190	214	-
13	139	290	-
14	284	317	-
15	199	322	-
16	136	161	-
17	98	121	-

a) Weeks post-inoculation, b) Metacercariae, c) Rabbit No.3 died at 10 WPI, 3 flukes from liver parenchyma & 2 from bile ducts recovered respectively.

(*Lymnaea ollula*) were put into a 6-mesh wire box (50 × 30 × 30 mm in size) and the whole box with the snails within was immersed into water in a polyethylene bag that was maintained in a glass beaker at 25°C. Ice was then added into glass beaker to induce emergence of cercariae from the snails by lowering the temperature. After the cold shock, the beaker with the snails in the bag was returned to 25°C condition. The emerging cercariae then encysted on the wall of polyethylene bag and the metacercariae were counted under a stereomicroscope. The metacercariae were kept at 10°C for 3 weeks before inoculations.

Three specific-pathogen-free eight weeks old New Zealand White rabbits (*Oryctolagus cuniculus*) were each fed a single dose of 12 or 20 metacercariae (MC) of *Fasciola* sp. Rabbit Nos. 1 and 2 were each fed 20 MC but rabbit No. 3 was fed with 12 MC, respectively. Metacercariae without capsule were spread on fresh cabbage leaves and given orally to the rabbits. Rabbits were given 70 g of pellet food (RC4, Oriental Yeast Co., Ltd., Tokyo, Japan) daily. Water was available *ad libitum*. The experimental animals were kept and handled according to the rules and regulations laid down by the Institutional Animal Care and Use Committee (IACUC) of Azabu University.

Fecal samples were collected once weekly from 6 weeks post-inoculation (WPI) and fluke egg counts were estimated using the beads sedimentation technique [2, 21, 22]. Briefly, one gram of feces was thoroughly mixed with 50 m*l* tap water and filtered through a 60-mesh sieve net. Then 3 grams of 500–710 μm diameter beads were added. The fecal suspension and glass beads were allowed to settle for 5 min and then rotated three times at a velocity 10 sec per rotation. Supernatant with debris was aspirated out and the packed material was mixed again with 50 m*l* of tap water. All fluid was then transferred into a V-shape bottom centrifuge tube. After allowing the contents to settle for 5 min, the supernatant was aspirated off gently and the resulting 2 m*l* of sediment at the bottom of the tube was transferred into a rectangular transparent chamber. The sediment in the chamber was examined under a microscope. (EPG=Total number of eggs count in chamber)

About 1 m*l* of blood sample was collected from the auricular vein of each rabbit before infection for use as baseline and on weekly intervals during the course of infection. The samples were allowed to clot and then centrifuged at 2,500 rpm for 10 min. Serum obtained was kept at −80°C until analyzed. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyltransferase (GGT) were measured with Spotchem^{TM} EZ dry chemical analyzer (Arkray Inc., Minneapolis, MN, U.S.A.) and commercial test kits (Arkray Inc.). Level are expressed as international units per liter (IU/*l*). Results are expressed as mean ± standard deviation (SD). Significant differences between infected and baseline value were determined by paired sample *t*-test, and value of $P<0.05$ was considered as significant. Alpha- fetoprotein (AFP) and carcinoembryonic antigen (CEA) were also measured for all the rabbit sera collected using the sandwich ELISA kit (GenWay Biotech Inc., San Diego, CA, U.S.A.).

At 8 WPI, fluke eggs were recovered for the first time from the feces of experimentally infected rabbits No.1 and No. 2. The weekly numbers of eggs counted are shown in Table 1. Rabbit No. 3 died at 10 WPI, 3 flukes were recovered from the migratory tunnels of the liver parenchyma and 2 flukes were recovered from bile ducts. The liver from this rabbit was pale with diffuse hemorrhagic patched over the parietal surface and no enlargement of bile duct was observed.

Fasciola infection has two distinct phases in which the signs and symptoms are different. The initial or parenchymal phase occurs when the parasite perforates the liver capsule and begins to migrate through the liver parenchyma towards the large biliary duct. After the ductal phase begins, parenchyma lesions resolve and eggs may be found in stools. Discrimination of the phases has been aided by measuring the activity of enzymes released by damaged hepatic cells in the serum. These may be used as markers of the different stages of *Fasciola* infection [7, 11]. Increases of ALT and AST have been previously reported to appear by 4–6 weeks post infection in sheep, buffalo and monkey [5–7, 24, 29]. In our study, significant increased in serum ALT and AST appeared at 3 WPI and reached a peak value at 6 WPI (Fig. 1). The elevation of these enzymes relates to the liver inflammatory state and to

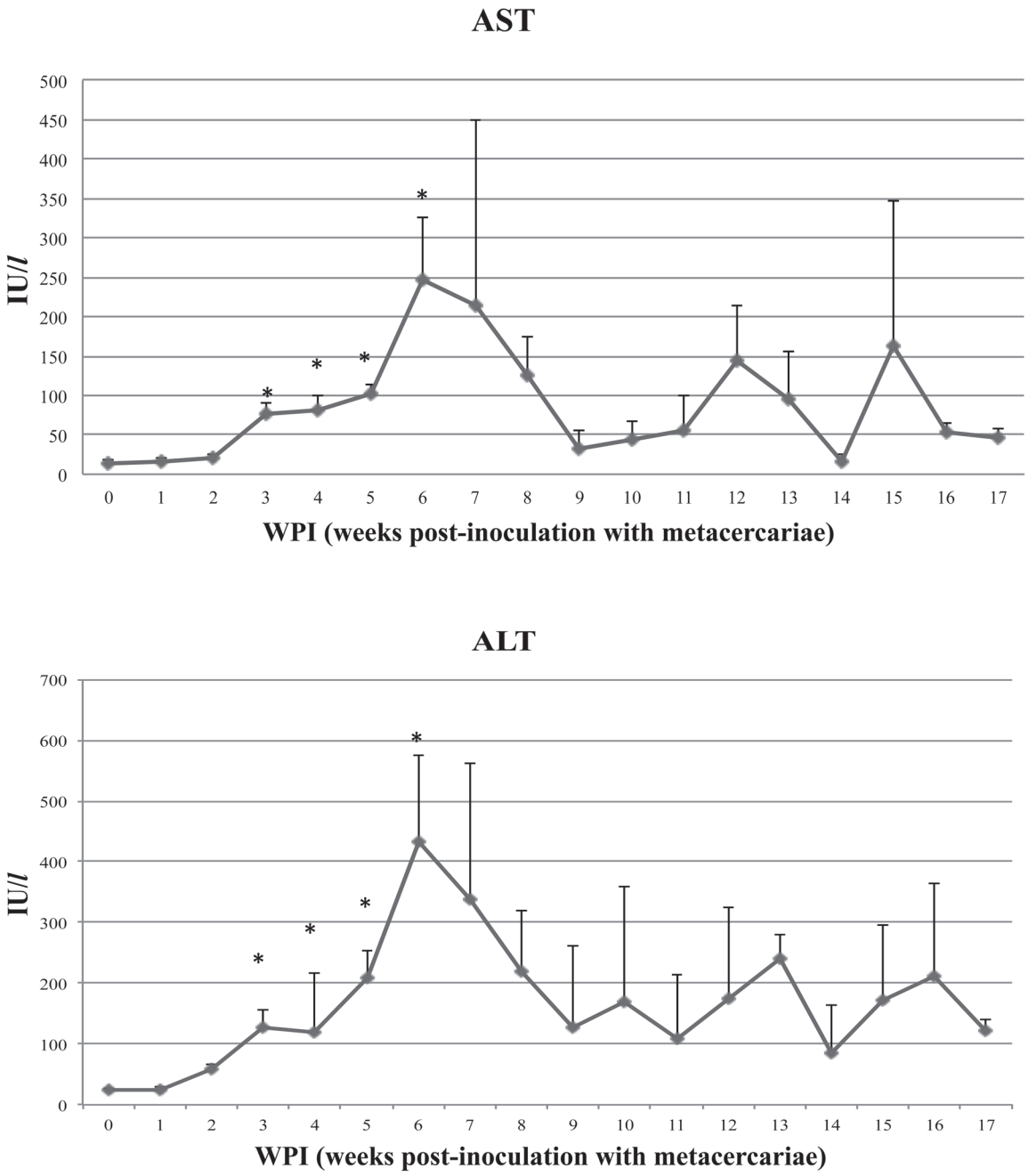

Fig. 1. Serum enzymes activities of AST and ALT of rabbits during the course of infection with *Fasciola* sp. (intermediate form). Data represent mean ± SD (n=3 at 0−10 WPI, n=2 at 11−17 WPI). An asterisk indicates significant difference (P<0.05) compared to baseline value.

tissue destruction provoked by parenchymal migration of juvenile flukes during the first stages of fascioliasis [6, 24]. At 7 WPI, ALT and AST progressively returned to normal values probably due to the migration of juvenile flukes to the bile ducts and since the liver is an organ that regenerates itself comparatively quickly ALT and AST returning to the normal level might reflect this observation [6, 7].

Yang *et al.* [29] reported an increase of GGT in buffalo at 8–26 WPI after daily infection with 60 metacercariae for over 20 days. Takemoto *et al.* [24] reported that serum ALP increased at 6–10 WPI and remained high until 17 WPI in monkeys infected with 20–100 metacercariae. On the contrary, we observed no significant increase in serum GGT nor ALP in our study with rabbits infected only once with 12–20 metacercariae (Fig. 2). This may imply that no injury of the bile ducts had occurred or that the adult flukes did not continue to destroy the biliary epithelial cell due to the hardening of the biliary duct wall. Moreover, the adult flukes might not be very motile, and thus might not be harmful to the biliary epithelium as compare with juvenile flukes [5].

Alpha- fetoprotein (AFP) and carcinoembryonic antigen (CEA) are used as the tumor markers for cholangiocarcinoma in human

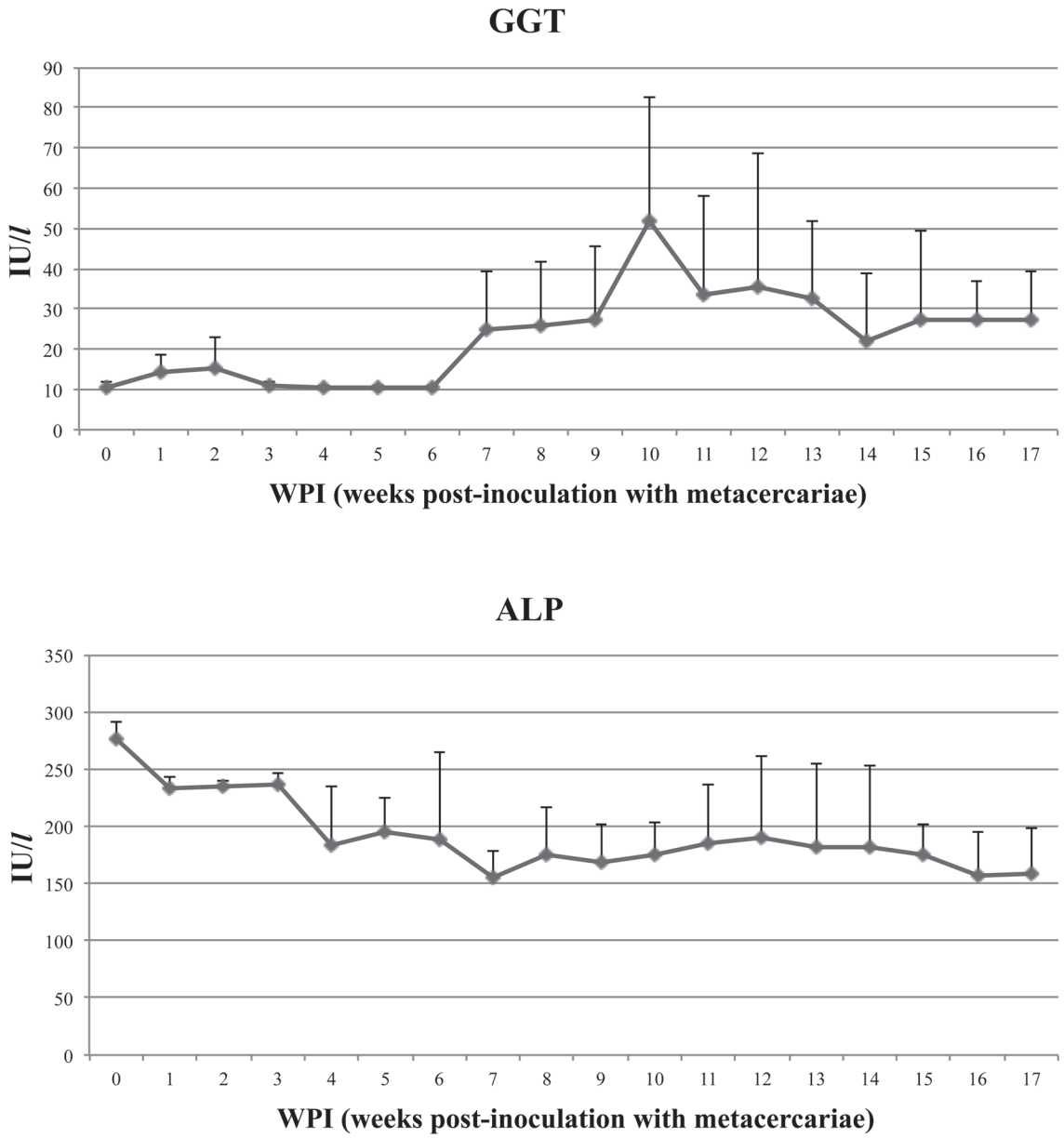

Fig. 2. Serum enzymes activities of GGT and ALP of rabbits during the course of infection with *Fasciola* sp. (intermediate form). Data represent mean ± SD (n=3 at 0−10 WPI, n=2 at 11−17 WPI).

[10, 13, 16]. There are reports, which suggested that the parasites that localizes in the bile duct such as, *Clonorchis sinensis*, and *Opisthorchis viverrini* can induce cholangiocarcinoma [19, 20, 26, 30]. Chen *et al.* [4] proved that rabbits can also produce AFP, albeit in viral infection. In this study, no change in the value of AFP and CEA was observed for all the rabbit sera. Our data are in agreement with previous case reports indicating that tumor markers including AFP and CEA were at normal value in human fascioliasis [1, 14, 15, 28]. This suggested that there might be no relationship between fascioliasis and cholangiocarcinoma, at least at the acute phase.

The *Fasciola* intermediate form may evolve its a new valid species with a given names but at the present moment, since the characterization is still being investigated we will leave the speciation subject for future discussion. Our data indicated that the serum levels of liver enzymes might be a useful parameter for understanding the host-parasite relationship in the final host. Although the number of rabbits seemed to be insufficient for statistical analysis after 11 WPI, we could obtain the trend of the serum enzymes in infected rabbit in this study. This trend could be used as indicators for the interpretation of the liver biochemical profiles of final host at the different stages of *Fasciola* sp. infection.

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