

Studies on *Eurytrema coelomaticum* I.

Preliminary Observations on the Biological Characters of *E. coelomaticum*

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Introduction

Infections of ruminants with two species under genus *Eurytrema* Looss, 1907, *E. pancreaticum* (Janson, 1889) Looss, 1907 and *E. coelomaticum* (Giard et Billet, 1892) Looss, 1907, so far, have been reported in Japan. The development of *E. pancreaticum* in the first and second intermediate hosts was clarified by Basch¹⁾ and Tang¹³⁾, respectively. After then, several investigators reported the life histories of those flukes.

In recent years, the heavy infections with a number of the flukes of species *E. coelomaticum* have broken out among the cattle in two pastures in Hokusatsu District, Kagoshima Prefecture, the most southern part of Japan.

The present paper deals with two problems; 1) the epidemiological survey on the life history of the flukes in the pastures and 2) the experimental observations on the biological characters of the flukes.

Materials and Methods

Land snails and tettigoniid grasshoppers were collected from the pastures mentioned above. These first and second intermediate hosts were dissected under a binocular microscope, and examined for larval *Eurytrema coelomaticum*. The seasonal variations of the infection rates of the larval flukes were observed in the molluscan and insect intermediaries.

6 Japanese black cattle, *Bos taurus* var. *domesticus*, reared in the above pastures were dissected. A number of the flukes were collected from the pancreatic duct of the cattle. Some of the flukes were used for preparing the stained specimens. The rests were incubated in the medium in which the inactivated calf serum at a rate of 20% was added to the medium NCTC 135 containing lactalbumin hydrolysate and yeast extract at the rates of 0.5% and 0.1%, respectively. The eggs, deposited in the medium by the flukes, were smeared on the surface of lettuce leaves, and were fed to six groups consisting of 20 of *Bradybaena similis* (Féussac) or *Acusta despecta sieboldiana* (Pfeiffer). The number of the hatched eggs in the droppings of snails was counted. The snails were dissected under a binocular microscope. The development of the larval flukes was observed using phase contrast microscope. The daughter sporocysts obtained from the infected snails were

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given to long-horned meadow grasshopper, *Conocephalus maculatus* le Guillon. The encystation of cercariae was observed in the meadow grasshoppers.

The metacercariae of the fluke were collected from the hemocoelom of the meadow grasshoppers. The metacercariae were agitated for half an hour at 37°C in 0.2% pepsin solution (Difco, 1: 10,000) in saline solution containing 0.1% hydrochloric acid, using magnetic stirrer. After then, the metacercariae were agitated again in Rinaldini solution containing trypsin at the rate of 0.5% for 15 minutes at 37°C on the magnetic stirrer. The excystment of the metacercariae *in vitro* was observed using an inverted microscope combined phase contrast apparatus.

1000 metacercariae were given orally to each of the two goats, 100 to each of 10 rabbits, 50 to each of 5 guinea pigs, 20 to each of the both 20 mice of ICR- and of dba-strains, and 10 to heterogenous nude mouse (BALB/c nu/+ mouse). The fecal examination was conducted to confirm the infection of the flukes in those animals, excepting the mice. The adult flukes collected from the experimentally infected animals were morphologically compared with those from naturally infected cattle and goats. All organs of the goat and mouse infected were examined histopathologically.

Results

1. Epidemiological survey of *Eurytrema coelomaticum*

The seasonal infection-rates of larval *E. coelomaticum* in both molluscan and insect intermediaries have been investigated in the two pastures in Hokusatsu District, Kagoshima Prefecture, Japan.

A. Survey for the infection in land snail

The land snails have gotten into an action with setting-in of the rainy season early in June in the pastures. 279 of *Bradybaena similaris* (Pfeiffer), 36 of *Acusta despecta sieboldiana* (Pfeiffer), 8 of *Satsuma japonica* (Pfeiffer) and 24 of *Euhadra herklotsi* (Martens) were collected from the pastures. Daughter sporocysts were found in 24 of 279 *B. similaris* (8.6%) and 3 of 36 *Acusta despecta sieboldiana* (8.3%). The infection-rates of the larval flukes in the land snails showed a great tendency to increase from early summer with the time elapsed, reaching their peak in the fall.

B. Survey for the infection in tettigoniid grasshoppers

4 species of tettigoniid grasshoppers, *Conocephalus maculatus* le Quillon, *Phaneroptera falcata* Scopoli, *Metrioptera hime* Furukawa and *Homorocoryphus nitidulus* Scopoli, were collected in the pastures, and were examined for the infection of metacercariae of the flukes. The metacercariae were found in the hemocoel of *C. maculatus* and *P. falcata*.

The long-horned grasshoppers, *C. maculatus*, come to be seen by us in the pastures early in June. The population of the grasshoppers reaches its peak in early fall, decreasing remarkably toward late fall. Small number of the grasshoppers were found still early in December in the pastures. In the season, they are observed to be slow in action on grass. On the other hand, the rate of infection with the metacercariae in the grasshoppers also tends to be increasing from early summer toward late fall. The metacercariae were found in 18.3% and 42.0% of *C. maculatus* early in July and early in December, respectively.

2. The experimental observations on *Eurytrema coelomaticum*

A number of adult flukes were obtained from the pancreatic duct of the naturally infected cattle. The flukes well cleaned were cultured in culture-flasks containing the culture medium. These flukes were active and deposited eggs, more than 100 days after incubation.

A. Development of the larva in *Bradybaena similaris*

Bradybaena similaris were infected with the eggs collected from the culture flasks. The eggs ingested together with lettuce leaf, were hatched in the lumen of digestive tract of the snail. The egg-shells hatched are eliminated together with feces. The miracidia covered with cilia are very active in movement, penetrate through the wall of intestine of the snail, and develop into the oval mother sporocysts lined with germinal cells in the peri-intestinal tissue. The multiplied germinal cells form many cellular masses in the mother sporocyst. Those cellular masses develop into daughter sporocysts. After then, mother sporocysts come to be degenerated and disintegrated. The massive germinal cells in the daughter sporocyst form many germinal balls. Those germinal balls develop into cercariae. The daughter sporocysts come to be dispersed inside the visceral sac by the disintegration of matured mother sporocyst, and migrate to the respiratory chamber of the snail host. The respiratory chamber was found to be packed with numerous daughter sporocysts about 150 days after infection. Thereafter, those daughter sporocysts begin to be expelled from the molluscan host. The cercaria is of a microcercus, dorso-ventrally flattened and elongatedly oval shaped and is 0.30~0.35 mm in length and 0.10~0.15 mm in the maximum width. The mouth of cercaria is surrounded with oral sucker, measuring 50~55 μ in diameter. The ventral sucker measuring 50~60 μ in diameter is situated on the middle portion of the body.

B. Development of the larva in *Conocephalus maculatus*

The fresh daughter sporocyst expelled from the land snails were given to long-horned meadow grasshoppers, *Conocephalus maculatus*. The daughter sporocysts devoured are disintegrated in the lumen of the digestive tract of the grasshopper. The cercariae are free from the daughter sporocyst, then penetrate into the hemocoel through the wall of intestine of the insect, and are encapsulated with the thin wall there. The wall of metacercarial cyst get gradually thicker. Three weeks later, the metacercariae are matured enough to be infective. The excretory bladder packing numerous granules are seen in the mature metacercaria. The cyst-wall of the fully developed metacercaria is transparent, flexible and tough, and averages 23 μ in thickness.

C. Excystment of the metacercaria

Metacercariae were agitated for half an hour at 37°C in 0.2% pepsin solution in saline solution containing 0.1% hydrochloric acid using magnetic stirrer. The cyst-wall became gelatinous and got swelled two to three times in thickness. After then, the metacercariae were agitated again in Rinaldini solution containing trypsin at a rate of 0.5% for 15 minutes at 37°C on the magnetic stirrer. A large part of metacercariae was excysted. The rest metacercariae were easily excysted by slight pressure or shock. The larvae removed from the cyst and fully extended, are shaped like elongated cucumber seed. The average size of their body is 880 μ in length and 255 μ in width. Their oral sucker and ventral sucker are 118 μ and 152 μ in average of the diameter. The excretory bladder containing granular substances was situated at a posterior part of the body. The flame-cell pattern is 2[(2+2+2)+(2+2+2)].

D. Development of *E. coelomaticum* in experimental animals

The metacercariae were orally given to 2 goats, 10 rabbits, 5 guinea pigs, 20 ICR-mice, 20 dba-mice and 1 BALB/c nu/+ mouse. The fecal examinations for the eggs of the flukes were made for all of the animals inoculated with the metacercariae, excepting mice. The eggs of the flukes were found to be eliminated in the feces of the 2 goats from 112 and 114 days after inoculation, respectively. No egg, however, was found in any of the rabbits and guinea pigs. The animals, excepting goats and BALB/c nu/+ mouse, were dissected 120 or 150 days after inoculation. The fluke was not found from the pancreas and other organs of those animals.

The female goat was sacrificed 180 days after inoculation. Pancreas did not macroscopically reveal remarkable lesion. 20 adult flukes were found in pancreatic duct. Several clumps consisting of several flukes were recognized in the peripheries of pancreatic ducts. The histological examination exhibited fibrous thickening of the wall of pancreatic duct and the proliferation of periductal connective tissues. The body size of adult flukes obtained from the goat is fixed to be 5.50 to 10.00 (average: 7.48) mm in length and 1.70 to 2.85 (av.: 2.22) mm in width. The diameters of oral and ventral suckers, and the ratio of diameter of oral sucker to that of ventral one, are 0.35 to 0.70 (av.: 0.52) mm, 0.52 to 0.95 (av.: 0.63) mm and 0.65 to 0.95 (av.: 0.82), respectively.

BALB/c nu/+mouse, which was given 10 metacercariae, was sacrificed 250 days after inoculation. 10 adult flukes were found in pancreas. The body size of 7 of the flukes is 6.50 to 9.85 (av.: 8.56) mm in length and 2.40 to 3.50 (av.: 3.14) mm in maximum width. The diameters of oral and ventral suckers and the diameter-ratio of oral sucker per ventral one are 0.50 to 0.82 (av.: 0.63) mm, 0.62 to 0.92 (av.: 0.80) mm and 0.67 to 0.97 (av.: 0.82), respectively.

In the histological examination of BALB/c nu/+mouse, the pancreatic duct is remarkably dilated by the pancreatic flukes packed in it. The wall of the pancreatic ducts is thickened by the proliferation of periductal connective tissues. The mucous membrane of the pancreatic ducts shows conspicuous papillary hyperplasia. Almost all the acinar cells reveal the vacuolation of their cytoplasm and the decreasing and disappearance of zymogen granules. The atrophy, degeneration and disappearance of the acinar cells are seen round the pancreatic duct. The periductal and interlobular fibrosis extends upon the adjacent acinar tissues which have been atrophic and degenerative. Namely, the pancreas reveals the findings of conspicuous pancreatitis interstitial chronica.

On the other hand, the flukes obtained from the cattle naturally infected with a number of *E. coelomaticum*, were measured. The size of the body is 4.0 to 8.0 (av.: 6.07) mm in length and 1.2 to 4.7 (av.: 2.55) mm in the maximum width. The diameter of the ventral sucker is almost equal to, or larger than, that of oral sucker. Namely, the body-size of the flukes obtained from the goat and mouse experimentally infected, is slightly larger than that of the flukes from the naturally infected cattle.

The flukes from the goat naturally infected with about 20 flukes of *E. pancreaticum* were measured. The body-size of the flukes is 7.60 to 12.00 (av.: 9.18) mm by 3.25 to 4.50 (av.: 3.88) mm. The diameter of the oral sucker of the flukes is larger than that of ventral one: the diameter of the oral sucker is 1.05 to 1.60 (av.: 1.26) mm, that of ventral sucker 0.90 to 1.43 (av.: 1.13) mm, and the diameter ratio of oral sucker per ventral one is 1.00 to 1.36 (av.: 1.13).

Discussion

Skvortsvo and Vol'f¹²⁾ who obtained immature sporocysts from the land snail, *Eulota lantzi* L., described it as a possible intermediate host of *Eurytrema pancreaticum*. Tang¹³⁾ succeeded first in obtaining the mature daughter sporocysts of *E. pancreaticum* fully developed in the land snails, *Bradybaena similaris* and *Cathaica revida sieboldiana*. After that, many investigators^{3, 5, 6, 9, 11, 14, 15, 17)} reconfirmed his work. Miyata⁸⁾ also succeeded in observing the development of molluscan stage of both *E. pancreaticum* and *E. coelomaticum* in *Fruticicola sieboldiana* and *Bradybaena similaris stimpsoni*. Recently, Tang and Tang¹⁷⁾ reported that the infection-rate of *E. coelomaticum* in *Bradybaena similaris* and *Cathaica ravidia sieboldiana* in Fu-zhon and Fu-jian, China is comparatively low, 0.54~7.96 (average: 3.52) % and 0~7.41 (av.: 0.23) %, respectively. In the present

survey, the first intermediate hosts of *E. coelomaticum* in the pastures of Kagoshima Prefecture, Japan, were noted to be *Bradybaena similaris* and *Acusta despecta sieboldiana*. The infection-rate of larval *E. coelomaticum* in *Bradybaena similaris* collected from the pastures was higher (8.6 %) than that reported by Tang and Tang¹⁷⁾.

Basch^{1,2)} first discovered that the second intermediate host of *E. pancreaticum* in Malaya is *Conocephalus maculatus*. Several investigators^{6,10,15,17)} clarified the insect intermediate host of *E. pancreaticum* in the respective localities where they were belonging to. Tang and Tang¹⁷⁾ reported that 6.68% of *C. maculatus* in the suburbs of Fu-zhon city were infected with *E. coelomaticum*. In the present survey, the second intermediate host of *E. coelomaticum* in the pastures were found to be *C. maculatus* and *Phaneroptera falcata*.

Concerning the seasonal infection-rates of the larval flukes in both molluscan and insect intermediate hosts, Tang and Tang¹⁷⁾ described that both intermediate hosts showed high incidence of infection in summer, reaching their peak in fall, and dropping down in winter. On the contrary, we observed that the infection-rate of larval *E. coelomaticum* in *C. maculatus* tended to increase from early summer toward late fall. It seems to us that this difference in the seasonal infection-rates of the intermediaries caused by observation lies on the basis of the different weathers in China and in Japan.

Basch¹⁾ has first achieved success in the artificial infection of goats with *E. pancreaticum*. Tang and Tang¹⁷⁾ also reported the experimental infection of *E. coelomaticum* in a kid. Chinone and Itagaki⁴⁾ observed the development of *E. pancreaticum* in experimentally infected rabbits and goats. They stated that some of the specimens identified as *E. coelomaticum* were conceivable fixed to be the developing stages of *E. pancreaticum*, because both ratios of the body-width to the body-length and the size of the ventral sucker to the oral one varied with the growth of the worm. In the present experiment, the metacercariae of *E. coelomaticum* were given to goats, rabbits, guinea pigs, ICR- and dba-mice and heterogenous nude mouse (BALB/c nu/+ mouse), and only the goats and heterogenous nude mouse were infected. It was considered to be necessary for confirming whether the rabbit was really susceptible to *E. coelomaticum* or not, because Chinone and Itagaki⁴⁾ reported that a calf was not infected with metacercariae of *E. pancreaticum* obtained from cattle.

There is no significant difference in body-size between the flukes obtained from the goat and those from the heterogenous nude mouse. It is generally considered that the parasites overcrowded in their host are stunted in size. The flukes from the goat and heterogenous nude mouse experimentally infected were larger than those from the cattle naturally infected with numerous flukes. The ranges of the body-size in the latter were all within that of *E. coelomaticum* reported by the previous authors. From the point view of using laboratory animal for the study of *E. coelomaticum*, it was to be especially interesting to us that the heterogenous nude mouse was revealed to be susceptible to *E. coelomaticum*. The regressive changes of acinar cells were seen in the pancreas of the infected mouse. From the above findings, it is considered that the parasite has severe pathogenicity for the acinar tissues of pancreas.

Summary

In recent years, heavy infection with *Eurytrema coelomaticum* has been brought forth among the cattle in two pastures in a northern part of Kagoshima Prefecture, Japan. Daughter sporocysts of *E. coelomaticum* were found in *Bradybaena similaris* and *Acusta despecta sieboldiana* collected from the pastures. Metacercariae of the flukes were found from *Conocephalus maculatus* and

Phaneroptera falcata in the pastures. The infection-rates of metacercariae in the grass-hoppers tend to be increasing from early summer toward late fall. The adult flukes obtained from the infected cattle were incubated *in vitro*, and they were active and deposited eggs more than 100 days after incubation. The eggs deposited in the medium were infective to *B. similaris* and *Acusta despecta sieboldiana*. The development of molluscan and insect stages of larval *E. coelomaticum* was observed in *B. similaris* and *C. maculatus*, respectively. Metacercariae of the fluke were given to goat, rabbits, guinea pigs, ICR- and dba-mice and BALB/c nu/+mouse. The goats and BALB/c nu/+mouse were infected with *E. coelomaticum*.

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Explanation of figures

Figures 1, 2, 4, 7, 9 and 10 were photographed by the use of a phase contrast microscope. Figures 13~15 are photomicrographs of specimens stained with hematoxylin-eosin.

- Fig. 1. Daughter sporocyst containing a number of cellular masses consisting of germinal cells. $\times 65$
- Fig. 2. Daughter sporocyst containing many germinal balls. $\times 150$
- Fig. 3. Cercariae in a daughter sporocyst. $\times 65$
- Fig. 4. Cercariae. $\times 150$
- Fig. 5. Metacercariae flowed over from the hemocoel of *Conocephalus maculatus* dissected. $\times 3.5$
- Fig. 6. Metacercaria. $\times 170$
- Fig. 7. Metacercaria having the capsule swelled by being treated with artificial gastric juice. $\times 170$
- Fig. 8. Larva excysted from the capsule of metacercaria. $\times 67$
- Fig. 9. Eggs of *Eurytrema coelomaticum*. $\times 670$
- Fig. 10. Excysted larva of *E. coelomaticum*. $\times 130$
- Fig. 11. Adult *E. pancreaticum* obtained from a naturally infected goat. $\times 8$
- Fig. 12. Adult *E. coelomaticum* obtained from BALB/c nu/+ mouse. $\times 10$
- Fig. 13. Adult *E. coelomaticum* having many eggs in the pancreas of BALB/c nu/+ mouse. $\times 80$
- Fig. 14. Pancreas of BALB/c nu/+ mouse. The acinar cells are atrophic and degenerated. The cells contain many vacuoles in their cytoplasm. Cellular infiltration is seen in interstitial and periductal connective tissues. $\times 65$
- Fig. 15. The periductal and interlobular fibrosis extends upon the adjacent degenerated acinar tissue in the pancreas of BALB/c nu/+ mouse. $\times 65$





