# CHAPTER 5

## Discussion

*F. gigantica* has been recognized as seriously medical trematodes in ruminant and human worldwide. Due to it can cause severely impact on liver of definitive hosts which mainly problematic on the production of animal farms (Dargie, 1987). In Chiang Mai Province, infection of fascioliasis in domestic cattle is still high. Based on our result, it can be confirmed that, the giant liver fluke found in Chiang Mai Province was identified as only *F. gigantica*. There was no any evidence that provides information concerning the infection of *F. hepatica* in northern Thailand. This result corresponds with the study of Sukhapesna *et al.* (1994) that revealed the main cause of fascioliasis in domestic ruminants from Thailand is *F. gigantica*. The prevalence of *F. gigantica* infecting domestic cattle in Chiang Mai Province was already determined. Prevalence of infection in *Bubalus bubalis* was higher than *Bos taurus*, with total prevalence of 65.71% and 46.67%, respectively. However, this finding is contradicted with the study of Sobhon *et al.* (1998) that demonstrate the prevalence of *F. gigantica* infection in cattle were only 4-24%, with highest incidences in the north and northeastern, and the lowest in the south. A few different of *F. gigantica* infection among *Bubalus bubalis* and *Bos taurus* was observed and it may depend on the preference of feeding behavior. *B. bubalis* can be feed a variety of grazing and aquatic plants and its can be feed food in both terrestrial and aquatic habitat whereas, *Bos taurus* was mostly cultured in farmland system that it has been greatly management and frequently treated by anthelmintics. Moreover, the incidence of *F. gigantica* found in Chiang Mai was higher than the other regions of Thailand (Sukhapesna *et al.*, 1994). The average prevalence of *F. gigantica* in cow and buffalo in Thailand was 11.8% (Srihakim and Pholpark, 1991). It may due to the *Bubalus bubalis* and *Bos taurus* from slaughterhouses of Chiang Mai Province was

mostly imported from neighboring countries and tended to increase which may be the point of higher fluke diversity in this region.

The present study has completely reported on the life history of the *F. gigantica* in Thailand. The pattern of life history consisted of six stages involving egg, miracidium, sporocyst, redia, cercaria, metacercaria, and adult stage, which is closely similar to the related species: *F. hepatica* (Andrews, 1999). In this study, the suitable hatching temperature ranged from 27-31ºC, at which range the eggs successfully developed and hatched after 12 days, while eggs of *F. hepatica* were hatched within 2 to 4 weeks at the temperature range of 23-26ºC (Bowman, 1995; Andrews, 1999). This indicated that the development of egg of *F. gigantica* in this study is rapid than *F. hepatica*, may be the higher temperature have effected on development and hatching of egg. A lower temperature resulted in a slower rate of development, while higher temperature results in a faster rate of development of the embryo in the egg (Andrews, 1999). After the miracidium hatched, they actively swam for search the several lymnaeid snail species, such as *Lymnaea columella*, *L. cousin*, *L. natalensis*, *L. truncatula* could sustain larval development (Dinnik and Dinnik, 1956; Salazar *et al*., 2006). The most frequently involved intermeadiate hosts are *L. auricularia rubiginosa* and *L. natalensis* (Kaufmann, 1966). *L. auricularia rubiginosa* was used as the experimental snail hosts in this study, which only one species of Lymnaeid snails has been reported serving as intermediate host of *F. gigantica* (Charoenchai *et al.*, 1997). This study is the first report of the development of *F. gigantica* larval stages in *L. auricularia rubiginosa* in Thailand. The miracidium then successfully penetrated the snail hosts, and developed to become sporocyst, redia, and cercaria. When the miracidium of *F. gigantica* failed to find a snail host, they were died within 24 hrs. This was also true of the miracidium of *F. hepatica* (Bowman, 1995). In infected-snails, the larval stages of *F. gigantica* in this study were completely developed and separated from the snails within 39 days PI which differ the reports of Dreyfuss and Rondelaud (1995) indicated that the cercariae shedding on 54 days PI at 23 ºC. The free-swimming cercaria contracted and successfully adhered on the rice plant or other substrate and suddenly formed the metacercaria cysts. The metacercaria encystment was suitable at high temperature of above 24 ºC (Shalaby *et al*., 2004). The life cycle was completed when the metacercariae were eaten by the

definitive host, such as a mammal. The life history trait of *F. gigantica* in Thailand was determine for the first time, which revealed that the larval stages of *F. gigantica* can be produced in snail hosts, *L. auriculalia rubiginosa*, and well encysted on rice plants. The adult worms can be allowed to develop to maturity in the experimental definitive host, dwarf hamster and albino mice, which indicated that *F. gigantica* can be successfully developed to maturity in other hosts besides ruminants and humans.

In the morphological study of the larval stage, *F. gigantica* eggs were found to be larger than the eggs of *F. hepatica* (Thomas, 1883). The miracidium of *F. gigantica* were also bigger than that of *F. hepatica* (Andrews, 1999). The sporocyst is oval shaped, and on average 0.13 mm in length and 0.10 mm in width. Redia was roughly cylindrical in shape, with two lateral projections at the posterior, and consisted of a mother redia and a daughter redia. The cercaria was tadpole-like with a discoidal body and a long tail. The oral sucker, ventral sucker, pharynx and prepharynx are present and have very conspicuous cystogenous glands and forked intestine. The cercariae are tadpole-like with a discoidal body and a long tail. They possess an oral sucker and a ventral sucker in the centre of their bodies and have very conspicuous cystogenous glands and forked intestine. The pharynx and prepharynx are presented. The metacercarial cyst can be easily identified using the morphological reference. These cysts were protected with capsules, which had a double thick wall that consisted of an outer and inner cyst, all of which were equal size to the cyst of the *F. hepatica* (Kaufmann, 1966). The cyst is white when it is laid, and is almost immediately infective to the definitive host. After a few days, the cyst gradually becomes yellow and dark in color.

This study is the first report on the worm recovery and maturity of *F. gigantica* in experimental hosts: dwarf hamsters (*P. campbelli*) and albino mice (*M. musculus*). The developmental model can be applied to other experimental hosts, and for which the acquired information can be used in the management, control and treatment of this parasite in ruminants and humans. The rates of parasitic incidence of this parasite in both experimental definitive hosts were found to be 100%. The results confirmed that experimental metacercariae were successfully developed in both hosts. The average worm recovery rates in dwarf hamsters and albino mice were found to be 36.00% and

35.83%, respectively and trend of discovery of both experimental hosts continuously declined. However, in dwarf hamsters were died on day 45 PI, while albino mice still alive in day 48 PI and they were died in day 52 PI. As a result, it was determined that dwarf hamsters and albino mice are also suitable for use as models for parasitic infections. But the disadvantages of dwarf hamster are inherent weakness and lowered resistance to parasitic infections. Therefore, some studies used bison as the experimental definitive host for *F. hepatica* (Foreyt and Drew, 2010). Moreover, the previous study used mice, black rat and hamster as the experimental hosts (Davies and Smyth, 1978; Valero *et al*., 1998; Keiser *et al*., 2006). The differences between worm recovery rates of *F. gigantica* in these experimental hosts have not been described in the previous study, and then in the further study should be more investigate. It could be seen that on day 45 PI, the dwarf hamsters died because of the heavy liver damage caused by the adult worms. Previous reports have involved the culturing of *F. hepatica* derived from mice and showed that the flukes revealed no further development in the genital rudiments (Davies and Smyth, 1978).

Developmental patterns of this parasite in albino mice and dwarf hamster found that sizes of body, oral and ventral suckers all continuously increased. On day 42 PI, the immature eggs were observed, which indicated that the *F. gigantica* had matured. An examination of the parasitic infection in goats found the parasite's eggs in the feces on days 64-70 PI (Taira and Saitoh, 2010). In this study, the parasite's eggs were not found due to the fact that the hosts had died before the adult worms could expel the eggs. The above results were different because the results were related to the liver fluke species, of which *F. hepatica* showed a period of maturity requiring 37 days (Dawes, 1962) and 40 days (Davies and Smyth, 1978).

By basing on the comparative study of nucleotide sequences of *F. gigantica* ITS-2 sequences, it was found that *F. gigantica* collected in Chiang Mai, Thailand are grouped with *F. gigantica* in GenBank while, *F. hepatica* and including other related-species are separated individually. The molecular analysis of the ITS-1 and ITS-2 genes of *Fasciola* spp. were commonly reported in Asia countries (Amer *et al.*, 2011). In accordance with the constructed phylogenetic trees, *F. gigantica* in this study showed highly relationship

with the groups of *F. gigantica* that acquired from GenBank. The K>4θ criterion proposed by Birky (2013) presented that the *F. gigantica* from Thailand (group A) are genetically closely with *F. gigantica* from other countries. Moreover the K>4θ criterion are indicated *F. gigantica* in this study diverge with *F. hepatica*. The degree of separation between these groups was sufficient for diagnosed as distinct species using four times rule for speciation (Walker *et al*., 2012). Our finding is in agreement with Amor *et al.* (2011) who reported confirmation *Fasciola* spp. using ITS-1 and ITS-2 sequences showed closely relationship of the Mauritanian samples with isolates of *F. gigantica* from different localities of Africa and Asia. Moreover NCBI databases can be used for compared with other species of trematodes in the family Fasciolidae (Le *et al*., 2007).

Even the adult worm of this fluke can generally be identified by using morphological characters (Ashrafi *et al.*, 2007), but its larval stages are still difficulties due to the similar morphological characters (Kaplan *et al.*, 1995). Thus, the accurate and sensitive approaches are needed for determining actual epidemiological situation during its developmental stages. *F. gigantica*-specific primers which successfully developed in this study have been approved as effective diagnostic tool for detecting in both larval and adult stages. In addition, our result confirmed the advantage of SCAR-marker and HAT-RAPD approaches for constructing specific DNA marker. Specifications of designed specific marker are FG-F (5'-TCC GTT CGT TTT CCC CTC TG-3') and FG-R (5'-GGG TTT CGC CCA TAC AGG AT-3') with length 20 bp of both primers and yielding a product size of 235 bp. The melting temperature (Tm) of both primers is 59.97 and 59.82 °C, respectively which this temperature is depends on the length of the DNA molecule and it is optimized for the melting primer in the reaction of PCR. Tm has correlated with annealing temperature in PCR reaction which generally using an annealing temperature lower 5 °C the melting temperature (Dieffenbach *et al.*, 1995). The annealing temperature of this specific primer used in PCR reaction is 55 °C.

These methods have frequently been introduced to use in several previous reports such as for detected *O. viverrini* (Parvatthi *et al*., 2008) and *H. taichui* (Wongsawad *et al*., 2009). However, a few differences were observed; first, the amount of $MgCl_2$ that much

higher reduced than in this study which it may depend on different buffer and *Taq* master mix. Second, the minimum DNA template needed in specific amplification; previous reports showed less amount of template (0.1 pg) while this study is higher (50 pg) (Wongsawad *et al*., 2009; Hamburger *et al*., 1998). Otherwise, most previous reports stated before never conducted on larval stages, whereas in this study, all developmental stages; miracedium, sporocyst, redia, cercaria, metacercaria and adult stages were completely included. Using of SCAR-marker derived from HAT-RAPD PCR approach result a reproducible, higher resolution and greater polymorphism output and importantly, lower cost, time and laborious consumption are also included (Wongsawad *et al*., 2009).

The determination of cercarial infection in field-collected snails showed negative result by revealing no specific DNA fragment was amplified. It is possible that all cercarial types tested are not the type of *F. gigantica* as described previously. It may be caused the sampling sites of snail intermediate host collection in this study have not enough, which did not covered epidemiological area of *F. gigantica* in Chiang Mai Province. Further studies should be collecting snail more than this study and cover all the area of Chiang Mai Province, especially area of culturing cows and buffaloes. However, specific primers developed in this study provide valuable utilization for the detection of *F. gigantica* in both adult and larval stages which usefulness for the prevention and epidemiological control program.