CHAPTER II
LITERATURE REVIEWS

2.1 *Opisthorchis viverrini*

2.1.1 Biology and morphology

Opisthorchis viverrini, a human liver fluke, is endemic in Thailand especially in Northeast, Lao PDR, Vietnam and Cambodia (IARC, 1994; Sithithaworn, Haswell-Elkins, 2003). The habitat of *O.viverrini* is in the hepatobiliary tract, bile duct and gallbladder. The classification of *O.viverrini* is as follow:

Kingdom	Animalia
Phylum	Platyhelminthes
Class	Trematoda
Subclass	Digenea
Order	Opisthorchiata
Family	Opisthorchiidae
Genus	*Opisthorchis*
Species	*Opisthorchis viverrini*

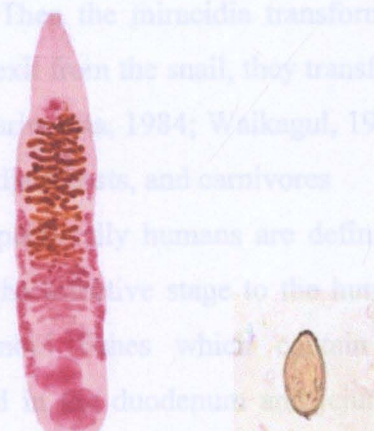
A B

Figure 1 Adult worm (A: original) and egg of *O.viverrini* (B: Kaewkes et al., 1991)

The adult worms of *O. viverrini* (Figure 1A) are monoecious, dorso-ventrally flattened, lancet-shaped, thin and transparent. The average size of fresh worms is 7.0 (5.4–10.2) × 1.5(0.8–1.9) mm. The oral sucker and the ventral sucker are at anterior of the body. It has two deeply lobe testes lying diagonal near the posterior extremity. The multilobe ovary is anterior to the testes. The vittelline glands are several columnous groups in the both sides of the body between the ventral sucker and testes. Excretory bladder is long, sac-like tubed, S-shaped and situates between the two testes. The embryonated eggs are oval or electric-bulb shaped, yellowish brown colored (Kaewkes, 2003). The average size is 27 µm × 15 µm. The eggs have prominent operculum, shoulder and knob. The eggshell surface is rough which is called musk-melon pattern (Kaewkes, 2003).

2.1.2 Life cycle

The adult worms of *O.viverrini* commonly live in the intrahepatic bile ducts and occasionally in extrahepatic bile ducts, gallbladder and pancreatic duct (Elkins et al., 1996) where they use the oral sucker and ventral sucker attach the epithelial lining. The adult flukes lay eggs in the biliary system, and are excreted in the feces, the embryonated eggs which contain the miracidium pass through with bile to the small intestine and excreted with feces in to the environment. The embryonated eggs are hatched after *Bithynia* snail of the family Bithyniidae (Kaewkes, 2003) which is the first intermediate host ingests them. Then the miracidia transform to sporocysts, rediae and cercariae. After the cercariae exit from the snail, they transform to metacercariae encysted in the cyprinoid fishes (Harinasuta, 1984; Waikagul, 1998). Many species of cyprinid fish act as the fisrt intermediate hosts, and carnivores (e.g cats and dogs) as reservoir hosts, but more specifically humans are definitive hosts (Parkin et al., 1993). The metacercariae are the infective stage to the humans who ingest raw or insufficiently cooked cyprinoid fishes which contain the metacercariae. Then the metacercariae are excysted in the duodenum and jejunum. The juvenile worms migrate up to the hepatic bile ducts and grow to adult worms and fertilization. Then the embryonated eggs pass with feces in to the environment. The life span of *O.viverrini* in human has been reported to be 25-30 years (Viranuvatti, Stitnimankarn, 1972).

2.1.3 The epidemiology of opisthorchiasis

Opisthorchiasis caused by *O.viverrini* which is one of risk factors for several hepatobiliary diseases and cholangiocarcinoma in humans has been recognized as a type-1 carcinogen since 1994 (IARC, 1994; Mayer, Fried, 2007). At least 40 million people are infected with this parasite worldwide. Two million people infected with the liver fluke *O.viverrini* in Lao PDR and eight million in Thailand (WHO, 1995; Sithithaworn, Haswell-Elkins, 2003). Regional liver fluke distribution ranks: the North (19.3%), the Northeast (15.7%), the Central (3.8%) and the South (0%). The decline in the northeast from 34.6% in 1981 to current levels (Jongsuksuntigul et al., 1992; Jongsuksuntigul, 2002) is attributed to intensive and continuous control activities (Jongsuksuntigul, Imsoomboon, the current issue). Especially in Khon Kaen province, Northeast Thailand (IARC, 1994) which is the highest endemic area of *O.viverrini* infection ranges from 2% to 71% (Sriamporn et al., 2004). In the endemic area, Ampher Chonnabot and Khon Kaen, the incidence of infection per year ranged between 19.4 and 46%. In children aged less than 5 years, the incidence ranged between 2.1 and 6.2%; the incidence among males was trending to be greater than females (Upatham et al., 1988). In 1993, three villages in Khon Kaen had an incidence range between 1.7 and 25% per 6 months (Saowakontha et al., 1993). In general, the youngest age groups (0 to 5 years) have the lowest prevalence and intensity, while teen infections are often plateaus (15 to19 years). In some areas, intensity of egg excretion trended to increase with age (Upatham et al., 1984) while worm burden decrease (Haswell-Elkins et al., 1991; Sithithaworn et al., 1991a) because of a late-developing immune response, lower parasite survival in heavily fibrosis bile ducts, death of parasites in heavily infected persons, or reduced exposure in the elderly. However heavier infections are more common among males (Haswell-Elkins et al., 1991).

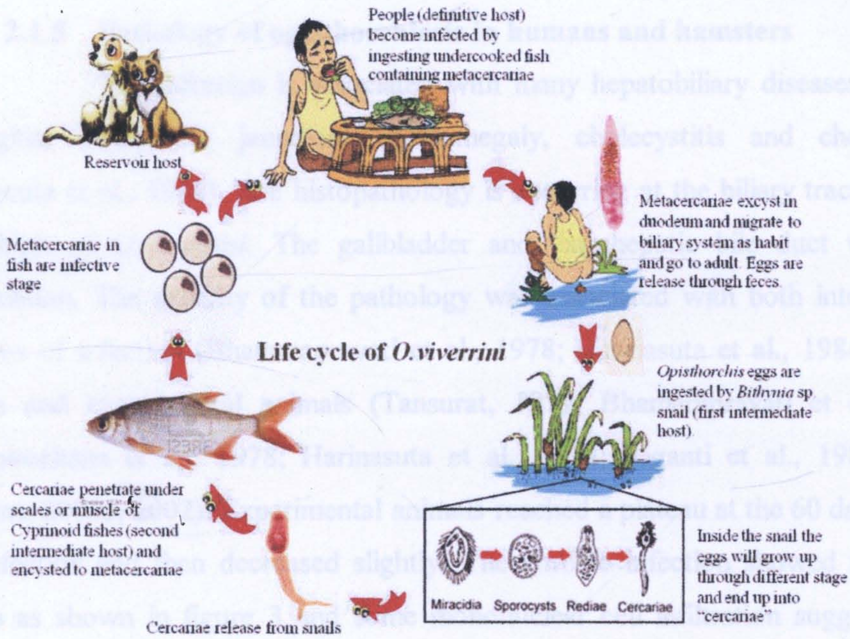

Figure 2 Life cycle of *O.viverrini* (Modified from WHO, 1995).

2.1.4 Opisthorchiosis in experimental animals

Opisthorchiosis or the liver fluke infection caused by eating infected raw fish with containing *O.viverrini* metacercariae, which is the infective stage. Many mammals can be infected with *O.viverrini* such as hamster which is widely used for many aspects in opisthorchiosis and cholangiocarcinoma development especially in the northeast Thailand (Ginovker, Zuevski, 1985; Ginovker et al., 1985; Adam et al., 1993; Sripa, Kaewkes, 2002; Keiser et al., 2006; Boonmars et al., 2008; Bychkov et al., 2008; Songserm et al., 2009). Jird or gerbil is one of animal that this parasite can develop to adult (Adam et al., 1993; Boonmars et al., 2008). Not only hamster and jird can be used but guinea pigs (Glumov, 1974 ; Ilyinskikh, et al., 1998) dogs (Schuster, 2007 ; Jessicaet al., 2010) and cats (Jessica et al., 2010) as well. Most of the goal of study of animal models is used instead human model, which has ethical limitation, for study many aspects such as mechanisms of infection, pathology, complication, treatment, control and prevention.

2.1.5 Pathology of opisthorchiasis in humans and hamsters

The infection is associated with many hepatobiliary diseases, such as cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis (Harinasuta et al., 1984). The histopathology is occurring at the biliary tract where is the habitat of *O.viverrini.* The gallbladder and extrahepatic bile duct was more inflammation. The severity of the pathology was associated with both intensity and durations of infection (Bhamarapravati et al., 1978; Harinasuta et al., 1984) in both humans and experimental animals (Tansurat, 1971; Bhamarapravati et al., 1978; Koompirochana et al., 1978; Harinasuta et al., 1984; Riganti et al., 1989; Sripa, Kaewkes, 2000a, 2002). Experimental animals reached a plateau at the 60 days post-infection and then decreased slightly. The chronic infection showed prominent fibrosis as shown in figure 3 and some mononuclear cell infiltration suggested that immunomodulation may occur (Bhamarapravati et al., 1978; Sripa, Kaewkes, 2002). Dilated bile ducts, hyperplasia epithelial lining and increase the number of goblet cells were observed. In chronic infection, periportal and periductal fibrosis was the most predominent histopathological change. Sripa, Kaewkes (2002) is the first report studied morphological changes of the gallbladder and extrahepatic bile ducts in hamsters infected with *O.viverrini*, the gallbladder and extrahepatic bile duct wall were slightly opaque, especially in heavy infected group as shown in figure 4. The severity of the opacity increased gradually with the duration of infection and striking lesions were observed in the extrahepatic bile ducts. These included ductal dilatation, increased opacity (thickened wall) may be evident on sonograms and computer tomography scans (Lim, 1990) and periductal nodule formation. Ultrastructural changes in the hepatocytes of male hamsters (*Mesocricetus auratus*) and jirds (*Meriones unguiculatus*) at 220 days after experimental infection with *O.viverrini* have been reported (Adam et al., 1993). The proliferation of the smooth endoplasmic reticulum (SER) is striking in the hepatocytes. The nuclei show lobe-like protrusions and are enlarged. The mitochondria are often dumbbell-shaped and show pathologic degenerations. And renal disease associated with *O.viverrini* infection has been reported in hamsters (Boonpucknavig et al., 1992).

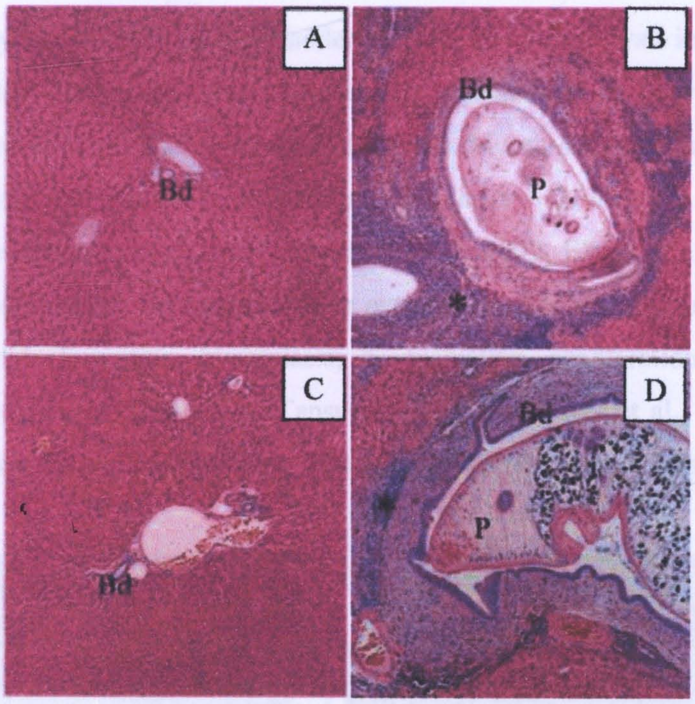

Figure 3 Histopathology change in infected hamster at 30 days and 60 days post-infection (B,D) compare with uninfected normal hamsters (A,C) (Boonjaraspinyo et al., 2009).

Human pathology, in the light infections, Tansurat (1971) found no detectable change in the biliary epithelium and periductal areas of the liver. However, in chronic infections, there was proliferation of epithelial cells with formation of varying degrees of periductal fibrosis. Riganti et al. (1989) described the predominant changes of infected liver as desquamation of the biliary epithelium, epithelial hyperplasia, bile duct hyperplasia, and periductal fibrosis. In addition, the cellular infiltrates consist of lymphocytes, monocytes, eosinophils and some plasma cells. Granulomatous inflammation in response to the parasite eggs that escape into the liver parenchyma, is occasionally seen in humans (Viranuvatti, Stitnimankarn, 1972; Riganti et al., 1989). Enlargement of the gallbladder is commonly found in opisthorchiasis (Riganti et al., 1989) and ultrasonographic studies (Dhiensiri et al., 1984; Elkins et al., 1990; Mairiang et al., 1992). Dhiensiri et al. (1984) described gallbladder abnormalities by ultrasound in patients with *O.viverrini* infection. Community-based data studies in Northeast Thailand using ultrasonography and cholecystography formed, an increase in the frequency and severity of gallbladder disease specifically wall irregularity and enlargement. Bile sludge is often seen in the gallbladder in heavy *O.viverrini* infections (Elkins et al., 1990; Mairiang et al., 1992). Moreover, poor function among apparently healthy individuals with moderate and heavy *O.viverrini* infection was observed (Haswell-Elkins et al., 1991; Mairiang et al., 1992; Elkins et al., 1996). On histopathological changes, the gallbladder or extrahepatic bile ducts in opisthorchiasis were adenomatous formation, hyperplasia, desquamation, proliferation of epithelial lining cells and chronic inflammation (Tansurat, 1971; Harinasuta et al., 1984; Riganti et al., 1989). Chronic cholecystitis was commonly observed in the adults.

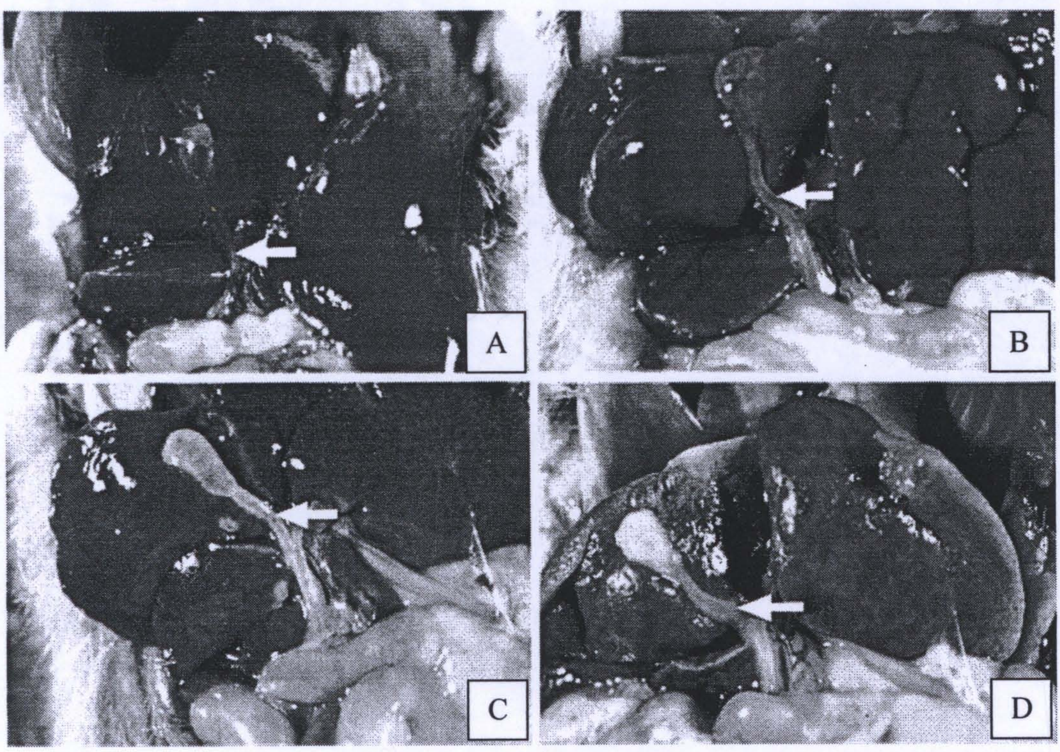

Figure 4 Gross appearances of the liver, gallbladder and extrahepatic bile duct in uninfected normal control (A) and infected hamsters at 30 (B), 90 (C) and 180 days (D) (Sripa, Kaewkes, 2002).

In *O. felineus* infection, there was also a report of opisthorchiasis–associated nephropathy (Lapteva, 1990). Acute renal failure in obstructive jaundice due to cholangiocarcinoma, which is associated with opisthorchiasis in Thailand, is observed in nearly all patients (Mairiang et al., 1990). Case report opisthorchiasis mimicking primary biliary cirrhosis by Melling et al. (2009) showed decompensated liver cirrhosis with hepatosplenomegaly. The routine postoperative histopathological examination of the explanted liver revealed a chronic liver fluke infection as causative agent of the end-stage liver disease.

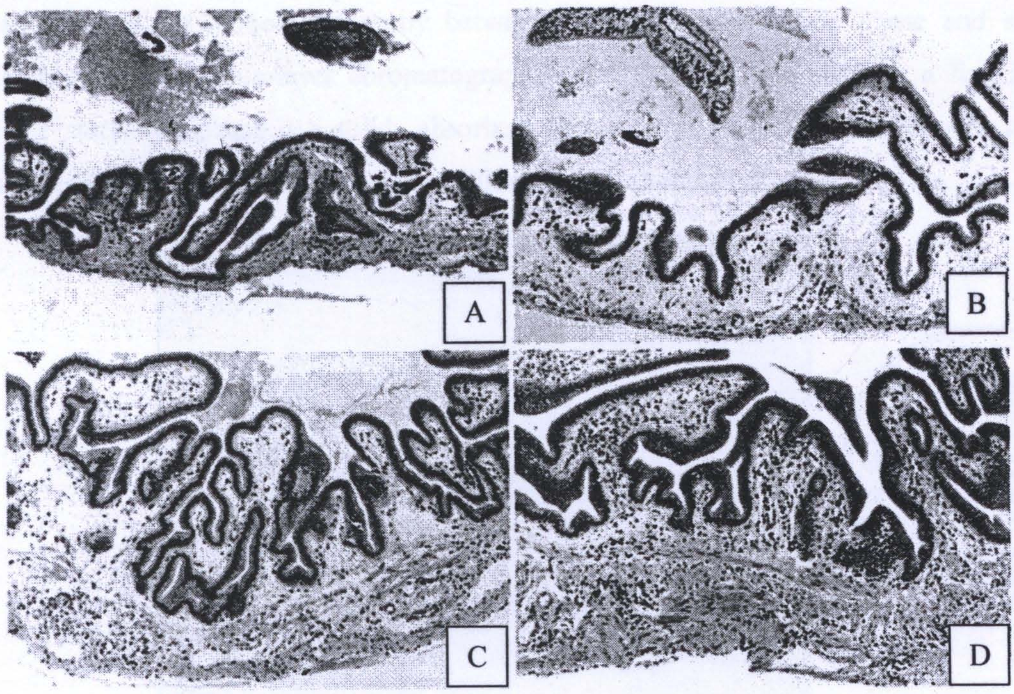

Figure 5 Histopathology changes of gallbladder in uninfected normal control (A) and infected hamsters at 30 (B), 90 (C) and 180 days (D) (Sripa, Kaewkes, 2002).

The pathogenesis of opisthorchiasis is due to the mechanical injury, metabolic product and immunopathology from the parasite and lead to desquamation, epithelial and adenomatous hyperplasia, goblet cell metaplasia, inflammation, periductal fibrosis and granuloma formation (Sripa, 2003). The pathological changes are depended on the intensity and the duration of the infection. The infections in older patients are usually found a large number of flukes (Harinasuta et al., 1984). Most of opisthorchiasis have no symptoms. Only 5 to10% of heavy infection cases has non-specific symptoms such as right upper quadrant abdominal pain, flatulence, fatigue and hot sensation over the abdomen (Pungpak et al., 1983; Upatham et al., 1984). Mild hepatomegaly occurs in 14%, mainly in the heavy infection group (egg counts >10,000 EPG).

2.2 Thin-layer chromatography

Chromatographic separations take advantage of the fact that different substances are partitioned differently between two phases, a mobile phase and a stationary phase. In thin-layer chromatography (TLC), the mobile phase is a liquid and the stationary phase is a solid absorbent (Egon, 1969) which is coated with a thin-layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose.

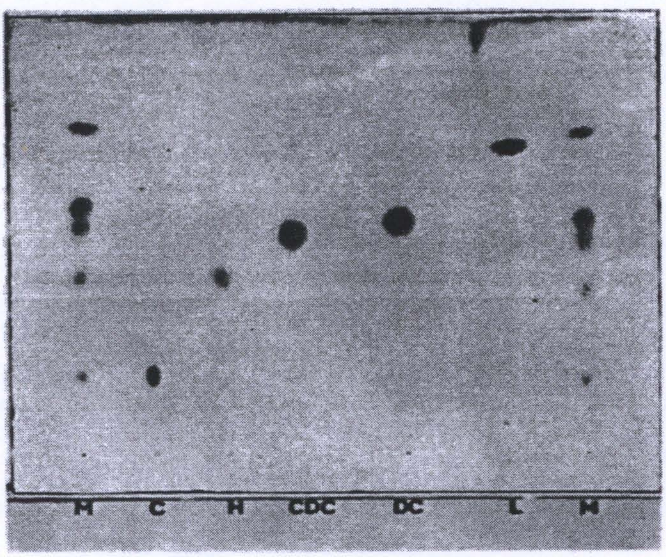

Figure 6 Separation of free bile acids by TLC technique. Free bile acids (10 pg; development time, 1 hour 30minutes) From left to right: mixture (M); cholic acids (CA); hyodeoxycholic (H); chenodeoxycholic acids (CDCA); deoxycholic acids (DC); lithocholic (L); Mixture (M). (Glinshirt, Koss and Morianz, 1960)

The sample is applied to the layer of adsorbent, near one edge, as a small spot of a solution. After the solvent has evaporated, the adsorbent-coated sheet is propped more or less vertically in a closed container, with the edge to which the spot was applied down. The spot on the thin-layer plate must be positioned above the level of the solvent in the container. If it is below the level of the solvent, the spot will be washed off the plate into the developing solvent. The solvent, which is in the bottom of the container creeps up the layer of adsorbent, passes over the spot, and as it continues up, effects a separation of the materials in the spot ("develops" the chromatogram). When the solvent front has nearly reached nearly the top of the adsorbent, the thin-layer plate is removed from the container (Figure 7).

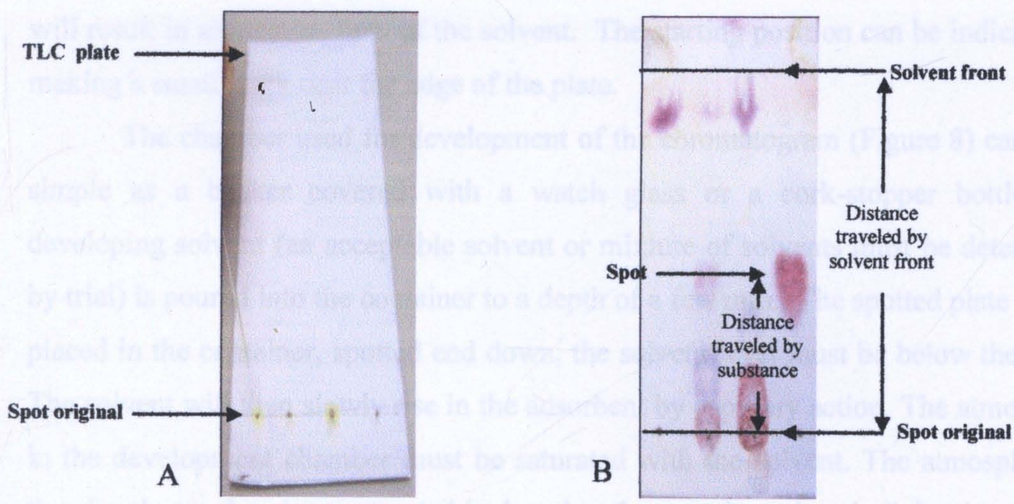

Figure 7 The sample is applied to the layer of adsorbent A: Position of the spot on a thin-layer plate. B: TLC plate showing distances traveled by the spot and the solvent after solvent front nearly reached the top of the adsorbent (original).

The distance traveled by a substance relative to the distance traveled by the solvent front depends upon the molecular structure of the substance, TLC can be used to identify substances as well as to separate them. The relationship between the distance traveled by the solvent front and the substance is usually expressed as the R_f value:

$$R_f = \frac{\text{Distance solute center of gravity moved}}{\text{Distance solvent front moved}}$$

The sample will be separated and applied as a small spot (1 to 2 mm diameters) of solution about 1 cm from the end of the plate opposite the handle. The addition may be made with a micropipette prepared by heating and drawing out a melting point capillary. As small a sample as possible should be used, since this will minimize tailing and overlap of spots, the lower limit is the ability to visualize the spots in the developed chromatogram. If the sample solution is very dilute, make several small applications in the same place, allowing the solvent to evaporate between additions. Do not disturb the adsorbent when you make the spots, since this will result in an uneven flow of the solvent. The starting position can be indicated by making a small mark near the edge of the plate.

The chamber used for development of the chromatogram (Figure 8) can be as simple as a beaker covered with a watch glass or a cork-stopper bottle. The developing solvent (an acceptable solvent or mixture of solvents must be determined by trial) is poured into the container to a depth of a few mm. The spotted plate is then placed in the container, spotted end down, the solvent level must be below the spots. The solvent will then slowly rise in the adsorbent by capillary action. The atmosphere in the development chamber must be saturated with the solvent. The atmosphere in the chamber is then kept saturated by keeping the container closed all the time except for the brief moment during which a plate is added or removed. If the compounds are colored, they are easy to see with the naked eye. If not a UV lamp is used.

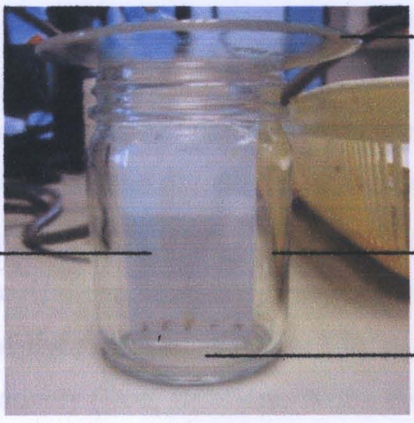

Figure 8 Developing chamber for thin-layer chromatography.

2.3 Bile acids

Bile acids are amphipathic structure synthesize from cholesterol (John,Chiang, 1998) that can divided in to two part, hydrophobic such as CA, CDCA, DCA, LTA and hydrophilic such as ursodeoxycholic acid (UDCA). In the live, the major bile acids biosynthesis pathway is initiated by cytocrome P450 enzyme such as cholesterol-7α-hydroxylase (CYP7A1) and sterol 27α-hydroxylase (CYP27A1) (Zimber, Gespach, 2008) through a classical pathway and alternative pathway resulting in formation of the main bile acids in human (Einarsson et al., 2000) are CA and CDCA, respectively (Zimber, Gespach, 2008).

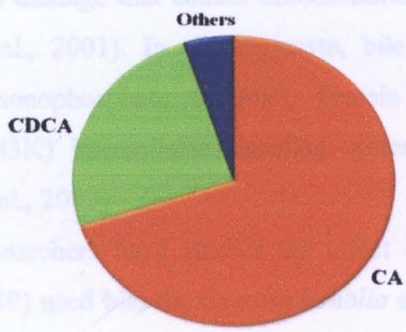

Figure 9 Distribution of cholic acids, chenodeoxycholic acids and precursors formed in primary cultures of human hepatocytes (Einarsson et al., 2000).

In normal condition CA and CDCA comprise approximately 70% and 25% of hepatic and biliary bile acids in culture of human hepatocyte (figure 9) (Einarsson et al., 2000). In the liver of healthy adults, the CA concentration is reported to be 14 to 20 nmol/g liver, whereas the CDCA concentration is 23 to 31 nmol/g liver. During cholestasis, a pathophysiological condition that results from obstruction of bile flow is a common manifestation of liver diseases, CA and CDCA levels are elevated to 120 and 85 nmol/g liver, respectively (Deo, Bandiera, 2008). Bile acids are divided into 3 groups; 1) primary bile acids, such as CA and CDCA, derived directly from cholesterol, 2) secondary bile acids such as DCA, LTA, and 3) tertiary bile acids UDCA (Javitt, 1994).

In cholangiocarcinoma, the proportion of CA is higher than CDCA and others (Changbumrung et al., 1990). When compared with healthy controls, cholangiocarcinoma and hepatocellular patients. Total bile acids are increased especially CA and CDCA (Changbumrung et al., 1990), no significant differance in opisthorchiasis cases (Virasak et al., 1988; Wongpaitoon et al., 1988)

The retention of hydrophobic bile acids in pathophysiological conditions, it is believed to play an important role in liver injury by inducing apoptosis or necrosis of hepatocytes (Attili et al., 1986) that are elevated the bile acids. Moreover, accumulation or high concentration of bile acids can cause liver cell damage (Fischer et al., 1996; Greim et al., 1971; Setchell et al., 1997) which is related to oxidative stress (Sokol et al., 1993) and inducing oxidative damage that causes mitochondrial dysfunction (Rodrigues et al., 2001; Yerushalmi et al., 2001). In cholangiocyte, bile acids alter calcium ion (Ca2+), cyclic adenosine monophosphate (cAMP), Protein kinase C (PKC) and Phosphoinositide 3-kinase (PI3K) intercellular signaling systems (Alpini et al., 2001; LeSage et al., 2001; Alpini et al., 2002)

In addition, many researchers have studies the effect of bile acids on parasite development; Gillin et al. (1989) used bile for *Giardia lamblia* excystation and found that human and porcine bile induced higher levels of *G. lamblia* encystation than bovine bile. The porcine bile had a hyocholate (H), rather than cholate, while bovine bile had less chenodeoxycholate and more deoxycholate than human bile. In *Clonorchis sinensis* infection, the activity of bacterial beta-glucuronidase was significantly higher in bile ducts, is in favour of the formation of pigment stone, this may explain why clonorchiasis is often complicated with cholelithiasis (Gou, 1990). Moreover, Kim et al. (2008) reported that bile stimulated the expressions of genes in *C. sinensis* cultivation. Newly excysted juveniles produce the genes involve in the generation of energy which used for migrating into the bile duct and the genes involve in modulating the regulatory signals of cell proliferation associated with adult development. All bile acids and conjugated bile acids were found to enhance activity and favor on newly excysted juveniles. The survival rate of *C. sinensis* is also lower in media containing more than 0.1% bile and the worm in lower bile media survived longer in higher bile media excepted in LCA, which was cell toxic (Li et al., 2008). So bile acids may plays an important physiological role in lipid absorption and secretion, and regulation of bile acids biosynthesis and cholesterol homeostasis (John, Chiang, 1998) and may necessary for parasite development.