Chapter 6
Liver Flukes: *Clonorchis* and *Opisthorchis*

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6.1 History and Taxonomy

Opisthorchiid flukes most commonly occur in the bile duct, gall bladder, and liver of their mammalian and avian hosts [1]. They are small to medium sized with 33 recognized genera in the family Opisthorchiidae. These are divided into 13 subfamilies [1, 2]. Both of the genera *Clonorchis* and *Opisthorchis* fall within the subfamily Opisthorchiinae. *Clonorchis sinensis* from East Asia and *Opisthorchis viverrini* from the Lower Mekong Basin are currently recognized as the most important human pathogens. Both are involved in the development of human cholangiocarcinoma (CCA) and have been classified as class one carcinogens by the International Agency for Research on Cancer, a part of the World Health Organization [3].

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The presence of *C. sinensis* and *O. viverrini* in East and continental Southeast Asia, respectively, is strongly correlated with the incidence of CCA particularly in northeast Thailand where there is the highest incidence worldwide [3]. Although *Opisthorchis felineus*, which occurs from Europe across to eastern Siberia, has not yet been recognized as a carcinogen, there is evidence suggesting that this is the case [4]. It is, nevertheless, a pathogen of considerable significance in its own right [5].

Opisthorchis lobatus, a new species recently found in freshwater fish in Lao PDR, may also cause zoonoses, but its role in humans is not known, and it will not be dealt with here [6].

Clonorchis sinensis was first described by J.F.P. McConnell in the August 21st issue of the Lancet in 1875 from postmortem specimens collected from the bile duct of a Chinese seaman who died in Calcutta, India. On September 18th of the same year T.S. Cobbold wrote a short letter, also published in the Lancet, indicating that from McConnell's description of the trematodes it was undoubtedly a new species which he then named *Distoma sinense*. This name was later revised by Looss [7], as *Clonorchis sinensis*, the type species for the genus. At the time of its discovery, McConnell noted "The morbid anatomy of the liver in this case seems unequivocally to point to the presence of the flukes in its biliary ducts as the exciting cause of the acute and extensive structural degeneration of the proper structure of that organ, and of that cholaemic condition induced by the obstruction of the biliary channels which appears to have been the immediate cause of death." clearly pointing out the pathological significance of this species.

The discovery of *Opisthorchis viverrini* initially followed a quite different path. Poirier [8] was the first to discover and describe this species which had been retrieved from the biliary ducts of a fishing cat (*Prionailurus viverrinus*) which had been kept in the Zoological Gardens attached to the Paris Natural History Museum. It was only over 20 years later that Leiper [9] described the first specimens from humans supplied by W.F.J. Kerr from Chiang Mai in the north of Thailand. In 1916 Kerr published a parasitologically more detailed paper listing 17 % of the 230 adult male prisoners examined to be infected. Interestingly, 74 % of those examined were Laotians who had lived in the Chiang Mai area for their whole lives—there were very few Thais in the sample group. In this paper Kerr originally identified the species as *Opisthorchis felineus*, but in a postscript indicates that they were identified by Leiper as *O. viverrini*. About a decade after Kerr's paper, Prommas [10] reported the first case of infection (again as *O. felineus*) from the northeast of Thailand at Roi-et while Bedier and Chesneau [11] reported high prevalences of 25 and 15 % from Thakhek and Vientiane, respectively, in Lao PDR.

Gurlt was the first to describe and illustrate *O. felineus* (subfamily Opisthorchiinae), although he confused it with *Distomum conus* Creplin 1825 [4]. It was only in 1885 that *O. felineus* which had been isolated from cats were described as a valid species by Rivolta as *Distomum felineum* [12]. It was later moved from the genus *Distomum* to *Opisthorchis*, created by Blanchard in 1895. This is the type genus for the family Opisthorchiidae Looss, 1899. Opisthorchiasis in humans caused by *O. felineus* was first described by Vinogradoff [13] from Siberia.

6.2 Current Status and Geographical Distribution

Human populations show high levels of infection with all three liver fluke species within each of their distributional ranges. Up to 680 million people worldwide are at risk of infection [14]. Recent estimates indicate that 45 million people living in Asia and Europe are infected, with approximately 35 million *C. sinensis* cases, 10 million *O. viverrini* cases, and 1.2 million *O. felineus* cases [15, 16].

Clonorchis sinensis is the most frequent human parasite of the three with 600 million people at risk of infection [14] in East Asia from mid-Vietnam through much of China, including Taiwan, into Korea and the far east of Russia [17, 18]. Although *C. sinensis* was previously endemic in Japan, the last human case was in 1991 and no autochthonous case has been reported since [19]. The second most common species is *O. viverrini* which is found along the Lower Mekong and its tributaries in the north and northeast of Thailand, Lao PDR, Cambodia, and southern Vietnam. Data for the last two countries are sparse and for *O. viverrini* most of our information comes from the northeast of Thailand with an increasing list of publications from Lao PDR [20–22]. The information which is available suggests that as many as 67 million people may be at risk of infection [14].

Although *O. felineus*, the European liver fluke, is the most poorly studied of the three species it has been reported from continental European countries except Finland, Norway, and Sweden. It does not occur in the UK. Animal hosts are wild and domestic carnivores [4] but humans probably play a significant role in parasite transmission [21]. Human cases have been reported to occur in Belarus, Germany, Greece, Italy, Poland, Romania, Russia, Spain, the Ukraine, the Baltic countries, Moldova, and Kazakhstan, but records from some of these countries are over 50 years old [4, 5, 23]. *O. felineus* represents a significant health problem in certain areas of Siberia where evidence indicates that its prevalence in both humans and in animals increases from west to east [23]. In the Ob-Irtysh basin, where the prevalence of infection peaks, it is of particular medical significance [23].

Although clonorchiasis is endemic to East Asia and opisthorchiasis to the Mekong area of Southeast Asia and parts of eastern Russia all can occasionally be found in non-endemic areas having been introduced by infected tourists, refugees or workers who have moved from endemic to non-endemic areas [16, 24–27]. The flukes have, however, not currently become endemic in these areas due to the lack of suitable intermediate hosts.

6.3 Biology and Life Cycle

Liver flukes are hermaphroditic trematodes which are dorsoventrally flattened. The body has an oral sucker situated anteriorly and a ventral sucker at mid-body. The differentiation of the species is based on morphology. The adult worms differ in the shape and position of their testes and the arrangement of the vitelline glands.

Fig. 6.1 Adult worms of the liver flukes. (**a**) *Opisthorchis viverrini* (**b**) *Clonorchis sinensis*

C. sinensis can be separated from the other two species by the presence of branched testes in a tandem position and the continuously distributed vitelline glands (Fig. 6.1). Although *O. viverrini* is similar to *O. felineus* in having lobed testes and a cluster vitelline gland, it differs by having deeper lobulation of and greater extremity of the testes and also lacks transversely compressed patterns of vitelline follicles. The size of the adult flukes depends on the species involved. *O. viverrini* is the smallest, measuring 5.5–10×0.77–1.65 mm. *O. felineus* is somewhat larger measuring 7–12×2–3 mm [5, 28] while *C. sinensis* is the largest measuring 10–25×3–5 mm. Variation in the size of the adults is density dependent, with individuals being smaller the higher the infestation, and also dependent on the diameter of the bile duct they inhabit [29].

The eggs of *C. sinensis*, *O. felineus*, and *O. viverrini* are morphologically similar making them difficult to distinguish from one another. The operculum of each species has a distinct shoulder while a small knob or comma shape appendage is found at the abopercular end [28]. The surface of the egg shell is rough and irregular, having been described as having a "musk-melon pattern" by scanning electron microscopy [30].

The transmission cycle of all three liver fluke species goes through three phases: (1) the infection of the snail first intermediate hosts via host feces, (2) cercarial release and finding fish second intermediate host for development of the infective metacardariae stage, and (3) ingestion of metacercariae in raw or partially cooked fish by humans. Petney et al. [21] argue that the three species

differ in terms of the relative significance of the zoonotic and anthroponotic components of the epidemiological cycle with *O. viverrini* having mainly human final hosts, *O. felineus* in Europe mainly wild carnivore hosts and *C. sinensis* and *O. felineus* in its Asian range a mixture of the two. This has considerable epidemiological significance, particularly in control and prevention programs.

The life cycles of all three species are very similar with a snail first intermediate host with usually low prevalences of infection, a fish second intermediate host with substantially higher levels of infection, and usually a carnivorous mammal as final host [31, 32]. The low prevalences of infection in the snail first intermediate hosts are, at least in part, compensated for by the often very high prevalences in fish, the infective phase for humans.

The distribution of each species is closely related to that of their hosts. This is particularly true of the snail first intermediate hosts which tend to be more restricted in the number of species used [17, 33, 34]. For *O. viverrini* the presence of snails of *Bithynia siamensis goniomphalos* in nearby freshwater sources is a prerequisite for the presence of the parasite in the human population [34, 35].

The importance of fecal contamination of fresh water sources inhabited by intermediate snail hosts by wild and domestic animal hosts (i.e., the zoonotic cycle) varies greatly between the three fluke species. In its European range fecal matter containing *O. felineus* eggs is almost exclusively found in wild and domestic carnivores. In areas with widespread human infection, fecal contamination of freshwater by infected animals is certainly reduced and in the case of *O. viverrini* probably only plays a minor role in infecting snails.

Clonorchis sinensis is a species with a relatively broad range of hosts sharing anthroponotic and zoonotic components in its epidemiological cycle [21]. *C. sinensis* is known to use eight main snail species as intermediate hosts. These come from five different families (Assimineidae, Bithyniidae, Hydrobiidae, Melaniidae, and Thiaridae) [19]. There is some geographic differentiation in the distribution and prevalence of *C. sinensis* in the different snail species. Prevalences vary locally and may be as high as 27 % for *Alocinma longicornis* in parts of Guangdong and 8 % for *Bithynia fuchsianus* in parts of Guangxi, although most values are substantially lower [19].

Once the snail has ingested the embryonated eggs, which are passed in the feces of the final host, the eggs hatch to release miracidia which then undergo development to sporocysts then rediae and finally cercariae in the snail. The tailed cercarial stage escapes from the snail host and actively swims to find a suitable fish second intermediate host which it then penetrates, losing its tail and encysting to become a metacercaria embedded either in the muscles of the fish or under the scales.

C. sinensis utilizes predominantly fish in 11 families, with 46 genera and 132 species, of which 32 genera and 71 species belong to the family Cyprinidae, but also several crustacean second intermediate hosts Lun et al. [19]. The fish include a number of species which are commonly used in aquaculture fish, including the common carp (*Cyprinis carpio*), the grass carp (*Ctenopharyngodon idellus*), the silver carp (*Hypophthalmichthys molitrix*), as well as tilapia (*Oreochromis mossambicus*) [19, 36–38].

The mammalian final hosts are infected when they eat raw fish containing the metacercariae of *C. sinensis*. This fluke has an unusually broad final host spectrum which includes human associated species such as cats and dogs, stock animals such as pigs and the brown rat (*Rattus norvegicus*), all of which are effective reservoir hosts, as well as a wide variety of wild fish eating carnivores and occasionally birds [19, 39]. Nevertheless, humans are considered to be the most important final host [19].

After being eaten, the metacercariae excyst in the host's duodenum and then move through the hepatopancreatic ampulla into the biliary ducts and towards the liver. Here, they attach to the mucosal lining and develop into hermaphrodite adults. These begin reproduction after about 3–4 weeks and may remain viable for years [18]. Attwood and Chou [40] reported that parasites survive up to 26 years in an infected human. In the case of humans, infection occurs through the deliberate ingestion of raw, partially cooked or fermented fish containing the infective metacercariae as part of tradition a food culture [41].

Although there are varying estimates of the number of humans infected with *C. sinensis* it appears that this number is increasing [16]. This is particularly the case in China where prevalence was estimated to be 4.7 million in the early 1990s to almost 13 million today [16, 42]. In the Republic of Korea the picture is variable with estimates of 4.6 % in 1971, 1.4 % in 1997, and 2.4 % in 2004 [43]. Unfortunately, few long-term records are available from Vietnam.

In addition to its direct influence on human health, *Clonorchis* infection in the second intermediate host can also reach substantial prevalences in aquaculture fish potentially limiting profitability in the aquaculture industry [14]. Chen et al. [44] found that freshwater fish in aquaculture had a 37.09 % prevalence of infection with a mean number of 10.7 cercariae/fish, while 3.07 % of shrimps from freshwater ponds carried on average a single metacercaria.

Molecular methods will probably extend our knowledge of the distribution of opisthorchid parasites. Traub et al. [45], using PCR-based technology, detected *C. sinensis* eggs in 23 % (5/22) of human feces which tested positive by microscopy as well as PCR for *Opisthorchis* like eggs. The samples came from a rural community in Eastern Thailand (Chachoengsao Province) extending the known range of *C. sinensis* substantially to the south and east of its recognized distribution in an area where it is sympatric with *O. viverrini*. Whether *C. sinensis* overlaps elsewhere with *O. viverrini* or *O. felineus* is currently unknown. *C. sinensis* is known to infect a variety of mammalian hosts including domestic dogs, cats, and pigs [18]. Dogs and cats can have high prevalences of infection (0.8–48.5 % in dogs, 0–64.1 in cats) which, however, varies considerably between endemic areas in China [17]. The higher prevalence in cats, as for *O. viverrini*, is probably due to their preference for eating fish. Pigs, which are omnivorous, had a prevalence of infection of 27 % in southern China [46].

O. viverrini is known only from three currently recognized taxa within a single snail genus, *Bithynia funiculata*, *B. siamensis goniomphalos*, and *B. s. siamensis*, from Southeast Asia [34]. In Thailand, all three taxa of *Bithynia* are found, namely, *B. funiculata* in the north, *B. s. siamensis* in the center, and *B. s. goniomphalos* in the northeast [47]. No regional separation of *Bithynia* snails has been reported in other parts of Southeast Asia, probably due to insufficient surveys [48].

Table 6.1 List of first intermediate hosts in Southeast Asia infected by cercariae of *O. viverrini*

Country	Snail	Sample size	% Prevalence	References
Thailand	*Bithynia funiculata*	352	0.30	[270]
	B. siamensis siamensis	2,800	1.60	[271]
	B. s. goniomphalos	5,729	0.45	[272]
	B. s. goniomphalos	1,382	3.04	[33]
	B. s. goniomphalos	537	0.37	[273]
	Bithynia snails	18,078	0.13	[51]
	B. s. goniomphalos	4,874	0.61–1.30	[274]
	B. s. goniomphalos	N/A	0.14	[275]
	B. s. goniomphalos	6,150	0.05	[276]
	B. s. goniomphalos	48,327	0.07	[50]
Cambodia	*B. s. siamensis*	406	0.25	[277]
Lao PDR	*B. s. goniomphalos*	3,142	2.01	[33]
	B. s. goniomphalos	81	2.47	[278]
	B. s. goniomphalos	3,913	0.60	[239]
	B. s. goniomphalos	2,000	0.95	[279]

The prevalence of *O. viverrini* infection in *Bithynia* snails is variable, with numerous collections being parasite free. If the parasite is present, cercarial release commonly occurs from about 0.1 to 2 % of individuals, but some collections have infection rates of 6–9 % (Table 6.1) [33, 34]. Snail population density is strongly seasonal, being highly abundant later in the rainy season, when reproduction occurs. At this time the *Bithynia* are extensively distributed in shallow water and rice fields. They can be found at a depth of at least 3 m, albeit in a much lower density [49]. During the dry season the population density crashes and the snails which survive are often found buried in the mud for seasonal aestivation [34, 50].

The snails are infected by ingesting the embryonated eggs of the parasite which are excreted with fecal matter. Indeed, human fecal bacterial contamination of fresh water bodies can act as an indicator for the seasonal transmission of *O. viverrini* eggs to snail intermediate hosts [51]. Once ingested, the eggs hatch to a miracidium which in turn develops to a sporocyst [28]. Once the sporocyst has developed within its snail host, it gives rise to numerous rediae which in turn produce numerous pleurolophocerous cercariae. After being released, they actively seek an appropriate fish second intermediate host, in the case of *O. viverrini* these belong to the family Cyprinidae [23, 32, 47].

The process of host finding by cercariae is complex. Free-swimming cercariae are very efficient at actively locating the appropriate species of fish in a large volume of water [52]. The intensity of infection in fish varies by season, species, individuals, and types of water bodies. Most metacercariae are distributed throughout the body of fish with some being found in the head. For *O. viverrini*, metacercarial burdens peak in winter (October–February) and become low in the rainy season and summer; thus, transmission of the parasite from fish to humans is probably seasonal.

Many cyprinid fish species have been reported as potential hosts for *O. viverrini* [16]. In Thailand, Lao PDR and Cambodia, at least 40 species of fish from 18 genera have been reported to serve as intermediate hosts of *O. viverrini*. Of these, the genera *Cyclocheilichthys*, *Puntius*, and *Hampala* are considered to be the most important [53]. For *O. viverrini*, the prevalence of infection in the fish second intermediate host is very much higher than in the *Bithynia* snail first intermediate hosts (Table 6.2). This is, however, fish species and locality dependent and ranges from 2.1 to 100 % [54]. For example, 30.4–97.1 % prevalence was found in *C. apagon* [55], 43.1–100 % in *C. armatus* [56, 57], 69.9–93.7 % in *P. leiacanthus* [58], and 33.3–74.1 % in *H. dispar* [56, 57]. The average number of metacercariae infecting fish varies from one to thousands with the highest intensity (average 1,989.8/fish) in *C. armatus* from Savannakhet, Lao PDR [57].

Humans are the dominant hosts for *O. viverrini*, while other domestic mammals, for instance dogs and cats, can act as reservoir hosts [47, 59]. Domestic cats have been found to have a relatively high prevalence of 35.5 % in the northeast of Thailand, making them potentially significant zoonotic sources of the disease during human based control programs [59]. Dogs have a much lower prevalence of 0.37 % [59]. Hamsters, rabbits, and guinea pigs are experimentally highly susceptible to infection [16]. There is no current information for native mammals and other fish-eating animals which may be also infected.

For *O. viverrini*, when fecal contamination from humans is reduced or eliminated by mass treatment and/or improved sanitation the prevalence of infection can be substantially reduced [60, 61]. However, complete elimination of infection may not be possible if domestic cat and dog reservoir hosts maintain the source of parasite and hence play a critical role in maintaining the life cycle. Based on the pattern of age-intensity profiles, *O. viverrini* may survive for 10–20 years [22].

In the case of *C. sinensis* or *O. viverrini*, both are known to cause significant health problems [47]. For these two species considerable epidemiological information is available: e.g., prevalence and intensity of infection increase with age and males tend to have a higher prevalence of infection than females [47]. Thus, hepatobiliary morbidity is more frequent in older individuals and males than in younger people and females. In addition, as is usual with many parasite species, the distribution in the host population is neither random nor uniform, but overdispersed with a few individuals harboring most of the worms [62]. Interestingly, some individuals appear to be predisposed to a heavy infection, with the intensity of infection returning to pretreatment levels after treatment [63]. Such epidemiological characteristics must be incorporated into the models on which control and eradication programs are based.

The infective metacercariae of all species discussed here are found only in their specific (mostly cyprinid) fish second intermediate hosts. These fish, when eaten raw, fermented or undercooked, act as the source of infection to humans and animal hosts [41]. Traditional dishes based on raw fish are the main sources of infection of *C. sinensis* throughout its range, for example, goi ca mai (raw fish salad) and slices of raw silver carp in Vietnam; yusheng, a raw fish salad in China; and sushi, sliced raw fish, in Korea and Japan [18]. The length of time which the metacercariae remain viable depends of the method of preparing the food.

Table 6.2 List of second intermediate hosts (Family Cyprinidae) in Southeast Asia infected by metacercariae of *O. viverrini*

Fish	% Prevalence	Country	References
Labiobarbus siamensis	51.3–100	Thailand, Cambodia	[277, 280, 281]
Cyclocheilichthys armatus	19.16–100	Thailand, Cambodia, Lao PDR	[53, 56, 57, 252, 280–286]
Hampala macrolepidota	2.6–100	Thailand, Cambodia, Lao PDR	[55, 239, 279, 283, 287–289]
Amblyrhynchichthys truncatus	100	Cambodia	[281]
Neolissochilus stracheyi	100	Lao PDR	[286]
Lobocheilus melanotaenia	100	Lao PDR	[288]
Puntius partipentazona	100	Thailand	[58]
Cyclocheilichthys apogon	25.0–97.1	Thailand, Cambodia, Lao PDR	[55, 58, 281, 286, 290]
Hampala dispar	6.49–94.80	Thailand, Cambodia, Lao PDR	[55–58, 239, 252, 277, 279, 281–283, 285, 286, 289–291]
Puntius brevis (*Puntius leiacanthus*)	14.0–93.7	Thailand, Cambodia, Lao PDR, Vietnam	[55, 57, 58, 239, 277, 281, 285, 286, 288, 292]
Poropuntius laoensis	90.0	Lao PDR	[288]
Puntius orphoides	31.0–90.0	Thailand, Cambodia, Lao PDR	[55, 252, 281, 282, 290, 291, 293]
Cyclocheilichthys repasson	10.0–80.0	Thailand, Cambodia, Lao PDR	[56, 57, 239, 277, 279, 283, 289]
Cyclocheilichthys enoplos	2.1–80.0	Cambodia, Lao PDR	[55, 56, 286, 288]

(continued)

Table 6.2 (continued)

Fish	% Prevalence	Country	References
Esomus metallicus	10.0–75.0	Thailand, Lao PDR	[57, 252]
Labiobarbus lineatus	3.0–69.6	Thailand, Lao PDR	[56, 252]
Barbonymus altus	30.0–66.7	Cambodia	[55, 281, 291]
Barbonymus schwanenfeldii	66.0	Cambodia	[281]
Puntioplites proctozysron	2.0–60.0	Thailand, Cambodia, Lao PDR	[55, 56, 252, 281–283, 286, 291, 293]
Thynnichthys thynnoides	3.7–59.7	Thailand, Cambodia	[55, 281, 287]
Cyclocheilichthys lagleri	58.2	Cambodia	[281]
Hypsibarbus lagleri	50.0	Lao PDR	[57]
Mystacoleucus marginatus	50.0	Lao PDR	[57]
Onychostoma elongatum	44.4	Lao PDR	[57]
Henicorhynchus lineatus	42.9	Lao PDR	[56]
Labeo chrysophekadion	40.0	Cambodia	[277, 293]
Oreichthys parvus	40.0	Lao PDR	[288]
Henicorhynchus lobatus	33.3	Cambodia	[281]
Hypsibarbus pierrei	33.3	Lao PDR	[286]
Hypsibarbus wetmorei	33.3	Lao PDR	[286]
Poropuntius deauratus	33.3	Lao PDR	[286]
Puntioplites falcifer	33.3	Lao PDR	[57]

Rasbora ourotacniatiran	33.3	Lao PDR	[288]
Osteochilus waandersii	30.5	Lao PDR	[56]
Carassius auratus	28.1	Vietnam	[292]
Cyclocheilichthys furcatus	25.0	Cambodia, Lao PDR	[286, 291]
Puntius viehoever	22.0	Thailand	[252]
Osteochilus hasselti	6.1–20.0	Thailand, Cambodia, Lao PDR	[57, 277, 281, 282, 291]
Puntius stoliczkanus	16.67	Thailand	[294]
Barbonymus gonionotus	2.0–16.1	Thailand, Cambodia, Lao PDR	[53, 239, 252, 279, 281, 287, 289, 291]
Onychostoma fusiforme	14.3	Lao PDR	[286]
Henicorhynchus siamensis	4.3–10.9	Thailand, Cambodia	[55, 281, 282]
Paralaubuca barroni	10.0	Lao PDR	[286]
Crossocheilus reticulatus	5.6	Cambodia	[281]
Rasbora spp.	4.3	Vietnam	[292]
Osteochilus sp.	4.0	Thailand	[252]

In Southeast Asia, particularly in Thailand and Lao PDR, the raw or partially cooked fish dishes which act as the source of the liver fluke infection can be grouped into three categories. Fresh raw fish dishes without heating are called "koi pla." These pose a high risk of infection. A moderate risk is presented by quickly fermented dish (1–2 days) known as "pla som." The last dish is fermented fish known as "pla ra" which normally requires long-term fermentation, but short-term and variable ingredients may provide favorable environments for metacercarial survival. Pla ra is a common ingredient for cooking in Southeast Asia, for example in papaya salad (som tum) [41]. In Lao PDR, the fermented fish is known as "pla dak." In other countries such as Cambodia raw fish are prepared as "pla hoc" which is similar to "pla som" and this may serve as a source of infection. The usual sources of *O. felineus* infection in Russia are dried or salted fish. Other dishes include sliced raw fish (stroganina), which is popular among native Siberians, and fish pickled in vinegar.

The number of *Bithynia* species present in the Eurasian area has changed from a single species, *Bithynia leachi*, to four morphologically similar species, *B. inflata*, *B. leachi*, *B. troscheli*, and *B. sibirica* [64] of which the first three of these can act as intermediate hosts for *O. felineus* [23]. As with *O. viverrini*, cyprinid fish also act as the exclusive second intermediate hosts for *O. felineus* [4]. These include the ide (*Leuciscus idus*), roach (*Rutilus rutilus*), European dace (*Leuciscus leuciscus*), tench (*Tinca tinca*), verhovka (*Leuciscus delineatus*), and silver crucian carp (*Carassius auratus gibelio*) [23]. After an outbreak of *O. felineus* infection in Italy, 83.1 % of tench from Lake Bolsena were found to be infected [65]. Apart from this outbreak humans are seldom infected in Europe probably because raw fish is not common in the human diet in this area [5]. However, prevalences can be very high in humans in the Asian distributional area of this species where raw fish are more commonly consumed [66].

In Europe, domestic cats and dogs can act as hosts [4, 67] in addition to a wide range of wild carnivores. These include several fox species, the raccoon dog and wolves, as well as *Martes* and *Mustela* spp., badger, otter and wolverine [4, 68]. Unlike *C. sinensis* and *O. viverrini*, *O. felineus* has also been reported from the Pinnipedia: the Caspian seal (*Pusa caspica*) from the brackish Caspian Sea, the bearded seal (*Erignathus barbatus*) from the Arctic, and the grey seal (*Halichoerus grypus*) [4, 23]. Non-carnivore and therefore presumably accidental hosts include chipmunks (*Eutamias sibiricus*) [23], beaver (*Castor fiber*), European water vole (*Arvicola terrestris*) and the brown rat (*Rattus norvegicus*), rabbit (*Oryctolagus cuniculus*), and wild and domestic pig (*Sus scrofa*) [4, 23].

6.4 Molecular Biology, Genetics, and Evolution

Advances in molecular biology have provided opportunities for increasing our understanding of the developmental and reproductive biology, as well as genetic diversity and evolution, of medically important trematodes including *Opisthorchis* and *Clonorchis*. They have also helped us to understand host–parasite interactions

and the pathogenesis of the diseases caused by these flukes. This has aided in the improvement of diagnostic methods, new drug treatments and potentially vaccine development. In addition, it is likely that new molecular data will help in examining theories on the molecular basis of chronic clonorchiasis and opisthorchiasis induced CCA. Below we will also discuss the cytogenetics, genomics, transcriptomics, and proteomics of these species.

6.4.1 Cytogenetic Analysis

The cytogenetic study of the liver flukes focuses on the study of the structure and function of the chromosomes. It includes the analysis of G-banded chromosomes, other cytogenetic banding techniques, as well as molecular cytogenetics, such as fluorescent in situ hybridization (FISH), and comparative genomic hybridization (CGH). The karyotypes of *C. sinensis* are reported to be either $2n=14$ (samples from the far east of Russia) [69] or $2n=56$ (samples from Korea and China) [70]. The $2n=56$ karyotype was described according to the number and morphology of chromosomes, probably being the octaploid form of typical opisthorchiid karyotype with $2n=14$ [69]. The karyotypes from Russia consisted of pairs of large metacentric and submetacentric chromosomes and five pairs of small chromosomes [69]. Those from Korea and China could be divided into two groups based on their sizes, with 8 pairs of large and 20 pairs of small chromosomes. They have the same number of 16 submetacentric and 8 subtelomeric pairs, but the Korean isolates have 3 metacentric and 1 metacentric/submetacentric pairs, whereas the Chinese isolates have two and two pairs. In addition, the mean total length of the diploid complements of the specimens collected in China is slightly longer than that of those collected in the Korea [70]. The question of the phylogenetic relationship between *C. sinensis* from Russia, China, and Korea therefore remains open. The liver fluke described in China and Korea could be an octaploid form of normal *C. sinensis* or possibly distinct species [69].

The karyotype of *O. felineus* collected from West Siberia was $2n=14$ and consisted of two pairs of large submetacentrics and five pairs of small chromosomes. There are three pairs of metacentric and four pairs of submetacentric chromosomes [71]. A comparison of the relative length and centromere indices of the chromosomes of these *O. felineus* did not reveal significant differences [71].

In contrast, the karyotype of *O. viverrini* is $2n=12$ and includes two pairs of large-submetacentrics, one pair of medium-sized submetacentrics, one pair of small-sized subtelocentrics or acrocentrics, and one pair of small-sized acrocentric chromosomes [72]. The medium-sized submetacentric chromosome of *O. viverrini* is probably the result of the fusion of two chromosomes from ancestral karyotypes [69]. However, the comparative analysis of mitotic and meiotic chromosomes by heterologous FISH revealed six pairs of chromosomes in the karyotype of *O. viverrini*, indicating a relatively recent chromosomal fusion event which took place in the formation of the modern karyotype [69]. However, none of the *O. viverrini*

chromosomes have shown any interstitial telomere sequences (ITSs) after FISH by telomeric DNA probe or PNA telomere probe [73]. More recently, the chromosome number, length and nomenclature of each chromosome were determined by scanning electron microscopy. The six chromosomes consist of one large metacentric, one medium-sized metacentric, two small-sized metacentric, one small-sized submetacentric, and one small-sized acrocentric chromosomes [74]. Moreover, the repetitive sequences show that despite the small size of *Opisthorchis* genomes, a large amount of interspersed repetitive DNA sequence is distributed along the euchromatic regions [75].

6.4.2 Genome

There are some reports on the genomic features characterizing *C. sinensis*, but very few on *O. viverrini* and *O. felineus*. The assembled genome of *C. sinensis* has a total size of 516 Mb with approximately 16,000 reliable protein-coding gene models. Genes for the complete pathways for glycolysis, the Krebs cycle and fatty acid metabolism were found, but key genes involved in fatty acid biosynthesis are missing from the genome, reflecting the fact that the liver fluke receives lipids from the bile of its host. Moreover, genes encoding proteases, kinases, and phosphatase enzymes, tegument and excretory–secretory products, host-binding proteins and receptors were also discovered. In addition, 53 genes related to sex determination, sex differentiation, and sexual reproduction were identified [76]. The genome of *C. sinensis* contained more than 100 copies of a long terminal-repeat retrotransposon (*CsRn1*) which belongs to the Ty3/gypsy-like long-terminal-repeat transposon family. The functional domains of Gag, proteinase, reverse transcriptase, RNase H, and subdomains of integrase are strongly conserved in *CsRn1*, which has been predicted to be mobile element based on structural considerations and from the presence of mRNA transcripts [77]. Insertions of *CsRn1* appear preferentially at repetitive and agenic chromosomal regions. Furthermore, *CsRn1* was reported to induce variations in the genome that may influence the evolution of *C. sinensis* [78]. The finding of such genomic characters of *C. sinensis* reveal the evolutionary interplay between parasite and host, which may be valuable for understanding host and parasite interactions [76]. In the case of *O. viverrini*, the estimated genomes size reported by real-time PCR was 75.95 Mb [79]. However, whole-genome sequencing of these liver flukes has not been reported.

The complete mitochondrial genomes of all three liver flukes have been successfully characterized. The mtDNA sequences of *O. viverrini*, *O. felineus*, and *C. sinensis* were variable and ranged between 13,510 and 14,277 bp and comprised 36 genes [80, 81]. Of these, 12 genes encoded for proteins, i.e., cytochrome c oxidase subunit 1 (CO1), CO2, CO3, NADH dehydrogenase subunit 1 (ND1), ND2, ND3, ND4, ND4L, ND5, ND6, cytochrome b, and ATPase subunit 6. Two genes encoded for ribosomal RNA, i.e., small subunit rRNA (*rrn*S) and large subunit rRNA (*rrn*L). The number of tRNA encoding genes varied between the different species, i.e., 20 genes for

O. viverrini and 22 genes for *O. felineus* and *C. sinensis* [80, 81]. The gene content and arrangement were almost identical between species. There were two noncoding regions, the long noncoding region (LNR) and the short noncoding region (SNR). For the *C. sinensis*, there is a lack of tandem repeat [80], whereas there was tandem repeat, interrupted in LNR region, in *O. felineus*. Moreover, when comparing the length of noncoding region of the mtDNA of *C. sinensis* from Russia and Vietnam there were significant differences between species [81].

6.4.3 Transcriptome

Expressed sequence tags (ESTs) for adult *C. sinensis* and *O. viverrini* have been reported with at least 3,000 and 4,194 ESTs, respectively, and have been registered in public dbEST databases [82, 83]. The most abundant genes in adult *C. sinensis* include cysteine proteases and mitochondrial genes, which may support biliary epithelia destruction by adult liver flukes to evade host immune attack [84, 85]. The second most abundant gene transcripts were proteins constituting muscular tissues, which enable adult flukes to abrade and feed on the biliary epithelium [86]. Vitelline precursor protein was the third most abundantly expressed gene product. It is responsible for hardening the eggshell encasing the germ cell and surrounding yolk cells [87].

The ESTs of the metacercarial stage of *C. sinensis* could be assembled into 322 genes. Those expressed most abundantly were for proteases and metabolic, transcription, and translation housekeeping proteins [82]. To obtain an insight in the developmental gene expression and regulation of *C. sinensis*, the adult and metacercariae ESTs of *C. sinensis* were compared. It was found that genes encoding structural and cytoskeletal proteins, transcription and translation machinery proteins, and energy metabolism-related proteins were highly expressed in *C. sinensis* metacercariae, while the other genes were highly expressed in the adult stage. These data may explain, to some extent at least, that *C. sinensis* metacercariae in fish hosts have a quite different physiology and metabolism compared with adult *C. sinensis* in mammals [82].

The most abundant genes in adult *O. viverrini* encoded for myoglobin, vitelline precursors, egg shell proteins, and glutathione-*S*-transferase. The other abundantly expressed genes encoded proteins involved in host–parasite relationships and included proteases, saposin-like proteins, and dynein light chains [88]. Homologues of some of the most abundantly represented proteins in *C. sinensis* and *O. viverrini* ESTs are cysteine protease, myoglobin, and vitelline B precursors, whereas others were overexpressed in each species [88]. The open reading frame (ORF) region in ESTs was also used to predict the expressed proteins in proteomic analysis. Such ORFs were generated from 4,194 available EST sequences of *O. viverrini* and subsequently analyzed for secretory signal sequence and the transmembrane domain. A total of 897 potential ORFs were identified, of which 78 were predicted to contain a secretory signal sequence and 42 to contained two or more transmembrane domains [83]. Proteases were highly presented in the *O. viverrini* transcripts

encoding secreted proteins, with five different cathepsins, a legumain, and an S1 type serine protease all predicted to contain a signal sequence [83]. In addition, more than 50 % of the predicted protein sequences of *C. sinensis* and *O. viverrini* were inferred to be homologues, reflecting their relatively close biological and physiological relationships [89]. Comparison of the predicted proteins of liver flukes and other trematodes, *S. japonicum*, *S. mansoni*, *F. hepatica* found that 29–31 % protein sequences were homologous [89]. However, this prediction of expressed proteins may not be satisfactory as some ORF encoded sequences might not express or express a low protein level. Thus, additional proteomic analyses are needed to provide more informative data on the proteins expressed in these liver flukes.

6.4.4 Proteome

Proteomic analyses should provide information on potential new and specific targets for treatment the infection. Moreover, the identification of parasitic-specific proteins could clearly facilitate the design of new tools for rapid and cheap diagnosis, which in turn could help breaking the transmission cycle of the parasite, as well as help in the identification of potential targets for vaccination, one of the best ways to control these parasite infections [90].

The adult stage of all three flukes dwells in the bile duct which provides an anaerobic environment where a large amount of exogenous glucose is used as a carbon source for energy metabolism. Thus, the most important endogenous proteins examined in the liver flukes are glycolytic enzymes which play an important role in the glycolysis pathway. Phosphoglycerate kinase (PGK), a glycolytic enzyme, was found extensively, localized in the muscular tissues of the oral and ventral suckers, ovary, testes, tegument, and intrauterine eggs of *C. sinensis* [91, 92]. The inhibitors of several glycolytic enzymes of *C. sinensis* have also been reported, e.g., vanadate can inhibit phosphoglycerate mutase (PGM) [93], whereas lactase dehydrogenase (LDH) was inhibited by Cu^{+2}, Fe^{+2}, and Zn^{+2} [94]. The cytosolic and mitochondrial malate dehydrogenases (cMDH and mMDH) of *C. sinensis* share low amino acid sequence homology (22 %) and these enzymes are differentially inhibited by 4.4′-bisdimethylamino diphenylcarbol. cMDH is more stable against heat and acidity than mMDH. Moreover, cMDH plays a pivotal role on the cytosolic side of the malate–aspartate shuttle. mMDH is a key enzyme in the tricarboxylic acid cycle and in the malate–aspartate shuttle. Thus, these glycolytic enzymes are required for the survival and pathogenesis of these liver flukes [95–97].

The mechanism of pathogenesis due to liver fluke infection mainly involves the interaction between parasite antigens and the host immune response [98]. Therefore, the excretory–secretory (ES) proteins and tegumental proteins of the liver fluke play crucial roles in host–parasite interactions, pathogenesis, and disease outcomes. Myoglobin is an abundant protein in the ES products of *C. sinensis*. It may play an oxygen-capturing role and then slowly release this oxygen to metabolic pathways in bile duct. Recombinant myoglobin reacted with the sera of *C. sinensis*-infected

rabbits and clonorchiasis patients. Lysophosphatidic acid phosphatase (LPAP), belonging to the acid phosphatase family, has been identified as an ES-antigen in adult *C. sinensis*. It shows high sensitivity and specificity in the serodiagnosis of human clonorchiasis [99]. The proteomic analyses by the 2D proteome mapping of *C. sinensis* ES products identified 62 protein spots including thioredoxin peroxidase, myoglobin, and a number cysteine proteases that were expressed abundantly [100]. More recently, Zheng et al. [101] have reported a proteome analysis of ES products of *C. sinensis* using LC-MS/MS analysis and found 110 proteins including 39 known functional proteins and 71 unknown proteins. The enzyme fructose 1,6-bisphosphate (Cs-FPBase) within the ES product was a potential causative agent of hepatic fibrosis [101].

A comparative proteomic analysis of the developmental stages from juvenile to adults of *O. viverrini* was made by 2D gel electrophoresis [102]. The total number of protein spots ranged between 210 and 239 according to the age of the worm (1–4 weeks). Only small differences in the pattern of protein spot were found during parasite maturation [102]. The secreted and surface-exposed proteomes of *O. viverrini* has also been reported [83]. The secretory proteins were analyzed using peptide OFFGEL electrophoresis (OGE) and (multiple reaction monitoring) MRM. A total of 25 proteins, 13 from MS/MS analysis of OGE and 12 from MRM identifications, were positively identified as constituents of Day 1 ES (in vitro culture of worms), whereas the remaining 18 proteins identified in Day 17 ES are a likely consequence of the culturing process [83]. Proteases were abundant, but proteolytic enzymes were underrepresented in the ES of *O. viverrini*. However, the prediction of the secretory proteins from a signal sequence or based on a transmembrane domain in the ESTs found 26 known proteins and 39 unknown proteins to be secretory proteins. Of these, only five, i.e., cathepsin F-like cysteine protease, cathepsin D, venom allergen-like protein 8, cystatin, and granulin were detected by proteomic identification [83]. Granulin, which is a homologue of human granulin, is a potent growth factor involved in cell proliferation and wound healing. A granulin of *O. viverrini* (*Ov*-GRN-1) was examined and found to be expressed in most parasite tissue, particularly the gut and tegument. *Ov*-GRN-1 is probably the major growth factor protein in the ES products secreted by *O. viverrini* that can induce the proliferation of host cells which may ultimately manifest in CCA [103].

The tegumental syncytium, which is the outermost surface of liver flukes, is considered to be very important for host response and parasite survival. Thus, it is generally seen as the most susceptible target for vaccines and drugs. Several tegumental proteins of the liver flukes have been characterized and identified. The tegumental protein of *C. sinensis*, CsTP31.8, has been proven to be an antigenic protein [104]. CsTP20.8 is expressed in adult worms and metacercariae but not at the egg stage. However, CsTP20.8 protein is considered to have limited valuable for the serodiagnosis of clonorchiasis because it shows only moderate sensitivity and although it has high specificity [105]. Another tegumental protein CsTP21.1 was identified from adult *C. sinensis* by bioinformatics analysis. It is localized in the tegument of adult worms [106]. Interestingly, CsTP21.1 is considered a trematode–nematode pan-specific antigen that could be useful for the development of a universal immunodiagnostic kit for human infection with trematodes and nematodes [106].

Membrane-spanning proteins of *O. viverrini* are predicted to include 28 known proteins belonging to the transporters/channels, protease/hydrolytic enzymes, structural/membrane organization proteins, and other miscellaneous proteins, as well eight unknown proteins based on transmembrane domains. However, proteomic identification found only four membrane proteins, i.e., ATP–ADP antiporter, Sm-TSP-2, succinate dehydrogenase, and succinate dehydrogenase complex subunit C expressed in *O. viverrini* [83].

6.4.5 Vaccine Development

It is clear that a vaccine against any of the major human pathogens within the Opisthorchiidae would greatly aid control measures, although progress in research in this direction, which has been under way for some time has been limited [107]. The goal for the development of a vaccine against *O. viverrini* and/or *C. sinensis* is not only to limit the pathologic sequels due to acute and chronic infections, but, as is the case with other carcinogenic pathogens such as human papilloma virus, it could also protect against cancer [108]. Genomic studies on both *O. viverrini* and *C. sinensis* may well facilitate the development of vaccines with time. As indicated above, the tegument is generally viewed as the most susceptible target for vaccines and drugs in liver flukes because it is a dynamic host-interactive layer with roles in nutrition, immune evasion and modulation, pathogenesis, excretion and signal transduction [109, 110]. Large proteins such as multifunctional secreted proteases and tegumental proteins have been identified as potential targets for the development of drugs and vaccines [76].

DNA vaccines against *C. sinensis* were produced by encoding cysteine proteinase and a fatty acid-binding protein and tested in a rat model. They showed 31.5 and 40.9 % protection efficacy, respectively [111]. In addition, an oral vaccine using *C. sinensis* tegumental protein 22.3 kDa fused with the *Bacillus subtilis* spore coat CotC showed 44.7 % protection in the rat model [112]. An *O. viverrini*-crude somatic antigen (CSAg) administered with complete Freund's adjuvant or alum was used to stimulate immune responses in *O. viverrini*-primed hamsters. The greatest protection was 48.4 % and elevated TGF-β induced by *O. viverrini* may play an important role in parasite survival [113]. The reported protection rate of the vaccine candidate molecules against these liver flukes is not satisfactory and more studies are required.

6.4.6 Phylogenetics, Systematics, and Genetic Diversity

A variety of molecular markers/techniques has been used to examine the genetic diversity of *Clonorchis* and *Opisthorchis* species at the interspecies and intraspecies levels. Although there are considerable similarities between *C. sinensis*, *O. viverrini*, and *O. felineus*, there is also a great deal of regional variation both within and

between species. Adaptation to differences in the environment and host selection process, as well as limited gene flow between different water sheds can all play a considerable role in determining the genetic constitution of each isolated species and population [114]. Although all three species are closely related, their systematic position remains controversial. Some reports indicate that *O. viverrini* is more closely related to *C. sinensis* than *O. felineus* when examined using 12 mitochondrial protein-coding genes [115] and the ninth intron region of the paramyosin gene [80]. By contrast, it has also been suggested that *C. sinensis* is more closely related to *O. felineus* than to *O. viverrini* based on ITS2 and mitochondrial DNA [116–118], or even that *C. sinensis* and *O. viverrini* are more closely related to one another than to *O. felineus* when examined by ITS and CO1 sequences [119]. Thus, the situation is far from clear and more powerful genetic markers together with greater sample sizes and more geographical isolates need to be studied for a comprehensive phylogenetic analysis among these liver flukes.

Considerable genetic diversity has been observed in *C. sinensis*, based on its geographic distribution within China, Korea and the Russian Federation, as well as among different reservoir hosts including human. Isoenzyme markers can be used to differentiate *C. sinensis* into the two populations from two different geographical isolates from Korea and China [120, 121]. However, the DNA regions of ribosomal DNA and mitochondrial DNA sequences were strongly conserved and nearly identical between different isolates [120, 122, 123]. In another study based on ITS1 sequencing, two levels of intraspecific variation, i.e., interindividual and intraindividual, were observed and these showed a "northern" and a "southern" genetic group of *C. sinensis* according to their distribution in China, Korea, and Russian Federation [124]. Moreover, the eggs of *C. sinensis* collected from a well preserved Chinese body which had been buried in 167 BC revealed the differences in the ITS1 sequence at 15 nucleotide positions compared to the present samples, suggesting sequence divergence through time [125]. More recently, the genetic variation and phylogeography of *C. sinensis* have been studied from two geographic localities in the Russian southern far east and compared to the other geographic localities from China, Korea, Japan, and Vietnam by CO1 sequence. A total of 18 haplotypes were observed. Of these four were common to Russian and Chinese isolates, and the other two were common to Russian and the other isolates. The Russian samples differed from those of the other localities in haplotype frequencies [126].

The role which animal reservoir hosts play in genetic variation of *C. sinensis* is currently being investigated. Both RAPD and MGE-PCR was used to examine the genetic variation among individual adult *C. sinensis* collected from cats and dogs in two geographical areas, Guangdong province in the South and Heilongjiang province in the North of China [117, 127]. Both revealed genetic polymorphisms among *C. sinensis* individuals from these hosts in each location. In a recent study from different geographical localities in Korea, as well as in China, using mitochondrial genes sequences, the genetic variation present in *C. sinensis* from naturally infected cats, dogs, rabbits, and humans was examined. Intraspecific nucleotide variation of the Korean population ranged between 0 and 1.6 % [117], whereas 0–1.7 % was found in the Chinese population [128].

The genetic diversity of *O. viverrini* has been intensively investigated based on a variety factors, e.g., spatial, temporal, and host factors. Unlike *C. sinensis*, there is no report of genetic variation between specimens collected from different reservoir hosts. An initial report was published on different geographical isolates by Ando et al. [129] using rDNA and mitochondrial DNA sequences but with a restricted sample size. The next study involved the establishment of 32 enzyme (allozymes) loci using multilocus enzyme electrophoresis (MEE) to comprehensively examine the genetic variation among natural populations of *O. viverrini* from 11 different geographical localities in Thailand and four localities in Lao PDR [130–132]. Two major lineages of *O. viverrini* were found, which could be additionally subdivided into at least six distinct genetic groups which correlated with five different wetland systems [132]. RAPD and microsatellite analyses in *O. viverrini* also showed significant differences between the isolates from Thailand and Lao PDR [133]. Interestingly, the MEE data provided evidence of potential coevolution between *O. viverrini* and its snail host, *B. s. goniomphalos*, as there was a high concordance of lineages and specific genetic groups [48, 132]. An additional *O. viverrini* genetic isolate from Savannakhet, Lao PDR was analyzed using 20 allozyme markers and also found to be associated with a specific wetland system [134].

Microsatellite markers and MEE have been used to explore the population genetics and systematics of *O. viverrini* from different geographical isolates [132, 135, 136]. In addition, *O. viverrini* populations collected from different years (temporal), as well as from different fish host species, was carried out by MEE [118, 132]. The level of genetic differentiation between the populations from Thailand and Lao PDR was very high, whereas it was low for comparisons among populations from Thailand. The same pattern was found among different fish host species and temporal populations [118, 132]. Based on the MEE and microsatellite analyses, *O. viverrini* populations almost always deviated from Hardy–Weinberg equilibrium with varying levels of heterozygote deficiencies [132, 137]. In addition, microsatellite markers could be used to examine the genetic differences among *O. viverrini* populations over small scale geographical distances within Khon Kaen Province, Thailand [136]. MEE was also be used to explore the genetic structure of *O. viverrini* populations at Vientiane Province, Lao PDR (Kiatsopit et al., unpublished). The analyses based on microsatellites, together with allozyme data, revealed that the predominant mode of reproduction in *O. viverrini* is selfing (inbreeding) rather than cross-fertilization (outbreeding). The demonstration of significant genetic heterogeneity, as well as biological variation between the different geographical isolates of *O. viverrini* from Thailand and Lao PDR, provide independent evidence that *O. viverrini* is a species complex [132, 138].

Genetic variation within *O. felineus* from different geographical localities was investigated using three different polymorphic genetic markers, i.e., CO1, CO3, and ITS1 sequences [139]. All *O. felineus* populations were classified into three geographically isolated groups, namely, from eastern Europe (the drainage basins of the Volga, the Don, and the Ural rivers), northern Asia (Siberia, the Ob-Irtysh and the Yenisei river basins), and Central Asia (Kazakh, the Nura-Sarysu Basin, part of the endorheic Aral-Caspian basin). Only low genetic differentiation between these geographically distinct European and Asian *O. felineus* population was observed.

This homogenization of population structure could result from potentially high levels of gene flow between populations, accompanied by active migrations of definitive hosts, including humans, during the Holocene [139]. More recently, ISSR and allozyme analyses have been used to examine genetic variation of *O. felineus* from six rivers of Western Siberia. In addition, ISSR was also used to explore the genetic variability of metacercariae of *O. felineus* collected from different fish host species [140]. Again, only a low degree of genetic polymorphism and differentiation among *O. felineus* population was observed. Southern *O. felineus* samples from the Tobol and Tura rivers showed higher polymorphism levels than the samples from rivers in northern part [140]. However, the metacercariae of *O. felineus* collected from different fish species showed no genetic differences [140]. The results so far suggest that population genetic data based on other genetic markers such as microsatellite DNA are required.

6.5 Diagnosis

The most common diagnostic method for fish-borne trematode, which involves finding eggs in fecal samples, seems still to be far from ideal. In low egg output and low prevalence situations, sensitivity is also low, and using this method is a puzzle that challenges scientific efforts.

6.5.1 Parasitological Methods

Fecal examination is the routine method used for the diagnosis of liver fluke infection. It has the advantages of the simplicity of sample collection and of being non-invasive. Once a fecal sample is available, the modified formalin–ether (or ethyl acetate) concentration technique (FECT) [141], the modified thick Kato smear [142], or Stoll's dilution egg count technique can be used [143]. Although these techniques are highly specific there are limitations because of the prepatent period of infection before eggs are produced, poor sensitivity when infection intensities are low, or intermittent egg excretion associated with bile duct obstruction. Both sensitivity and specificity vary depending on the method used but also on the experience of the examiner. The diagnostic value of these methods lies in their ability to detect relatively light infections, which occur in the majority of infected individuals, and in individuals recently treated with praziquantel. As a single examination does not necessarily provide diagnostic certainty, repeated examinations are needed to improve diagnostic sensitivity. Thus, three consecutive Kato–Katz thick smears are more sensitive than a single examination by FECT [144]. However, even using such repeated stool examination there can be a discrepancy between egg count and worm detection so that a false negative diagnosis remains a real possibility.

In an autopsy study, adult *O. viverrini* were recovered directly from 139 livers. Examination of postmortem fecal samples from these individuals showed that only 67 % were positive for *O. viverrini* infection. The detection limit using normal fecal examination was estimated to be 20 worms or approximately 1,000 epg. Individuals with low infection intensities and limited egg output are likely to be underdiagnosed by as much as ~20 % [62]. Although there is some evidence of density-dependent fecundity, there is in general a linear relationship between fecal egg count and worm burden.

Diagnostic kits which reduce processing time (Parasep SF) have also become available; however, these show a lower sensitivity than FECT although they have a higher sensitivity than the simple smear method (Sithithaworn, unpublished). The performance of this kit is comparable with that of the widely used Kato–Katz method.

Eggs can also be detected during treatment of bile duct obstruction either in bile from nasobiliary or percutaneous transhepatic biliary drainage (PTBD) or in the duodenal fluid. Adult worms are ejected during expulsion chemotherapy [145–148]. Similar data to those available for *O. viverrini* are now also available for *C. sinensis*. These indicate that this species lays more eggs than *O. viverrini* (514 epg/worm), probably due larger size of adult worms [149]. Worm burdens determined by expulsion chemotherapy ranged from 1 to about 100 worms for *C. sinensis* [149, 150].

In some endemic areas for liver flukes, for example in Southeast Asia, intestinal flukes coexist leading to a potential diagnostic problem if conventional fecal examination is used. The eggs of *O. viverrini*, which are identified by their characteristic rough and thick egg shells are very similar to the eggs of several species of other food-borne trematodes belonging to the families Opisthorchiidae, Heterophyidae, and Lecithodendriidae. The latter two families are commonly referred to as minute intestinal flukes (MIF) due to their small size compared to the liver flukes [28, 151, 152]. These species are, like the liver flukes, fish-borne trematodes (FBT) or fish-borne zoonotic trematode (FZT) [153–155]. The similarity between the eggs of these other FBT species and those of *O. viverrini* and *C. sinensis* can substantially increase the likelihood of a false positive diagnosis, depending on the prevalence of these species. They thus reduce diagnostic specificity and the identification of adult worms and PCR confirmation may be necessary for correct species identification in areas where a number of species exist.

6.5.2 Immunological Methods

Several serological tests for clonorchiasis and opisthorchiasis have been developed for use in a diagnostic assay with greater sensitivity and specificity than fecal examination. These include the intradermal test (IDT), immunoelectrophoresis (IEP), indirect hemagglutination assay (IHA), indirect fluorescent antibody test (IFAT), and indirect enzyme-linked immunosorbent assay (indirect ELISA) [17, 156, 157]. Indirect ELISA is commonly preferred for the detection of antibodies although, due to the complexity of the antigen, neither sensitivity nor specificity is constant. Crude somatic extracts of adult worms of both *O. viverrini* and *C. sinensis* used for ELISA provide higher

sensitivities than fecal examination [17, 158, 159], while ES antigens show a superior or equivalent performance to the crude antigen [160, 161]. Interestingly, antigen extracted from the *Bithynia* intermediate host snails has also been used as an antibody detecting antigen for the diagnosis of human *Opisthorchis* infection [162, 163], although the value of such rests has yet to be evaluated.

Recombinant antigen for serum antibody detection by ELISA has been produced from eggs and egg shells [159, 164]. In addition, the propeptide of cathepsin L, glutathione *S*-transferases, adenylate kinase 3, phosphoglycerated kinases, PGM, LPAP, and cathepsin B, cathepsin F, cathepsin L-like, legumain, taurocyamine kinase have been characterized and show better diagnostic sensitivity and specificity over conventional fecal examination diagnostic methods [91, 106, 164–168]. Nevertheless, the increased specificity and reduced cross reactivity of these proteins need to be tested under field conditions before they can be judged good enough to replace the commonly used native crude antigen [17]. The detection by ELISA of antibodies in non-fecal clinical samples such as urine and saliva has been considered and saliva found to be of potential use for the serodiagnosis of opisthorchiasis [169].

One drawback of antibody-based detection is the inability of this method to differentiate between past and present infections because of the persistence of antibodies in the in the patient even after a cure has been effected [170–172]. One way of overcoming this problem is to use an antigen-based detection which indicates if current infection is present [173–175]. Monoclonal antibody (mAb)-based systems offer increased diagnostic sensitivity, as they are able to detect secretory products from only a few adult worms. This is effective in low-scale infections when eggs are not detectable in fecal samples [174]. This has been corroborated in an autopsy study [62]. Studies in animal models for *C. sinensis* [176] and *O. viverrini* (Duanngai unpublished) showed promising results. Recently it was suggested that coproantigen detection is useful for detecting positive cases, again especially when fecal examination negative, although the antigen level is also correlated with the intensity of infection [177]. This may be a useful approach for the detection of mild infections and for the evaluation of the effectiveness of pharmaceutical cure.

6.5.3 Molecular Methods

A number of target genes from both *C. sinensis* and *O. viverrini* have been tested for their diagnostic suitability including satellite DNA, ITS1, ITS2, and mitochondrial DNA. These were used for both conventional PCR and real time PCR diagnosis showing high specificity but variable sensitivity [17, 157, 178]. The detection of *O. viverrini* egg DNA in human stools using PCR and based on primers complementary to the repeat DNA element showed a specificity of 98 % and a sensitivity of 100 % for moderate-to heavy infections with more than 1,000 EPG. In light infections with less than 200 EPG the sensitivity was reduced to only 68 % [179, 180]. More recently, the retrotransposon of *O. viverrini* (*OV-RTE-1*) has been found to be the new alternative genetic marker of high sensitivity and specificity for the PCR diagnosis of opisthorchiasis [181]. Another PCR-based study using the same target

DNA showed low sensitivity (50 %) at high egg counts of more than 1,000 EPG in stool samples from Lao PDR [182]. However, if the quality of the DNA was improved by using cetyltrimethylammonium bromide during its preparation (CTAB) to remove PCR inhibitors the sensitivity was increased to 79 % [183]. PCR-positive tests occurred in a 29 % of cases which were parasite negative in this study using the conventional fecal examination method indicating its potential diagnostic value for light infections. Another *O. viverrini*-specific primer pair was established which was able to detect adult worms with 10–12 ng of DNA, and metacercariae when three or more occurred in a fish sample [184]. Loop-mediated isothermal amplification (LAMP) has been established for the detection of both *O. viverrini* and *C. sinensis* with a higher sensitivity than conventional PCR [185–187].

Species-specific PCRs are now also available to distinguish between the three species of liver fluke: *O. viverrini* [129, 179], *O. felineus* [188], and *C. sinensis* [189]. In addition, several genetic markers/approaches involving conventional PCR, PCR-RFLP, multiplex PCR, real-time PCR and multiplex ligation-depended probe amplification (MLPA) pyrosequencing can be used to differentiate between species involved [189–192].

The molecular methods discussed above will contribute significantly towards a more effective and accurate diagnosis of trematode infections, although further simplification of the tests and an understanding of cost-effectiveness under various socioeconomic scenarios is needed. In addition, the validation of DNA positive test results is required, although evidence from animal models is accumulating and supported human studies [193, 194].

Real-time PCR can also now be used to quantify the intensity of infection with *C. sinensis* [99]. In addition, molecular identification techniques can be used in cases of multiparasite infections in a single host [6, 191].

Such approaches can also be used in a food security setting to test for the presence of liver flukes in aquaculture or native fisheries products, particularly for export [184, 186, 195].

Due to their high specificity, such molecular diagnostic tests are likely to play an increasingly significant role in anthelminthic drug efficacy evaluations, the rigorous monitoring of reinfection patterns, and to investigate changes in the endemic range of the liver flukes [45, 55].

6.6 Consequence of Infection

6.6.1 Pathogenesis, Pathology, and Morbidity

Liver fluke infection causes significant pathological changes to the bile ducts which the worms inhabit. The pathology can also extend to affect both the liver and gall bladder [18, 47]. Syrian golden hamsters provide a suitable animal model to examine these changes [196, 197]. During the early phase of infection with *O. viverrini* there is an acute inflammatory reaction in the large intrahepatic bile ducts as well as portal connective tissue. Once the infection has become chronic (at about 30 days

post infection) hyperplasia and adenomatous formations of the bile duct epithelium occur [198]. Granulomatous responses to both the adult flukes as well as to the eggs which they produce lead to periductal fibrosis and scarring. This is the most prominent feature during the chronic stage of infection [196]. The extensive fibrosis is associated with a significant increase in the synthesis of and the hepatic content of collagen [199, 200]. With the onset of the chronic phase of infection the inflammatory responses become less severe suggesting that immune modulation may occur. Fibrotic tissue accumulates due to repair dysfunction and an imbalance in synthesis and degradation of the fibrotic tissue. These factors may lead to cell proliferation which, in the presence of cofactors, significantly contributes to cancer development [201]. In humans, periductal fibrosis is a significant cause of hepatobiliary disease and leads to an increased risk of CCA development [202, 203]. In *O. viverrini* patients with advanced periductal fibrosis there was an eight times higher level of IL-6 responses to *O. viverrini*-excretory/secretory products than in patients without fibrosis, indicating the role of IL-6 in the pathogenesis of advanced periductal fibrosis in opisthorchiasis [204].

Chronic infection by the liver flukes corroborated by a marked humoral immune response indicated by the presence of parasite-specific IgG, IgA, and IgE in the serum and bile of humans infected with *O. viverrini* [205, 206]. Although the IgG level against CSAg correlated with hepatobiliary abnormalities diagnosed by ultrasonography, there was weak correlation with the intensity of infection [207].

The bile ducts which harbor the adult worms show the most significant and potentially dangerous gross and microscopic pathological changes in both *O. viverrini* and *C. sinensis* infections but development is long-term taking up to 7–15 years for *O. viverrini* [208]. Immunomodulation during both the acute and chronic phases of infection is responsible for the pathological changes observed [18].

Light infections may be inapparent with no significant symptoms. Pathology depends on both the duration and the intensity of infection as well as to the susceptibility of the host [17, 47, 209, 210]. For heavy infections the peripheral bile ducts may become thickened beneath the fibrotic capsule of the liver. A recent outbreak of opisthorchiasis in Italy caused by *O. felineus* infection presented as a febrile syndrome with eosinophilia and cholestasis [211]. This outbreak is interesting as 37 (82 %) of the 45 infected individuals showed symptoms of the disease and 8 (17.7 %) were admitted to hospital for treatment.

As indicated above, inapparent infections are common with only about 5 % morbidity occurs among infected individuals [212]. Once the symptoms become apparent they are usually nonspecific, involving general abdominal discomfort. In such cases hepatobiliary abnormalities and/or CCA can usually be detected by ultrasonography [203, 213].

6.6.2 Liver Flukes and Cholangiocarcinoma

CCA is a cancer of the epithelial cells in the bile ducts arising along either the intrahepatic or extrahepatic biliary tree [214, 215], although studies on molecular pathogenesis are currently confined to the intrahepatic CCA type. CCA is responsible for

as much as 15 % of liver cancers worldwide, most of which are associated with trematode infection [216, 217]. Large-scale epidemiological studies of CCA indicate an increase in both the incidence and mortality rates. Currently CCA is the second most frequent primary liver cancer [218]. The highest incidence of CCA worldwide occurs in northeast Thailand [219]. In addition to CCA induced by either *C. sinensis* or *O. viverrini*, early observations indicate that around 400 cases of this disease currently occur every year in patients heavily infected with *Opithorchis felineus* [220].

The induction of cancer by these liver flukes appears to be dependent on a variety of factors including host genetic background, past exposure to infection as determined by elevated *O. viverrini* antibody levels, liver cirrhosis, chronic infection with hepatitis C virus and heavy alcohol consumption [221].

The association between *O. viverrini* and CCA was first determined in a hospital-based, case–control study conducted in Thailand in the late 1980s [222]. A total of 103 patients with CCA were compared with an equal number of age- and sex-matched controls and elevated *O. viverrini* antibody titers were positively correlated with an increased risk of CCA [222]. This was confirmed in a repeat study based on 129 cases the cancer. This study indicated that the population-attributable risk is as high as 88 % in endemic areas [223]. Another a case control study, this time on *C. sinensis* from Korea, compared 41 patients with CCA with 406 controls and found a similarly strong association between liver fluke eggs in fecal samples and CCA [224]. A recent meta-analysis including 912 cases and 4,909 controls confirmed this association [225]. The population-attributable risk was lower than that calculated for *O. viverrini* but was nevertheless 27.9 % for men and 16.2 % for women.

In patients with a *C. sinensis* infection the formation of calculi in the intrahepatic biliary passages is a characteristic pathological change. This may be associated with suppurative cholangitis, cholecystitis, and biliary abscess or so-called cholangiohepatitis. It can eventually lead to the development of primary liver cancer, especially CCA. The occurrence of calculi is probably caused by bile stagnation, which in turn causes mechanical obstruction by *C. sinensis* worms and eggs in the bile ducts. The calculi in the intrahepatic and extrahepatic bile ducts are made up of bilirubin and calcium salts. The formation of such pigment stones in clonorchiasis is thought to be due to bile stagnation leading to changes in the composition of bilirubin, cholesterol, phospholipid, bile acid and the activity of bacterial glucuronidase. The goblet cell metaplasia of the bile duct epithelium is responsible for the high content of mucous secretion in the bile. This mucin-rich bile in conjunction with the worms and eggs not only cause cholestasis but also provide a suitable environment for secondary bacterial infection. This is usually due to *Escherichia coli* which cause ascending cholangitis from the intestine [18]. Studies on *C. sinensis* indicate that this species also stimulates biliary epithelial hyperplasia [226], which is considered to play a significant role in carcinogenesis [197, 227]. Clonorchiasis-associated CCA involves substantial mucin secretion, usually accompanied by extensive fibrosis [228, 229]. Although the larger bile ducts are only slightly enlarged and fibrotic, they are commonly blocked by adult worms or calcium bilirubinate stones [230]. Clonorchiasis-associated CCA has develops in a discrete nodular or confluent mass in which smaller

ducts with adenomatous hyperplasia undergo malignant transformation occur [231]. Chronic inflammation is of particular significance for the induction of CCA due to oxidative and nitrative DNA damage [232].

Although most of these studies indicate that liver flukes cause tissue damage by mechanical and chemical irritation, some recent studies suggest that parasite-specific immune responses may also play a major role [232]. A genetic polymorphism in the detoxifying enzyme glutathione-*S*-transferase (GSTM1) in association with seropositivity for opisthorchiasis was found to modify the cancer risk factor for CCA [223]. Thus, gene–environment interactions (current or past infection of *O. viverrini* infection) can play a significant role in individual susceptibility to CCA.

Carcinogenesis of CCA is still not clearly understood; however, it appears to be a multistage process with a variety of factors being involved of which chronic infections and persistent inflammation are predominant [233]. It is also possible that nitric oxide (NO), which can generate DNA-reactive agents and N-nitrosamines, is involved [232]. Excess NO production plays an important role in a number of pathological processes, including the induction of cancer (see [232]). If a host becomes infected with a liver fluke, macrophages and other cell types (e.g., mast cells, eosinophils, and epithelial cells) are activated by parasite specific T cells and cytokines and synthesize NO from L-arginine via the induction of iNOS with the aim of eliminating the intruder. Nitric oxide is not only cytotoxic but also genotoxic by reacting with superoxide to form the highly reactive peroxynitrite which leads to oxidative and nitrative DNA damage via the formation of 8-oxodG and 8-nitroguanine [234]. These can be used to indicate DNA damage in the affected tissues. The overproduction of NO caused by *O. viverrini* infection can also lead to the endogenous nitrosation of amine precursors to form potentially carcinogenic N-nitrosamines such as *N*-dimethylnitrosamine (NDMA) [235]. NDMA, which is a carcinogenic product of the nitrosation reaction, has been detected in the urine of *O. viverrini*-infected subjects. It seems to be associated with a lymphoproliferative response to active liver fluke antigens which ceases after praziquantel treatment and the death of the parasites [235]. During an active *O. viverrini* infection of either hamsters (the animal model) or humans, an isoform of cytochrome P-450 (CYP) enzymes, CYP2A6 is formed [236, 237]. NDMA requires metabolic activation, mainly by CYP2E1 and CYP2A6, before becoming carcinogenic. It is hypothesized that this increase in CYP2A6-related enzyme activity in *O. viverrini*-infected individuals is an important link between inflammatory processes due to chronic liver fluke infection and a high risk for CCA.

6.7 Epidemiology

A somewhat dated national survey carried out by the Ministry of Public Health in Thailand in 2001 showed that helminth infections are common with a country wide total prevalence of 22.5 %. Of the species involved hookworms are the most common (11.4 %), while *O. viverrini* ranked second with an average prevalence of

9.6 %, although the central and southern areas of the country showed a very limited presence of the parasite [238]. In the northeast, there is substantial variation in the prevalence of opisthorchiasis among provinces, ranging from 4 to 33 % [238]. In Lao PDR, *O. viverrini* is common in the lowlands among people with close ethnic ties to the majority of the northeast Thai population probably due to high levels of partially enforced migration from Lao PDR into Thailand in the past [239]. The prevalence in certain areas is as high as 36–60 % [15]. This is much higher than previous records indicate [240, 241]. The presence of mixed infections including *O. viverrini* and heterophyid and lecithodendriid flukes found in communities along the Mekong River, potentially make conventional fecal diagnosis difficult [151].

Limited information on the incidence of infection in endemic communities in Thailand is available [61, 63, 242]. In a study of three villages in Khon Kaen Province, the incidence was 1.7–25 % over a 6 month period [61]. In a central Thai village containing a migrant population from the northeast of the country the incidence was 21.6 % per year [243]. The high levels of incidence in some villages correspond with the high prevalences in some areas. For example, with an incidence of 40 % per year, only 6 years are required for the prevalence of an originally uninfected cohort to exceed 95 % [244]. In northeast Thailand information on the rate of reinfection after treatment also show a high incidence of (re)infection. After a pretreatment prevalence of 55.1 %, it took 1 year for the prevalence to return to 54.8 % [242]. Upatham et al. [63] reported that in an area in Chonnabot, Khon Kaen Province, where 97.4 % of villagers were infected, the prevalence had reached 94 % 1 year post praziquantel treatment. It is significant that individuals with a high pretreatment intensity of infection tended to have a high intensity of reinfection. This may be a predisposition to heavy infection in some individuals. This hypothesis is supported by evidence from other parasites, such as *Ascaris lumbricoides* [245], *Necator americanus* [246], *Trichuris trichiura* [247], and *Schistosoma mansoni* [248]. Rapid reinfection after treatment shows little evidence for protective immunity, although this may occur in some individuals.

Although the rates of *O. viverrini* and *C. sinensis* infection vary considerably between villages, the pattern of infection is similar. Infection is age-dependent with the youngest age groups (0–5 years) having a low prevalence and intensity of infection. These increase through the preteen and early teenage years, often reaching a plateau in late teenagers (e.g., 15–19 years). In some areas, the intensity of eggs released increased with age [212], but the worm burden declined after the age of 50–60 [62, 249]. A number of possible reasons have been suggested for this decline including the late-development of an immune response, lower parasite survival in more heavily fibrosed bile ducts, death of parasite in heavily infected people, or reduced exposure to infection in older age groups. Infection in infants may be due to mothers feeding them raw fish which is often finely ground [212, 250, 251]. However, the reported intensities of infection under the age of four are invariably low and there is little evidence that young children experience frequent exposure to infection.

In general, the prevalence and average intensity of *O. viverrini* infection is either not sex related or is slightly higher among males compared to females [212, 249,

251, 252], although more heavy infections may be found among males than females. This is also the case for *C. sinensis* [253]. Males could therefore be more at risk of significant pathology, including cancer, as this increases in a nonlinear fashion with infection [207, 254].

As with other helminths, *O. viverrini*, and probably all of the liver flukes dealt with here, is highly overdispersed with the majority of worms being found in only a few heavily infected individuals [146]. The maximum worm load was 565 with a mean of 85 (SD = 154). Haswell-Elkins et al. [249] observed that 81 % of 11,000 worms recovered after treatment of 246 village residents were expelled by just 25 individuals (10 % of the sample population). The highest burdens were over 100 worms. Interestingly, a number of individuals who did not expel worms were nevertheless positive for eggs. In an autopsy study in Khon Kaen in which the worm burden was accurately measured, Sithithaworn et al. [62] found that 30 out of 181 cadavers contained 66 % of all the worms recovered and that only 13 people (7 %) had worm burdens greater than 400.

6.8 Treatment

Treatment programs vary considerably between countries. In general, infection with *O. felineus* is so limited in Europe where only local control is necessary if a particular community or group is infected [5]. In Thailand, a trial liver fluke control program was developed as early as 1967 in Sakon Nakhon Province [60]. This, as with other control programs in Thailand, was based on the selective treatment of infected individuals as opposed to mass treatment. One of the limitations of this approach is that although the drug of choice, praziquantel, has a high efficacy (90–95 %) and there is no evidence of drug resistance, the reinfection rate is high. This suggests that control by chemotherapy alone is unlikely to be completely successful, which is supported by the "residual" prevalence found in many areas after control measures were conducted [60]. With the advent of praziquantel in the mid-1970s, which is effective in about 90 % of cases, the duration and toxicity problems were largely eliminated [255]. The recommended daily dose for treatment of *C. sinensis* was 3 × 25 mg/kg × 1 day with cure rate of 85 % and egg reduction rate of 99.7 % [256], for *O. viverrini* the dose of 40 mg/kg with cure rate of 90 % and egg reduction rate of >99.7 [255, 257] and for *O. felineus* the dose of 3 × 25 mg/kg × 1 day with cure rate of 90 % and egg reduction rate of 100 % [258, 259].

Recent data based on higher sensitivity methods such as PCR, however, suggest that the treatment efficacy may be lower than previous estimates has suggested, an area which clearly requires urgent investigation. Nevertheless, treatment with praziquantel usually leads to the elimination of symptoms. As an alternative to praziquantel, tribendimidine has been examined and it gave an efficacy comparable to praziquantel in the treatment of *C. sinensis* infection and resulted in fewer adverse events [260] but more study is required.

6.9 Prevention and Control

Methods of prevention and control aim at breaking the transmission cycle to humans. The millions of people infected and at risk of infection, as well as the direct and indirect economic losses resulting from liver fluke infection indicate the great importance of implementing effective and long lasting prevention and control measures. There have been a number of attempts to do this both via direct treatment and also education aimed at reducing or elimination the consumption of raw or partially cooked fish. For *O. viverrini*, when fecal contamination from humans is reduced or eliminated by mass treatment and/or improved sanitation, the prevalence of infection can be substantially reduced [22]. A control program initiated in 1989 in certain provinces, particularly in the north and northeast of the country, resulted in a reduction in prevalence from 35.6 % in 1988 to 8.7–9.4 % during 2001–2009 [15, 261]. However, complete elimination of infection may not be possible if domestic cat and dog reservoir hosts maintain the source of parasite therefore playing a critical role in maintaining the life cycle. The difficulty involved in detecting infected cases with only a light infection (<1,000 EPG) and the problem of reinfection after treatment also present serious problems to effective, long-term control. At the social level, education has proven both difficult to implement and ineffective to reduce the consumption of raw or partially cooked fish in Thailand [22].

An easy and promising method of preventing infection is to kill the infective metacercariae before fish are consumed. Unfortunately, as the consumption of raw or partially cooked fish is a deeply imbedded tradition in areas where opisthorchiasis and clonorchiasis are most common, the relevant populations have proven refractory to change [41].

In particularly Thailand and Lao PDR there are three types of fish dishes which act as a source of infection with *O. viverrini*. “Koi pla” is prepared from fresh raw fish which are seasoned with lemon juice and spices and consumed without heating, posing a high risk of infection. “Pla som,” which is a dish made out of fish which have been fermented for 1–2 days is poses a moderate risk, while. “Pla ra” which undergoes less than the usual long-term, although less risky, may provide a favorable environment for metacercarial survival [41]. Other Mekong countries have their traditional potential sources of infection: fermented “pla dak” in Lao PDR, raw “pla hoc” in Cambodia which is similar to “pla som,” “goi ca mai” (raw fish salad) and slices of raw silver carp in Vietnam, a raw fish salad in China, and sushi in Korea [18]. In Russia, *O. felineus* infection may come through eating dried or salted fish or sliced raw fish (“stroganina”) which is popular among native Siberians, as well as fish pickled in vinegar.

Salted fish is generally considered ready in a day or two. It has been shown, however, that metacercariae remain viable under high salt concentrations for up to 2 weeks. The popular dishes in Russia and Eastern Europe of slightly salted fish are not safe at all. The same is true for dried fish where greater than 12 days of drying are required to kill 99 % of metacercariae. Given that the weight of fish and the temperature of drying are not constant even at fish plants or after 12 day period,

dried fish remain dangerous for consumption. Cold smoking has similar effects to those of drying and salting [262].

A recent investigation in fish farms in Lao PDR supported by Food and Agriculture Organization (FAO) demonstrated that some carp species commonly cultured in fish ponds contained *O. viverrini* metacercariae (unpublished data). This preliminary result suggests that apart from captured fish, culture fish can provide an additional source of infection to consumers and thus urgently need attention control body to ensure food safety.

Control efforts are primarily focused on the reduction and elimination of parasite transmission by ensuring proper food preparation, promoting the development of improved diagnostic techniques, providing chemotherapy, and improving sanitation. A combination of health education, mass treatment, and governmental aid could significantly reduce liver fluke infection. Emphasis on health education should be placed on the younger generation in school as a part of the conventional education curriculum.

Interrupting the life cycle of the parasite has always been regarded as a promising way of disease control, for example application of molluscicides (chemicals that kill snails) to control snail populations [34, 263]. Low concentrations of certain molluscicides (e.g., phenasal, niclosamide) are lethal for infected snails, sublethal for uninfected ones, and, presumedly, nontoxic for other animals [263]. The biggest case against this approach is that it involves interference of the ecosystem which can have dramatic consequences including potential toxicity to fish in rice fields [263, 264]. Economic factors also negate its usage as the application of molluscicides is practical only for small water bodies since the costs of treating big areas are extremely high. Additionally, it has been shown that snail populations are restored in about 5 years, hence repeated treatments are necessary. Therefore, decontamination of aquatic bodies has been abandoned in Russia.

In order to achieve overall, long-term control of liver flukes a multidisciplinary approach is necessary. This must aim at breaking the transmission cycle at the level of the first and second intermediate hosts, i.e., at the level of general hygiene as well as at the fisheries and aquaculture levels. This must also be extended to the food production and distribution industries. Of great significance, is that the population at risk must be educated as to how infection occurs and how it can thus be avoided, and to the risks of infection, particularly the development of CCA, should it occur.

The FAO recognizes the necessity of determining the importance of aquaculture in comparison with capture fisheries in the likelihood of human infection with food-borne trematodes. This is a primary requirement in food safety assurance from aquaculture at both the domestic and international trade levels. Hazard Analysis and Critical Control Point (HACCP) methodologies are already available to assist control approaches at the food production stage; however, more work is required taking into account the economics of the costs accruing through implementation of these approaches.

Thailand is considered the hot spot of opisthorchiasis and CCA and the public health importance is acknowledged. In order to prioritize the health problems associated with liver fluke infection, an estimation of burden of disease (BOD) in Thailand was instigated in 1999 and data are available from 2004 [265].

Generally BOD is based on two measurements, namely, Disability Adjusted Life Year (DALY) and, when accurate mortality data are lacking, estimated number of Years of Life Loss (YLL). DALY is a summary measure of population health for setting priorities since this measure combines both fatal and nonfatal health outcomes. BOD estimates are far from complete, especially in developing countries where resources and budget are not adequate. The BOD study in Thailand has identified a short list of the top 20 diseases based on mortality, DALY and YLL. Of these only the top ten diseases were selected. Liver cancer ranks fifth in males and eighth in females. A total of 27,500 people die every year of liver cancer, while YLL is estimated to be 400,000. Both of these figures show the high significance of opisthorchiasis, and CCA, as a public health problem in Thailand.

Thailand was one of the first nations to initiate a program of liver fluke control. This was based initially on funding supplied by USAID as early as 1950. This was followed up by a Thai government program supported by the Deutsche Gesellschaft für Technische Zusammenarbeit (German Society for Technical Cooperation, GTZ). These programs have been successful in reducing the prevalence of *O. viverrini* infection from approximately 63.6 % in 1984–1987 to 9.6 % in the year 2001 [60]. In spite of this success, a nationwide survey showed that there was still a residual prevalence of 8.7 % in 2009. The northeast of Thailand currently still has the highest prevalence with 16.6 % with northern Thailand at 10 %. Both central (1.3 %) and southern Thailand (0.1 %) have low infection prevalences [15]. These data indicate that although the control programs have been successful in reducing the prevalence, they have not eliminated the infection.

The control programs to date have relied on the use of praziquantel (40 mg/kg) curing (>95% cure rate) infected individuals. Most infected individuals involved in the control programs are now estimated to have only light infections (epg <1,000). Treatment with praziquantel, although it is successful in killing adult worms, does not prevent reinfection. Indeed, evidence is accumulating to suggest that it may compromise the immune system or cause liver complications when used repeatedly for reinfections [266]. This leads us to the conclusion that the current control programs require urgent modification.

Both short-term and a long-term program components should be reassessed. The short-term program requires a modification in the current selective treatment strategy by including traditional methods applied with increased accuracy, as well as molecular diagnostics. Multiple stool samples taken from an individual can be analyses using the Kato–Katz method or the more sensitive the formalin–ethyl acetate method. In addition, the cure rate based on the administration of praziquantel must be regularly monitored. This is particularly relevant given the different genetic groups of parasites present in different areas [20]. Control approaches must also include zoonotic cycles in carnivore reservoir hosts as they will increase in significance as human prevalences decrease.

One of the most important aspects in the long-term strategy involves an education program based on food safely. Current programs have shown that long-term, continuous education is required as raw or partially cooked fish consumption is a deeply rooted, raw attitude in the areas where it occurs [41]. Ziegler et al. [267]

recommend school-based health education for young children in order to imprint the importance of food preparation and hygiene in relation to public health. Such educational programs can be promoted through participatory activities in schools. It is anticipated that the information will not only become part of the child's background knowledge as they grow to adulthood but also be discussed at home during the period of schooling. Such a program should have a major impact at the family, extended family, and village levels. An approach of this intensity and magnitude will have the potential for a long-term impact that is not present with national and international selective treatment strategies.

Moreover, the one health approach recommended by the world Health organization is a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of health care for humans, animals and the environment is applicable for liver fluke control. This helps improving our understanding of the social, economic and ecological dimensions of the liver fluke transmission including opisthorchiasis in Thailand and Southeast Asian countries.

Long-term strategies, and indeed a good deal more research, are needed to overcome the dynamic situation caused by land use and climatic changes either taking place or predicted for the Thailand. Such changes are often coupled with dynamic changes in parasite transmission [268, 269]. Given the public health significance of *O. viverrini* infection, the Thai Ministry of Public Health has recently initiated the "Esan agenda: eradicate the liver fluke to reduce CCA" which is primarily aimed at screening for CCA patients. If the cancer is recognized sufficiently early, curative surgery may lead to an effective cure. Such a strategy, however, does not attack the problem at its roots as comparatively very few people are involved compared with the population at risk based on infection with *O. viverrini*. There are currently at least 26 million people at risk of infection in the north and northeast of Thailand. Any control program aimed at reducing the long-term burden of *Opisthorchis* infection must address this population group as the initiation point for the effective control of opisthorchiasis and its associated CCA.

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