CHAPTER SEVEN

# Recent Advances in the Diagnosis and Detection of *Opisthorchis viverrini* Sensu Lato in Human and Intermediate Hosts for Use in Control and Elimination Programs

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## Abstract

Opisthorchiasis is a neglected tropical disease, caused by infection with the fish-borne trematode *Opisthorchis viverrini* sensu lato that afflicts more than 10 million people in Southeast Asia, including Thailand, Lao PDR, Vietnam and Cambodia. The disease is characterized by a chronic infection that induces hepatobiliary inflammation, especially periductal fibrosis, which can be detected by ultrasonography. This chronic inflammation eventually leads to cholangiocarcinoma (CCA), a usually fatal bile duct cancer that develops in approximately 1% of *O. viverrini*—infected individuals. In Thailand alone, CCA kills up to 20,000 people every year and is therefore of substantial public health importance. Its socioeconomic impacts on impoverished families and communities are considerable. To reduce *O. viverrini*—associated morbidity and CCA, the primary intervention measures focus on opisthorchiasis control and elimination. Accurate diagnoses of *O. viverrini* infection, in both mammalian, snail and fish intermediate hosts, are important for achieving these goals. Despite extensive efforts over several decades to find sensitive and specific diagnostics for opisthorchiasis, a simple and robust diagnostic method is still required. Here we review earlier and current developments in the search for new diagnostics for opisthorchiasis, with practical applications in the research laboratory, the clinic and the field. Of the methods currently available, the urine antigen assay shows considerable potential for the diagnosis and screening of opisthorchiasis. Nevertheless, these new assays require validation, determination of their cost-effectiveness when applied for mass screening in an endemic setting in support of policy decisions for national public health programs aimed at the control and elimination of opisthorchiasis.

## 1. INTRODUCTION

Opisthorchiasis, caused by *Opisthorchis viverrini* sensu lato (*O. viverrini* species complex) infection, is a major public health problem in mainland Southeast Asia particularly Thailand, Lao PDR, Vietnam, Cambodia and Myanmar (Sithithaworn et al., 2012; Aung et al., 2017). Unlike other food-borne trematodiasis, infection with this liver fluke induces hepatobiliary disease and bile duct cancer (cholangiocarcinoma, CCA), and *O. viverrini* was designated as group I carcinogen (IARC, 2012). The extent and impact of *O. viverrini*—associated CCA were not well recognized until recently. In Thailand alone an estimated 20,000+ people die annually of this disease

(Bundhamcharoen et al., 2011; Khuntikeo et al., 2016). To date, there are several known risk factors for CCA, but the most fundamental factor is liver fluke infection (Sithithaworn et al., 2014). To reduce CCA incidence and its resulting fatality, the primary focus has aimed at prevention, control and elimination of the liver fluke in the endemic communities. Under the current transmission landscape of opisthorchiasis, light infections predominate and only a few people carry heavy infections. To screen the light infection group, improved diagnostic methods are required to replace or supplement the traditional methods which have been used for several decades. A robust diagnostic method also serves as an important tool for parasite prevention and control by mass chemotherapy. Currently, there are three major methods in use to diagnose *O. viverrini* infection in humans, as well as in other animal hosts, namely parasitological, immunological and molecular. Furthermore, accurate diagnostic methods are required to detect and confirm *O. viverrini* infection in humans and in animal hosts, including the other live stages in the life cycle such as cercariae in *Bithynia* snails, free-living cercaria in water and metacercariae infecting the second intermediate host, cyprinid fish (Fig. 1). In addition, since only praziquantel is used for chemotherapeutic control, other alternatives are required.

Thus, this chapter aims to review and update procedures for the diagnosis and detection of the various life stages of *O. viverrini*, as well as their implications for epidemiological studies and parasite control.

## 2. HUMAN DIAGNOSIS

### 2.1 Conventional Methods

The classical diagnosis of *O. viverrini* infection is recovery of adult worms for morphological identification. Many studies have obtained adult worms by searching for expelled worms in faecal specimens at postanthelmintic treatment (Ramsay et al., 1989; Elkins et al., 1991; Radomyos et al., 1994; Sato et al., 2015). Worm recovery by postanthelmintic treatment or expulsion chemotherapy may not be efficient as unknown proportions of worms were not recovered, and hence there were egg-positive cases who expelled no worms (Elkins et al., 1991). The worm recovery method by expulsion chemotherapy is suitable for species confirmation (Chai et al., 2005; Lovis et al., 2009), but for diagnostic purposes it may be difficult to efficiently handle and process large faecal volumes. By contrast, worm burden can be accurately recovered by collecting the worms directly from

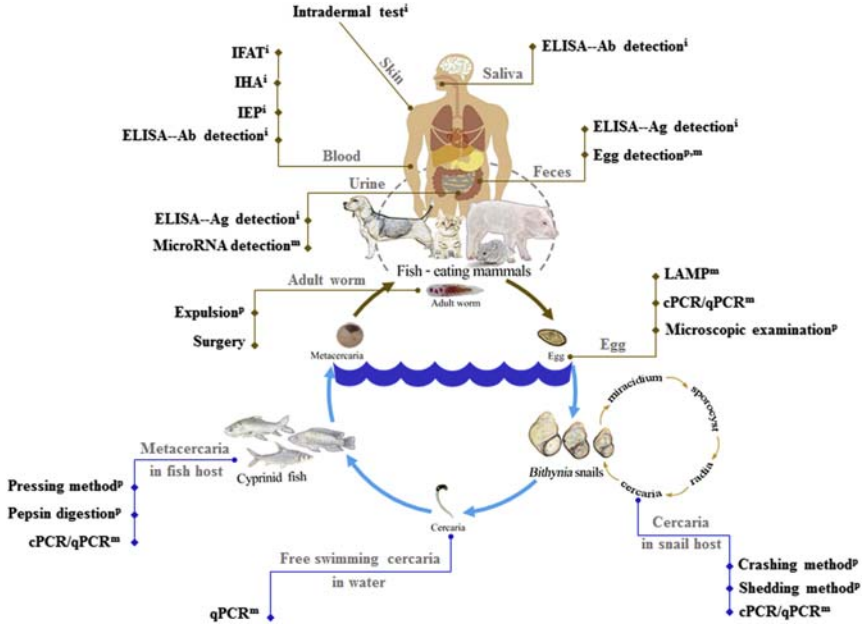


**Figure 1** The methods for diagnosis of *Opisthorchis viverrini* infection through its life cycle. p = parasitological method, i = immunological method, m = molecular method. *ELISA*, enzyme-linked immunosorbent assay; *PCR*, polymerase chain reaction.

the liver by postmortem autopsy (Sithithaworn et al., 1991). Based on autopsy study, the detection limit for finding eggs in the faeces using the standard formalin-ethyl acetate concentration technique (FECT) was estimated to require 20 worms or approximately 100 or less EPG. Individuals with low infection intensities and limited egg output are likely to be underdiagnosed by microscopic examination by as much as ~20% (Sithithaworn et al., 1991).

Similar to other intestinal helminthiases, the conventional diagnostic method is egg detection in faeces in human and animal hosts. The detection of eggs in faeces by microscopic examination is considered to be the gold standard for opisthorchiasis diagnosis. In addition, eggs in faecal samples can be detected during treatment of bile duct obstruction either in the bile, from nasobiliary, percutaneous transhepatobilbiary drainage, or in the duodenal fluid (Sithithaworn and Haswell-Elkins, 2003; Saijuntha et al., 2014). Occasionally, eggs can be found in biopsy materials or tissues resected during surgery or at necropsy, which can then be examined directly or can

be fixed and examined as stained histological sections for diagnosis and pathological purposes (Riganti et al., 1988, 1989).

However, the egg of *O. viverrini* which can be identified by characteristic rough and thick eggshell is very similar to the minute/small 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 (MIFs) due to the small size of the adult worms compared with the liver flukes (Chai et al., 2005; De et al., 2011). These species are, like the liver flukes, fish-borne trematodes (FBTs) or fish-borne zoonotic trematode (Lan-Anh et al., 2009; Phan et al., 2010a,b). The similarity to the eggs of these FBT species and those of *O. viverrini*, which often coexist in the same community, can substantially increase the likelihood of false positive diagnosis, depending on the prevalence of these species. Thus, apart from adult worm recovery for identification, the accurate diagnosis and differentiation of the minute eggs requires expertise of the microscopists. Parasitological techniques have been progressively developed and improved for easier diagnosis and differentiation of *O. viverrini* eggs from those of other flukes. For instance, fat extraction using diethyl ether or ethyl acetate to eliminate faecal debris from the sediment has made it cleaner for easier microscopic examination, such as in FECT (Elkins et al., 1990). Special staining of faecal specimens or sedimentation with potassium permanganate (Sukontason et al., 1999) or methylene blue (Pasuralertsakul et al., 2005) has been used to highlight the different characteristics of *O. viverrini* eggs from other morphologically similar eggs, such as those of *Haplorchis taichui*, *Phaneropsolus bonnie*, *P. molenkampi* and *Stellantchasmas falcatus*.

Because the collection of faecal samples is not an invasive procedure, detection of eggs in faecal samples is commonly used in routine work for the diagnosis of *O. viverrini* infection. A number of methods are available for faecal examination, including a direct smear, the modified thick Kato smear or Kato-Katz (KK) (Hong et al., 2003; Johansen et al., 2015), the modified FECT (Elkins et al., 1990), Stoll's dilution egg count technique (Viyanant et al., 1983) and recently the commercial faecal concentrator kit (Laoprom et al., 2016a). The KK method has been recommended for the diagnosis of liver fluke infections for both opisthorchiasis and clonorchiasis (infection with *Clonorchis sinensis*). For more accurate diagnosis, a repetitive examination on consecutive 3-day faecal samples by KK or a single examination by FECT was suggested (Sayasone et al., 2015). To circumvent the drawbacks of both KK and FECT, the faecal concentrator

kit (mini Parasep, Diasys, UK), which is a closed concentrating system, provides an increased ease of use and presents a reduced health hazard compared with conventional concentration methods. The initial test suggested that it is slightly less sensitive than FECT, but it gave better diagnostic accuracy than the simple smear method for the diagnosis of *O. viverrini* infection and for other parasitic infections (Laoprom et al., 2016a). Although the kit offers a safer and faster way to conduct the concentration procedure and is commonly used in field studies, there are considerable disadvantages in terms of cost and diagnostic accuracy compared with the KK method.

It is known that animal reservoir hosts (cats and dogs) are important in maintaining the *O. viverrini* life cycle and spreading the parasite (Enes et al., 2010; Pumidonming et al., 2016). Thus, the diagnosis and treatment of *O. viverrini* infection in these companion animals are important for the prevention and control of opisthorchiasis in endemic areas. The conventional diagnostic method is again the detection of eggs in faecal samples. It is known that these domestic animals often harbour multiple parasite infections, thus careful identification is required particularly for species that have a similar egg morphology to human parasites. For example, *Opisthorchis tenuicollis* has been recovered in pigs from Mumbai, India (Jawalagatti et al., 2016), *Opisthorchis lobatus* metacercariae were recovered from snakehead fish in Cambodia and can develop to adult worms in hamsters (Thaenkham et al., 2011), *Opisthorchis caninus*, can develop to adult worms in rhesus monkeys (Pande and Shukla, 1974), as well as an *Opisthorchis*-like fluke discovered in ducks (Dao et al., 2014) and other birds in Vietnam. This species was molecularly identified as an *Opisthorchis* sibling species of *O. viverrini* (Dao et al., 2016). Application of more accurate diagnostic methods to assess the true prevalence of infection in these animal reservoir hosts, such as immunological or molecular methods, is not available.

## 2.2 Immunological Methods

Immunodiagnostic tests have the advantage of being applicable during all stages of the disease, and consequently they are commonly used for the diagnosis of infections in field situations. Several serological tests for human opisthorchiasis have been developed for use in a diagnostic assay with greater sensitivity and specificity than the faecal examination technique. These include the intradermal test, immunoelectrophoresis, indirect haemagglutination assay, indirect fluorescent antibody test and indirect enzyme-linked

immunosorbent assay (indirect ELISA) (Wongratanacheewin et al., 2003). For these diagnostic methods, the type(s) of antigen (Ag) and reacting antibody (Ab) play an important role in the sensitivity and specificity of the test. Thus, selection of an appropriate antigen and antibody molecule for immunological diagnosis is a crucial step. There are, however, many candidate molecules that could serve as antigen or induced antibody for the diagnosis of *O. viverrini* infection (Fig. 2).

Immunological parameters have been used as biomarkers for parasite-induced pathologies. Measurements of delayed-type hypersensitivity responses to unrelated antigens, and liver fluke-specific IgG and IgA levels in serum were correlated with the biliary tract abnormalities of residents from an endemic community, as determined by ultrasonography (Haswell-Elkins et al., 1991; Mairiang et al., 1992). Immune responsiveness to unrelated antigens did not vary with the intensity of parasite infection or disease status. Of all the variables, IgG levels were most markedly elevated in disease cases compared with normal subjects and were closely associated with gall bladder size and dysfunction. This is consistent with the hypothesis that an immunopathological mechanism is involved in opisthorchiasis and

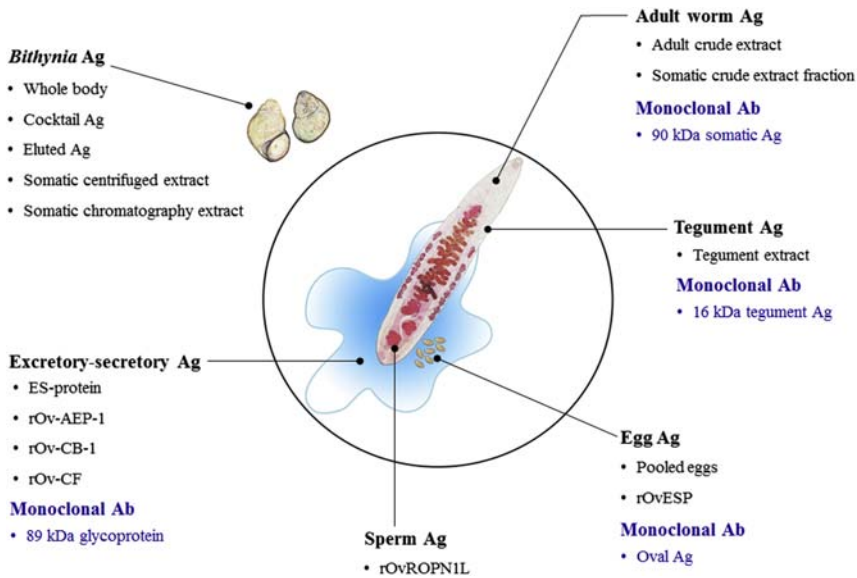


**Figure 2** Antigen and antibody used for immunodiagnosis of *Opisthorchis viverrini* infection. *AEP*, asparaginyl endopeptidase; *CB*, cathepsin B; *CF*, cathepsin F; *ES*, excretory–secretory.

suggests that antibody levels may be useful in screening populations for fluke-associated hepatobiliary disease (Haswell-Elkins et al., 1991; Sripa et al., 2012a). The persistency level of parasite-specific IgG in serum has been utilized to indicate past *O. viverrini* infection and risk factor for CCA (Sithithaworn et al., 2014).

On the serodiagnosis front, several types of liver fluke-derived antigens and recombinant proteins were evaluated for diagnostic usefulness. Crude somatic extract of adult *O. viverrini* was the most common antigen used for antibody detection by ELISA (Haswell-Elkins et al., 1992; Wongratanacheewin et al., 2003), providing higher diagnostic performance than faecal examination (Poopyruchpong et al., 1990; Wongsaroj et al., 2001). The composition of somatic extract of adult *O. viverrini* was identified by radioimmunoprecipitation and polyacrylamide gel electrophoresis. It was composed of protein sizes of >116, 89, 78 and 20 kDa. Several proteins in the extract reacted specifically to human opisthorchiasis serum. A protein with molecular weight 89 kDa was found the most specific antigen for the diagnosis of *O. viverrini* infections in humans (Wongratanacheewin and Sirisinha, 1986). This antigenic protein was dominant in the excretory–secretory proteins (ES product) from adult worms (Wongratanacheewin et al., 1988).

An extraction of surface tegument proteins also reacted specifically to sera of human opisthorchiasis, i.e., >116, 108, 64, 38, 34, 20 and 16–17 kDa proteins. Of these, the 16–17 kDa protein revealed the highest concentration, but low immunogenicity compared with 89 kDa protein (Sirisinha et al., 1990). Production of these parasite-derived antigens, especially the ES antigen, is costly because it requires both a laboratory and an experimental animal facility. As such, the supply of antigens for research and commercial exploitation is limited.

With the advances in liver fluke genomics and proteomics, several recombinant antigenic molecules have been examined and assessed for their diagnostic potential. The recombinant antigen produced from eggs and the glycine tyrosine–rich eggshell proteins (rOvESP) of *O. viverrini* were used for serum antibody detection by ELISA (Wongsaroj et al., 2001; Ruangsittichai et al., 2006). Proteins in the ES product examined and synthetic peptide antigen produced were asparaginyl endopeptidase (rOv-AEP-1), cathepsin B-1 protease (rOv-CB-1) and cathepsin F (rOv-CF) (Laha et al., 2008; Sripa et al., 2012b; Teimoori et al., 2015), as well as the rhophilin-associated tail protein of sperm (rOvROPN1L) (Rattanachan et al., 2014). In addition, the propeptide of cathepsin F and cathepsin L-like

has been characterized and showed better diagnostic sensitivity and specificity to the conventional faecal examination methods (Kaewpitoon et al., 2008; Pinlaor et al., 2009). As more recombinant proteins are produced, such as of the *C. sinensis* proteins (Li et al., 2011), a suitable antigen(s) for better diagnosis and screening of the high risk human population should be forthcoming in the near future.

As a snail-borne trematode, *O. viverrini* has an intimate evolutionary relationship with its *Bithynia* snail intermediate hosts (Saijuntha et al., 2007). Most interestingly, there is a common antigenic protein in the parasite and its snail host: antigen extracted from the *Bithynia* snails can be used to detect a specific antibody for diagnosis of human *O. viverrini* infection. The association between *O. viverrini* and its snail intermediate hosts, *Bithynia funiculata*, *Bithynia siamensis siamensis* and *Bithynia siamensis goniomphalos*, was determined based on the correlation between shared antigens and infection rates. Crude extract of *O. viverrini* adult worms produced the same number of two common precipitin bands of four different electrophoretic mobilities against anti-*B. funiculata* and anti-*B. s. siamensis* sera and one precipitin band in the antigen well region against anti-*B. s. goniomphalos* serum. The three snail antigens produced the same number of two common precipitin bands against anti-*O. viverrini* serum (Chanawong et al., 1990). Four batches of crude antigens, i.e., adult *O. viverrini* including whole body, head–foot and visceral mass of *B. funiculata* were analyzed by IgG-ELISA. *B. funiculata* antigen was the most effective for the diagnosis of *O. viverrini* infection. However, cross-reactions may occur with sera from patients with paragonimiasis and strongyloidiasis (Watthanakulpanich et al., 1997). Cocktail antigen was prepared using Sephacryl S-200 HR gel filtration chromatography with electroeluted *Bithynia* snail antigens being found at 53 kDa from polyacrylamide gel. The eluted antigens have a higher sensitivity and specificity for diagnosis using indirect ELISA. However, cross reactions with the cocktail antigen were seen in some other infections such as hymenolepsiasis and strongyloidiasis (Waikagul et al., 2002). In a subsequent study, total IgG and IgG1-4 were selectively reactive to antigens of *B. s. goniomphalos* extract derived from liquid-phase isoelectric focusing (IFE). Antigens (Iso-AgF) from 20 IEF fractions were selected based on a high ELISA-OD ratio between pooled-positive and pooled-negative sera. Iso-AgF 7, 7, 6, 2 and 10 resulted in high OD-ratios to total IgG, IgG1, 2, 3 and 4, respectively. Iso-AgF7 to IgG1 showed the best result, with sensitivity, specificity, positive and negative predictive value of 100%, 96%, 86% and 100%, respectively. Low cross-reactivity of IgG1 was found in

one case each of gnathostomiasis, trichinellosis, toxocariasis, angiostrongyliasis, bancroftian filariasis, enterobiasis, neurocysticercosis and taeniasis (Pakdee et al., 2010).

Taken together, the *Bithynia* snail antigens approach here may become a promising alternative to the parasite-derived antigens. It is, however, important that any subsequent research should be based within the molecular/genetic framework of the discovery that *B. s. goniomphalos* is a species complex (Saijuntha et al., 2007; Laoprom et al., 2012; Kiatsopit et al., 2014) with may cryptic species associated with cryptic species in the *O. viverrini* species complex in defined wetland systems.

Blood collection by venipuncture requires either a phlebotomist or trained nurse and is an invasive method of sample collection. Alternatively, clinical samples such as urine and saliva are easier to collect; this is done noninvasively and is better accepted in the human population. Antibody detection by ELISA in nonblood samples, such as urine and saliva, has received considerable attention. ELISA used to detect IgG and IgG4 in the urine of opisthorchiasis patients revealed that IgG detection is a promising diagnostic method for opisthorchiasis (Tesana et al., 2007). Moreover, the detection of urine IgG specific to *O. viverrini* antigen may indicate a degree of renal pathology in opisthorchiasis patients, in addition to a greater risk of developing cholangicarcinoma (Saichua et al., 2013). In another study, the patient's saliva was found to have more potential for use in antibody detection than urine (Sawangsoda et al., 2012), and the level of saliva antibody was correlated with an active opisthorchiasis (Chaiyarit et al., 2011).

As opposed to antibody detection, antigen detection is one of the attractive alternatives in the diagnosis of opisthorchiasis. Antigen detection reflects the current status of infection and is relevant for treatment and control. Several reports have tested the usefulness of determining the presence of ES products of *O. viverrini* in faeces. An 89 kDa antigen in the ES products has been found to be a promising candidate for coproantigen detection by ELISA (Amornpunt et al., 1991; Sirisinha et al., 1991). A monoclonal antibody—based enzyme-linked immunosorbent assay (MAb-ELISA) has been developed to detect antigen for the diagnosis of ongoing opisthorchiasis. Specific monoclonal antibodies have been produced against *O. viverrini* antigenic peptides such as 16 kDa tegument protein, 90 kDa somatic protein and 89 kDa glycoprotein (Amornpunt et al., 1991). The sensitivity was higher than microscopic detection of *O. viverrini* eggs (Sirisinha et al., 1995). Another study indicated sensitivity 31% and specificity of 100%

(Chaicumpa et al., 1992). On the other hand, Sirisinha et al. (1991) reported a sensitivity and specificity using MAb-ELISA of 57.1% and 70.5%, respectively, using a monoclonal antibody reactive to the 89 kDa ES antigen of *O. viverrini*. Treatment of faecal samples with modified trichloroacetic acid improved detection sensitivity of MAb-ELISA to 52 ng/mL and provided a quantitative diagnosis (Watwiengkam et al., 2013). Recently, coproantigen detection by sandwich ELISA using recombinant *O. viverrini* cathepsin F chicken IgY in combination with rabbit IgG antibody to the somatic *O. viverrini* antigens was investigated. This method showed a detection limit of 70 ng/mL by spiking the parasite antigens into control faeces. When applied for the diagnosis, the IgY-based sandwich ELISA exhibited sensitivity and specificity of 93.3% and 76.7%, respectively (Teimoori et al., 2017). To change from faecal to urine samples, recently, a novel urinary antigen detection method was established for the quantitative diagnosis of opisthorchiasis by MAb-ELISA with 81% sensitivity and 70% specificity when compared with the current gold standard diagnostic method (Worasith et al., 2015). The advantage of using urine samples is the noninvasiveness of sample collection and the high acceptability by individuals. These antigen detection methods offer many advantages, being simple, reproducible and allowing for the simultaneous analysis of many samples without any special equipment. This technique may be used for mass screening, especially in new endemic areas. It also detects only current infections.

## 2.3 Molecular Methods

There are, nevertheless, limitations to the parasitological and immunological methods of diagnosis, such as the difficulty of differentiating the eggs of *O. viverrini* from those of other fish-borne trematodes, the detection of light infections, the limited immune response period and cross-reactions in immunodiagnosis. Molecular diagnosis represents an alternative choice for solving these problems. A number of target genes from *O. viverrini* have been tested for their diagnostic suitability, including satellite DNA, ITS1, ITS2, 28S rRNA, mitochondrial DNA and microRNA. Several genetic markers/techniques involving conventional polymerase chain reaction (PCR) alone or combined with other techniques, e.g., PCR-restriction fragment length polymorphism (RFLP), multiplex PCR, pyrosequencing and LAMP and real-time PCR, can potentially be used to differentiate *O. viverrini* from other species (Table 1). In addition, molecular identification techniques can be used in cases of multiparasite infections in a single host (Sato et al., 2009; Thaenkham et al., 2011). The molecular methods

**Table 1** Molecular Diagnosis of *Opisthorchis viverrini* Infection

| Molecular Method | DNA Target | Specimens | Purpose of Study | References |
|---|---|---|---|---|
| cPCR | pOV-A6 DNA | Egg spite in hamster feces | Diagnosis/detection | Wongratanachewin et al. (2001) |
| cPCR | pOV-A6 DNA | Feces | Diagnosis/detection | Stensvold et al. (2006) and Duenngai et al. (2008) |
| cPCR | pOVCRA26-5 DNA | Fish tissue/ metacercariae | Diagnosis/detection | Parvathi et al. (2008) |
| cPCR | pOVCRA26-5 DNA | Feces | Diagnosis/detection | Umesha et al. (2008) |
| cPCR | pOV-A6 DNA | Snail and fish tissue | Diagnosis/detection | Maleewong et al. (2003) |
| mPCR | Mitochondrial DNA | Adult, metacercariae and egg | Differential diagnosis | Le et al. (2006) |
| cPCR combined qPCR | CO1 | Feces | Detection | Lamaningao et al. (2017) |
| qPCR | CO1 | Feces | Diagnosis/detection | Hashizume et al. (2017) |
| mqPCR and HRMA | ND2 | Feces | Differential diagnosis | Kaewkong et al. (2013) |
| TaqMan-qPCR | RPLO gene | Liver tissue | Detection | Suksumek et al. (2008) |
| FRET-PCR | pOv-A6 DNA | Feces | Detection | Intapan et al. (2009) |
| FRET-PCR and melting curve analysis | pOv-A6 DNA | Infected fish | Detection | Intapan et al. (2008) |
| FRET-PCR and melting curve analysis | pOV-A6 DNA | Feces | Differential diagnosis | Janwan et al. (2011) |
| FRET-PCR | pOV-A6 DNA | Infected Bithynia snails | Detection | Sri-Aroon et al. (2011) |
| FRET-PCR and melting curve analysis | ND2 | Feces | Differential diagnosis | Sanpool et al. (2012) |
| qPCR and HRMA | CO1 | Adult DNA | Differential diagnosis | Cai et al. (2014) |
| cPCR and pyrosequencing | 28S rRNA | Adult DNA and feces | Differential diagnosis | Tantrawatpan et al. (2014) |
| cPCR, PCR-RFLP and DNA sequencing | 18S-ITS1-5.8S rDNA | Adult DNA | Differential diagnosis | Kang et al. (2007) |

| | | | | |
|---|---|---|---|---|
| cPCR and DNA sequencing | CO1 | Feces | Confirmation identification | Aung et al. (2017) |
| cPCR and DNA sequencing | ITS2 | Cercaria from infected snails | Confirmation identification | Boonmekam et al. (2017) |
| cPCR, DNA sequencing and qPCR | CO1 | Feces | Differential diagnosis | Lamaningao et al. (2017) |
| cPCR and DNA sequencing | CO1 | Adult DNA | Confirmation identification | Vo et al. (2014) |
| cPCR and DNA sequencing | CO1 and ND1 | Feces | Diagnosis and identification | Buathong et al. (2015, 2017) |
| cPCR, PCR-RFLP and DNA sequencing | ITS2 | Feces | Diagnosis/identification | Traub et al. (2009) and Buathong et al. (2017) |
| Loop-mediated isothermal amplification | ND1 | Feces | Diagnosis | Le at al. (2012) |
| | ITS1 | Feces | Diagnosis | Arimatsu et al. (2012, 2015) |
| | Microsatellites (OVMS6) | Feces | Diagnosis | Arimatsu et al. (2015) |
| qPCR | MicroRNA | Urine | Diagnosis | Silakit et al. (2017) |
| cPCR | Retrotransposon DNA (OV-RTE-1) | Adult DNA and feces | Diagnosis | Thi Phung et al. (2014) |
| qPCR | CO1 | Water | Detection in environment | Hashizume et al. (2017) |
| Multiplex ligation-dependent probe amplification | ITS1 | Adult DNA | Differential diagnosis | Sun et al. (2011) |
| HAT-RAPD | Unknown | Adult DNA | Differentiation | Wongsawad and Wongsawad (2012) |

*dqPCR*, duplex real-time PCR; *FRET*, fluorescence resonance energy transfer; *HAT-RAPD*, high annealing temperature-random amplified polymorphic DNA; *mqPCR*, multiplex real-time PCR; *PCR*, polymerase chain reaction; *RFLP*, restriction fragment length polymorphism.

discussed below 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 are needed. In addition, the validation of DNA positive test results is required, although evidence from animal models is accumulating and is supported by human studies (Rahman et al., 2011; Duenngai et al., 2013). Such approaches can also be used in a food security assessment to test for the presence of liver flukes in aquaculture or native fisheries products, particularly for export (Parvathi et al., 2007, 2008; Cai et al., 2010). Due to their high specificity, such molecular diagnostic tests are likely to play an increasing role in anthelminthic drug efficacy evaluations, the rigorous monitoring of reinfection patterns, and to investigate changes in the endemic range of the liver flukes (Touch et al., 2009; Chen et al., 2010).

The early period of the molecular diagnosis of *O. viverrini* infection focused on developing species-specific primers for detecting *O. viverrini* DNA. The repeat DNA element (pOV-A6 DNA) was shown to be a good molecular marker for this purpose. Primer OV-6 was proven to be specific for pOV-A6 DNA of *O. viverrini* with high sensitivity and specificity (Wongratancheewin et al., 2001). 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 1000 EPG. In light infections with less than 200 EPG, the sensitivity was reduced to only 68% (Wongratancheewin et al., 2001, 2002). In another, subsequent PCR-based study using the same target DNA, pOV-A6 showed low sensitivity (50%) at high egg counts of more than 1000 EPG in faecal samples from Lao PDR (Stensvold et al., 2006). 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% (Duenngai et al., 2008). The pOVCRA26-5 DNA method was subsequently developed and used for specific detection of *O. viverrini* DNA in human faeces with high sensitivity and specificity (Umesha et al., 2008). More recently, the retrotransposon of *O. viverrini* (*OV-RTE-1*) was found to be a new alternative genetic marker of high sensitivity and specificity for the PCR diagnosis of opisthorchiasis. PCR-positive tests occurred in 29% of cases that were parasite-negative using the conventional faecal examination method, indicating its potential diagnostic value for light infections (Thi Phung et al., 2014). PCR methods

targeting the CO1 and ND1 genes to detect *O. viverrini* in faecal specimens have been developed. The sensitivities of PCR for CO1 and ND1 were 89.1% and 71.7%, respectively, in specimens containing greater than 100 EPG *O. viverrini* eggs (Lovis et al., 2009; Buathong et al., 2015).

Conventional PCR combined with other techniques such as RFLP (PCR-RFLP) of 18S-ITS1-5.8S rDNA can be used to reliably differentiate between *O. viverrini*, *C. sinensis* and *Opisthorchis felineus* infections (Kang et al., 2008). PCR-RFLP of the ITS2 region can be used to differentiate *O. viverrini* from *C. sinensis*. This method was used to detect *C. sinensis* DNA from Central Thailand (Traub et al., 2009). Subsequently, use of PCR-RFLP of the ITS2 region and DNA sequencing of CO1 and ND1 genes for confirmation of the identification of *O. viverrini*-like eggs detected in faecal samples of the villagers from Chachoengsao Province, Central Thailand found no *C. sinensis* in this endemic *O. viverrini* area (Buathong et al., 2017), as previously reported by Traub et al. (2009). The HAT-RAPD PCR method was used to differentiate *O. viverrini* from *H. taichui*. Two pairs of specific primers were designed to amplify *H. taichui* and *O. viverrini*. These generated a specific PCR products of 170 and 319 bp, respectively, with no cross-reaction with any of the other parasites tested (Wongsawad and Wongsawad, 2012). A combination of cPCR and real-time PCR (qPCR) of the CO1 region for detecting and discriminating between *O. viverrini* and *H. taichui* infections was developed for testing clinical stool samples and exhibited a sensitivity of 100% and specificity for *O. viverrini* and *H. taichui* of 71% and 91%, respectively (Lamaningao et al., 2017).

Another PCR-based molecular identification method is multiplex ligation–dependent probe amplification using the ITS1 region, which allows rapid and specific detection of single nucleotide acid differences between *O. viverrini*, *C. sinensis* and *O. felineus* (Sun et al., 2011). A PCR assay coupled with DNA pyrosequencing was developed for the identification of FBTs, including *O. viverrini*. A region of 28S rRNA with a length of 46–47 nucleotides contained variable positions for species-specific identification that were selected for primer design and PCR amplification. This method can differentiate *O. viverrini* from the other FBTs, i.e., *C. sinensis*, *H. taichui*, *H. pumilio* and *S. falcatus* by seven nucleotide positions (Tantrawatpan et al., 2014). The multiplex approach is potentially useful not only for human diagnosis but also for fisheries product inspection.

Loop-mediated isothermal amplification (LAMP) is another potential molecular method that is widely used for the detection and identification of parasitic trematodes. It has been established for the detection of *O. viverrini* with a higher sensitivity than microscopic examination and conventional PCR. The mitochondrial ND1 region was used by LAMP to detect *O. viverrini* DNA (mito-OvLAMP) using four LAMP primers. The specificity was 100% when tested with *C. sinensis*, *H. pumilio*, *H. taichui*, *Fasciola hepatica* and *F. gigantica*. The limit of DNA detection was very low at 100 fg, indicating 10 to 100 times more sensitivity than cPCR (Le et al., 2012). Although the ITS1 region was selected for LAMP analysis (ITS1-LAMP) with five primers this revealed high sensitivity but low specificity because other parasites were also amplified (Arimatsu et al., 2012, 2015). Thus, the other species-specific region, namely microsatellite 6 (OVMS6), was selected for LAMP analysis (HNB-LAMP). This showed a specifically amplified region of DNA from *O. viverrini*, but not from *C. sinensis*, *O. felineus*, *Centrocestus caninus*, *H. taichui*, *F. gigantica* or *Haplorchoides* sp. This had sensitivity 1000 times higher than ITS1-LAMP, which only needed 1 ng of DNA to be detected (Arimatsu et al., 2015).

Recently, real-time or quantitative PCR (rt-PCR or qPCR) has been widely used for the diagnosis of human parasitic infections. TaqMan real-time PCR assay was used to detect *O. viverrini* DNA in the liver tissue of hepatocellular carcinoma (HCC) and CCA patients (Suksumek et al., 2008). Real-time fluorescence resonance energy transfer (FRET) PCR was consequently developed to detect the pOV-A6 DNA of *O. viverrini* in human faecal samples with high sensitivity and specificity (Intapan et al., 2009; Janwan et al., 2011), as well as to differentiate *O. viverrini* and *C. sinensis* eggs in human faecal samples by real-time FRET PCR with a melting curve analysis of the ND2 region (Sanpool et al., 2012). Multiplex real-time PCR coupled with high-resolution melting (HRM) analysis of the ND2 region for the differentiation of *O. viverrini* and *C. sinensis* eggs in faecal samples has been developed (Kaewkong et al., 2013). An integrating assay of real-time PCR and HRM analysis of the CO1 region for the specific detection and rapid identification of *C. sinensis* and *O. viverrini* has also been developed (Cai et al., 2014). A new real-time PCR of the CO1 region has been developed for the detection of *O. viverrini* DNA in human faecal and environmental water samples in Lao PDR (Hashizume et al., 2017).

The availability of highly specific molecular methods has led to the idea of multiple parasite detection that can give a complete transmission

picture of NTDs allowing more suitable and better prevention and control (Llewellyn et al., 2016; Meurs et al., 2017).

## 3. DETECTION FROM THE INTERMEDIATE HOSTS AND FROM THE ENVIRONMENT

To complete its life cycle, *O. viverrini* needs two intermediate hosts, i.e., the first is *Bithynia* snails and the second is freshwater cyprinid fish. Knowledge of the prevalence of infection in these intermediate hosts is essential to confirm parasite endemicity and transmission dynamics. For transmission, the developed stages need to pass through environment between hosts to reach the new hosts, i.e., eggs have to enter the freshwater and wait to be eaten by *Bithynia* snails. In these snails, they develop into cercariae that are shed into the water as free-swimming cercariae and seek a cyprinid fish in which they become metacercariae. Thus, our ability to detect the various stages *O. viverrini* within the species complex is critical for our understanding of the eco-epidemiology of this parasite.

### 3.1 Snails

The intramolluscan developmental stages of *O. viverrini* in *Bithynia* snails consist of miracidium, sporocyst, redia and cercaria. Of these stages, the cercariae are the most easily detected as they shed into the water to find their next host (Kiatsopit et al., 2012). Usually, snails are individually placed in small containers and incubated for some time under constant illumination and temperature to induce shedding. After this, the emerged cercariae can be identified under a stereomicroscope and their prevalence recorded. Cercariae can be stained by 0.5% neutral red and identified according to their specific characteristics as pleurolophocercus cercaria (formed of a body and tail), the body has two suckers, a primitive gut, two eye spots, five pairs of penetration glands, cystogenous glands and an excretory bladder. The tail is simple and longer than the body and is provided with cubicular sheath. The cercariae do not emerge during darkness, but low-intensity light could induce a release (Phongsasakulchoti et al., 2005). The seasonal pattern of *O. viverrini* s.l. cercarial emergence reveals that the hot-dry season shows the highest prevalence, which declines in the rainy and cool-dry seasons. Peak cercarial emergence occurred between 08:00 and 10:00 h during the rainy and cool-dry seasons and between 10:00 and 12:00 h during the hot-dry season (Kiatsopit et al., 2012; Laoprom et al., 2016b). Light intensity is the most important

stimulus determining the quantity of *O. viverrini* cercariae shed from naturally infected *Bithynia* snails. Snails were evaluated for cercariae output every hour after exposure to various light intensities for a total period of 7 h. The same infected snail was tested under different intensities of light: in the dark, and at 1000, 3000 and 5000 lx. The data showed that under exposure to 1000 and 3000 lx of light, the average percentage and number of cercariae released were higher than when exposed to 5000 lx during the first 2 h of the experiment. In contrast, under higher illumination (5000 lx) a longer time (6 h) was required to stimulate the peak emergence of cercariae. Among the three intensities of light, exposure at 1000 lx induced the highest average number of released cercariae per snail and the highest percentage of cercarial emergence within the first 2 h (54.86%), followed by exposure at 3000 lx (25.58%) and 5000 lx (7.78%). The results suggest that the light intensity of 1000 lx for 2 h would be optimal for *O. viverrini* cercarial shedding from naturally infected *B. s. goniomphalos* snails (Kaewkes et al., 2012).

To increase the accuracy of nondestructive method and also to detect prepatent infections with trematode larvae, multiple sequential shedding for several days or weeks can be used (Studer and Poulin, 2012). Other methods are rarely used as they are time-consuming, for example, enzymatic electrophoresis of the snail's digestive glands, which allows the detection and identification of immature stage infection (Caron et al., 2008). Trematode cercarial release as a detection method has been criticized as inaccurately by several studies (Born-Torrijos et al., 2014). A very low prevalence of *O. viverrini* infection in *Bithynia* snails (estimated lower than 0.01%) has been reported using this detection method in several endemic areas (Ngern-klun et al., 2006). Moreover, in some cases snails containing mature cercariae did not shed (Stunkard and Hinchliffe, 1952). However, there are reports of varying prevalence of *O. viverrini* infection in *Bithynia* snails in some endemic areas in Thailand, Lao PDR and Vietnam (Kiatsopit et al., 2012; Dao et al., 2017), which could reflect that we are dealing with cryptic species of both that may have coevolved and may have differing biological properties/responses etc. In Vietnam, *O. viverrini* cercariae were observed in *B. s. goniomphalos* and *B. funiculata*, at infection rates of 0.86% and 0.14%, respectively (Dao et al., 2017).

Investigating infection levels in snails has already highlighted the importance of PCR-based techniques (Born-Torrijos et al., 2012). The real-time FRET PCR method of pOV-A6 DNA revealed an *O. viverrini* infection rate in snails of 8.52%, whereas using the cercarial shedding method this

was 2.47% (Sri-Aroon et al., 2011). The conventional PCR of pOV-A6 showed a high sensitivity and specificity to *O. viverrini* cercarial DNA in infected *Bithynia* snails (Maleewong et al., 2003). Moreover, based on currently available DNA sequences of *O. viverrini* submitted in GenBank, confirmation of the identification of *O. viverrini* becomes easier using blast DNA sequences. For example, the ITS2 DNA sequence was used for confirmation of the identification of *O. viverrini* cercariae shed by *Bithynia* from Cambodia (Boonmekam et al., 2017). Moreover, there are several molecular markers/methods that specifically detect *O. viverrini* DNA, such as LAMP, but this has not yet been applied to cercarial DNA in infected *Bithynia*. Thus, future research may require the application of modern molecular techniques, e.g., next generation sequencing for *O. viverrini* DNA detection in *Bithynia*.

## 3.2 Fish

The infection rate of *O. viverrini* in fish is usually higher than in the first intermediate hosts, *Bithynia* snails (Sithithaworn and Haswell-Elkins, 2003; Petney et al., 2012). Detection of *O. viverrini* metacercariae in their fish hosts is based on morphological identification (Chai et al., 2014). The method of recovering metacercariae from fish is usually done by using the pepsin digestion method (Sithithaworn et al., 1997; Srisawangwong et al., 1997). The sediment contains the metacercariae which can be identified under a stereomicroscope. In a study on cyprinid fish of 13 species collected from 20 provinces in northeastern Thailand, *O. viverrini* metacercariae were found in six species, *Cyclocheilichthys armatus*, *Systomus rubripinnis* (=*Puntius orphoides*), *Hampala dispar*, *Gymnostomus siamensis*, *Osteochilus vittatus* (=*Osteochilus hasselti*) and *Puntioplites proctozysron* (fish taxonomy based on Kottelat, 2013). Among the sites where *O. viverrini* metacercariae—infected fish were found, 70.0% were dams, 23.7% were ponds/lakes and 7.7% were rivers (Pinlaor et al., 2013). *O. viverrini*—like metacercariae were found in 10 fish species representing both Cyprinidae and noncyprinid families. The prevalence of *O. viverrini* infection in fish was significantly associated with species. *Carassius auratus*, a fish species commonly eaten raw, *Rasbora aurotaenia* and *Puntius brevis* had the highest prevalences of 74.0%, 55.8% and 31.6%, respectively. Sharing of the same snail and fish intermediate host species was found for *O. viverrini* and an *O. viverrini* duck genotype that are sympatric in central Vietnam (Dao et al., 2017). A total of 1479 freshwater cyprinid fish comprising 20 different species was surveyed for *O. viverrini* metacercariae in southern Cambodia. *O. viverrini*—metacercariae

were found in 10 species with the infection rate ranging 2.1%–66.7%. Adult worms were obtained by experimental infection in hamsters. The liver flukes found in this study were identified as *O. viverrini* by the morphological features of adult worms, and this identification was confirmed by partial CO1 sequencing of the metacercariae (Touch et al., 2009).

Because the morphology of fish-borne trematode metacercariae, i.e., *O. viverrini* and MIFs such as *H. taichui*, are similar, molecular confirmation is sometimes required. The PCR procedure for the detection of *O. viverrini* in natural and experimentally infected cyprinid fish was developed by Pitaksakulrat et al. (2013). This procedure was based on primers designed from pOV-A6 DNA (Wongratanacheewin et al., 2001). The detection was accomplished in host tissue homogenates to which a single metacercaria was introduced. PCR can detect from as little as a single metacercaria in a fish sample. The method gave 100% detectability for all samples (Maleewong et al., 2003). This assay is specific, with no cross-reactions with other trematodes commonly found in fish, including the closely related species *C. sinensis*. The sensitivity of the assay was determined $10^{-12}$ ng of *O. viverrini* DNA, whereas in artificially spiked fish meat, three metacercariae could be detected (Parvathi et al., 2008). The real-time FRET PCR of pOV-A6 DNA could also detect as little as a single metacercaria artificially inoculated in fish samples (Intapan et al., 2008).

## 3.3 Environment

The life cycle stages of *O. viverrini* need a period in the external environment, i.e., soil and water, for transmission between hosts of the adult worms who produce eggs and of cercariae. Thus, eggs and cercariae should be detectable in these. Due to the small size of the eggs and cercariae, to directly observe them is extremely difficult, thus the most efficient method is DNA detection using molecular methods. This so-called environmental DNA (eDNA) indicates the presence of organisms contaminating in environmental water or soil samples (Ficetola et al., 2008; Minamoto et al., 2012). Currently there has only been limited work on the detection of *O. viverrini* DNA in the environment. A species-specific real-time PCR assay using the CO1 region for detecting *O. viverrini* using eDNA was developed and the efficacy of eDNA analysis and its applicability in the field were tested using a total of 94 environmental water samples collected from 44 sites in Savannakhet, Lao PDR during May and October 2015 and February 2016. *O. viverrini* eDNA was detected in five samples by real-time PCR, indicating the presence of the fluke in the area and the risk

of infection for individuals consuming fish from these water sources (Hashizume et al., 2017). It remains to be seen whether such an application is related to transmission in endemic communities.

## 4. IMPLICATIONS FOR EPIDEMIOLOGY, PREVENTION AND CONTROL

Accurate and reliable diagnostic methods are essential for epidemiological studies and play an important role in developing prevention and control strategies for diseases at the community and clinical levels. The diagnostic standard for active opisthorchiasis is the presence of viable eggs in faeces. Several conventional methods have been used such as Stoll's dilution egg count, FECT and KK. While quantitative FECT has been preferred because of the sample storage time after being fixed in formalin (Elkins et al., 1990), KK has been used extensively for cross-sectional surveys and monitoring drug efficacies (Sayasone et al., 2015, 2016, 2017, 2018) due to simplicity of the techniques. All of these conventional microscopy—based methods have a common weak point as they are operator dependent and hence diagnostic accuracy depends largely on the experience of the microscopist and to a lesser extent on sample preparation. To circumvent the low sensitivity of the faecal examination method, repeated sample examinations over 3 days or a single FECT has been recommended (Sayasone et al., 2015). FECT analyzes a greater specimen sample of up to 2 g of faeces but requires laboratory equipment that is not always available in most field settings. KK, on the other hand, uses a smaller sample specimen (approx. 41 mg) but requires no special equipment except the regents and the microscope. The all in one tube faecal concentrator kit (with fixative and built in filter system) offers an intermediate between FECT and KK using approximately 0.5 g of faecal material. Field trials for screening for opisthorchiasis using the faecal concentrator kit (mini Parasep, Diasys, UK) were initiated and the kit provided results comparable to that of FECT (Laoprom et al., 2016a). A similar comparison with KK was evaluated and the kit gave a comparable diagnostic performance (Kopolrat, unpublished data). Although a more in-depth study is needed, the fact that the faecal concentrator kit can be kept over several months after being suspended and fixed in formalin similar to FECT means that it offers some advantages over the KK method. From the public health perspective, these conventional faecal examination methods require experience microscopist and also an adequate laboratory facility. These methods are thus far

from ideal for field work in a resource poor setting so that a simplified method is needed to facilitate and enhance control efforts.

The Thai Ministry of Public Health (MOPH) launched a national policy to control opisthorchiasis and CCA to be operated from 2015 to 2026. In 2015, the targeted population screened by faecal examination (Kato-thick smear) involved 76,000 people in 84 subdistricts in 27 provinces in the north, northeast and central Thailand. The urine assay for antigen detection based from our previous work (Worasith et al., 2015) was applied in 23 subdistricts in addition to faecal examination ($n > 20,000$). This initial survey at base line showed that the average prevalence of opisthorchiasis was 11% by faecal examination (combining Kato thick smear and FECT), but this proved to be 41% by urine antigen assay (Sithithaworn, unpublished data). The availability of a sensitive urine assay has, for the first time, allowed us to map the prevalence of opisthorchiasis without the need of stool collection. Using the urine assay, 1 year after the treatment of infected individuals with praziquantel, the prevalence was reduced substantially, but reinfection and new infections reached approximately 6%–10%. Due to the low sensitivity of faecal examination, even by quantitative FECT, data for reinfection and new infection are very limited. Obviously more research is needed for a better and improved diagnosis of opisthorchiasis by molecular, as well as serological techniques. The possibility of the antigen detection in urine has made the detail study transmission dynamics and surveillance much more feasible. Consequently, the results indicate strongly that repeated treatment is required to control and eventually eliminate the parasite in the community. The situation is, to a certain extent, similar to the case of urine antigen detection in schistosomiasis in which schistosomiasis is more prevalent than previously thought creating uncertainty among public health policy makers (Colley et al., 2013). According to the Thai MOPH policy, the cumulative targets for *O. viverrini* screening over the next 10 years are >4 million people. The currently used method for urine assay by ELISA is clearly not practical for handling such a massive screening target. The simplified method, such as using the rapid point of care test format, is required and is being developed for future use in Thailand and for application in other endemic countries.

An important public health aspect of monitoring, control and elimination programs is the detection of *O. viverrini* infections in the snail and fish hosts. Data on snail and fish infections enable the identification of environmental contamination during control and elimination programs.

Fully patent infections in these hosts are detected by parasite recoveries and by molecular parasitological techniques such as PCR or LAMP assays (Parvathi et al., 2008; Duenngai et al., 2008; Hashizume et al., 2017; Arimatsu et al., 2012). Thus, comprehensive diagnosis and detection methods for humans, animal reservoir and intermediate hosts are essential for opsithorchiasis control and elimination programs.

## 5. DRUG TREATMENT

Chemotherapy, such as praziquantel, has been used for opisthorchiasis treatment and chemotherapeutic control for decades because of its high efficiency, safety margin and reasonable price. The World Health Organization has recommended praziquantel as the drug of choice for opisthorchiasis treatment (WHO, 2006, 2011; WPRO, 2017). However, alternative drugs are being studied. Of these, tribendimidine is the best candidate to date. It shows a promising treatment response (Soukhathammavong et al., 2011). However, an effective treatment alone is not able to control and eliminate opisthorchiasis. The most difficult problem is the traditional and deep-rooted eating behaviour (raw attitudes) and lifestyle of people in the endemic areas (Grundy-Warr et al., 2012). Hence, drug treatment together with integrated control measures is required (Sripa et al., 2015).

### 5.1 Praziquantel

Praziquantel was discovered in the mid-1970s. From its veterinary use in the beginning, it was developed for trematode and cestode treatment in humans (Lee and Chai, 1985). Praziquantel is a broad-spectrum anthelmintic drug, in tablet form, that has a high safety margin. It is the drug of choice for various food-borne trematodes and schistosomes (WHO, 2006, 2011; WPRO, 2017). The active form of drug increases calcium influx to cells, which leads to the damage of tegument cells and vitelline cells of the parasites. Changing parasitic antigenicity and its metabolism, such as glucose influx, release of lactase, glycogen and decrease of ATP, results from disturbance of intracellular calcium homoeostasis (Day et al., 1992). Subsequently, this biochemical reaction leads to muscle contraction, paralysis and perforation of the tegument causing loss of glucose and amino acid, vacuolization and ultimately rupture of the parasite (Andrew and Harder, 1988; Chan et al., 2012; Doenhoff et al., 2008).

The chemical formula of praziquantel is 2-(cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinolin-4-one with a molecular weight of 312.3. Although praziquantel has been used for decades, only few pharmacokinetic studies have been reported from humans. The phamacokinetics of praziquantel was summarized by Olliaro et al. (2014). Although it is rapidly and almost completely absorbed, its bioavailability is low and varies between individuals. Oral administration, particularly with food intake, showed a greater pharmacokinetic variability than the intravenous route because of changes in gastric blood flow and pH. The drug can distribute throughout the body and can be concentrated more in the internal organs, particularly the liver and kidneys. Praziquantel can cross the blood—brain barrier and pass into breast milk. It is metabolized in the liver through the CYP system. The main metabolite in humans is trans-4-hydroxypraziquantel. Other metabolites include dehydrogenated and monohydroxylated forms. Excretion of praziquantel mostly occurs through the kidneys.

Praziquantel has been used as 'Preventive Chemotherapy' (WHO, 2006). The recommended dosage used to be 25 mg per kg of body weight, 3 times a day on 2 consecutive days or 40 mg per kg of body weight in a single administration with a repeat interval of 6 or 12 months (WHO, 2011). Currently, the WPRO has just launched a new recommendation of a single dose of 40 mg/kg (WPRO, 2017). The clinical trials of anthelmintic drugs for opisthorchiasis have been carried out mainly in Lao PDR. Sayasone et al. (2017) reported that a single dose of 40 mg/kg in an endemic area is preferable because of its effective outcome and less adverse effects. A flat dose response was observed with praziquantel in light infections and no benefit of multiple doses over a single dose (Sayasone et al., 2017). Although mass drug treatment or universal treatment is included in the WHO guidelines (WHO, 2011), selective chemotherapy or 'Test and Treat' is very effective in Thailand.

Epidemiological and animal studies suggest that repeated infection with *O. viverrini* followed by multiple treatments with praziquantel increases the risk of CCA development (Pinlaor et al., 2004; Kamsa-Ard et al., 2015). As a result, only infected individuals should receive this drug. Therefore, a history of regularly eating raw freshwater fish or fermented fish and/or alcohol consumption will help in determining the target population (WHO, 2011). Praziquantel has different adverse effects, including headache,

abdominal pain, dizziness, nausea and urticaria (Keiser and Utzinger, 2004; WHO, 2011). These are usually caused by the substances released from dying worms and systemic effects. These will be more severe in heavy infected individuals and in the population that have not received any previous treatment (WHO, 2011). To prevent adverse effects, postprandial administration after dinner is recommended (personal experience). Drug resistance issues for praziquantel have not been reported in liver fluke; however, there is a report of low cure rate (CR) (29%) for the drug for other parasites such as *C. sinensis* in Vietnam (Tinga et al., 1999).

## 5.2 Tribendimidine

Tribendimidine is a new candidate for *O. viverrini* treatment. It was first synthesized in China during mid-1980s. Like pyrantel and levamisole, tribendimidine is an L-subtype nicotinic acetylcholine receptor antagonist (Hu et al., 2009; Xiao et al., 2013). It shows a high efficacy against several kinds of helminths (Xiao et al., 2005). In an animal model it decreased *O. viverrini* intensity in hamsters by 95.7% (Keiser et al., 2007). Many clinical studies of tribendimidine conducted in Lao PDR show interesting and promising results. Its efficacy is as good as that of praziquantel. The egg reduction rates (ERRs) of tribendimidine and praziquantel are 99.3% and 98.4%, respectively (Soukhathammavong et al., 2011). A dosage of 100−600 mg/kg showed high therapeutic efficacy in adults and adolescents, while the effective dose for children (8−14 years) was 100 mg/kg. Lower doses give a lower therapeutic efficacy, while the efficacy did not increase when administered with more than 100 mg/kg. Adverse effects could occur 3 h after treatment. These included vertigo, headache, nausea and fatigue. Children reported headache, vertigo and fatigue (Sayasone et al., 2016). Sayasone et al. (2018) conducted a comparative study using an open-label, randomized, noninferiority, phase 2 trial to test the effects of tribendimidine and praziquantel on children (8−14 year) and adults (including adolescents). One oral dose of tribendimidine (200 mg for children, 400 mg for adolescents and adults) and two oral doses of praziquantel (50 mg/kg body weight and 25 mg/kg body weight, 6 h apart) were compared. The results showed that the CR for tribendimidine was slightly lower than that for praziquantel, but that the ERR was not different. The authors prefer tribendimidine because of its efficacy, broad spectrum and safety. However, tribendimidine is a new drug and needs to be approved by a stringent regulatory authority such as FDA.

### 5.3 Other Treatments

Few studies have been conducted on new trematocidal drugs for opisthorchiasis. These include peroxidic drugs, for example, artemisinins and synthetic trioxolanes. It seems that the therapeutic results were not satisfied. A dose of 400 mg/kg for artesunate and artemether led to worm-burden reductions in hamsters of 77.6% and 65.5%, respectively. Adverse effects were reported when using a dosage over 400 mg/kg (Keiser et al., 2006). In people, the ERR for a mefloquine, artesunate and mefloquine–artesunate combination was below 50%. Adverse effects such as nausea, vomiting, vertigo and anxiety were reported in patients taking mefloquine and mefloquine–artesunate (Soukhathammavong et al., 2011).

Medicinal plants have been studied as possible treatments for opisthorchiasis in vitro and in vivo, such as *Garcinia mangostana* (purple mangosteen) extract (Aukkanimart et al., 2015), *Thunbergia laurifolia* (blue trumpet vine) (Wonkchalee et al., 2013) and curcumin (Charoensuk et al., 2011). It seems that the medicinal plants could be used as a supplement for their antioxidant and antiinflammatory effects, including reduce adverse effects from chemotherapy. A combination or supplement of these with an anthelmintic drug such as praziquantel is now in the research phase (Wonkchalee et al., 2013; Charoensuk et al., 2016).

## 6. CONCLUSION AND FUTURE RESEARCH

Advances in diagnosis will play a pivotal role in the control and eventual elimination of opisthorchiasis, a risk factor for CCA that affects communities of the lowest socioeconomic status in the Mekong region of Southeast Asia. To achieve the elimination goal for opisthorchiasis and its associated CCA, an effective and practical diagnostic test should be inexpensive and easy to use, preferably in a point of care setting. Of the different methods currently available, parasitology-based techniques are logically challenged and have the major limitation of low diagnostic accuracy, especially for the low-intensity infections in low endemic areas. Recent advances in imaging technology for liver fluke egg identification may help to improve the diagnostic performance of this conventional approach (Jiménez et al., 2016). Difficulties in differentiating between current and past infections and the inability to assess therapeutic responses are major limitations of conventional antibody detection methods, although

recent advances such as the development of recombinant-based antibody detection have improved these. Molecular methods yield highly specific results, but more simplification for field application is needed. Among the recent diagnostic developments, the urine antigen assay has shown potential in overcoming some of these challenges, as it can be applied noninvasively. However, further development into a rapid 'point of care' test is necessary such that it can be used for mass screening and so that it is applicable in a resource-poor setting. Moreover, the direct and molecular detection of infected snail and fish intermediate hosts and environmental methods to identify sites of active transmission will provide effective ways of monitoring opisthorchiasis in different geographical locations, especially in Thailand, where disease elimination is now being implemented. Finally, to date, drug treatment for opisthorchiasis has relied solely on praziquantel. Alternative drugs such as tribendimidine have now emerged as potential alternatives, but more tests in endemic areas are required.

Due to the uniqueness of the opisthorchiasis-CCA aetiology in Southeast Asia, which differs markedly from that of Western countries, many new and specific research ventures are required to battle these diseases. A badly needed research outcome is to facilitate the screening of the risk population for CCA. An additional research dimension is to expand and modify the control program being implemented in Thailand into nearby countries where *O. viverrini* is endemic, such that comprehensive programs can be applied and a future opisthorchiasis free community can be achieved.

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