THE DIAGNOSIS OF HUMAN OPISTHORCHIASIS

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Abstract. Opisthorchiasis viverrini is a liver fluke infection causing a serious public health problem in Thailand, Lao PDR, Cambodia, and South Vietnam because it acts as a strong promoter of cholangiocarcinoma. The diagnosis of human opisthorchiasis is based on four approaches: clinical manifestations, parasitological, molecular biological, and immunological methods. These methods have advantages and disadvantages. Clinical manifestations of the patients are practically indistinguishable from those of other liver diseases. The features of the *O. viverrini* eggs are, by light microscopy, difficult to differentiate from those of other minute intestinal flukes' eggs. Polymerase chain reaction (PCR) is very complicated, needs special and expensive apparatus, and is time-consuming; it is, however, highly sensitive and specific. Immunological testing is the method of choice: the techniques are applicable to both routine laboratory work and field or epidemiological studies. Of these tests, enzyme-linked immunosorbent assay (ELISA) and immunoelectrotransfer blot assay are often used for the detection of *O. viverrini*-specific antigens (coproantigens) and antibodies (IgM, IgG, IgA, or IgE). Monoclonal antibodies are prepared to detect coproantigens, while the crude somatic and excretory-secretory antigens from the adult worms, metacercariae, eggs, and snail intermediate hosts are prepared in order to detect antibodies in sera. To eliminate the cross reactions between parasites, the appropriate amount, type, and efficacy of antigens or antibodies preparation should be considered. In this paper, the advantages and disadvantages of the four diagnostic methods are discussed.

INTRODUCTION

Opisthorchiasis viverrini is a liver fluke infection caused by *Opisthorchis viverrini*. The word *opisthorchis* is derived from the Greek *opisthen* + *orchis*. *Opisthen* means behind or at the back; *orchis* means testicle. *Opisthorchis* is a genus of trematodes (flukes) that have two testes at the posterior end and are parasitic in the biliary tracts of birds and mammals, including humans (Bennington, 1984). Human infection is caused by ingestion of raw or undercooked freshwater fish that harbor viable metacercarial cysts, the infective stage of the parasite. It is highly prevalent in Northeast and North Thailand (Jongsuksuntigul and Imsomboon, 1997; Radomyos *et al*, 1998), Lao PDR (Kobayashi *et al*, 2000), Cambodia (Harinasuta, 1969), and South Vietnam (Waikagul, 1996). Actually, mortality due to the parasites alone appears to be uncommon, but the association between this disease and the chemical interaction of nitrosamines in food and tobacco may be etiological factors in the development of cholangiocarcinoma or cancer of the bile duct epithelium, which is the cause of death (Mitacek *et al*, 1999). Opisthorchiasis is a serious public health problem that should not be ignored. Furthermore, it is a non-opportunistic parasitic infection commonly found in Thai HIV-infected people regardless of immune status with or without diarrhea (Wiwanitkit, 2001). Clinical manifestations, parasitological, molecular biological, and immunological techniques provide the main methods for the diagnosis of human opisthorchiasis. This paper presents an overview of the advantages and disadvantages of these methods.

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CLINICAL MANIFESTATIONS

Clinical signs and symptoms are proportional to the intensity and duration of infection. Light and moderate infections are symptomless. Clinical features vary from mild to severe. The signs and symptoms are vague gastro-intestinal complaints, flatulence, anorexia, lassitude, weight loss, dull pain in the right hypochondrium, a hot cutaneous sensation of the abdomen, and enlargement of the liver with some tenderness. In a few cases, the manifestations are severe. There is relapsing cholangitis, the patient is seriously ill and may succumb to septic shock. Cholangiocarcinoma, gallstones, and obstructive jaundice are not unusual associations (Harinasuta *et al*, 1984). However, these non-specific clinical manifestations mean that it is difficult to arrive at a safe differential diagnosis without stool examination.

PARASITOLOGICAL METHODS

Stool examination methods, recommended for the

detection of trematode eggs, can be used for the detection of *Opisthorchis* eggs; these methods include direct smears and sedimentation. However, the opisthorchid eggs are similar to the eggs of tiny intestinal flukes. After recovering the eggs from stool samples, their appearance must be studied carefully; several methods may be used to help distinguish the liver fluke eggs. The principle that underpins these methods is the differentiation of the features of the eggs and adult worms by microscopy.

In patients with severe opisthorchiasis and complete biliary obstruction, *O. viverrini* eggs are not able to pass into the stool. However, numerous eggs and flukes are found in the biliary system during surgery. Therefore, the diagnosis and severity of the infection in these patients cannot be based on the stool egg output (Pungpak *et al*, 1985).

Egg recovery

Direct simple smear is the simplest technique for routine use in hospitals. However, the eggs of *O. viverrini* and other minute intestinal flukes of the families Heterophyidae and Lecithodendriidae are practically indistinguishable one from another by this technique (Ditrich *et al*, 1990; Kaewkes *et al*, 1991; Tesana *et al*, 1991). The intestinal flukes that produce the *O. viverrini*-like eggs found in Thai people are *Haplorchis taichui*, *H. yokogawai*, *H. pumilio*, *Stellantchasmus falcatus*, *Centrocestus caninus* (these five species are in the family Heterophyidae), *Prosthodendrium molenkampi*, *Phaneropsolus bonnei* (these two species are in the family Lecithodendriidae), and *Plagiorchis harinasutai* (family Plagiorchiidae) (Radomyos *et al*, 1994; 1998). Since these eggs are similar in size and shape (small, oval, operculated) under a light microscope, their features can be misidentified. Consequently, the measurements of prevalence and intensity are incorrect (Tesana *et al*, 1991). In addition, it is difficult to determine the effectiveness of campaigns for *Opisthorchis* control (Kaewkes *et al*, 1991).

Iodine staining is used to determine the morphological features that distinguished *O. viverrini* from lecithodendriid eggs in human feces. Under a light microscope, the features are more conspicuous than when they are unstained and examined at low magnification (100x). An iodophilic body, a large mass at the posterior end of miracidium that stains brown in iodine solution, is usually found inside the embryonated stage of lecithodendriid eggs, whereas it is much smaller and very rarely found inside *O. viverrini* eggs. Also, the shell surface, knob, operculum, shoulder, shape, and size of these eggs are different when they are in the embryonated stage. However, partially embryonated, unembryonated, and malformed eggs are still very difficult to identify because their characteristics cannot be observed clearly (Kaewkes *et al*, 1991).

Potassium permanganate staining has been developed for differentiating the eggshells of *O. viverrini*, *H. taichui*, and *P. bonnei* by light microscopy at low magnification. The method is simple, rapid, cheap, and able to differentiate the eggshell patterns almost as accurately as scanning electron microscopy; moreover, the method is suitable for routine and field-survey examinations and may help to establish the true prevalence and intensity of opisthorchiasis; the method is not appropriate for the differentiation of the intestinal protozoa because the nucleus cannot be detected (Sukontason *et al*, 1999). However, the eggs that are usually examined by this technique are from adult worms rather than from the feces of patients. Repeated ether extraction improves the potassium permanganate technique, although ether is both flammable and exposive - major disadvantages; moreover, considerable risks are involved in its storage, use, and disposal (Piangjai *et al*, 2000).

The ultrastructure of the eggshell surface using a scanning electron microscope (SEM) can be used to differentiate all stages of eggs, including deformed eggs. SEM shows that the *O. viverrini* eggshell has a musk-melon pattern with prominent shoulders and a long knob. However, this complicated technique is not practicable in either field-survey or routine diagnostic settings (Kaewkes *et al*, 1991; Tesana *et al*, 1991).

Adult worm detection

Because of the similarity of many trematode eggs, it is difficult to be certain of a diagnosis that is based on fecal eggs unless worms are recovered from stool specimens after treatment. The adult worms can be stained and identified by their morphology under a stereoscopic microscope or a light microscope in the case of the minute worms. However, in order to ensure that live worms are obtained and not left to degenerate in the natural bowel movement a purgative must be used (Radomyos *et al*, 1998).

MOLECULAR BIOLOGICAL METHODS

A polymerase chain reaction (PCR) technique has been used for the detection of *O. viverrini* eggs in the stool of experimental animals. It gave very high sensitivity (100%), and it could detect even a single egg in artificially-inoculated feces (Wongratanacheewin *et al*, 2001). It was much more sensitive than the stool examination method and, in addition, it was highly specific, showing no cross-

reaction with heterophyid flukes (Wongratanacheewin *et al*, 2001). Therefore, it is useful for detecting light infections and monitoring a therapeutic study. It could be further modified as a test of confirmation of the diagnosis of human opisthorchiasis in the future. However, this technique requires expensive equipment, several reagents, complicated steps, and the time required to get to the final result is greater than that needed for the conventional parasitological methods.

IMMUNOLOGICAL METHODS

The principle of these immunological methods is diagnosis on the basis of antigen-antibody interactions. Since 1965, several techniques have been developed for the diagnosis of human opisthorchiasis.

Monoclonal antibody-based enzyme-linked immunosorbent assay (MAb-ELISA)

The method has been applied to detect *O. viverrini* metabolic antigens in feces or coproantigens using a single clone of a specific monoclonal antibody (MAb). This antigen-antibody reaction is conducted in the wells of microtitration plates and determined by colorimetric assay after adding the anti-human immunoglobulin conjugated with an enzyme and its substrate. It is more sensitive than microscopic examination for detecting early and light infections because it is estimated to be sensitive enough to detect the antigen excreted by a single mature fluke (Sirisinha *et al*, 1991b; 1995). This MAb does not cross-react with other human liver flukes such as *O. felineus* and *Clonorchis sinensis* or the minute intestinal flukes such as *Haplorchis* and *Centrocestus* species (Amornpunt *et al*, 1991; Sirisinha *et al*, 1992). Therefore, it is sufficiently specific for the diagnosis of opisthorchiasis, even though there is slight cross-reactivity with *Paragonimus heterotremus*. This technique is useful for routine work, field work, epidemiological studies, and monitoring therapeutic studies since a large number of samples can be analysed rapidly at the same time (Sirisinha *et al*, 1995). It does not require experienced technicians, in contrast to parasitological methods. As it does not require elaborate and expensive equipment, it is generally less expensive than PCR. Unlike the antibody detection methods, MAb-ELISA detects only current infections. Nevertheless, it requires the complicated steps of MAb preparation (Billings *et al*, 1990; Amornpunt *et al*, 1991; Chaicumpa *et al*, 1991) and one more process of fecal sample extraction before detection (Chaicumpa *et al*, 1992).

Indirect enzyme-linked immunosorbent assay (Indirect ELISA)

This technique has been used to detect specific antibodies to *O. viverrini*; for example, IgG, IgA, IgM, and IgE, in patients' sera and bile by using crude somatic extract, tegument extract, and excretory-secretory product (soluble metabolic products) antigens from adult worms, metacercariae, and eggs of *O. viverrini* coated into the wells of microtitration plates before determining by ELISA technique that is similar to MAb-ELISA. In contrast, the preparations of these antigen-based detectors for indirect ELISA are less complicated than those required for MAb; furthermore, this assay could directly detect antibodies in serum or bile without extraction steps (Srivatanakul *et al*, 1985; Wongratanacheewin *et al*, 1988a; Sirisinha *et al*, 1990; Elkins *et al*, 1991; Ditrich *et al*, 1991; Akai *et al*, 1994b; Itoh *et al*, 1994; Sakolvaree *et al*, 1997). Because there was a significant correlation between the serum IgG antibody level and the severity of infection (Wongratanacheewin *et al*, 1988a), high antibody titers were useful for determining the prognosis of cholangiocarcinoma (Srivatanakul *et al*, 1985; Akai *et al*, 1994a; Itoh *et al*, 1994). In addition, this assay is appropriate for use as a screening test for epidemiological studies (Elkins *et al*, 1991).

Owing to the relationship between *O. viverrini* adult worms and their snail intermediate hosts, antigen sharing takes place (Chanawong *et al*, 1990); snail antigen has been found to be as good as *O. viverrini* antigen (Watthanakulpanich *et al*, 1997). Consequently, using crude somatic antigens from snail intermediate hosts provides an alternative method of opisthorchiasis detection. This kind of antigen has various advantages: a large amount of snail material can be obtained rapidly; a stable source of antigen can be accessed conveniently, resulting in lower costs than the preparation of parasite antigens, which also requires the maintenance of a complex life cycle; the antigens can be readily maintained in a laboratory (Rivera-Marrero and Hillyer, 1985; Watthanakulpanich *et al*, 1997).

However, indirect ELISA has two disadvantages that cannot be overcome. Firstly, current and past infections cannot be distinguished since serum antibodies are known to persist after the parasites have been eliminated by anthelmintic treatment (Wongratanacheewin *et al*, 1988b; 2001; Akai *et al*, 1995). Secondly, it is unavoidable the cross reactions between *O. viverrini* and several other parasitic infections (Sirisinha *et al*, 1990; Ditrich *et al*, 1991; Akai *et al*, 1995; Sakolvaree *et al*, 1997; Watthanakulpanich *et al*, 1997; Wongratanacheewin *et al*, 2001). Even though partially purified antigens of *O. viverrini* can improve the sensitivity and specificity of indirect ELISA (Poopyruchpong *et al*, 1990), these antigens remain cross-reactive with other parasites.

Indirect hemagglutination (IHA) and lectin immuno test (LIT)

Both techniques determine the presence of specific antibodies to *O. viverrini* in sera. IHA uses sheep red blood cells sensitized with *O. viverrini* antigen to be the detector; positive results are indicated by hemagglutination. LIT uses *O. viverrini* antigen directly as the detector; the absence of hemagglutination is regarded as a positive result. In comparison with indirect ELISA, IHA and LIT are less complicated and easier to interpret by the naked eyes. Nevertheless, it appears that indirect ELISA is more effective in assessing the therapeutic efficacy of anti-opisthorchiasis drugs than IHA and LIT (Thammapalerd *et al*, 1988). Unfortunately, these three assays produce unavoidable false positive results due to the cross reactions with other parasites.

Enzyme-linked immunoelectrotransfer blot (EITB) or the electroimmunotransfer blot (EITB) or the immunoblot (IB) technique

The EITB technique uses *O. viverrini* antigens to detect specific antibodies in sera. The type of antigen detectors and target antibodies are as same as those used for indirect ELISA. The antigen proteins are separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto nitrocellulose membranes. After cutting the membranes into many thin strips according to each lane of loaded antigens, the strips are incubated with an individual serum and then processed as for indirect ELISA. EITB is better than indirect ELISA because EITB can differentiate between *O. viverrini* and *H. taichui* infections because of the differences in immunoblot patterns (Ditrich *et al*, 1991). However, there are some disadvantages of EITB: the method cannot tell a current from a past infection (Ditrich *et al*, 1991); there are unavoidable cross reactions with other parasitic infections (Akai *et al*, 1994); neurotoxic and carcinogenic substances are used as reagents (Laemmli, 1970; Towbin *et al*, 1979).

SUMMARY

Apart from the patients' clinical manifestations, parasitological, molecular biological, and immunological methods are very helpful in the diagnosis of human opisthorchiasis; however, their advantages and disadvantages should be considered carefully. At present, egg recovery by stool examination, a parasitological method, is the fundamental technique used in routine laboratories, even though false positives can occur because the features of *O. viverrini* eggs resemble those of other minute intestinal fluke eggs. While molecular biological methods are being developed, immunological methods will remain useful for confirmation of diagnoses. In order to eliminate the cross reactions with other parasites and distinguish a current from a past infection, both MAb-ELISA and indirect ELISA should be used simultaneously. A number of researches are attempting to modify and improve these methods. For this reason, safe, simple, convenient, rapid, cheap, sensitive, specific, accurate, and reliable diagnostic tests ought to become available in the future.

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REFERENCES

Akai PS, Pungpak S, Chaicumpa W, *et al*. Serum antibody responses in opisthorchiasis. *Int J Parasitol* 1995; 25: 971-3.

Akai PS, Pungpak S, Chaicumpa W, Viroj K, Bunnag D, Befus AD. Serum antibody response to *Opisthorchis viverrini* antigen as a marker for opisthorchiasis-associated cholangiocarcinoma. *Trans R Soc Trop Med Hyg* 1994a; 88: 471-4.

Akai PS, Pungpak S, Kitikoon V, Bunnag D, Befus AD. Possible protective immunity in human opisthorchiasis. *Parasite Immunol* 1994b; 16: 279-88.

Amornpunt S, Sarasombath S, Sirisinha S. Production and characterization of monoclonal antibodies against the excretory-secretory antigen of the liver fluke (*Opisthorchis viverrini*). *Int J Parasitol* 1991; 21: 421-8.

Bennington JL. Saunders dictionary and encyclopedia of laboratory medicine and technology. Canada: WB Saunders, 1984: 1105.

Billings PB, Utsakhit N, Sirisinha S. Monoclonal antibodies against *Opisthorchis viverrini* antigens. *Parasite Immunol* 1990; 12: 545-57.

Chaicumpa W, Ruangkunaporn Y, Kalambaheti T, *et al*. Specific monoclonal antibodies to *Opisthorchis viverrini*. *Int J Parasitol* 1991; 21: 969-74.

Chaicumpa W, Ybanex L, Kitikoon V, *et al*. Detection of *Opisthorchis viverrini* antigens in stools using

specific monoclonal antibody. *Int J Parasitol* 1992; 22: 527-31.

Chanawong A, Waikagul J, Thammapalerd N. Detection of shared antigens of human liver flukes, *Opisthorchis viverrini* and its snail host, *Bithynia spp. Trop Med Parasitol* 1990; 4: 419-21.

Ditrich O, Giboda M, Sterba J. Species determination of eggs of opisthorchiid and heterophyid flukes using scanning electron microscopy. *Angew Parasitol* 1990; 31 : 3-9.

Ditrich O, Kopacek P, Giboda M, Gutvirth J, Scholz T. Serological differentiation of human small fluke infections using *Opisthorchis viverrini* and *Haplorchis taichui* antigens. *Southeast Asian J Trop Med Public Health* 1991; 22: 174-8.

Elkins DB, Sithithaworn P, Haswell-Elkins M, Kaewkes S, Awacharagan P, Wongratanacheewin S. *Opisthorchis viverrini*: relationships between egg counts, worms recovered and antibody levels within an endemic community in northeast Thailand. *Parasitology* 1991; 102: 283-8.

Harinasuta C. Opisthorchiasis in Thailand: A review. In: Harinasuta C, ed. Proceedings of the Fourth Southeast Asian Seminar on Parasitology and Tropical Medicine, Schistosomiasis and other Snail Transmitted Helminthiasis. Bangkok, Thailand, 1969: 253.

Harinasuta T, Riganti M, Bunnag D. *Opisthorchis viverrini* infection: pathogenesis and clinical features. *Arzneimittelforschung* 1984; 34: 1167-9.

Itoh M, Pairojkul C, Thamawit W, *et al*. Association of antibodies to *Opisthorchis viverrini* with hepatobiliary disease in northeastern Thailand. *Am J Trop Med Hyg* 1994; 51: 424-9.

Jongsuksuntigul P, Imsomboon T. The impact of a decade long opisthorchiasis control program in northeastern Thailand. *Southeast Asian J Trop Med Public Health* 1997; 28: 551-6Ê

Kaewkes S, Elkins DB, Sithithaworn P, Haswell-Elkins MR. Comparative studies on the morphology of the eggs of *Opisthorchis viverrini* and Lecithodendriid trematodes. *Southeast Asian J Trop Med Public Health* 1991; 22: 623-36.

Kobayashi J, Vannachone B, Sato Y, Manivong K, Nambanya S, Inthakone S. An epidemiological study on *Opisthorchis viverrini* infection in Lao villages. *Southeast Asian J Trop Med Public Health* 2000; 31: 128-32.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.

Mitacek EJ, Brunnemann KD, Suttajit M, *et al*. Exposure to N-nitroso compounds in a population of high liver cancer regions in Thailand: volatile nitrosamine (VNA) levels in Thai food. *Food Chem Toxicol* 1999; 37: 297-305.

Piangjai S, Sukontason K, Sukontason K, Methanitikorn R. Enhancing identification of *Opisthorchis viverrini* eggs by repeated ether extraction. *J Trop Med Parasitol* 2000; 23: 32-4.

Poopyruchpong N, Viyanant V, Upatham ES, Srivatanakul P. Diagnosis of opisthorchiasis by enzyme-linked immunosorbent assay using partially purified antigens. *Asian Pac J Allergy Immunol* 1990; 8: 27-31.

Pungpak S, Riganti M, Bunnag D, Harinasuta T. Clinical features in severe opisthorchiasis viverrini. *Southeast Asian J Trop Med Public Health* 1985; 16: 405-9.

Radomyos P, Radomyos B, Tungtrongchitr A. Multi-infection with helminths in adults from northeast Thailand as determined by post-treatment fecal examination of adult worms. *Trop Med Parasitol* 1994; 45: 133-5.

Radomyos B, Wongsaroj T, Wilairatana P, *et al*. Opisthorchiasis and intestinal fluke infections in northern Thailand. *Southeast Asian J Trop Med Public Health* 1998; 29: 123-7.

Rivera-Marrero CA, Hillyer GV. Isolation and partial characterization of shared antigens of *Biomphalaria glabrata* and *Schistosoma mansoni* and their evaluation by ELISA and the EITB. *J Parasitol* 1985; 71: 547-55.

Sakolvaree Y, Ybanez L, Chaicumpa W. Parasites elicited cross-reacting antibodies to *Opisthorchis viverrini*. *Asian Pac J Allergy Immunol* 1997; 15: 115-22.

Sirisinha S, Chawengkirttikul R, Haswell-Elkins MR, Elkins DB, Kaewkes S, Sithithaworn P. Evaluation of a monoclonal antibody-based enzyme-linked immunosorbent assay for the diagnosis of *Opisthorchis viverrini* infection in an endemic area. *Am J Trop Med Hyg* 1995; 52: 521-4.

Sirisinha S, Chawengkirttikul R, Sermswan R. Immunodiagnosis of opisthorchiasis. *Southeast Asian J Trop Med Public Health* 1991a; 22: 179-83.

Sirisinha S, Chawengkirttikul R, Sermswan R,

Amornpunt S, Mongkolsuk S, Panyim S. Detection of *Opisthorchis viverrini* by monoclonal antibody-based ELISA and DNA hybridization. *Am J Trop Med Hyg* 1991b; 44: 140-5.

Sirisinha S, Chawengkirttikul R, Tayapiwatana C, *et al*. Specific and cross-reactive monoclonal antibodies to the 89-kDa antigen of *Opisthorchis viverrini*. *Southeast Asian J Trop Med Public Health* 1992; 23: 489-90.

Sirisinha S, Sahassananda D, Bunnag D, Rim HJ. Immunological analysis of *Opisthorchis* and *Clonorchis* antigens. *J Helminthol* 1990; 64: 133-8.

Srivatanakul P, Viyanant V, Kurathong S, Tiwawech D. Enzyme-linked immunosorbent assay for detection of *Opisthorchis viverrini* infection. *Southeast Asian J Trop Med Public Health* 1985; 16: 234-9.

Sukontason K, Piangjai S, Sukontason K, Chaithong U. Potassium permanganate staining for differentiation the surface morphology of *Opisthorchis viverrini*, *Haplorchis taichui* and *Phaneropsolus bonnei* eggs. *Southeast Asian J Trop Med Public Health* 1999; 30: 371-4.

Tesana S, Srisawangwong T, Kaewkes S, Sithithaworn P, Kanla P, Arunyanart C. Eggshell morphology of the small eggs of human trematodes in Thailand. *Southeast Asian J Trop Med Public Health* 1991; 22: 631-6.

Thammapalerd N, Tharavanij S, Nacapunchai D, Bunnag D, Radomyos P, Prasertsiriroj V. Detection of antibodies against *Opisthorchis viverrini* in patients before and after treatment with praziquantel. *Southeast Asian J Trop Med Public Health* 1988; 19: 101-8.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 1979; 76: 4350-4.

Waikagul J. The Trematodes: Family Opisthorchiidae. In: Waikagul J, ed. Medical Helminthology. Bangkok : Faculty of Tropical Medicine, Mahidol University, 1996: 158.

Watthanakulpanich D, Waikagul J, Anantaphruti MT, Dekumyoy P. Evaluation of *Bithynia funiculata* snail antigens by ELISA-serodiagnosis of human opisthorchiasis. *Southeast Asian J Trop Med Public Health* 1997; 28: 593-8.

Wiwanitkit V. Intestinal parasitic infections in Thai HIV-infected patients with different immunity status. *BMC Gastroenterol* 2001; 1: 3.

Wongratanacheewin S, Bunnag D, Vaeusorn N, Sirisinha S. Characterization of humoral immune response in the serum and bile of patients with opisthorchiasis and its application in immuno-diagnosis. *Am J Trop Med Hyg* 1988a; 38: 356-62.

Wongratanacheewin S, Charupatana C, Bunnag D, Sirisinha S. Effect of praziquantel treatment on antibody levels and lymphoproliferative responses in patients with opisthorchiasis. *Southeast Asian J Trop Med Public Health* 1988b; 19: 109-16.

Wongratanacheewin S, Pumidonming W, Sermswan RW, Maleewong W. Development of a PCR-based method for the detection of *Opisthorchis viverrini* in experimentally infected hamsters. *Parasitology* 2001; 122: 175-80.