THE DIAGNOSIS OF HUMAN OPISTHORCHIASIS

Natsuda Jamornthanyawat

Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

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 sonomic 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.

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.

INTRODUCTION

Opisthorchiasis viverrini is a liver fluke infection caused by *Opisthorchis viverrini*. The word *opisthorchis* is derived from the Greek *opisten* + *orchis*. *Opisten* 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 (Jongsusuntigul 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.

Correspondence: Natsuda Jamornthanyawat, Department of Helminthology, Faculty of Tropical Medicine, 420/6 Rajvithi Road, Bangkok 10400, Thailand.
Tel: 66 (0) 2246 9000-12; Fax: 66 (0) 2246 8340

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
diagnosis of opisthorchiasis

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 mor-
phological features that distinguished O. viverrini from
lecithodendrid 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 lecithodendrid 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
expensive - major disadvantages; moreover, consi-
derable 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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