IMMUNODIAGNOSIS OF OPISTHORCHIASIS

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Abstract. Monoclonal antibody-based enzyme-linked immunosorbent assay and DNA dot blot hybridization techniques were developed and evaluated for their potential in the detection of *Opisthorchis viverrini*. A mixture of IgG monoclonal antibodies specific for the 89 kDa metabolic product of *O. viverrini* was captured on a microtiter plate by rabbit anti-mouse IgG and used in a sandwich ELISA for the detection of soluble parasite antigen in the feces of patients with opisthorchiasis. As little as 0.1 ng of the antigen could be detected. A specific *O. viverrini* DNA probe was used in a dot blot hybridization of parasite DNA. The labeled probe could detect DNA released from as few as five *O. viverrini* eggs. Both approaches were highly specific for *O. viverrini* and their sensitivity appeared to be comparable with that of the classical parasitological method. Preliminary data obtained from a field trial showed that these two methods have potential in the diagnosis of opisthorchiasis. Moreover, the limited data currently available showed that it is possible to use these methods to detect the presence of *O. viverrini* metacercariae in naturally infected fish.

INTRODUCTION

Liver fluke infection is still an important endemic disease in many parts of the world including Southeast Asian countries. It has been estimated that at least 40–50 million people are currently infected. The two important parasites causing the disease in humans residing in this part of the world are *Opisthorchis viverrini* and *Clonorchis sinensis*. While clonorchiasis is prevalent in China, Hong Kong, Korea, Taiwan and Japan, opisthorchiasis caused by *O. viverrini* is found largely in Thailand and Laos. On the other hand, both opisthorchiasis and clonorchiasis have been reported on occasions from non-endemic areas including North America and Western Europe and, at present an increasing number of cases has been diagnosed and reported. One reason for such an increase is the increasing number of immigrants and refugees from endemic areas in the last few years. Another possible reason is the importation of fish from endemic areas. For example, the disease has been reported in native Hawaiians as a result of consumption of imported fish from endemic areas.

The current method for the diagnosis of opisthorchiasis is based on the demonstration of eggs in feces (Sirisinha, 1986). However, a microscopic examination for the presence of eggs is laborious and time-consuming and is reliable only in the hands of experienced personnel. Even with the latter situation, a false positive can be expected in individuals concurrently infected with minute intestinal flukes (Radomyos *et al*, 1984). It was recently reported that indirect ELISA for the detection of serum antibody in patients was just as sensitive (Srivatanakul *et al*, 1985), but because illdefined antigens were used, false positives are unavoidable. However, encouraging results using more refined antigens have been reported (Wongratanacheewin *et al*, 1988; Poopyruchpong *et al*, 1990). Unfortunately, these antigens could not be made available in mass quantity for routine use, and a further attempt to clone genes expressing these specific epitopes was unsuccessful (Sermswan, 1990). Furthermore, the detection of antibodies cannot readily distinguish present from past infections as the antibodies are known to persists long after the parasites have been eliminated by anthelminthic treatment (Wongratanacheewin *et al*, 1989). The purpose of the present study was to develop alternative approaches for the detection of *O. viverrini* in suspected specimens and to evaluate their applications in the diagnosis of opisthorchiasis and in detecting parasite contamination in food products prepared from naturally infected inter-

mediate host.

MATERIALS AND METHODS

Production of monoclonal antibodies

Monoclonal antibodies (MAbs) specific for the *O. viverrini* 89–kDa metabolic product were produced in BALB/c mice and hybrids were cloned and tested as previously described (Billings *et al*, 1990; Amornpunt *et al*, submitted). The MAbs were screened against a variety of crude somatic and metabolic antigens prepared from closely related and unrelated parasites. Those reacted specifically with *O. viverrini* antigen were used in this study. A mixture of 3 IgG1 MAbs were used in the monoclonal antibody-based ELISA for the detection of soluble *O. viverrini* antigen in suspected specimens.

Monoclonal antibody-based ELISA (MAb-ELISA)

A sandwich ELISA was developed for the detection of soluble parasite antigen in the feces of patients with opisthorchiasis as previously described (Sirisinha *et al*, submitted). In this test, a MAb mixture was captured on the microtiter plates previously coated with the IgG fraction of rabbit anti-mouse IgG. Appropriately diluted unknown specimens containing 89-kDa antigen was added and the antigen captured with the MAb on the plate and its presence was subsequently detected with biotinylated polyclonal rabbit IgG to *O. viverrini* metabolic products. The method was found to be highly sensitive and could detect the antigen at a concentration as low as 1–2 ng/ml.

Construction of DNA probe

DNA probe specific for *O. viverrini* DNA was prepared from the genomic DNA extracted from adult worms collected from experimentally infected hamsters by the proteinase K/SDS method exactly as described by Sermswan *et al* (submitted). One of the recombinant plasmids, referred to as pOV-A6, was tested for its specificity and finally evaluated for it diagnostic potential.

Hybridization procedure for the detection of *O. viverrini* DNA

The protocol for the dot blot hybridization designed for the detection of parasite DNA in suspected specimens was similar to the one described previously (Sirisinha *et al*, submitted). In brief, the DNA released from eggs or other stages of life cycle (eg, metacercariae) was applied onto a nitrocellulose membrane. The membrane was then washed in appropiate solution and placed in a prehybridization solution containing heat denatured salmon sperm DNA (to block non-specific binding sites). Subsequently, ^{32}P-labeled pOV-A6 probe was added. After incubation, the membrane was washed, blotted dry and exposed to an X-ray film. The method was sensitive for the detection of genomic DNA at a level as low as 25 pg/30 µl.

Fecal specimens

Feces from a small number of patients with moderate to heavy infections with *O. viverrini* were made available through the courtesy of Professor D Bunnag. These were patients attending the Outpatient Clinic of the Hospital for Tropical Diseases (Faculty of Tropical Medicine, Mahidol University, Thailand). Microscopic examinations for intestinal parasites by Stoll's egg count method were routinely performed for all patients. Specimens were also collected from apparently healthy individuals in a village in the endemic area of infection and examined for fluke eggs by a quantitative formalin-ether technic (kindly performed by Dr M Haswell-Elkin (Queensland Institute of Medical Research, Brisbane. Australia, and Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand). Fecal specimens were also available from a group of apparently healthy adults resided in non-endemic areas of infection and whose stools were found to be negative for intestinal parasites by routine microscopic examination.

O. viverrini antigens

Crude somatic extract and metabolic products of adult flukes were prepared as described (Wongratanacheewin *et al*, 1988). Eggs were obtained by gently teasing the uterus of adult flukes and metacercariae were collected from a

peptic digest of naturally infected fish.

RESULTS

Using metabolic products as standard reference, the MAb-ELISA was able to detect the presence of as little as 0.1 ng of the antigen in 50–µl volume. Results presented in Table 1 showed that with this antigen detection method, all 5 stool specimens (tested at a dilution of 1:10) from these moderately to heavily infected patients gave positive results. However, it was not possible to correlate the intensity of the reaction with the number of eggs. Stools from healthy individuals from non-endemic areas of infection were all negative. Positive and negative specimens could be readily distinguished from one another by visual inspection of the color intensity, thus no special equipment is needed by this approach. Similarly, the DNA probe constructed for this study could detect the DNA released from eggs in the feces of all 5 patients (Fig 1). However, with one patient, the intensity of the reaction was rather low, but was positive with prolonged exposure during autoradiagraphy.

In a limited field trial using specimens from apparently healthy individuals from endemic area, promising results were also obtained. In these individuals, the microscopic examinations were carried out using concentration technique and because of the similarity between *O. viverrini* eggs and those of other minute intestinal flukes, no attempt was made to distinguish them from

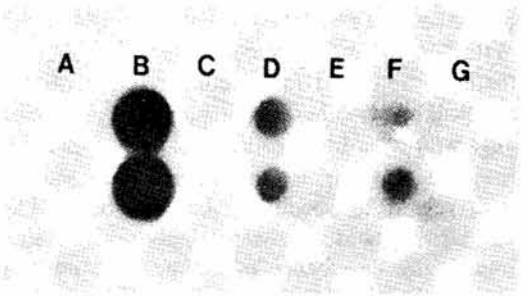

Fig 1 – Antoradiagram of specimens from 5 patients with opisthorchiasis (A and G were from *O. viverrini* negative negative specimens; B to F were *O. viverrini* positive specimens). The faint spot obtained with specimen C was too faint to be satisfactorily photographed. It could be visualize upon prolonged exposure.

one another and all such eggs were recorded as *O. viverrini* eggs. Even with this limitation, the results from microscopic egg count paralleled those of antigen and DNA detection methods. Of the 31 specimens analyzed, 14 were positive by microscopic examination, 13 positive for soluble antigen by MAb-ELISA and 15 positive for DNA by dot blot hybridization (Sirisinha *et al*, 1990). This gave a percentage positive between 42 and 48. However, not all specimens positive by microscopic examination were positive by the other two methods and vice versa.

DISCUSSION

The results presented in this study showed potential of the two proposed diagnostic methods

Table 1

Detection of *O. viverrini* in patients by MAb-ELISA and dot blot hybridization.

Specimen No.	Microscopic egg count* (EPG)	MAb-ELISA	Dot blot hybridization
1	14,000	+	+
2	1,600	+	±
3	4,000	±	+
4	2,000	+	+
5	46,000	+	+

* Stoll's technic

for detecting *O. viverrini* in unknown specimens. With the limited data currently available, the methods appeared to be comparable with the classical microscopic examination for the parasite eggs with regard to sensitivity. The two methods may be superior to the microscopic method with regard to specificity because both MAb and DNA probes used were highly specific, thus minimizing the chance of getting false positive results. This is advantageous in a situation where there is a concurrent mixed infection with other intestinal flukes whose eggs cannot be readily distinguished from those of *O. viverrini*. Moreover, the proposed methods do not require experienced personnel and can handle a large number of samples at a time. The MAb-based ELISA for the detection of soluble parasite antigen in fecal specimens does not require elaborate or expensive equipment as positive results can be readily spotted by naked eyes. The DNA hybridization method described in this study however requires the use of radioactive material and can be hazard to the environment but it is possible to develop a non-radioactive probe to be used in the future.

Although steps required for both methods may appear complicated, one can process a large number of samples at a time. On the average, the time required for an individual sample is considerably less than that required for the microscopic method. Between the two proposed methods, sample preparation for the DNA probe is more time-consuming and, therefore, the MAb-based ELISA may have the advantage.

Slight discrepancy of the results obtained with these two proposed methods was not unexpected in view of the fact that the two methods detect different parasite components. The MAb-ELISA detects the parasite metabolic product which appears in feces prior to the time the eggs appear in the feces. It is, therefore, useful for early detection of infection. However, we feel that a more extensive evaluation using a larger sample size should be made to be certain of their reproducibility and reliability in the field. We have recently obtained data showing that in addition to detecting *O. viverrini* in the fecal specimens from patients or other suspected individuals, the methods can be readily adapted for detecting *O. viverrini* metacercariae. The latter can be useful in tracing parasites in uncooked or poorly cooked food products. Moreover, it may be possible to use these methods for detecting cercariae in snails, or metacercariae in fish or other intermediate hosts, thus providing an additional tool for epidemiological study.

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