

# RECENT ADVANCES IN DIAGNOSIS OF PARAGONIMIASIS

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**Abstract.** Paragonimiasis in endemic areas can be diagnosed by clinical symptoms. However, the diagnosis should always be confirmed by microscopic examination of the sputum or stool in order to find *Paragonimus* eggs. Within recent years marked advances in diagnosis of paragonimiasis have been made. Two new approaches comprising a genetic probe and immunological tests have been developed with claims to be as good or better than microscopic examinations. This report reviews these two areas, especially in paragonimiasis caused by *Paragonimus heterotremus* and *P. westermani*. In addition, problem areas in assay development are discussed.

## INTRODUCTION

The lung fluke, *Paragonimus*, is a harmful parasite, causing paragonimiasis in man and animals, mainly in Asia, some parts of West Africa and South and Central America. Up to the present time, there are at least 40 species reported in the world (Bunnag and Harinasuta, 1984). Fourteen species of *Paragonimus* are known to infect humans. However, from the medical point of view, *P. westermani* infection is the most common elsewhere while *P. heterotremus* is the etiologic agent of human paragonimiasis in Thailand (Vanijanonta *et al.*, 1981). Diagnosis of paragonimiasis is based on clinical features as well as laboratory tests. The current mean is based on the demonstration of *Paragonimus* eggs in the feces and/or sputa by microscopic examination (Yokogawa, 1965).

However, microscopic examination even by expert microscopists is time-consuming and labor-intensive. In addition, the result can be negative in early or extra-pulmonary infection. To overcome some of the above shortcomings, two new approaches comprising a genetic probe and immunological tests have been developed with claims to be as good or even better than microscopic examinations. In this review, recent advances (especially in paragonimiasis caused by *P. heterotremus* and *P. westermani*) in these two areas are highlighted to serve as a guide to the selection of methods for use in the laboratory or the field.

## DIAGNOSIS OF PARAGONIMIASIS HETEROTREMUS

### Immunological tests

In 1990, Pariyanonda and others evaluated enzyme-linked immunosorbent assay (ELISA) and indirect hemagglutination tests (IHA) for serodiagnosis of human paragonimiasis heterotremus using homologous adult worm extract as the antigen. IgG-ELISA was found to be the most sensitive, being positive in all paragonimiasis sera tested while IHA and IgA-ELISA gave 88% and 59% positive rates, respectively. Cross reactivity in IgG-ELISA was detected with fascioliasis sera, with an overall assay specificity of 97%. It was suggested that IgG-ELISA was a reliable serodiagnostic test. Later, adult somatic antigen extract of *P. heterotremus* was compared with excretory-secretory (ES) antigen in an ELISA for serodiagnosis of human paragonimiasis (Maleewong *et al.*, 1990). The absorbance values in ELISA using the adult worm somatic antigens were not significantly different from the values obtained using ES antigens ( $p > 0.05$ ). The sensitivity of the ELISA using either antigen was 100%, but the specificity was 96% with somatic and 98% with ES antigens, respectively, due to a cross reaction with fascioliasis sera. It also appears that both somatic and ES antigens are effective antigens for use in serodiagnosis.

As with most parasites, little is known about specific immunogen(s) of *P. heterotremus*. The antigenic components of a *P. heterotremus* saline extract were revealed by SDS-PAGE and immunoblot analysis using sera from 32 patients with *P. heterotremus* infection, 60 with other helminthiasis and 15 normal human sera (Maleewong *et al*, 1991). It was found that the worm extract consisted of more than 13 polypeptides, among which 5 components were clearly recognized by paragonimiasis sera. These components had approximately molecular mass of < 12.3, 12.3, 18.5, 31.5 and 38 kDa. Only the 31.5 kDa component was recognized by all of the 32 paragonimiasis sera. Sera from other helminthic infections and from uninfected persons did not produce this specific band with the worm extract. Recently, we studied the antigenic components of the *P. heterotremus* metabolic product by SDS-PAGE and immunoblot analysis (Maleewong *et al*, 1992a). It was found that the metabolic products comprised more than 8 major polypeptides. Immunoblot analysis revealed 11 components which were clearly recognized by sera of patients with paragonimiasis. These antigenic components had molecular mass ranging from less than 12.3 kDa to 144 kDa. One antigenic band, *ie* 31.5 kDa was found to give a consistent reaction with the sera (97 % sensitivity). Only sera of patients with fascioliasis reacted with antigenic bands of 18.5, 38 and 56 kDa. The above findings suggested that the 31.5 kDa component may be eligible for diagnostic use.

We also used ELISA and immunoblot analysis to determine IgG antibody levels in patients infected with *P. heterotremus* from Thailand before and after treatment with Praziquantel (Maleewong *et al*, 1992b). An IgG-ELISA showed a substantial reduction one year after the treatment. SDS-PAGE and immunoblot analysis showed that *P. heterotremus* adult worm extracts were highly complex, consisting of more than 9 antigenic bands with molecular mass ranging from < 12.3 kDa to 123 kDa. Two bands of 18.5 kDa and 31.5 kDa were found to show consistent reactions with all serum samples from the pre-treatment group. There was a marked reduction in the intensity of the reaction of the 31.5 kDa band with each serum sample from patients post-treatment. The other bands disappeared after one year of treatment.

Although significant progress has been made in improvement and development of procedures for parasitic infections, it is generally accepted that specificity and sensitivity of the immunodiagnostic

procedures aimed at detecting specific antibodies depends on purity and specificity of the antigens and the kinds of serological test used. Worms and their products are antigenically more complex than viruses and bacteria and also exist in different stages within the host, thus expressing a degree of cross-reactivity with antigens of other parasites.

Indrawati *et al* (1991) used crude and partially purified adult worm antigens for serodiagnosis of human paragonimiasis caused by *P. heterotremus*. Adult *P. heterotremus* were recovered from the lungs and pleural cavity of cats orally infected with metacercariae. The worms were ground and extracted with distilled water. The soluble crude antigen (CA) contained about 40% proteins which could be fractionated by gel filtration on Sephadex G-200 into three profiles namely F1, F2 and F3. The CA and its Sephadex profiles were used in an ELISA for detecting antibodies to *P. heterotremus* in three groups of patients, *ie*. patients whose sputa and/or feces revealed *P. heterotremus* eggs, patients with other parasitic infections, bacterial proven tuberculosis patients and healthy, parasite-free controls. The sensitivity and specificity of the assay when the F1 was used as the antigen were 100%. Immunoblot analysis revealed that the specific antigen of *P. heterotremus* was a non-protein component of Mr 35 kDa. Various in specific antigenic bands, reported above by different investigators, may be due to different methods of antigen preparations as well as some other details of the experiments.

Later, we partially purified the crude extract of adult *P. heterotremus* by preparative isoelectric focusing onto a Rotofor preparative isoelectric focusing cell (Bio-Rad, USA) (Wongkham *et al*, 1994). The fractions at approximately pH 5 which contained a specific antigen with a relative molecular mass of 31.5 kDa were pooled and used in an ELISA and immunoblot analysis for diagnosis of human paragonimiasis. The sensitivity and specificity of ELISA were found to be 100 % and 99 %, respectively. The band of 31.5 kDa antigenic component was found to give consistent reaction with paragonimiasis sera. The sensitivity, specificity and predictive values (positive and negative) of immunoblot analysis for the 31.5 kDa band were all 100%.

Another approach which has been successful at purifying potential antigens in antibody detection assays is the use of specific monoclonal antibodies (MAbs) as immunoabsorbents to recover specific

antigens from the crude antigens. Our experiments on the production of specific MABs against *P. heterotremus* were constructed. These studied were the production of specific MABs against the excretory-secretory (ES) and somatic adult extract antigens of the lung fluke, *P. heterotremus*. The ES antigen was obtained from culturing of the adult worms in cultured medium *in vitro*. From three cell fusions, large numbers of hybridomas secreting IgM or IgG antibodies against *P. heterotremus* ES antigens were obtained. When screened against a panel of homologous and heterologous antigens by ELISA, these MABs exhibited four different groups of reactivities. Approximately 15% of MABs were highly specific for *P. heterotremus* (Group 1) and the remainder cross-reacted with *Opisthorchis viverrini* and *Dirofilaria immitis* (Group 2), *O. viverrini* and *Taenia saginata* (Group 3) and *O. viverrini*, *D. immitis* and *T. saginata* (Group 4). Characterization of the relevant antigens of these MABs were performed using SDS-PAGE and immunoblotting. The *P. heterotremus* specific MABs gave immune complex bands against 22 and 31.5 kDa polypeptides, while some were negative against the ES antigen. By indirect immunofluorescent test (IFA) using sections of adult worms as the antigen, these MABs from clones ES-6F6, ES-10F2, ES-11C6, ES-3F7, ES-4E4, ES-7D6, ES-9F10 and ES-10G4 reacted only with intestinal mucosa and their contents.

When the MABs were produced against somatic antigens of the lung fluke, *P. heterotremus*, many clones were obtained but 20 clones secreting high amounts of MABs against *P. heterotremus* in the cultured medium as revealed by ELISA against the homologous antigens were selected for further study. The MABs produced by these clones were exclusively IgG isotypes. By testing against *P. heterotremus* and a panel of other heterologous antigens, these MABs could be categorized into five groups. MAB group 1 consisted of three MABs, namely SOM-5D3, SOM-11F4 and SOM-4G6 and all were specific to *P. heterotremus* somatic antigen. MAB groups 2-5 reacted to *P. heterotremus* somatic antigen as well as one or more of the other parasite antigens, ie *T. saginata*, *Ech<sup>o</sup> inostoma malayanum* and *P. westermani*. Nine hybridoma clones of groups 1-5 which had high ELISA activities were selected and further characterized by immunoblotting and IFA. Three of them (SOM-4G6, SOM-2E6 and SOM-22B5) were found to react with an antigen with the molecular mass of 12.3 kDa and gave bright fluorescence over thinner muscle underlying the tegument and intestinal ceca, muscle layer and

the reproductive organs but failed to react with the tegument and vitelline glands of *P. heterotremus*. Two MABs (SOM-8E9 and SOM-22E10) were found to react with the antigen with Mr of 17.5 kDa and gave strong fluorescent reaction over the tegument and the circular and longitudinal muscular layers lying just beneath the tegument. Two MABs (SOM-11E4 and SOM-6D5) recognized the 29 kDa polypeptide and reacted with the component intimately associated with all major muscular systems, vitelline glands and the tegument of the worm. One MAB which reacted with multiple bands of the somatic antigen by immunoblotting gave bright fluorescence over the muscular layers. One remaining MAB which reacted with the 66 kDa of somatic antigen of *P. heterotremus* failed to produce fluorescence in the worm section.

Each *P. heterotremus* specific MAB against ES or somatic antigen (MAB from clone ES-10F2 which gave immune complex bands with 22 and 31.5 kDa polypeptides and MAB from SOM-4G6 which gave immune complex bands with 12.3 kDa polypeptide) was used as immunoadsorbent to recover the respective antigens from the crude extract of *P. heterotremus* by affinity chromatography. These affinity purified antigens were then used as the immunodiagnostic reagents for detection of *P. heterotremus* specific antibodies by the indirect ELISA in 30 sera of patients with paragonimiasis heterotremus. The specific IgG antibodies could not be detected by ELISA in control subjects. The sensitivity, specificity and positive and negative predictive value at the 19% prevalence of the disease of this test against the affinity purified ES antigen were all 100%. On the contrary, system using the affinity purified somatic antigen gave sensitivity, specificity and positive and negative predictive values of 73.7%, 99.2%, 95.6% and 94.0%, respectively.

Although the indirect ELISA for detecting specific antibodies developed in our study was more specific and sensitive than those in previous studies, serological methods cannot readily distinguish present from past infection as serum antibodies are known to persist after the parasites have been eliminated by antihelminthic treatment (Maleewong *et al*, 1992b). Antigen detecting assay incorporating such MABs might constitute a better diagnosis test for active paragonimiasis. We also developed a two site polyclonal-monoclonal antibody based sandwich ELISA (PAB-MAB based sandwich ELISA) and evaluated for use in the detection of *P. heterotremus* antigen in the feces of 19 experimentally infected cats as a model.

Each of the specific MAbs (ES-10F2 and SOM-4G6) was used as an antibody probe for the PAB-MAB based sandwich ELISA. For the detection of *P. heterotremus* ES antigen in the specimens, the sensitivity and specificity of this test were 73.7% and 100%, respectively. However, the sensitivity of the *P. heterotremus* somatic antigen detection was low, only 2 of 19 infected stools were positive.

#### Genetic probe

Another model in our study for the diagnosis of *P. heterotremus* infection was the development of a sensitive, specific DNA probe and used in a DNA hybridization assay for detection of the parasite DNA in the feces of experimentally infected cats. The DNA probe containing 1,500 base pairs specific against *P. heterotremus* was obtained from screening, by DNA hybridization, of a genomic DNA library of the organism constructed in a pKS+ plasmid vector. The probe was used in a dot blot hybridization assay for the detection of the parasite DNA. The labeled probe detected *P. heterotremus* DNA released from as few as five eggs or from two metacercariae. The sensitivity and specificity were found to be 100%. DNA hybridization using this specific DNA probe might be used as a taxonomic aid, in parasite identification for diagnosis and epidemiology.

#### Diagnosis of paragonimiasis westermani

In earlier work, Slemenda and others (1988) revealed a sensitive and specific immunoblot assay and used to rapidly and accurately diagnose paragonimiasis. The immunoreactivity of a complex *Paragonimus westermani* chaffee antigen was evaluated by SDS-PAGE and immunoblot analysis. Probing with pooled human serum from proven *Paragonimus* infections revealed many bands including a significant antibody response to an approximately 8 kDa protein. These results indicate that the immunoblot for paragonimiasis, which used a comparatively crude antigen, is highly sensitive (96%) and specific (99%).

Waikagul (1989) used antigens from 4 species of *Paragonimus* in ELISA for the diagnosis of paragonimiasis in 50 patients with *P. westermani*, 6 patients with *P. miyazakii*, 64 patients with other helminthic infections and 17 healthy blood donors. There was no significant difference between the ELISA values of the same sera tested against crude antigens prepared from *P. westermani*, *P. miyazakii*, *P. heterotremus* and *P. siamensis*.

Recently immunodiagnosis in pleural effusion of paragonimiasis has been studied (Yokogawa *et al*, 1976; Ikeda *et al*, 1992). Yokogawa and others (1976) compared total IgE levels in serum samples from five patients with paragonimiasis miyazakii or westermani with those in pleural effusions and found that the levels in pleural effusions were higher. Moreover, Ikeda and others (1992) used an ELISA to measure *P. westermani* specific IgE and IgG in seven patients with paragonimiasis westermani, and compared the antibody levels detected in sera by ELISA with those in pleural effusion. The sensitivity to adult ES antigen was compared with the sensitivity to whole worm extract, and the former was more sensitive in both IgE-ELISA and IgG-ELISA. Both parasite-specific IgE and IgG could be detected by ELISA at levels much higher than those in control subjects using ES antigen. When specific IgE and IgG levels in sera and pleural effusion of individual patients were compared, the latter had higher values. The difference between levels of specific IgE in pleural effusion and serum did not correlate with that of specific IgG. These results indicate that specific local IgE and IgG antibodies *ie*, in the lung and that pleural effusions from patients with paragonimiasis are more suitable than serum for immunodiagnosis.

The production of MAbs have numerous applications in current biotechnology. The combined application of MAb and ELISA has the potential for improving the immunological detection of parasitic diseases. An attempt to utilize the MAb in an ELISA-inhibition test for improving the specificity of micro-ELISA in diagnosis of paragonimiasis westermani was revealed (Yong *et al*, 1993). By cell fusion, one hybridoma clone secreting anti-*P. westermani* specific MAb was selected (Pwa-14), which reacted on bands of 28 kDa, 42.5 kDa, 89 kDa and 120.5 kDa. IFA showed Pwa-14 was located at the vitelline follicles. By micro-ELISA, 100% of 22 paragonimiasis cases were found positive, but 5 of 40 clonorchiasis cases (12.5%) and 3 of 26 cysticercosis cases (7.7%) showed false positive. None of 10 sparganosis patients or 28 normal controls reacted positively. On the other hand, by ELISA-inhibition test using a *P. westermani* specific MAb, 100% of paragonimiasis cases were found positive, and there were no positive in cysticercosis, sparganosis cases or normal controls, except 2 (5.0%) false-positive sera of 40 clonorchiasis cases. The ELISA-inhibition test using a MAb showed higher specificity in comparison with micro-ELISA for serodiagnosis of human paragonimiasis westermani.



Zhang and others (1991) described the development of MAbs to metacercarial and adult worm stage-specific antigens of *P. westermani* and reported preliminary studies suggesting that an antigen-detection assay incorporating such MAbs might constitute a sensitive and specific diagnostic test for active paragonimiasis westermani. Later, the evaluation of the diagnostic potential of a dot-ELISA based on some of these *P. westermani* stage-specific MAbs was reported (Zhang *et al*, 1993). Sera from all donors with parasitologically confirmed infections with *P. westermani* contained adult worm antigens, as did a high proportion of sera from persons suspected to be infected with this parasite. A smaller proportion of these sera also contained metacercarial stage-specific antigens. Sera from donors with other helminth infections, with confirmed pulmonary tuberculosis, or from healthy Chinese donors were nonreactive in the assay. Treatment of experimentally infected animals with Praziquantel triggered a marked but transient increase in serum levels of adult *P. westermani* antigens, which then gradually disappeared with in the next two months.

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