

Immuno-PCR for Detection of Antigen to *Angiostrongylus cantonensis* Circulating Fifth-Stage Worms

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Background: Definitive diagnosis of infestation with *Angiostrongylus cantonensis* is difficult because the parasitic nematode is undetectable in the cerebrospinal fluid (CSF) of one-half of afflicted patients and the diagnostic sensitivity of ELISA for circulating worm antigens in patient sera is low. We studied immuno-PCR as a diagnostic tool.

Methods: We studied 30 controls and 60 afflicted patients (30 confirmed by parasitologic analysis of CSF). We used a monoclonal antibody to capture circulating *A. cantonensis* antigens in serum samples. A DNA label generated by PCR amplification with biotinylated primer was bound by use of streptavidin to a biotinylated third antibody. Circulating antigens sandwiched by monoclonal antibody were detected by PCR amplification of the DNA label.

Results: The detection limit of the ELISA was 100–1000 times higher than that of the immuno-PCR. The concentrations of circulating antigens in patients were markedly higher than those in controls (Wilcoxon rank-sum test, $P < 0.001$). At a cutoff of 0.1 ng/L, sensitivity and specificity for immunodiagnosis of patients with angiostrongyliasis by immuno-PCR were 98% (95% confidence interval, 91–99%) and 100% (93–100%), respectively. The test was positive in all parasitologically confirmed cases.

Conclusions: Immuno-PCR is a promising technique for diagnosis of *A. cantonensis* infestation.

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Angiostrongylus cantonensis, a nematode that is the most common infectious cause of eosinophilic meningitis and meningoencephalitis both worldwide and in areas of Southeast Asia and many Pacific Islands, is one of the most important zoonotic parasites in Taiwan (1, 2). Definitive diagnosis of angiostrongyliasis is established by the detection of *A. cantonensis*-specific worms in the cerebrospinal fluid (CSF)⁴ of patients suffering from meningitis or meningoencephalitis (3). The diagnostic process is challenging; even with the advent of an improved spinal-tap technique, which enhances recovery of *A. cantonensis*, the worm is detected in the CSF of only 50% of infested patients (4).

This low detection rate has prompted efforts to develop immunologically based detection methods. The suitability of a various antigens for the detection of serum antibodies has been explored, including antigens associated with the adult worm, the third- or fifth-stage larvae of *A. cantonensis* (AcL₃ and AcL₅, respectively), and certain metabolic processes (5–11). Most methods have not displayed sufficient antigenic specificity to be of reliable diagnostic value. To avoid the diagnostic liability of antigenic cross-reactivity between *A. cantonensis* and other helminthic species, partially purified and purified *A. cantonensis* antigens have been used to detect angiostrongyliasis-affected patients (12–17). The purification of these antigens, typically accomplished by column chromatography, is a time-consuming process because crude antigens typically require passage through several columns to appropriately prepare them for immunodiagnostic use. In another approach, specific monoclonal antibodies (mAbs) against antigens of the AcL₃ or AcL₅ larval stages of *A. cantonensis* have been prepared and used to detect circulating antigen in a double-antibody

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⁴ Nonstandard abbreviations: mAb, monoclonal antibody; AcL₃ and AcL₅, third- and fifth-stage larvae, respectively, of *A. cantonensis*; CSF, cerebrospinal fluid; PBS, phosphate-buffered saline; and TETBS, Tris-buffered saline containing EDTA and Tween 20.

sandwich ELISA (18, 19). The specificity of this method is high, but the detection sensitivity remains unsuitably low.

In seeking an alternative approach, we have considered the use of immuno-PCR. The immuno-PCR assay is similar in operation to the ELISA; both detect an antigen-antibody reaction. However, instead of an enzyme conjugated to an antibody, as is used in ELISA, immuno-PCR uses a reporter DNA target that is amplified to diagnostic concentrations by PCR (20, 21). To date, immuno-PCR has been used for the sensitive immunodetection of a variety of soluble protein molecules from infectious pathogens and compounds, including *Helicobacter pylori* and *Clostridium botulinum* exotoxin A (22–24). The present study was initiated with the aim of using immuno-PCR to detect AcL₅ circulating antigen in the serum of patients with eosinophilic meningitis or meningoencephalitis and to estimate the diagnostic accuracy of the method.

Materials and Methods

ANTIGEN PREPARATION

AcL₃ was collected from infested *Biomphalaria glabrata* snails after they were mechanically minced and digested with use of artificial gastric juice (17). Hybrid mice were individually infested with 50 larvae via an oral stomach tube. The mice were raised in an air-conditioned laboratory animal center (22 ± 1 °C and 50% ± 10% relative humidity). Three weeks after infestation, each mouse was sacrificed with an excess of ether, and AcL₅ were recovered from the brain. Larval components were extracted with cold 0.15 mol/L phosphate-buffered saline (PBS; pH 7.2) and delipidated with ether as described previously (25). The extracts were stored at –70 °C.

PURIFICATION OF mAbs

The hybridomas AcJ1 and AcJ20 were used. AcJ1 secretes IgG2a, and AcJ20 secretes IgG1. Both antibodies recognize specific AcL₅ antigens with a molecular mass of 204 kDa (17). These hybridomas were injected into the peritoneum of pristane-primed BALB/c mice. Antibody-enriched ascites were collected and purified through a protein A-Sepharose CL4B immunosorbent column (Pharmacia Fine Chemicals AB), with 0.1 mol/L sodium citrate–citric acid buffers (pH 4.5 and 6.5) used to elute IgG2a and IgG1, respectively. Antibody-containing eluates were immediately pooled and neutralized with 1 mol/L Tris to pH 7.0, then desalted by passage through a prepacked PD-10 column (Sephadex G-25; Pharmacia Fine Chemicals). mAbs were concentrated by lyophilization and then adjusted to a concentration of 10 mg/L with 0.15 mol/L PBS.

PURIFICATION OF ANTIGEN

AcL₅ antigen was purified by an immuno-adsorption technique (26). Briefly, purified mAb was mixed with swollen, cyanogen bromide-activated Sepharose 4B gel and incubated for 2 h. Gels were packed into a 2 × 15 cm column, and were washed and mixed with 1 mol/L ethanolamine (pH 8.0) for 1 h. Noncovalently adsorbed

proteins were washed out with 0.15 mol/L PBS. AcL₅ extracts were eluted with PBS at a flow rate of 8 mL/h until the eluate was free of extract. The bound components were then eluted with cold 0.1 mol/L glycine–hydrochloric acid buffer, neutralized with 0.15 mol/L glycine solution (pH 11.0), and dialyzed with PBS. The protein concentration of the purified antigen was detected by a specific protein assay (Bio-Rad). Aliquots of antigen were stored at –70 °C until required.

COLLECTION OF SPECIMENS

Patients in the Department of Pediatrics at Chong-Ho Memorial Hospital, Kaohsiung Medical University (Taiwan, ROC), with eosinophilic meningitis or meningoencephalitis with clinical syndromes including severe headache, stiffness of the neck, and intermittent fever and eosinophil counts >8% of leukocytes in CSF smears were involved in this study. Serum specimens were acquired during March 2000 and November 2002 from 60 consecutive patients, 21 females and 39 males [mean (SD) age, 7.0 (1.7) years]. In 30 patients, worms were found in the CSF. Control sera were collected from 30 healthy volunteers [13 females and 17 males; 7.2 (2.6) years]. All serum samples were randomly assigned numbers and stored at –70 °C until used.

BIOTINYLATION OF mAbs

The IgG1 mAb of the AcJ20 hybridoma was biotinylated as described by Hnatowich et al. (27). We reacted 1 mg of IgG1 mAb with 0.1 mg of sulfo-*N*-hydroxysuccinimide-Lc-biotin in 1 mL 0.15 mol/L PBS for 30 min at room temperature. After termination of the reaction, biotinylated IgG1 mAb was obtained by dialysis of the reactants.

BIOTINYLATED DNA

Biotinylated DNA was prepared as described by Sano et al. (20). The biotinylated pUC 19 was a linear 2.67-kb *Hind*III-*Acc*I fragment in which one biotin molecule had been incorporated at its *Hind*III terminus by a filling-in reaction with Sequenase version 2.0 DNA Polymerase (United States Biological) in the presence of a biotinylated nucleotide (biotin-14-deoxyadenosine triphosphate; BRL). The product was purified by use of a G-50 column (Pharmacia Fine Chemicals).

DETECTION OF ANTIGEN BY ELISA

The double-antibody sandwich ELISA technique described in detail elsewhere (25) was used to detect circulating antigens of *A. cantonensis*. Briefly, IgG2a mAb secreted by the AcJ1 hybridoma was adjusted to a concentration of 10 mg/L and applied to the wells of a microtiter plate (cat. no. 3912; Falcon). Adhesion of the antibody occurred during an overnight incubation at 4 °C. Unbound antibody was removed by washing with PBS containing 0.5 mL/L Tween 20. The reaction was then terminated by blocking with 10 g/L bovine serum albumin.

In the ELISA procedure, diluted serum or a preparation of purified antigen was added and incubated for 1 h. After a wash to remove unbound components, biotinylated IgG1 diluted to 1 mg/L was added to each well and incubated for 1 h. Unbound antibody was removed by washing each well eight times. After the final wash, a streptavidin–alkaline phosphatase conjugate diluted 1:1000 was added to each well. After incubation for 1 h, the wells were washed four times, and *p*-nitrophenyl phosphate substrate was added. The plates were incubated for 15 min in the dark to allow for color development of the enzymatically cleaved substrate, and the absorbances of the well contents were read at 405 nm with a colorimeter (MR 5000; Dynatech).

IMMUNO-PCR

Immuno-PCR was carried out according to the description of Sano et al. (20). The analysts were blinded to the whether samples came from patients and controls when evaluating all serum specimens by immuno-PCR. A schematic representation of this method is shown in Fig. 1. Briefly, purified IgG2a mAb (10 mg/L) was coated on the surface of wells of flat-bottomed immuno-PCR plates (Model 6511; Costar Corporation) overnight at 4 °C. Unbound antibody was removed by washing with Tris-buffered saline, wells were blocked for 1 h at 37 °C with 10 g/L bovine serum albumin in Tris-buffered saline containing 0.1 mmol/L EDTA and 1 g/L salmon-sperm DNA. The plates were then washed five times with the above buffer containing 1 mL/L Tween 20 (TETBS), after which, a 1:20 dilution of serum or purified antigen diluted with TETBS containing 1 g/L bovine serum albumin and 0.1 g/L salmon-sperm DNA were then applied to wells for 1 h at 37 °C. After the wells were washed 12 times with TETBS, biotinylated IgG1 (1 mg/L) was added for 1 h at 37 °C. After 12 washes with TETBS, streptavidin (1 mg/L) was added for 1 h at 37 °C. After another 12 washes with the same buffer, 1×10^{-19} mol/ μ L biotinylated PUC 19 was added for 30 min at room temperature. Wells were washed with TETBS 25 times and distilled water 3 times, and then 25 μ L of distilled water was added to each well,

followed by 5 μ L of the PCR mixture, 5 μ L of 1.25 mol/L deoxyribonucleoside triphosphates, 5 μ L of 20 μ mol/L 3' primer (5'-GTT TTC CCA GTC ACG AC-3'), 5 μ L of 20 μ mol/L 5' primer (5'-AGC GGA TAA CAA TTT CAC ACA GGA-3'), and 5 μ L (0.5 U) of *Taq* DNA polymerase mixture. We then layered of 25 μ L of mineral oil on the top of the mixture in each well and subjected the mixtures to PCR under the following conditions: denaturation at 94 °C for 15 s, 35 cycles of annealing at 55 °C for 10 s and extension at 74 °C for 30 s, and a final extension for 5 min at 74 °C. We then electrophoresed 5 μ L of the PCR product on a 3% agarose gel containing ethidium bromide.

STATISTICAL ANALYSIS

All concentrations of Acl₅ antigen in serum specimens detected by immuno-PCR are given as the mean (SD). The statistical significance of the differences in concentrations between patients with eosinophilic meningitis or meningoencephalitis and controls was assessed by means of Wilcoxon rank-sum test. Analysis of difference were also carried out between parasitologically confirmed patients and patients who displayed only clinical syndromes. $P < 0.05$ was considered significant. The sensitivity and specificity for immunodiagnosis of patients with angiostrongyliasis by immuno-PCR were assessed according to the methods of Newcombe (28) for 95% confidence intervals. Three samples of known concentration were tested in 20 replicates at one time to assess intraassay precision.

Results

INTRAASSAY PRECISION

Because all samples in this study were analyzed at one time, the intraassay precision of immuno-PCR for detection of circulating Acl₅ antigen in patients was estimated previously. The means (SD) of known antigen concentrations were 5 (0.14), 60 (1.44), and 120 (3.6) ng/L, respectively. The CV for this assessment were 2.8%, 2.4%, and 3.0%, respectively.

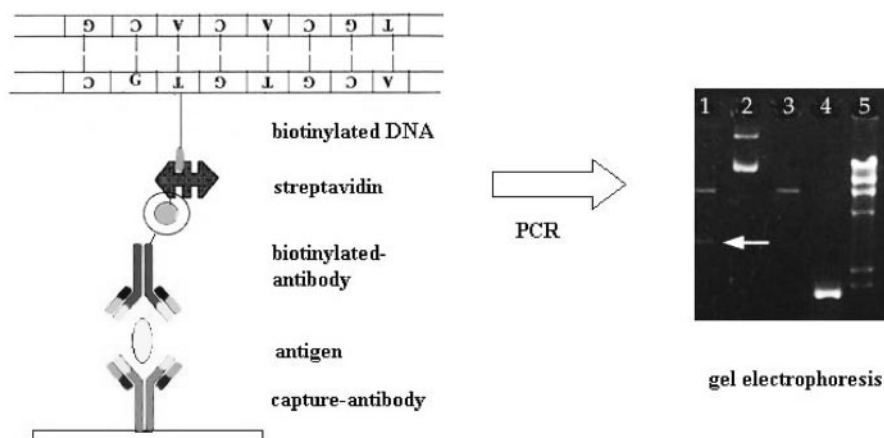


Fig. 1. Schematic representation of the immuno-PCR assay.

IgG2a mAb immobilized on an immuno-PCR plate is used to capture antigen sandwiched with biotinylated IgG1 mAb. Finally, streptavidin, used as a bridge, links the antigen–antibody complex to a biotinylated DNA. The amount of antigen is then quantified by PCR amplification of the reporter DNA.

COMPARISON OF THE DETECTION LIMITS OF ELISA AND IMMUNO-PCR

In this immuno-PCR system, a single 150-bp DNA band was routinely evident (Fig. 2A) with 0.1 ng/L purified antigen. No DNA product was observed for the negative controls. By contrast, the ELISA (Fig. 2B) did not detect antigen concentrations <10 ng/L. A high-circulating-antibody-titer serum from a patient suffering from angiostrongyliasis was tested in both assays.

As seen in Fig. 3A, immuno-PCR successfully detected antibody in dilutions up to 10^5 , whereas the ELISA failed to detect antibody in dilutions $>10^2$ (Fig. 3B).

IMMUNO-PCR DETECTION OF CIRCULATING ANTIGENS IN SERUM OF ANGIOSTRONGYLIASIS PATIENTS

A calibration curve for the AcL₅ 204-kDa antigen is shown in Fig. 4. The concentrations of antigen in patients known to harbor the parasite were 3–600 ng/L, whereas they were <0.1–120 ng/L in those suspected of harboring the parasite. Sera from noninfested controls contained <0.1 ng/L (Fig. 5).

The median (range) circulating antigen concentrations, as shown in Table 1, were 25 (3–600) ng/L and 39 (<0.1–120) ng/L in parasitologically confirmed patients

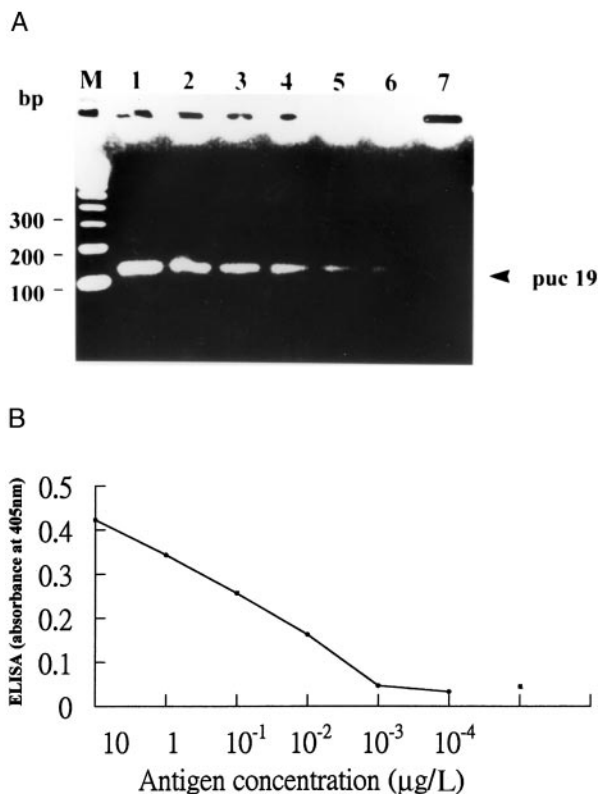


Fig. 2. Detection of purified 204-kDa AcL₅ antigens by the AcJ₂₀ mAb in immuno-PCR (A) and ELISA (B), respectively.

(A), purified antigen was serially diluted 10-fold from 10 µg/L (lane 1) to 0.1 ng/L (lane 6). Lane M, molecular markers; lane 7, antigen-negative control. (B), ● beyond the line, antigen-negative control. Limits of detection for the immuno-PCR and ELISA were 0.1 and 10 ng/L, respectively.

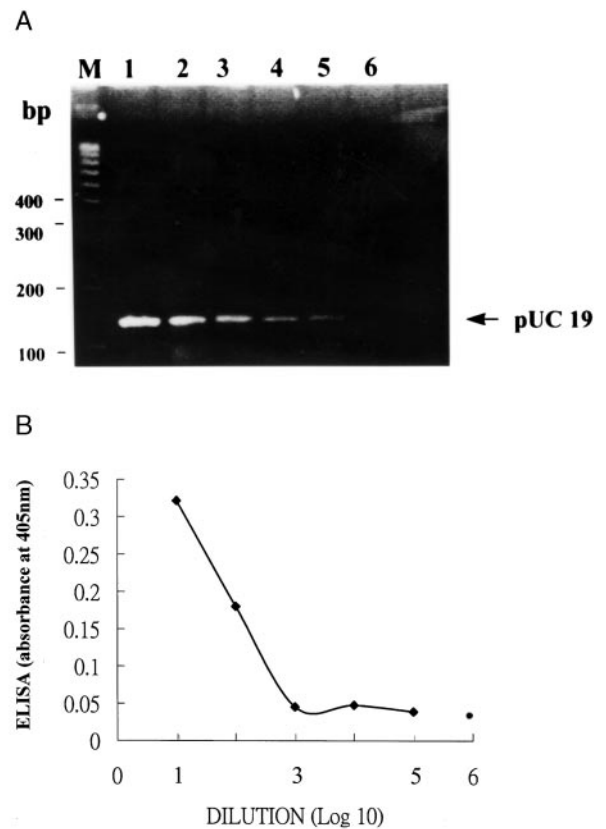


Fig. 3. Detection of circulating 204-kDa AcL₅ antigens in serum specimens from patient with *A. cantonensis* infestations by immuno-PCR (A) and double-antibody sandwich ELISA (B), respectively.

Serum was serially diluted 10-fold from 1×10^{-1} to 1×10^{-5} . (A), lane M, molecular markers. Lane 6, noninfested control serum. (B), serial dilution 6, noninfested control serum. The dilution limits for immuno-PCR and ELISA were 10^{-5} and 10^{-2} , respectively.

and symptomatic patients, respectively. Antigen concentrations in both groups of patients were significantly higher than in the noninfested control group ($P < 0.001$). Additionally, the antigen concentrations in parasitologically confirmed and symptomatic patients were significantly different ($P < 0.05$).

DIAGNOSTIC SENSITIVITY AND SPECIFICITY OF IMMUNO-PCR

At a cutoff of 0.1 ng/L, all controls were negative, and 59 of 60 patients (98%) were positive, including all 30 patients in whom parasite had been identified in the CSF. The 95% confidence intervals for sensitivity and specificity were 91–99% and 93–100%, respectively.

Discussion

When ingested by humans, AcL₃ penetrate the intestinal wall and then migrate to the brain and spinal cord within a period of 12 h. The speed of progression of the infestation necessitates rapid detection of the parasite if meaningful therapy is to be commenced. A definitive diagnosis for patients with this parasitic infestation can be based on

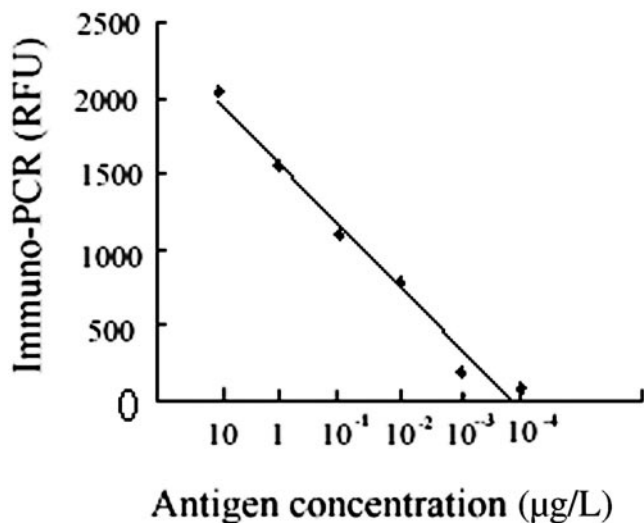


Fig. 4. Immuno-PCR calibration curve constructed with AcJ₂₀ mAb against purified 204-kDa AcL₅ antigens.

Detection values for the immuno-PCR are expressed as relative fluorescence units (RFU).

the presence of worms in the CSF. This diagnosis requires a highly trained physician to collect the sample. Even then, the method of collection by lumbar puncture poses a risk to the patient. Furthermore, because *A. cantonensis* larvae can develop into AcL₅ in the brain ventricle, serum antigen concentrations may remain very low, hampering diagnosis.

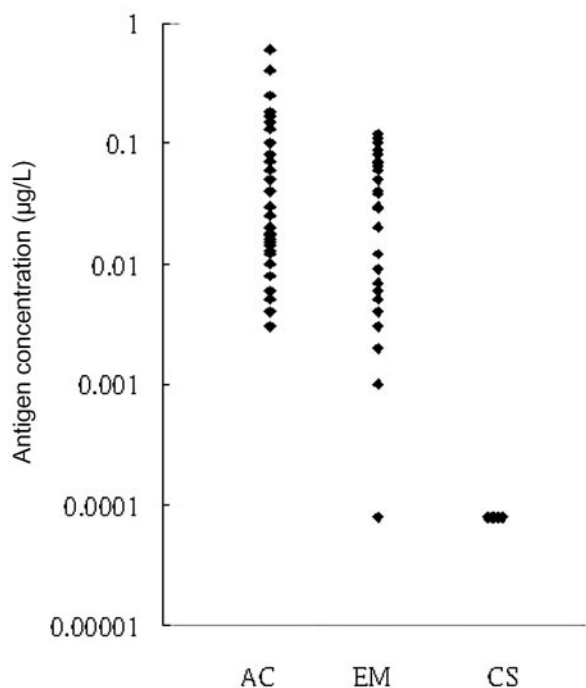


Fig. 5. Distribution of the 204-kDa AcL₅ antigen in serum from patients with eosinophilic meningitis or meningoencephalitis.

AC, patients confirmed to have the parasite; EM, patients displaying only clinical syndromes; CS, controls. Antigen concentrations in controls were all below the detection limit (<0.1 ng/L).

Table 1. Antigen concentration in serum specimens from patients with eosinophilic meningitis or meningoencephalitis and control individuals.

Group	Serum antigen concentration, ng/L	P
All patients (n = 60)		
Median	30	
Range	<0.1–600	
Controls (n = 30)	<0.1	<0.001
Parasitologically confirmed patients (n = 30)		
Median	25	
Range	3–600	
Patients with only clinical syndromes (n = 30)		
Median	39	<0.05
Range	<0.1–120	

The diagnostic challenges posed by CSF collection and a low antigen concentration in the serum of patients have spurred efforts to develop alternative, sensitive methods for the detection of *A. cantonensis*. Sano et al. (20) developed a highly sensitive serum antigen detection system that combines an ELISA and several DNA oligonucleotide reporters for exponential amplification by PCR. We exploited this hybrid technology to detect patients with angiostrongyliasis cantonensis. The method was successful in detecting the strikingly increased concentrations of soluble antigens characteristic of *A. cantonensis* infestation, relative to noninfested controls.

In this study, the AcJ1 mAb was used to capture the circulating antigen in serum, after which the biotinylated AcJ20 mAb bound to the antigen. Free streptavidin was then used to attach biotinylated DNA to the biotinylated AcJ20 mAb. Finally, this complex was amplified by PCR to quantities resolvable by agarose gel electrophoresis. Development of this immuno-PCR technique to aid in the diagnosis of angiostrongyliasis cantonensis was technically challenging. Initially, even the negative control for the first mAb occasionally generated some nonspecific amplification (29), which likely arose as a consequence of the concentration of the biotinylated DNA used for the reaction. A serial 10-fold dilution of the reactive biotinylated DNA revealed that if the DNA concentration exceeded 1×10^{-19} mol/µL, nonspecific amplification occurred. Thus, this concentration became the optimum.

The sensitivity and specificity of ELISA is influenced by insufficient blocking, and this nonspecific binding may be minimized by saturating the remaining adsorptive surfaces of the assay plate with blocking proteins, such as those in nonfat dry milk (30). Consistent with these observations, we found that signal-to-noise ratios improved when fetal calf serum was used as a blocking reagent in the assay. To minimize nonspecific binding, we used bovine serum albumin plus salmon-sperm DNA as blocking agents. These agents have been used for the same purpose by others (20, 31–33). As reported by others

(20, 24, 31), we also minimized the signal-to-noise ratio by extensively washing assay plate wells with detergent-containing buffer during experimental manipulations.

The choice of pUC 19 DNA as a reporter molecule was based on an earlier study (20). The recombinant nature of pUC 19, which contains an ampicillin resistance gene, *lacZ*, and a restriction enzyme site, makes the chance of encountering the vector or a closely related sequence in human biological specimens virtually zero. This aids in preventing DNA cross-contamination problems. Furthermore, the specificity of immuno-PCR is dependent on the quality of the first antibody used in the process. In our study, we used the AcJ1 mAb, which is highly specific for the AcL₅ antigen, as the capture antibody.

Sensitivity and specificity are the most important factors in the evaluation of the reliability of a diagnostic technique for parasitic infestations (34). High sensitivity (91%) and specificity (98%) for detecting serum antibodies in patients with eosinophilic meningitis or meningoencephalitis have been obtained by an ELISA incorporating a purified AcL₅ antigen with a molecular mass of 204 kDa and a corresponding mAb (17). However, delays in the appearance of antibodies after infestation and the persistence of antibodies after cure have restricted the diagnostic reliability of serum antibody detection in patients with these parasitic diseases (18).

The abilities and limitations of mAbs, as described above, led to the adaptation of a double-antibody sandwich ELISA technique as a means of detecting circulating antigens in patient specimens. In an immunoprecipitation analysis, a 204-kDa AcL₅ antigen could be detected by circulating antibodies in the serum of rats in early stages of experimental infestation (35). Indeed, two mAbs recognizing this antigen have been prepared previously (36) and have been applied for detection of circulating antigen in patients (19). A sandwich ELISA using these mAbs for detection of circulating antigen in the serum of patients achieved high specificity (100%). However, the sensitivity, only 81%, was problematic (19).

In conclusion, in the present study, immuno-PCR, like ELISA, detected circulating 204-kDa AcL₅ antigens in the serum of patients with eosinophilic meningitis or meningoencephalitis with 100% specificity. In contrast to the ELISA, however, the sensitivity of the immuno-PCR was 100% (95% confidence interval, 93–100%) for patients known to have the parasitic worm in CSF specimens and 96.7 (83–99)% for patients who displayed only clinical syndromes. Thus, the double-determinant immuno-PCR system demonstrates greater sensitivity than appears to be the case for any existing antigen detection system and may become the assay of choice for the routine diagnosis of patients with *A. cantonensis* infestation.

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