

Method for detecting circulating *Toxocara canis* antigen and its application in human serum samples

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ABSTRACT

Diagnosis of larval migrans (LM) is usually done by immunodiagnostic methods. These methods, however, simply show the presence or absence of antibody but not the active infection of the patients. Therefore, we aimed to establish a diagnostic method for detecting circulating *Toxocara canis* antigen using a sandwich-ELISA. Monoclonal antibodies (MAb) were produced against the excretory-secretory (ES) antigen of second-stage *T. canis* larvae. Among the MAbs obtained, we selected one MAb (TCMAb12; molecular weight, 30-80 kDa, IgG) for use in the sandwich ELISA. The cross-reactivity of the sandwich-ELISA against thirteen different kinds of parasite antigens were examined. The results revealed that the antibody reacted with *T. canis* ES antigen, *T. canis* female antigen, and *T. canis* second-stage larvae antigen, but did not react with any other antigens. From results obtained using an ES antigen concentration standard curve, we confirmed that the detection limit of the sandwich-ELISA was 5 ng/ml, which provides sufficient sensitivity for the diagnosis of toxocariasis (LM). We applied the method to suspected toxocariasis patients and examined the circulating antigen in their sera. We used nine serum samples collected from patients with suspected toxocariasis based on both their clinical symptoms and high antibody titers. Overall, five sera showed antigen-positive reactions, while the remaining four were negative. These results indicated that about 44.0% of the antibody-positive patients were antigen-negative, not ongoing active infection. The results obtained using this technique would provide us for understanding toxocariasis.

Keywords: *Toxocara canis*, sandwich ELISA, monoclonal antibody, excretory-secretory antigen

INTRODUCTION

Toxocara canis, a parasite whose definitive host is the dog, is distributed worldwide.¹⁻⁶ In man, this nematode causes visceral larva migrans (VLM) characterized by hepatomegaly, pulmonary infiltration and eosinophilia. However, little is known about the actual status of invading larva migrans caused by *T. canis* because of its diagnostic difficulties. Usually, ordinary parasitic infections are diagnosed by fecal examination. However, in the case of toxocariasis, a cause of larva migrans, eggs are never excreted in human feces. Therefore, toxocariasis is diagnosed using immunological or genetic diagnostic methods. In particular, a wide variety of immunological methods have been reported, including fluorescent antibody test, hemagglutination test, and ELISA.^{2,7} Of these methods, it has been reported that the fluorescent antibody test has poor sensitivity and the hemagglutination test has low specificity, and have, therefore, raised doubts about their utility.⁷ Even with the ELISA, cross-reactivity to other antibodies have been observed when extract or adult antigens were used.⁸ However, since 1975, after the first attempt of *in-vitro*

culture⁹ second-stage of *T. canis* larvae to prepare an excretory-secretory (ES) antigen, serodiagnosis of toxocariasis using this ES antigen has become a common method. Although these conventional immunological diagnostic methods based on antibody detection, the results obtained from such methods are not always indicative of conditions in the actual infection. Namely, antibodies are sometimes detected despite eradication of parasites; in such cases, false positive results are obtained. To overcome this drawback, detection of circulating antigens in the blood has been investigated.¹⁰⁻¹²

Sandwich ELISA is done using antibody sensitized microtiter plate, then adding a test serum to form an antigen-antibody complex, addition of conjugate and finally quantifying the antigen using a ELISA reader. This method can be used in variety of combinations but with different degree of sensitivity. Bowman *et al.*¹⁰ described a method sensitizing a plate with anti-ES polyclonal antibody, and detecting the antigen using an enzyme-labeled anti-ES polyclonal antibody. Cuellar *et al.*¹¹ and Yokoi *et al.*¹² described methods sensitizing a plate with in house monoclonal antibodies, and using a

Table-1: Cross-reactivity of TCMAB 12 against other helminthic antigens by sandwich ELISA

No	Antigens	OD value
1	<i>Toxocara canis</i> ES antigen	0.950
2	<i>Toxocara canis</i> male antigen	0.136
3	<i>Toxocara canis</i> female antigen	0.900
4	<i>Toxocara canis</i> embryonated egg antigen	0.116
5	<i>Toxocara canis</i> second-stage larva antigen	0.404
6	<i>Toxocara cati</i> male antigen	0.091
7	<i>Toxocara cati</i> female antigen	0.227
8	<i>Toxocara cati</i> embryonated egg antigen	0.128
9	<i>Ascaris suum</i> adult antigen	0.270
10	<i>Dirofilaria immitis</i> adult antigen	0.153
11	<i>Angiostrongylus cantonensis</i> adult antigen	0.099
12	<i>Trichinella spiralis</i> encysted larva antigen	0.101
13	<i>Centrocestus armatus</i> metacercariae antigen	0.131

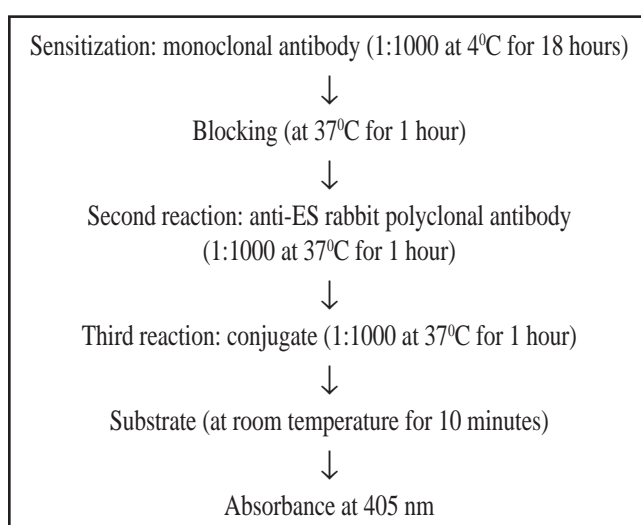
commercially available enzyme-conjugated antibody. Bowman *et al.*¹⁰ studied the ways to increase the sensitivity by pre-treatments, such as heat treatment of serum samples with the addition of polyethylene glycol (PEG) or ethylenediaminetetraacetic acid (EDTA). Even the monoclonal antibodies are known to cross-react with other helminth antigens.¹³ Yokoi *et al.*¹² performed cross-reactivity tests of monoclonal antibodies they obtained, using four kinds of antigens, but appeared that the choice of antigens have not been adequately investigated.

Regarding the sensitivity of sandwich ELISA, the detection limit was reported to be 20 ng/ml¹⁴ or 4 ng/ml,¹² but as precise procedures were not shown in either study reproducibility of these studies is limited. The purpose of this study, therefore, was to prepare monoclonal antibodies specific for *T. canis*, and experiment using these antibodies with an aim to increase the sensitivity of sandwich ELISA for the detection of antigen and to clarify the true sense (status) of toxocarasis.

MATERIALS AND METHODS

Experimental animals: Monoclonal antibodies were prepared using female BALB/c mice (SLC, Shizuoka, Japan) at four weeks of age. Ascitic fluid was collected from female BALB/c mice at seven weeks of age. Anti-ES polyclonal antibodies were prepared using female JW/CSK rabbits (SLC) at six weeks of age.

Myeloma cells: Myeloma cells (P3-X63-Ag8-U1) used for this study were kindly supplied from the Department of Virology, Kobe University Graduate School of Health

**Fig. 1:** Procedure of sandwich ELISA for antigen detection

Sciences, and have been maintained in our laboratory. For the cultivation, we used RPMI 1640 medium (Wako, Osaka, Japan) containing with 20% fetal bovine serum (Funakoshi, Tokyo, Japan), 5×10^{-3} M 2-mercapto ethanol (Biomedical, Eschwege, Germany), 200 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), and 1% penicillin-streptomycin solution (SIGMA, St. Louis, USA). The medium was exchanged every three days.

Toxocara antigens: The *T. canis* ES antigen was prepared according to the method of De Savigny.⁹ *T. canis* second-stage larvae were cultured in RPMI-1640 for four weeks. The supernatant (medium was exchanged every week) was recovered and concentrated to yield the ES antigen. Cross-reactivity was tested using the male antigen, female antigen, embryonated egg antigen, second-stage larvae antigen, and ES antigen of *T. canis*, male antigen, female antigen, embryonated egg antigen of *Toxocara cati*, the adult antigens of *Ascaris suum*, *Dirofilaria immitis*, *Angiostrongylus cantonensis*, the encysted larval antigen of *Trichinella spiralis*, and the metacercariae antigen of *Centrocestus armatus*. These antigens were prepared by washing each parasite with phosphate buffered saline (PBS; Nissui, Tokyo, Japan) three times, then milling them in a mortar, suspending them in PBS, and centrifuging the suspension at $1,500 \times g$ for 10 minutes, the supernatant thus obtained was used as the antigen. In the case of metacercariae and larvae, their suspension in PBS was sonicated for 10 minutes, centrifuged in the same manner as described above, and the resulting supernatant was used. Each antigen prepared was used in the following experiments after adjusting its protein concentration to 5 mg/ml by using the Folin-Lowry method.

Anti-ES rabbit polyclonal antibody: The *T. canis* second-stage larvae antigen, along with Freund's complete adjuvant (Biomedicals), was subcutaneously injected in

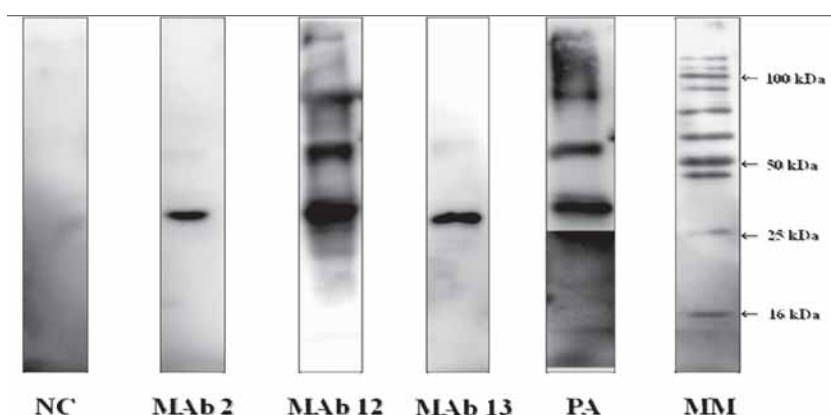


Fig. 2. Western blotting analysis. NC: negative control, MAb 2: TCMAB 2, MAb 12: TCMAB 12, MAb 13: TCMAB 13, PA: polyclonal antibody, MM: molecular marker

a rabbit. This first immunization was followed by two booster doses at 2- to 3-week intervals. After confirmation of a sufficient rise in antibody titer by ELISA, the whole blood was drawn from the rabbit, and serum was separated for use as the anti-ES rabbit polyclonal antibody.

Positive or negative samples for sandwich ELISA: The ES antigen was diluted to obtain a concentration of 0.5, 1, 5, 10, 50, or 100 ng/ml using a PBS solution containing 0.05% Tween 20 (Wako) and 1% bovine serum albumin (SIGMA) (PBS-TB solution). The 100-fold dilutions of 20 human serum samples in the PBS-TB solution were used for sandwich ELISA (negative samples). In the experiments, the negative samples were used after absorption of antigens by the TCMAB 12. Nine antibody-positive human sera against *T. canis* were confirmed by the ELISA beforehand.

ELISA test: A 96-well microtiter plate (Nunc, Roskilde, Denmark) was sensitized with 5 mg/ml of ES antigen at 4°C for 18 hours. After the 100-fold diluted serum was reacted at 37°C for 1 hour, horseradish peroxidase-conjugated sheep anti-mouse IgG (Abcam, Cambridge, U.K.), previously diluted at 1,000-fold, was reacted at 37°C for 1 hour. The absorbance was determined using a microplate reader (Thermo, Helsinki, Finland) at 405 nm.

Preparation of monoclonal antibodies: Monoclonal antibodies were prepared in accordance with the method of Yokoi *et al.*¹² Specifically, a BALB/c mouse was inoculated with a previously prepared mixture of 2,000 ng of the ES antigen and Freund's complete adjuvant by intraperitoneal injection. This first immunization was followed by two booster doses at 2- to 3-week intervals, and a marked rise in antibody titer in the immunized mouse was confirmed by ELISA. For the final immunization, 2,000 ng of the ES antigen was injected

intraperitoneally. Three days after the last immunization, the mouse was sacrificed and the spleen was aseptically recovered, which was co-cultured with myeloma cells in a 10:5 ratio for hybridization. Subsequently, antibodies within the medium were confirmed by the ELISA, after which limiting dilution was twice performed. The cloned cells thus obtained were considered to be monoclonal antibody-producing cells. Finally, 1×10^6 antibody-producing cells were given to a BALB/c mouse by intraperitoneal injection, and the ascitic fluid obtained was used as the monoclonal antibody. The class and

subclass of the monoclonal antibody obtained were determined using an immunoglobulin typing kit (Wako).

Western blot: The ES antigen (0.1 mg/ml) was electrophoresed on each track of 12% polyacrylamide gel. The electrophoregram was transferred to a nitrocellulose membrane. TCMAB 2, 12, and 13 monoclonal antibodies were used in 100-fold dilutions as the first antibodies. Analysis was performed using the LAS-1,000 analyzer (Fuji Film, Tokyo, Japan) with peroxidase-conjugated sheep anti-mouse IgG (MBL, Aichi, Japan) as the second antibody.

Sandwich ELISA for antigen detection: Sandwich ELISA was performed using the procedure shown in Fig. 1. Specifically, a 96-well microplate was sensitized with a 1,000-fold dilution of the monoclonal antibody (4°C, 18 hours), which was followed by blocking with 3% bovine serum albumin at 37°C for 1 hour. After the first reaction at 37°C for 1 hour, the anti-ES rabbit polyclonal antibody was used for the second reaction (at 37°C for 1 hour). The complex was captured (at room temperature for 10 minutes) using horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed, California, USA) and absorbance was determined using a microplate reader (405 nm).

RESULTS

In the present study, four kinds of monoclonal antibodies against the *T. canis* ES antigen were obtained, of which three were of the IgG1 subclass and one was of the IgM subclass. We designated these four monoclonal antibodies as TCMAB 2, 12, and 13 (IgG1 subclass), and as the TCMAB 24 (IgM subclass). TCMAB 2, 12, and 13 were used in the western blot analysis and TCMAB 12 was used in the cross-reactivity experiments described below.

Western blot analysis detected three bands from TCMAB

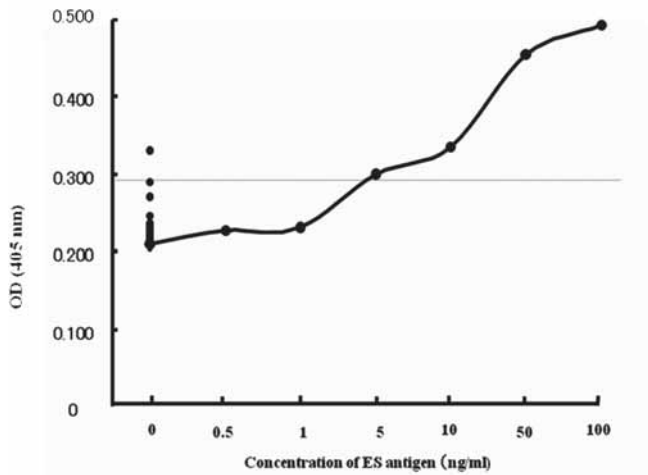


Fig. 3: Relationship between ES antigen concentration and absorbance value. Dot line () shows a cutoff line, which was obtained by the following formula: mean absorbance value of 20 negative sera + 2 standard deviations

12 at positions corresponding to molecular weights of 80, 50, and 30 kDa. By contrast, one band from TCMAB 2 and 13 were detected at around 30 kDa. The medium was used as the negative control, and produced no bands (Fig. 2). Although TCMAB 2 and 13 were considered to be used for further study, we could not use them because of low potency of antibody titer, and/or the low yield of ascitic fluid of mice.

TCMAB 12 was examined its cross-reactivity using 13 kinds of antigens (five kinds of *T. canis*-related antigens, three kinds of *T. cati*-related antigens, and five kinds of other helminth antigens) by sandwich ELISA (Table-1). TCMAB 12 reacted to female antigen, second-stage larvae antigen, and ES antigen of *T. canis*, but did not react to any other antigens. These results showed that the TCMAB 12 monoclonal antibody was specific for *T. canis*.

Hence, we used TCMAB 12 in the subsequent sandwich ELISA. We performed the sandwich ELISA using 20 kinds of negative sample, and obtained a mean absorbance (OD) at 405 nm of 0.233 with a standard deviation of 0.031. We established the cutoff value for the present method to be 0.295 as mean + 2 standard deviations. Accordingly, in the present experiments, we judged that OD values of more than 0.295 were positive, and those of less than 0.295 were negative (Fig. 3). With this criterion, experiments using positive samples produced OD values showing a nearly linear distribution with dose dependency. Because the OD value obtained at 5 ng/ml was 0.299, the antigen detection limit of our method was considered to be 5 ng/ml (Fig. 3).

Of the nine antibody-positive sera examined, four sera were negative for antigen by the sandwich ELISA. Our result revealed that these four patients were not active

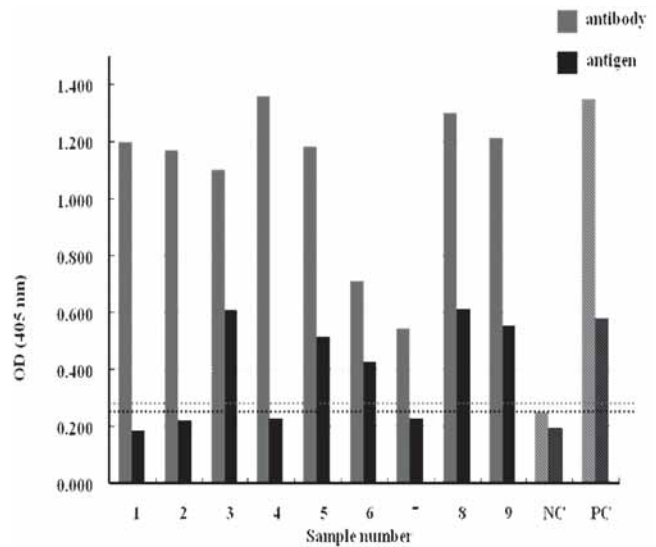


Fig. 4: Antigen levels in 9 antibody positive human serum. NC: negative control, PC: positive control

infection but revealed the remaining five patients were the true sense of toxocarasis (Fig. 4).

DISCUSSION

Studies reporting the preparation of monoclonal antibodies specific for the *T. canis* have been presented by Cuellar *et al.*,¹¹ Yokoi *et al.*,¹² and Maizels *et al.*¹⁵ Cuellar *et al.*¹¹ examined changes of circulating antigen and antibody levels in sera of mice infected with *T. canis* second-stage larvae, by sandwich ELISA using a monoclonal antibody, and confirmed rises in the levels of both the antigen and antibody from just after the infection. They concluded, however, that sandwich ELISA was useful in monitoring the course of infection, because administration of anthelmintic resulted in a clear reduction in the antigen level, while the antibody remained relatively constant. A similar approach was also reported by Robertson *et al.*¹⁴

Kennedy *et al.*¹³ reported that they detected two to four bands from monoclonal antibodies using polyacrylamide gel electrophoresis (SDS-PAGE). Yokoi *et al.*¹² reported that they detected one band using Western blots. Detection of only one band indicates that one epitope of ES antigen is recognized; detection of more than one band indicates that several epitopes are recognized. Provided that all these epitopes are specific sites for the parasite to be detected, the finding is valuable for diagnostic purposes. We detected three bands from TCMAB 12, all of which were free from cross-reactivity with parasites other than *T. canis*; hence, we used TCMAB 12 as the monoclonal antibody for the sandwich ELISA.

It is well known that in making a diagnosis based on the antigen-antibody reaction, nonspecific reactions, and

cross-reactions can affect diagnostic results.^{8,16,17} Cuellar *et al.*¹⁶ reported that cross-reactions occurred between antisera from mice infected with *T. canis* adult worms and the ES antigens of *Ascaris suum* or *Toxascaris leonina*. Kennedy *et al.*¹³ examined the reactions between a number of monoclonal antibodies they prepared and second-stage larva antigen by the indirect fluorescent antibody test, and reported that some antibodies were reactive and others were not; in agreement with our results. TCMAB 12 was thought to be fully applicable to the diagnoses of *T. canis* by detecting antigens in blood flow on the basis of its yields, reactivity, and other factors.

Regarding methods of sandwich ELISA for detecting circulating antigens, a wide variety of combinations of antibody, conjugation, and their dilution rates, for example, have been reported.^{12,14,18} Luo *et al.*¹⁸ reported on a system for detecting antigens by sensitizing a polyclonal antibody, and sandwiching the antigen with a horseradish peroxidase-conjugated polyclonal antibody. However, their method was shown to produce cross-reactivity with the *Ascaris lumbricoides* antigen, raising a question about the serological diagnosis using this method alone. According to Kennedy *et al.*,¹³ the use of a monoclonal antibody for sensitization is recommended because cross-reactions are likely to occur due to the similar molecular weights of the ES antigens of *T. canis* and *T. cati*. Yokoi *et al.*¹² evaluated a method of sandwiching an antigen with monoclonal antibodies, and showed decreased cross-reactivity and high diagnostic efficacy, unlike with a polyclonal antibody. Hence, we used a method based on the report by Yokoi *et al.*¹² and obtained a detection sensitivity of 5 ng/ml. This is nearly the same level obtained by Yokoi *et al.*¹², and may be sufficient to detect ES antigens in serum. Cuellar *et al.*¹¹ attempted but failed to detect circulating antigens in rabbits infected with 2,000 larvae. Other authors reported that only very small amounts of antigens were obtained from experimentally infected animals,¹⁰ further investigation will be necessary.

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