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Detection of *Echinococcus multilocularis* Coproantigens in Experimentally Infected Dogs Using Murine Monoclonal Antibody Against Adult Worms

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Abstract

Diagnosis of *Echinococcus multilocularis* infection in definitive host by detection of coproantigens was performed in experimentally infected dogs.

A sandwich enzyme-linked immunosorbent assay (ELISA) using rabbit polyclonal antibodies against excretory/secretory (ES) antigens of *E. multilocularis* adult worms and murine monoclonal antibody, designated EmA9, against somatic antigens of the adult worm was used for the detection of coproantigens. The specificity of the test was evaluated using feces of animals infected with *Taenia taeniaeformis, T. crassiceps, Trichuris vulpis* and *Toxocara canis*. No cross-reaction was observed. Sensitivity was evaluated from results of a serial twofold dilutions of *E. multilocularis* antigen. The lower limit of detection was 4 ng *E. multilocularis* antigen g^{-1} of feces. Detection of coproantigens using EmA9 in feces of three dogs experimentally infected with *E. multilocularis* was carried out almost daily until autopsy. Coproantigens were detected as early as 3–5 days postinfection (PI) and increased steadily until autopsy on day 21 PI. Anthelmintic treatment of one dog on day 17 PI by praziquantel resulted in the disappearance of the antigens from feces. Thus, an accurate diagnosis could be made using this method.

Detection of circulating antibodies was performed in parallel with the coproantigen test. Antibody levels of two dogs increased steadily from day 8 PI until day 21 PI, but antibody levels of one dog which was treated with praziquantel were apparently high throughout the experimental infection.

From these results, detection of coproantigens using EmA9 is a more effective diagnostic method than detection of circulating antibodies because of the capability to diagnose infection during the prepatent phase and it provides a good estimate of the actual status of infection in the host. In addition, freezing and subsequent heating of the feces did not influence the sensitivity of coproantigen detection by ELISA, which prevent accidental infection, by heating fecal samples before the test is performed.

Key words: *Echinococcus multilocularis*; coproantigen; monoclonal antibody; immunodiagnosis; dog; ELISA.

Introduction

Accurate diagnosis of *Echinococcus* infection in definitive hosts, especially dogs, has always been an important component in a hydatid disease control program. Diagnosis of *Echinococcus* adult worm infection in definitive hosts has been performed by detection of proglottids and/or eggs in feces (FAO, 1982; Gemmell and Lawson, 1986; Gemmell, 1987). However, it is often inaccurate because the parasites during pre-patency seldom excrete eggs and their excretion in feces during patency is irregular. Arecoline purging of dogs with subsequent fecal examination has been widely used, but this technique also have some disadvantages, such as a risk of infection

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to man and having no effect or side-effect on some dogs (FAO, 1982; Rickard and Arundel, 1985).

The employment of immunodiagnostic methods has been alternatively proposed in a recent report, for the detection of specific antibodies in serum samples or cestodes antigens in feces, i.e., coproantigens.

Serological studies have been performed for the diagnosis of *E. granulosus* infection in dogs (Jenkins and Rickard, 1985, 1986a, b; Gasser *et al.*, 1988, 1993) and of *E. multilocularis* in foxes (Gottstein *et al.*, 1991a). It was shown that serodiagnosis using enzyme-linked immunosorbent assay (ELISA) is superior to fecal examination in sensitivity, and that it is reliable in terms of specificity (Jenkins and Rickard, 1985, 1986a, b; Gasser *et al.*, 1988, 1993). However, antibody detection does not always reflect the current intestinal *Echinococcus* infection. Antibodies are not detectable in the early infection and persist for long periods even after elimination of the parasites. On the other hand, excretion of *Echinococcus* antigens in feces of hosts may correspond to the presence of parasites in the intestine and antigen release cease after the elimination of parasite.

Detection of cestode coproantigens has been studied in *Echinococcus, Taenia* and *Hymenolepis* infected definitive hosts (Allan and Craig, 1989; Allan *et al.*, 1990, 1992; Pelazes *et al.*, 1990, 1991, 1992; Maass *et al.*, 1991). These studies showed that sandwich ELISA using polyclonal antibodies against the parasites antigens is able to detect coproantigens and it is specific at the genus level. In addition, coproantigen is detectable during pre-patency or when excretion of eggs is not observed. Alternatively, detection of parasite-specific DNA using highly sensitive techniques such as polymerase chain reaction has also been proposed for diagnosis of *Taenia* or *Echinococcus* infection in definitive hosts (Gottstein and Mowatt, 1991; Gottstein *et al.*, 1991b; Bretagne *et al.*, 1993; Gasser and Chilton, 1995). However, excretion of eggs or cells from worms in feces is indespensable for this method. Therefore, a combination of DNA method with coproantigen detection may be a good tool for reliable diagnosis.

Kohno *et al.* (1995) produced monoclonal antibodies against adult *E. multilocularis* somatic antigens for the diagnosis of the infected definitive hosts and showed that one monoclonal antibody, designated EmA9, can detect the coproantigens in dogs experimentally infected with *E. multilocularis*. They suggested that sandwich ELISA using MoAb is more sensitive than the older methods for coproantigen detection and have a potential to be good tool for immunodiagnosis of *E. multilocularis* infected definitive hosts. In the present study, we performed further examination using EmA9 for the coproantigen detection in experimentally infected dogs and discussed its sensitivity and specificity in sandwich ELISA.

Materials and Methods

Parasite infections

E. multilocularis protoscoleces were prepared from hydatid cysts of experimentally infected gray red-backed vole, which has been maintained using dogs and voles in Hokkaido Institute of Hygiene. Three helminth-free dogs were dosed with approximately 200,000 protoscoleces orally. The feces from these dogs before infection and postinfection were used in subsequent experiments. One dog (No. 3) was treated with praziquantel (Bayer Japan Co. Ltd., Tokyo, Japan) (5 mg/kg) at 17 days PI. All dogs were autopsied at 21 days PI and infected worms were recovered as described by Thompson and Eckert (1982). Recovered worms were then counted and used for preparation of ES antigens.

ES antigens

Equipment, Hank's balanced saline and media used were all sterilized. *E. multilocularis* adult worms recovered from dogs at 21 days PI were washed ten times in Hank's balanced salt solution (pH. 7.4) containing antibiotics (penicillin 10^5 IU/l, streptomycin 1 g/l), and followed by three times washing in Medium 199 (Gibco Laboratories, New York, USA) (pH. 7.4) containing glucose (4.5 g/l), KCl (0.2 g/l), penicillin (10^5 IU/l), streptomycin (1 g/l) and $NaHCO_3$, and subsequently cultured. Approximately 1,000 worms were cultivated in 50 ml medium (i.e., Medium 199) and incubated at 37°C in CO_2 incubator (Sanyo Forma Scientific Model 3161, Marietta, USA). The medium was replaced every 6 hours. ES antigens were prepared by concentration of the collected culture medium using polyvinylpyrrolidone

(PVP-360, Sigma, St. Louis, USA) followed by dialysis with distilled water. Protein concentrations were assessed by a Bio-Rad protein assay kit using bovine γ-globulin as standard.

Somatic antigens

E. multilocularis somatic antigens were prepared for detection of circulating antibody in sera. The procedures are similar to that described previously by Kohno et al. (1995).

Fecal samples

Fecal samples were collected daily from three dogs experimentally infected with *E. multilocularis* from the day of infection and until 21 days PI. All samples were placed at 4°C for one or two days prior to preparation. Coproantigens were basically prepared as described by Deplazes et al. (1990). Briefly, PBS containing 0.3% Tween 20 (= sample buffer) was added in a 1:4 ratio to the fecal samples. This suspension was ultrasonicated for 30 seconds (40 W) and subsequently centrifuged at 3,000 g for 15 min. The supernatant was stored at –20°C until used.

To examine the stability of coproantigen, fecal samples were stored at –20°C for one month and subsequently boiled for 15 min. Then fecal supernatants were prepared as previously described and used for coproantigen detection.

To examine cross-reactivity of the test, feces of animals infected with other parasite species were also used. Feces were obtained from three dogs correspondingly infected with *Taenia crassiceps, Toxocara canis* and *Trichuris vulpis*, as well as from a cat infected with *Taenia taeniaeformis*. All feces were used during patency. From each fecal sample, a fecal solution was prepared as described above.

Antibodies

Rabbit anti-*E. multilocularis* ES antibody was produced as described by Kohno *et al.* (1995), with *E. multilocularis* ES antigen for immunization.

Monoclonal antibody, EmA9, against *E. multilocularis* adult worm somatic antigens produced by Kohno *et al.* (1995) was used in the present study.

Sandwich ELISA for the detection of coproantigens

Each well of flat-bottomed microtitre plates (Greiner, Frickenhausen, W. Germany) as coated with 50 μl of 25 μg/ml rabbit anti-*E. multilocularis* ES antibody in 0.05 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.6) and left overnight at 4°C. The remaining procedures is similar to that described previously by Kohno *et al.* (1995). The test was read at OD 490nm using an ELISA reader (Corona Electric MTP-12, Katsuta, Japan).

ELISA for detection of circulating antibody

ELISA for detection of circulating antibody in sera of dogs was basically performed as described by Kohno *et al.* (1995), and is summarized briefly as follows: Flat-bottomed microtitre plates were coated at 50 μl/well with 5 μg/ml somatic antigens of *E. multilocularis* in coating buffer and left overnight at 4°C. For blocking, 5% BSA-PBS containing 0.1% Triton X-100 (30 min at room temperature) and 5% skim milk-PBS (1 hour at 37°C) were used. Then, a 1:200 dilution of sera were added to each well and a 1:2,000 dilution of peroxidase conjugated goat anti-dog IgG (Zymed Laboratories, San Francisco, USA) were added for detection of antibodies that reacted to the *E. multilocularis* antigen. Finally, substrate solution (o-phenylene diamine) were added and the plates were read at OD 490nm.

Results

Animal infections

The number of worms recovered from small intestines of three dogs (No. 1–3) at 21 days PI were 57,500 (dog No. 1), 137,000 (dog No. 2) and 0 (dog No. 3). Worms were not recovered from dog No. 3 because of treatment with praziquantel at 17 days PI.

Specificity evaluation

The results of specificity evaluation are shown in Fig. 1. All sandwich ELISA tests using feces of animals infected with other parasites, i.e., *Taenia taeniaeformis, T. crassiceps, Trichuris vulpis* and *Toxocara canis*, showed a negative reaction. The cut-off point for discriminating a negative from a positive reaction was determined by calculating the mean OD value ($\bar{X}$) of fecal samples from helminth-free seven dogs plus 4 SD.

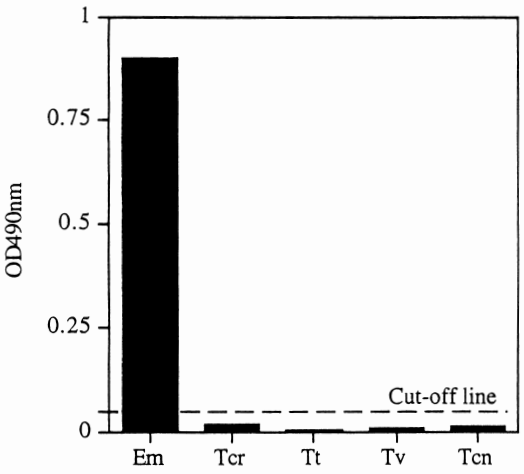

Fig. 1 Specificity evaluation of sandwich ELISA using fecal samples of animals infected with various parasites. Em, fecal sample of *Echinococcus multilocularis* infected dog; Tcr, *Taenia crassiceps*; Tv, *Trichuris vulpis*; Tcn, *Toxocara canis*; Tt, fecal sample of *Taenia taeniaeformis* infected cat. The cut-off line was determined as described in the text.

Sensitivity evaluation

E. multilocularis ES antigen was diluted twofold serially either in sample buffer or in fecal solution prepared from negative control, and assessed by sandwich ELISA (Fig. 2). The cut-off point was determined as described above. The test detected at least 0.5 ng antigen ml^{-1} in sample buffer and 1 ng antigen ml^{-1} in a diluted (1:4) fecal sample, equivalent to 4 ng antigen g^{-1} of feces.

Effect of fecal treatment on E. multilocularis *coproantigen*

Influence of freezing and heating of feces prior to processing of the fecal solution was examined using feces obtained from helminth-free dogs and experimentally infected dog (14 or 15 days PI). The sensitivity of sandwich ELISA was not influenced by both freezing and heating (Table 1).

Coproantigen detection in experimentally infected dogs

The results of the coproantigen detection by sandwich ELISA in dogs experimentally infected with *E. multilocularis* are shown in Fig. 3.

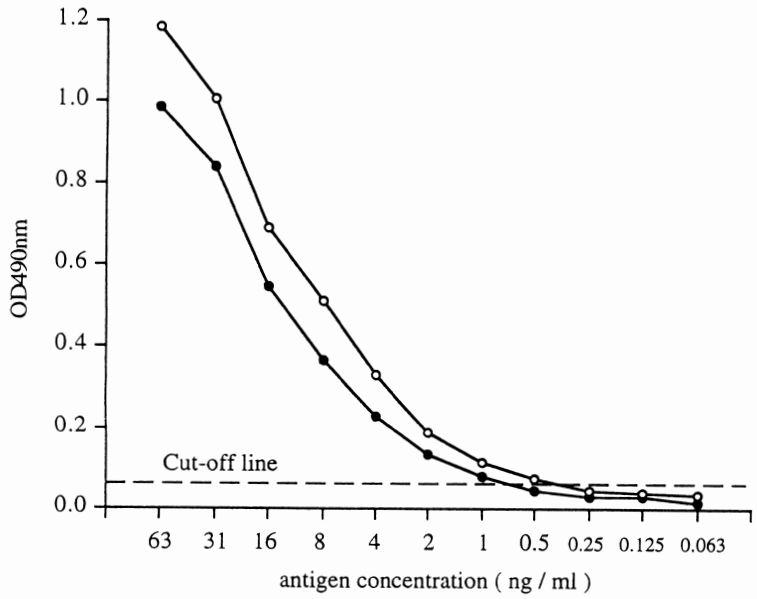

Fig. 2 Sensitivity evaluation of sandwich ELISA using serial twofold dilutions of *E. multilocularis* ES antigen. The antigen was diluted in sample buffer (○) or in fecal solution (●). The cut-off line was determined as described in the text.

Table 1 Influence of freezing and heating of feces on sandwich ELISA sensitivity. Feces obtained from helminth-free dog and *E. multilocularis* infected dog (14 or 15 days PI) were used in ELISA

Final treatment	ELISA OD value (490nm)	
	feces of non-infected dog	feces of infected dog
no treatment	0.049	0.892*
–20°C for 1 month	0.005	0.999†
–20°C for 1 month/Boiling for 10 min	0.04	1.239†

*feces at 15 days PI, †feces at 14 days PI.

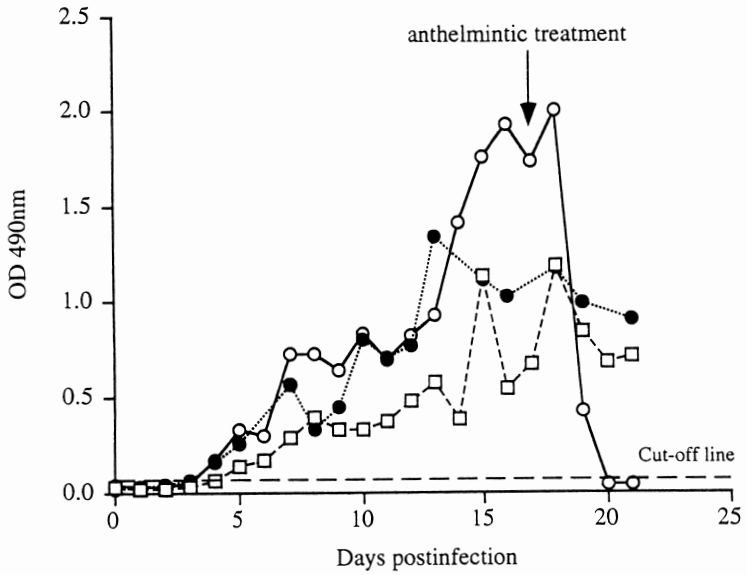

Fig. 3. Detection of coproantigen by sandwich ELISA in feces of three experimentally infected dogs. Dog No. 1 (□), Dog No. 2 (●), Dog No. 3 (○). Dog No. 3 was treated with praziquantel at 17 days PI. The cut-off line was determined as described in the text.

Coproantigens were detected in all dogs from 3 or 4 days PI until autopsy. OD value of dog No. 2 infected with 137,000 worms at autopsy tend to be higher than that of dog No. 1 which was infected with 57,500 worms at autopsy. OD value of dog No. 3 treated by praziquantel at 17 days PI increased one day after treatment and subsequently decreased two days after treatment and was negative after 3 days.

Detection of circulating antibody

Results of circulating antibody detection are shown in Fig. 4. Antibody levels of dog No. 1 and dog No. 2 increased steadily from day 8 PI until day 21 PI, but antibody levels of dog No. 3 which was treated with praziquantel were apparently high throughout the experimental infection.

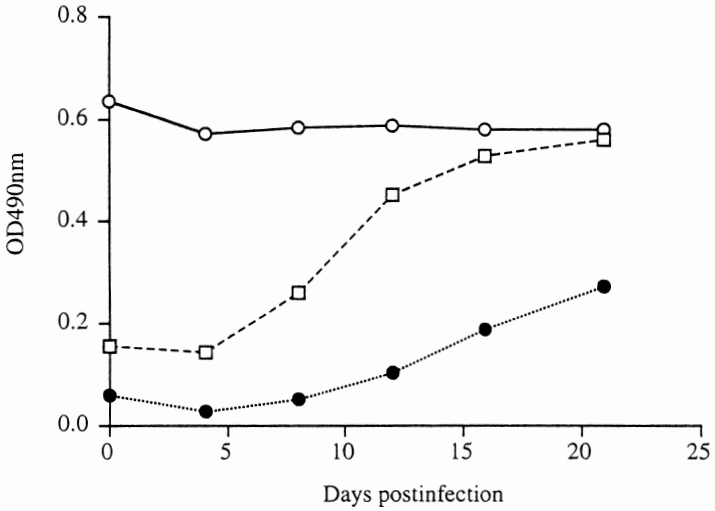

Fig. 4 Antibody response of three *E. multilocularis* infected dogs against *E. multilocularis* somatic antigens. Dog No. 1 (□), Dog No. 2 (●), Dog No. 3 (○). Dog No. 3 was treated with praziquantel at 17 days PI.

Discussion

Coproantigen detection by sandwich ELISA has been generally performed using polyclonal antibodies. Kohno *et al.* (1995) described the detection of *E. multilocularis* coproantigen using the monoclonal antibody, EmA9, but further examination was required to evaluate the specificity and sensitivity of the test. In the present study, we examined the reliability of the test for use in routine diagnosis. To evaluate the specificity of the present ELISA using EmA9, we used four fecal samples obtained from dogs infected with helminths other than *Echinococcus*. No cross-reaction was observed in all these samples. Thus, EmA9 is thought to be specific not only to *E. multilocularis* somatic antigen as described by Kohno *et al.* (1995), but also specific to the coproantigen. However, the specificity of the test need further evaluation by using more fecal samples obtained from dogs infected with more helminth species, especially cestodes, except *Echinococcus*. Cross-reactivity to the antigen to species of the same genus, i.e., *E. granulosus*, has not been examined. Since coproantigen detection by sandwich ELISA has been used for both cestodes (Deplazes *et al.*, 1992), immunodiagnosis of *E. granulosus* infected dogs using our methods may be possible.

In terms of sensitivity, the test detects at least 0.5 ng antigen ml^{-1} in sample buffer or 1 ng antigen ml^{-1} in a diluted (1:4) fecal sample. The high sensitivity of the test may be based on a low cut-off value. Since EmA9 has high specificity, OD values of negative controls were very low resulting in a low cut-off value.

The comparatively low sensitivity using fecal supernatants may be due to host proteases in feces which could detach the capture antibody from solid phase, i.e., ELISA plate (Viscidi *et al.*, 1984; Hanvanich *et al.*, 1985). To prevent the lowering of sensitivity, fecal dilution with high protein solutions (ex. 50% FCS) or low pH solution and heating of feces have been recommended (Viscidi *et al.*, 1984; Hanvanich *et al.*, 1985). These treatments may be needed in the present ELISA to maintain high sensitivity.

Kohno *et al.* (1995) suggested that EmA9 recognize the carbohydrate epitope of the antigen that is generally heat-stable. We examined the influence of freezing and heating of feces prior to preparation of the fecal solution. Coproantigens detected by the present ELISA were quite stable and sensitivity of

the test was not influenced by fecal treatment. Thus, the present method prevent accidental infection by heating fecal samples to kill eggs before the test is performed. In addition, proteases in heated feces are inhibited in their activity which may result in high sensitivity of the test.

In all experimentally infected dogs, the coproantigens were detected as early as 3 or 4 days PI. The present test using EmA9 also showed high sensitivity as described by Kohno *et al.* (1995). In addition, elimination of the worm using anthelmintic was followed by a rapid disappearance of coproantigens. Thus, an accurate diagnosis which reflect the current intestinal *Echinococcus* infection could be made using this method. An increase in ELISA OD value one day after anthelmintic treatment may be due to the break-up of worms by praziquantel (Bossche, 1985). OD values of dog No. 2 tend to be higher than that of Dog No. 1. This may reflect differences in worm burden between infected dogs. Further examination to clarify the minimum number of infecting worms to detect coproantigens will be necessary.

Detection of circulating antibody in experimentally infected dogs did not reflect current infection. Especially, antibody levels of dog No. 3 were apparently high throughout the experimental infection in spite of elimination of worms anthelmintic treatment. Persistence of circulating antibodies may be due to previous infection of *E. multilocularis* or other parasites (i.e., in the latter case, the antibody is cross-reacting). In addition, antibody response of dog No. 1 and No. 2 is not observed early in the infection. Thus, antibody detection for diagnosis of infected dogs is not always accurate and unsuitable during the early stage of infection.

The present sandwich ELISA for coproantigen detection using monoclonal antibody, EmA9, in dogs infected with *E. multilocularis* may improve routine diagnosis due to the high sensitivity and specificity. In addition, the coproantigens are quite stable to freezing and heating. Therefore, further simplification of the test will lead to field use. For this purpose, further examination is required to clarify the sensitivity and specificity of the test under field conditions.

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