Specificity of Commercially Available *Entamoeba histolytica* Antigen in Enzyme-Linked Immunosorbent Assay (ELISA) to Sera from Dogs with Various Parasitic Infections

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Amebiasis caused by *Entamoeba histolytica* is suspected as one of the important zoonoses, since it causes severe amebic dysentery or amebic liver abscess in humans and non-human primates [5]. Although the prevalence of *E. histolytica* in the dog has been reported to range from 1% to about 8% in various parts of the world [1–3, 6, 10, 12, 18], canine cases reported in Japan were mostly sporadic [16] . However, prevalence of canine entamebiasis histolytica is not precisely known because of the following reasons: The parasite is usually non-pathogenic for canidae [5, 16], therefore, special attention has not been paid to *E. histolytica* infection in dogs, and the stool examination, which is of low sensitivity to detection, is the only method available for the diagnosis of *E. histolytica* infection in dogs. This situation underscores the need of a reliable immunodiagnostic method in dogs. There has been no report so far on the immunodiagnosis of *E. histolytica* infection in dog. Recently, enzyme-linked immunosorbent assay (ELISA) has been proved effective in the serodiagnosis of entamebiasis histolytica in humans, since the development of axenic culture of *E. histolytica* provided the highly specific antigen [4, 13]. Since the cross-reactivity in immunodiagnosis has been frequently observed in parasitic infections, species-specificity is important for a reliable immunodiagnostic method [11, 17]. In the present study, species-specificity of *E. histolytica* antigen in immunodiagnosis with sera of dogs, which were naturally or experimentally infected with various species of parasites, was investigated by ELISA.

A micro-ELISA technique described previously [9] was utilized with modifications. Assay was performed in wells on a micro-ELISA plate (M129A, Dynatech, West Germany). Commercial amebic antigen for complement fixation (CF) test (Virion, Switzerland) reconstituted with distilled water was used for ELISA [8]. The optimum condition for ELISA in the present study was determined by the checker-board titration. The wells of plate were sensitized in a moist chamber with 50 μl of antigen at a concentration of 5 μg/m*l* of 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 37°C for 1 hr. The wells were rinsed with 0.01 M phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/T) three times for 5 min each. Fifty μl test sera diluted at 1:100 with PBS/T and placed in a sensitized well as primary antibody were incubated at 37°C for 30 min. The wells were rinsed again. Then the conjugate, horseradish peroxidase conjugated anti-dog immunoglobulin (Ig) G (H + L) rabbit serum (Cappel, U.S.A.), diluted at 1:3,000 with PBS/T was placed in the well as the secondary antibody and incubated at 37°C for 30 min. After washing, 100 μl of substrate was placed in the well at 37°C for 30 min to allow enzymes to react. As the substrate, 30 mg of ABTS (Sigma, U.S.A.), 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), was dissolved in a mixture of 50 m*l* of 0.1 M citric acid, 50 m*l* of 0.1 M sodium phosphate dibasic and 10 μl of 30% H_2O_2. Reaction was stopped by adding 25 μl of 1.25% sodium fluoride solution, and absorbance was read by at a wave length of 415 nm.

Immunized serum was used as a positive standard in ELISA [13, 14]. To obtain immunized serum, a dog was immunized by subcutaneous injection of the amebic antigen for CF test (500 μg protein / 0.5 m*l*) emulsified in an equal volume of complete Freund's adjuvant. The same emulsion was given to the dog as a booster shot 2 weeks later. The positive standard serum used was collected 4 and 6 weeks after the first immunization and pooled. The antibody specificity of positive standard serum to amebic antigen for CF test and PBS extract antigen, whose immunodiagnostic reliability has been evaluated in human entamebiasis, was confirmed by Ouchterlony's immunodiffusion mehtod in gel using serum obtained from a human patient. Negative reference sera were obtained from dogs free of parasites. All the sera were stored at −40°C until use. In the present method of ELISA, the absorbance, represented by optical density (O.D.), of positive standard serum at a dilution of 1:1,000 was around 1.4 and that of negative pooled sera diluted at 1:100 was less than 0.04. Since the O.D. values fluctuated under the test conditions, reaction with positive standard serum was repeated on each plate to correct the readings [7].

Sample sera were obtained from dogs naturally or experimentally infected with 12 species of protozoa and/or helminths. Parasitic infections were confirmed by detection of parasite directly in the body, feces and/or blood of dogs except for *Toxoplasma gondii. Toxoplasma gondii* infection was diagnosed by antibody titer against *T. gondii* antigen measured by the latex agglutination test. Details of infected dogs are shown in Table 1.

Sera from 81 dogs with various parasitic infections were diluted at 1:100 with PBS/T and duplicate samples were used for ELISA against *E. histolytica* antigen. The O.D. values of each serum sample tested are illustrated in Fig. 1.

Table 1. The dogs infected with various species of parasites used for ELISA against *Entamoeba histolytica* antigen

Species of parasite	No. of dogs tested	Range of estimated age (Yrs)	Range of body weight (kg)	Degree of infection
Protozoa:				
Isospora spp.	10	0.5–4.5	5.0–10.5	300–312,900[a]
Giardia spp.	2	4.0–4.5	5.0–11.0	++
Trichomonas sp.	1	3.0	6.0	1,300[b]
Toxoplasma gondii	10	0.5–6.0	5.7–20.0	1:32–1:512[c]
Nematoda:				
Trichuris vulpis	11	1.5–4.0	7.3–10.5	1–332 worms
Ancylostoma caninum	10	1.0–4.0	7.6–15.7	8–341 worms
Toxocara canis	10	0.5–3.0	3.6–15.1	2–17 worms
Dirofilaria immitis	10	0.5–7.0	4.0–10.0	7–76 adults 0–31,400[d]
Trematoda:				
Metagonimus yokogawai	1	3.0	6.7	8 flukes
Paragonimus westermani	3	1.0–3.0	7.6–8.8	18–28 flukes
Cestoda:				
Spirometra erinacei	3	0.5–4.5	4.0–7.9	2–3 worms
Dipylidium caninum	10	1.5–5.5	5.5–20.0	2–39 worms

a) OPG.
b) Number of parasites in 1 g of feces.
c) Antibody titer measured by the latex agglutination test.
d) Density of microfilariae in 1 m*l* of blood.

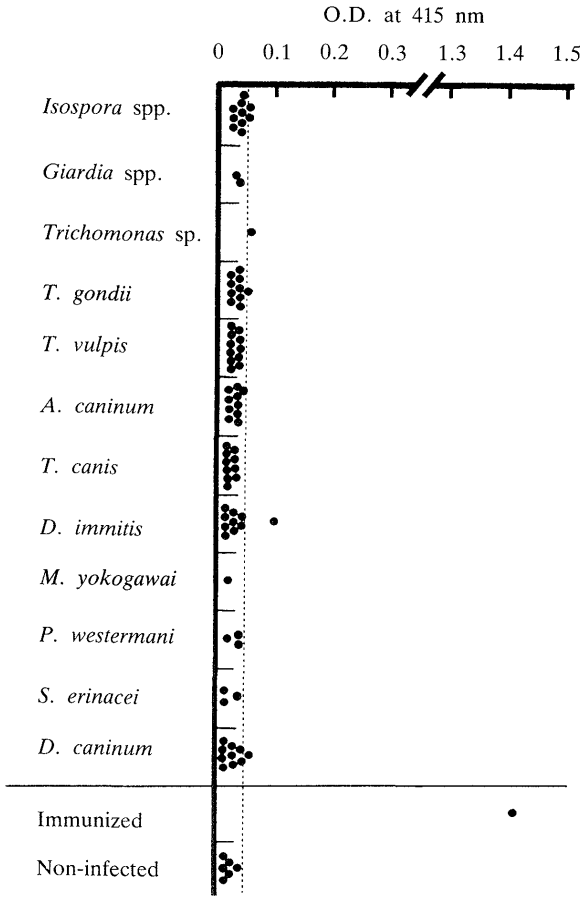

Fig. 1. Distribution of absorbance (optical density at 415 nm) in ELISA with *Entamoeba histolytica* antigen and sera obtained from dogs which were naturally or experimentally infected (1:100 serum dilution) with varied parasites and immunized (1:1,000 serum dilution) with *E. histolytica* antigen.

Satisfactory and reasonable specificity of *E. histolytica* antigen was demonstrated against sera from varied protozoan and helminthic infections examined. The O.D. value of 75 (93%) in 81 serum samples from infected dogs fell into the range of those of non-infected sera. Although one of 10 *Dirofilaria immitis* infected dogs showed the highest O. D. value as shown in Fig. 1, no cross-reactivity was observed in 70 dogs infected with *D. immitis* in a later study (data not shown). The results indicated that ELISA investigated in the present study is less cross-reactive to other parasite infections, and would be expected as a specific immunodiagnostic technique of *E. histolytica* infection in dogs.

Several immunodiagnostic techniques have been proposed so far to entamebiasis histolytica in humans [11, 19], since stool examination is not reliable and troublesome in technique. Recently, ELISA has been noted of value for seroepidemiological investigation as well as for the diagnosis of the amebic infection [13].

However, the preparation of sufficient antigens of *E. histolytica* for ELISA is not so easy, and their supply from the maintenance laboratories is not always feasible. For this reason, the commercial amebic antigen for CF test was used in the present study to obtain successful results. Commercial amebic antigen for CF test was proved to be species-specific by its less cross-reactivity with other

parasitic infections. This finding suggests that the commercial CF antigen would be suitable for ELISA. Nagano *et al.* tried to use CF antigen for ELISA against human sera with amebic infection to obtain excellent results [8].

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