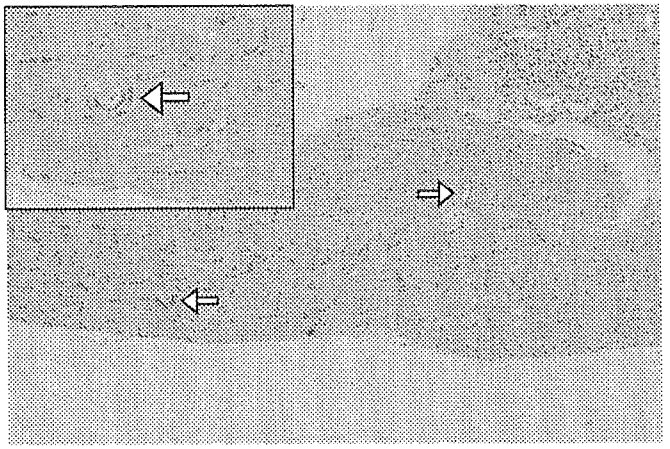

Fig. 6.4. Histopathological observation of the optic chiasma of the Mongolian gerbil following intracranial inoculation with *Toxocara canis* larvae: two migrating larvae (arrows) in the optic chiasma and one at higher magnification (arrow).

central nervous system to the eye via the optic chiasma (see Chapter 9, this volume).

Neurologic toxocariasis in Mongolian gerbils

Mongolian gerbils infected with *Toxocara*, both *T. canis* and *T. cati*, show gait difficulty and progressive ataxia (Akao *et al.*, 2003). The onset of disease occurred 50 days post-infection. Neurological abnormalities developed in six of 13 gerbils (46%) infected with *T. canis*, and in five of seven gerbils infected with *T. cati* (71%). Clinical signs included swinging gait while attempting to stand on their hind legs, circulating movement in the same direction, difficulty in normal positioning of the head, paraplegia of the hind limbs and urinary incontinence. Despite severe illness, they show a good appetite until they lapsed into a coma.

Histopathologically, the cerebellum was the most affected area of the brain in these gerbils. Loss of Purkinje cells, glial nerve fibres and nerve sheaths were characteristic and common findings. There were no apparent pathologic changes in the brain except in the cerebellum. Clearly, these morphological changes could be responsible for the neurologic disorders observed. Migrating larvae were seen in the affected cerebellum, but larvae and lesions also existed independently, suggesting that some of the degenerative changes might be the result of indirect effects of the larvae. Future investigations should include an analysis of the interaction of nerve cells with the excretory-secretory products of *T. canis in vitro*.

Conclusions

Toxocara spp. are ubiquitous parasites in both developed and developing countries, and are responsible for one of the most challenging zoonotic parasitic infections worldwide. Further, many of the issues concerning the pathogenesis of *Toxocara* infections, such as the reactivation mechanism of arrested larvae in skeletal muscle and the therapeutic advantage of steroid use in ocular toxocariasis, are poorly understood or controversial. It is hoped that the present gerbil model will contribute to the development of improved diagnostic and therapeutic approaches for toxocariasis, since this model allows us to test these approaches experimentally. Our ongoing research will continue to focus on human toxocariasis and will add to our understanding of the basic process of host-parasite relationships in nematode parasites.

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Development of membrane-based tests for the detection of urinary antigens and antibodies in human toxoplasmosis: preliminary studies in Ghanaian patients

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Abstract

Two membrane-based ELISA systems were used in detecting *Toxoplasma* antigens and anti-*Toxoplasma* antibodies in urine samples collected from 54 ophthalmology (22 suggestive active and 32 suggestive past infection) patients and 26 pregnant women attending obstetrics/gynaecology clinic (OGP), suspected of toxoplasmosis by eye examination, past medical records and questionnaire, respectively, in Ghana from mid-February to April 2002. The antigen detecting ELISA was able to demonstrate antigen in 100% (22/22) ophthalmology (active infection) and 62.5% (20/32) ophthalmology (past infection) patients, and 42% (11/26) of OGP which included 3 that were sero-negative prior to and during this study, giving an overall prevalence of 66.3% (53/80). The urinary antigen positive samples also included 6 that were negative for both the Dye Test (DT) and latex agglutination test (LAT). Antigen was not detected in the urine of 22 normal (sero-negative for antibodies to *Toxoplasma*) individuals. The membrane-based urinary antibody detecting sandwich ELISA also detected anti-*Toxoplasma* antibodies in 100% (22/22) of ophthalmology (active infection) and 81.3% (26/32) of ophthalmology (past infection) patients, a total of 89% (48/54); and 80.8% (21/26) of OGP with an overall prevalence of 86.3% (69/80), including 7 ophthalmology patients' samples that were sero-negative for both DT and LAT. Antibody sero-positivity of the samples was determined by DT as 87% (47/54) in ophthalmology patients and 73.1% (19/26) in pregnant women, LAT as 85.2% (46/54) and 65.4% (17/26), and an overall prevalence as 82.5%

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(66/80) and 78.8% (63/80), respectively. The membrane-based ELISA systems appear promising but need to be investigated further for its efficacy as reliable diagnostic tests.

Keywords: *Toxoplasma* infection; Antigen; Human urine; Membrane-based ELISA; Ghana

1. Introduction

High prevalence of anti-*Toxoplasma* sero-positivity in a cross-section of the Ghanaian populace as well as evidence of congenital disease caused by *Toxoplasma gondii* infections have been reported in Ghana (Anteson et al., 1978a,b, 1980). Among the existing diagnostic tests for toxoplasmosis, the most readily available one to clinicians in Ghana is the commercial latex agglutination test (LAT), which detects mainly IgG antibodies indicative of past or old infections. According to Luft and Remington (1988), the frequent occurrence of *Toxoplasma* infection in immunocompromised patients and the difficulty in establishing the diagnosis without brain biopsy has necessitated the development of new, non-invasive methods for diagnosis of the disease. Furthermore, *Toxoplasma* antigens have been demonstrated in serum samples from acutely infected animals and humans (Raizman and Neva, 1975; Van Knapen and Panggabean, 1977; Araujo et al., 1980; Araujo and Remington, 1980; Trunen, 1983; Lindenschmidt, 1985; Asai et al., 1987) and also in urine from mice acutely infected with *T. gondii* as well as from human patients with acute toxoplasmic encephalitis (Huskinson et al., 1989). However, the methods used were rather elaborate and expensive which may not be suitable for routine examination. The risk of congenital infection, for example, in this era of rapid spread of HIV/AIDS, necessitate periodical routine examination of all females of childbearing potential, which may not be cost-effective in the Ghanaian environment. Earlier, Bosompem et al. (1996, 1997) had reported the employment of a membrane-based ELISA to detect antigens in the urine of urinary schistosomiasis patients and its convenience and cost-effectiveness in mass screening of suspected patients in the field. In this study we explored the usefulness of two membrane-based ELISA systems in diagnosing recent infection in suspected toxoplasmosis patients.

2. Patients and methods

2.1. Patients

A total of 80 Ghanaians who consented were involved in this study. Fifty-four of them were patients attending four ophthalmology clinics across the country and they included 30 females and 24 males aged 10–70 years. The patients mostly reported with aching eyes and blurred vision and presented with symptoms, such as traumatic and posterior uveitis and signs suggestive of active *Toxoplasma* retinochoroiditis and well demarcated scars upon examination (Holliman, 1990), which was defined as the presence of intraocular inflammation in association with a retinochoroidal scar and an active adjoining retinitis (Guerina et al., 1994). Twenty-two out of the 54 patients (41%) had eye lesions suggestive of active infections and others had scars suggestive of old infection.

The other 26 patients were pregnant women in their second or third trimester attending obstetrics/gynaecology clinic (OGP) and aged 22–44 years. All of them had experienced spontaneous abortions and/or stillbirth (by questionnaire), 18 of them had been sero-positive and 8 sero-negative, by a commercial LAT (according to their past medical records) 6 months to 4 years before this study.

As control 22 people, 14 males and 8 females aged 15–60 years who were considered healthy by questionnaire and screening were included in the study. All of them had tested sero-negative (by LAT) for toxoplasmosis 3 months to 2 years, before this study.

2.2. Collection and analysis of blood, urine and stool

Approximately, 3–5 ml of blood was collected from each of the individuals involved in the study. Thick and thin blood smears were prepared on microscope glass slides, processed and examined microscopically

for haemoparasites. Sera were also obtained from the blood and tested for anti-*Toxoxplasma* sero-positivity by the Dye Test (DT) and LAT.

A total of about 25–80 ml of urine was collected from the same individuals who had given blood and were also provided with containers to produce stool samples. Each urine sample was centrifuged at 400 × *g* and the deposits were examined microscopically for *Schistosoma haematobium* ova and the supernatant tested for *Toxoplasma* antigens and anti-*Toxoplasma* antibodies using a simple designed membrane-based ELISA. Stool specimens were examined for *Schistosoma mansoni* ova using the Kato method (Katz et al., 1972) and the formalin–ether sedimentation technique for the detection of other intestinal parasites' ova, cysts and oocysts of other related coccidian species like *Cryptosporidium parvum.*

2.3. Serological tests

DT (Sabin and Feldman, 1948; Beverly and Beattie, 1952; Handman and Remington, 1980) was performed with modification. Briefly, peritoneal exudates were obtained from mice inoculated with the RH strain of *T. gondii* 3 days previously. *Toxoplasma* suspensions rich mainly in extra cellular parasites were used in the test within 20 min after harvesting. Briefly, 20 μl of test sera diluted four-fold in normal saline in appropriately labelled eppendorf tubes were added with 40 μl of activator serum (normal human serum) and then 20 μl of suspension of tachyzoites in PBS (pH 7.4) in each tube and incubated in a water bath at 37 °C for 1 h after brief shaking. Then, 60 μl of 1% methylene blue dye in borate buffer (pH 11.0) were added to the tubes and allowed to stand at room temperature for 10 min. The tubes were kept at 4 °C until results were read.

The commercially available LAT (Toxotest®-MT Eiken, Tokyo, Japan) was performed according to the manufacturer's instructions.

2.4. Infection of mice

Infection of mice was carried out as by Handman and Remington (1980) with modification. Briefly, tachyzoites of the RH strain of *T. gondii* obtained from mouse peritoneal exudates (described below) were fixed in 0.1% formol–saline at 4 °C for 30 min and washed with excess PBS (pH 7.2). Eight to twelve weeks old ddY mice were bled a day before infection and sub-cutaneously injected with 2×10^3 fixed tachyzoites, and bled at days 5, 10, 15 and 20 after infection when antibody titer in DT was between 1:16 and 1:10,000. Pooled sera were stored in aliquots at −20 °C until used.

2.5. Preparation of Toxoplasma crude antigen

Toxoplasma antigen was prepared according to the method of Araujo and Remington (1980) with modification. Crude tachyzoite antigen was prepared using *T. gondii* tachyzoites from the peritoneal exudates of mice infected 3 days earlier with the RH strain. Pooled exudates were centrifuged at 1000 × *g* for 10 min in a refrigerated centrifuge at 4 °C. The sediment containing cells and parasites was re-suspended in cold PBS (pH 7.2), washed three times with PBS and filtered (Remington et al., 1970). Parasites were pelleted and frozen at −30 °C overnight. The frozen pellet containing approximately 2×10^7 tachyzoites was re-suspended with distilled water and the suspension subjected to repeated freezing (−80 °C for 30 min) and thawing before sonicating on ice using a Branson Sonifier 250 (Branson Sonic Power Company, CT, USA) set at 60 cycles for 45 s. The resulting suspension was centrifuged at 1000 × *g* for 20 min and then at 3600 × *g* for 30 min. After demonstrating the presence of *Toxoplasma* antigens by Ouchterlony (1976), protein concentration was estimated at 0.8 mg/ml using Bio-Rad Protein Assay Reagent according to the manufacturer's protocol (Bio-Rad Laboratories, CA, USA). Aliquots of the preparation were stored at −30 °C until used.

2.6. Urinary antigen detecting ELISA procedure

The urinary antigen detecting ELISA (UAgE) was performed according to Bosompem et al. (1997) with modification as follows. Methanol wetted polyvinylidene difluoride (PVDF) membrane (Atto Company Ltd., Tokyo, Japan) strips were incubated in test urine for 36 h at 4 °C, rinsed with Tris-buffered saline (TBS) (50 mM Tris and 150 mM NaCl, pH 7.2) and then blocked for 45 min in blocking solution consisting of 5% skimmed milk/TBS. The strips were transferred into *T. gondii* infected mouse serum (1:100) for 1 h, washed three times each by 5-min incubation in TBS and then incubated in goat anti-mouse polyclonal

IgG + A + M conjugated to horse-raddish peroxidase (HRPO, 1:5000, Catalog no. 1999, Sigma–Aldrich, Japan) for 1 h. The strips were washed three times each by 10-min incubation in TBS and then incubated in substrate solution [98.65% (w/v) (3,3-diaminobenzidine), 0.25% (v/v) H_2O_2 and 15.4% (w/v) $NiCl_2$ in TBS] for 1 min. A bluish black reaction represented positive results while negative results remained colourless. The assay was run at room temperature and reagents were diluted in 0.1% blocking solution, unless otherwise stated.

2.7. Detection of type of immunoglobulins bound by Toxoplasma antigens

The UAgE described above was repeated with multiple membrane strips incubated in each urine sample. At the conjugate incubation level, strips from each sample were distributed into appropriately diluted goat anti-mouse monoclonal IgG-, IgA- and IgM-HRPO (Catalog no. 1999, Sigma–Aldrich, Japan), respectively.

2.8. Urinary antibody detecting sandwich ELISA procedure

The urinary antibody detecting ELISA (UAbE) was performed as described above with the following modifications. Briefly, methanol wetted PVDF membrane strips were incubated in test urine for 36 h at 4 °C. Strips were removed and rinsed with TBS and blocked. They were then rinsed in TBS and transferred into *T. gondii* tachyzoite lysate diluted appropriately in PBS (5 μg/ml) for 1 h at room temperature. They were washed three times each by 5-min incubation in TBS and then incubated in *T. gondii* infected mouse serum and the procedure from here was the same as for the antigen detecting system.

2.9. Determination of optimal working dilutions of murine serum and goat anti-mouse horse-raddish peroxidase conjugate used in the membrane-based ELISA

Pooled murine *T. gondii* infected serum was tested in membrane-based ELISA as follows. Briefly, *Toxoplasma* tachyzoite lysate-captured PVDF membrane strips were rinsed in TBS, pH 7.2 for 2 min and different strips incubated with test serum and then goat anti-mouse polyclonal (IgG + IgA + IgM) horse-raddish peroxidase conjugate in a checkerboard, from 1:50 to 1:1000 and 1:1200 to 1:20,000 at two-fold dilutions in blocking solution, respectively. The respective dilutions that gave the most intense colour reactions by visual assessment were used in the membrane-based ELISA. The procedure was repeated using goat anti-mouse monoclonal (IgG); (IgA); and (IgM) horse-raddish peroxidase conjugate separately to ascertain the inclusion of all the necessary immunoglobulins and in sufficient amounts using visual assessment of the intensity of colour reactivity.

2.10. Sensitivity and specificity of the tests

The percentage sensitivity and specificity of LAT and membrane-based tests were calculated, using DT as the gold standard, as follows (see Bosompem et al., 1996):

$$\text{sensitivity (\%)} = \frac{\text{number of individuals positive by the test that were also positive by DT}}{\text{total number of individuals testing positive by DT}} \times 100$$

$$\text{specificity (\%)} = \frac{\text{number of individuals negative by the test that were also negative by DT}}{\text{total number of individuals testing negative by DT}} \times 100$$

3. Results

3.1. Results of stool, blood smears and urine deposit examination

Two out of the 26 (7.8%) pregnant women and 36 out of the 54 (66.7%) ophthalmology (22 active and 14 past infection) patients, submitted stool samples, the overall being 38/80 (47.5%). None of the 38 stool samples were found to contain any *S. mansoni* eggs, however, ova of

hookworms were identified in 3 (7.9%) ophthalmology (2 active and 1 past infection) patients' samples. *Plasmodium falciparum* was the only haemoparasite detected in blood smears from 5/80 (6.3%) individuals including four ophthalmology (3 active and 1 past infection) patients and one pregnant woman. *S. haematobium* ova were demonstrated in urine sample deposits of 6/80 (7.5%) ophthalmology (3 active and 3 past infection) patients who included 2 that were also *P. falciparum* positive. No cysts or oocysts were detected in any of the 62 faecal samples collected.

None of the parasites mentioned above were found in any of the 22 stool specimens from the normal controls except 2 of them that had ascariasis.

3.2. Detection of anti-Toxoplasma antibody by DT, LAT and UAbE

Results of the membrane-based tests are presented in Fig. 1. The use of $NiCl_2$ in the membrane-based ELISA resulted in a bluish black positive reaction, which was easier to differentiate from a negative result, than a brown positive result without its addition. Thus, membrane strips that appeared coloured after incubation in test urine samples with haematuria demonstrated clearly as positives (strips d and e) or negatives (strips j and k). Control strips (strips l and m) remained colourless. In both systems, positive results showed varying intensity of colouration of membrane strips, which were graded from strong to weak positive.

Prevalence of anti-*Toxoplasma* antibodies in sera and urine as determined by the three different tests, is presented in Table 1. The overall prevalence values were not statistically different ($P > 0.05$). Three samples, which were DT-positive could not be determined clearly as positive or negative by LAT, 2 of them were from obstetrics and 1 from ophthalmology (past infection) patients, respectively.

Table 1
Prevalence of anti-*Toxoplasma* antibody as determined by the three different tests

Test	Source of sample	Positive	Negative	Ind[a]	Prevalence (%)
DT[b]	OP[c]	47	7	0	87.0
	OGP[d]	19	7	0	73.1
LAT[e]	OP	46	7	1	85.2
	OGP	17	7	2	65.4
UAbE[f]	OP	48	6	0	89.0
	OGP	21	5	0	80.8

[a] Indeterminate: positivity or negativity could not be determined.
[b] Sabin–Feldman Dye Test.
[c] Samples from ophthalmology patients ($n = 54$).
[d] Samples from pregnant women attending obstetrics/gynaecology clinic ($n = 26$).
[e] Latex agglutination test.
[f] Urinary antibody detecting ELISA.

Urinary antibody detection by UAbE as compared with DT and LAT from obstetrics/gynaecology (OGP) as well as ophthalmology (active and past infection) patients' samples is presented in Table 2. As shown in Table 2, 17/22 (77.3%) suggestive active cases and 30/32 (93.8%) of the past-toxoplasmosis cases were confirmed. Also, 19/26 (73.1%) of the pregnant women showed positive serology. Seven out of eight OGP that were previously sero-negative remained so during this study.

Using DT as gold standard, the global sensitivity and specificity of LAT and UAbE on the total num-

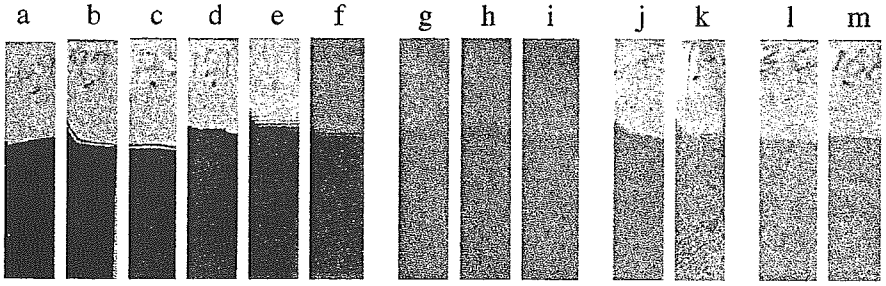

Fig. 1. Results of membrane-based ELISA showing colourization of PVDF membrane strips. Strips (a–f) indicate strong positive results; strips (g–i) indicate very weak positive results; strips (j and k) were incubated with urine from negative samples; strips (l and m) were not incubated with urine.

Table 2
Results of UAbE as compared with DT and LAT

Test	Result	UAbE[a]						Total ($n=80$)
		OP-active infection[b] ($n=22$)		OP-past infection[c] ($n=32$)		OGP[d] ($n=26$)		
		Pos[e]	Neg[f]	Pos	Neg	Pos	Neg	
DT[g]	Pos	17	0	24	6	16	3	66
	Neg	5	0	2	0	5	2	14
LAT[h]	Pos	17	0	23	6	15	2	63
	Neg	5	0	2	0	5	2	14
	Ind[i]	0	0	1	0	1	1	3

[a] Urinary antibody detecting ELISA.
[b] Samples from ophthalmology patients with active toxoplasmosis.
[c] Samples from ophthalmology patients with past toxoplasmosis.
[d] Samples from pregnant women attending obstetrics/gynaecology clinic.
[e] Number positive by the tests from that group of patients.
[f] Number negative by the tests from that group of patients.
[g] Sabin–Feldman Dye Test.
[h] Latex agglutination test.
[i] Indeterminate: positivity or negativity could not be determined.

ber of samples examined in this study (including the 80 toxoplasmosis suspected group of patients and the 22 normal individuals) were determined. The sensitivity and specificity for LAT were 96.0% (63/66) and 92.0% (33/36), and for UAbE, 86.4% (57/66) and 67.0% (24/36), respectively.

All the 22 control samples were negative for DT, LAT and UAbE.

3.3. Detection of *Toxoplasma* urinary antigens by UAgE

The UAgE detected antigens in 78.0% (42/54) and 42.3% (11/26) of the ophthalmology and obstetrics/gynaecology urine samples, respectively, and an overall prevalence of 66.3% (53/80) as shown in Table 3. The 42 UAgE-positive samples included 100% (22/22) of patients that presented with eye disorders suggestive of active *Toxoplasma* infection and 62.5% (20/32) of the suggestive past infection cases.

Results of UAgE as compared with DT are shown in Table 4. All of the 11 UAgE-positive obstetrics/gynaecology patients were also DT-positive and antigens from 7 of them recognized murine-IgGs only, mostly in trace amounts whilst 1 bound both murine-IgA and IgG. In addition, six (five ophthalmology active infection and one OGP) samples, which were negative by both DT and LAT were positive by UAgE and bound by both murine-IgA and IgG (four samples), and IgG only (two samples). All six were positive for urinary antibodies. Samples from five out of seven ophthalmology (active infection) patients all of which were negative by both DT and LAT were positive by UAgE, and the immunoglobulins bound by the antigens were murine-IgA and/or IgG. Two of them were positive for both murine-IgA and IgG and, three for IgG only. On the other hand, all seven samples tested positive for urinary antibodies.

Table 3
Detecting *Toxoplasma gondii* urinary antigens in samples from ophthalmology and obstetrics patients using the membrane-based ELISA (UAgE)

Source of sample	UAgE[a]		Prevalence (%)
	Positive	Negative	
OP-active infection[b] ($n=22$)	22	0	100.0
OP-past infection[c] ($n=32$)	20	12	62.5
OGP[d] ($n=26$)	11	15	42.3
Total ($n=80$)	53	27	66.3

[a] Urinary antigen detecting ELISA.
[b] Samples from ophthalmology patients with active toxoplasmosis.
[c] Samples from ophthalmology patients with past toxoplasmosis.
[d] Samples from pregnant women attending obstetrics/gynaecology clinic.

Table 4
Results of UAgE as compared with DT

Test	Result	UAgE[a]						Total ($n=80$)
		OP-active infection[b] ($n=22$)		OP-past infection[c] ($n=32$)		OGP[d] ($n=26$)		
		Pos[e]	Neg[f]	Pos	Neg	Pos	Neg	
DT[g]	Pos	17	0	17	10	11	11	66
	Neg	5	0	3	2	0	4	14
Total		22	0	20	12	11	15	80

[a] Urinary antigen detecting ELISA.
[b] Samples from ophthalmology patients with active toxoplasmosis.
[c] Samples from ophthalmology patients with past toxoplasmosis.
[d] Samples from pregnant women attending obstetrics/gynaecology clinic.
[e] Number positive by the tests from that group of patients.
[f] Number negative by the tests from that group of patients.
[g] Sabin–Feldman Dye Test.

Serum and urine samples from one patient tested positive for all the tests and the antigens recognized murine-IgM in trace amounts in addition to IgA (++) and IgG (+) (data not shown). The individual involved was a 30-year-old male ophthalmology (active infection) patient who presented with traumatic uveitis. All the 22 urine samples from control individuals were negative by UAgE.

3.4. Probable influence of detected parasites on UAbE and UAgE

All the three ophthalmology (active infection) patients with *S. haematobium* infection, one of whom was also habouring *P. falciparum*, were positive for UAbE and UAgE but one was negative for DT. Two of the other three *S. haematobium* infected ophthalmology (past infection) patients were negative for both UAbE and UAgE and the remaining one who had *P. falciparum* infection in addition was UAbE positive only, although all three were sero-positive. Two of the ophthalmology (active infection) hookworm infested patients' samples were both positive by UAbE and UAgE but one was sero-positive and the other sero-negative. The remaining one (past infection) was sero-negative but positive by UAbE and UAgE.

4. Discussion

Detection of toxoplasmosis in the early stages of infection could pave the way for early treatment and consequently effective control of the disease. In addition, a relatively simple, cheap and easy-to-perform test could encourage routine mass screening of people, especially, women of potential childbearing age which will help control congenital transmission as well as to determine the sero-conversion rate among such women in Ghana. The present study is the first to employ membrane-based ELISA to detect *Toxoplasma* antigens and anti-*Toxoplasma* antibodies from the urine samples of such a large number of patients since Huskinson et al. (1989) reported the detection of *Toxoplasma* antigens in urine samples from patients with acute toxoplasmic encephalitis and acquired immunodeficiency syndrome in preliminary studies.

In this study, the two membrane-based ELISA systems were successfully used for the detection of *Toxoplasma* antigens and anti-*Toxoplasma* antibodies in the urine of infected individuals. Although sensitivity of UAbE (86.4%) compared with DT (gold standard) on one hand and with LAT (96.0%) on the other, was not statistically different ($P>0.05$), its specificity (67.0%) was quite low and might not be considered as a reliable diagnostic method for *Toxoplasma* infection, nevertheless, its performance could be enhanced in further studies. It was useful for comparative purposes in this study. For example, in the present study, detection of anti-*Toxoplasma* antibodies by UAbE in the urine samples of seven patients who were anti-*Toxoplasma* antibody sero-negative by both DT and LAT could be due to IgM and/or IgA antibodies which is suggestive of ongoing infections as has been reported by Bessieres et al. (1992) that, IgA antibodies associated with IgM

antibodies indicate acute infection because they are not usual in acquired immunity and very rare in chronic infections. They further reported that the presence of IgA together with IgG antibodies in circulation indicate recent acquired infection because IgA antibodies persist over 3 or 4 months following infection in acquired toxoplasmosis and low titers of IgG antibody are usual in patients with active toxoplasmic chorioretinitis, and IgM antibody cannot usually be detected.

Direct statistical comparison of the performance of UAgE with DT on one hand and with LAT on the other may be erroneous since the respective tests are detecting different components in circulation. Moreover, antibodies once produced against antigens are known to remain in circulation for a prolonged period even after drug therapy whilst antigens gradually reduce in quantity and go out of circulation. However, detection of *Toxoplasma* antigens in urine from patients whose sera were negative for anti-*Toxoplasma* antibodies is suggestive of early stage of fresh (or primary) infection, which was evident in this study. In other words, DT sero-negative serum samples that were positive by UAbE and/or UAgE could possibly be due to a putative active infection (IgM/IgA+ but IgG−). Additionally, in the UAgE system, antigens from samples that indicated weak positive results bound to murine-IgM and IgA antibodies, which appear in circulation earlier than IgG. Among the patients whose sera were positive for both DT and LAT but negative for urinary antigens by UAgE were two with high IgG titers (1:1024–1:2048). This is expected because both DT and LAT are reported to measure primarily IgG antibodies low titers of which may persist for life whilst some patients have persistently high titers (e.g. 1:1000–1:4000) for years. Consequently, the titer does not correlate with the severity of illness (Anderson and Remington, 1975). The comparable efficacy of DT and LAT in determining seropositivity was evident in this study (Table 1).

Considering the high prevalence of parasitic diseases like falciparum malaria, schistosomiases and soil-transmitted helminthiasis in Ghana, and more so with the existence of a urinary antigen detecting membrane-based dipstick assay for urinary schistosomiasis (Bosompem et al., 1997), it was necessary to clarify possible cross-reactions due to any of these parasites' antigens. None of the parasites detected do seem to have influenced the results of the membrane-based tests since urine samples from some individuals with mixed parasitic infections were negative for those tests.

Cryptosporidiosis is in Ghana but no comprehensive studies have been conducted. Addy and Aikins-Bekoe (1986) recorded a prevalence of 12.9% (61/474) in children from 2 to 60 months old and prior to our study a hospital-based research conducted by Otchere in 2001, using the same method on watery and normal formed faecal specimens from Ghanaian patients, yielded no oocysts (personal communication). That no oocysts were detected in any of the stool samples by the formalin–ether sedimentation technique was not surprising but does not rule out also inclusion of patients with antigenemia for, and/or antibodies against other relevant coccidian species like *C. parvum*, *Isospora belli* and *Cyclospora cayetanensis*. Current research methods like the use of fluorescein isothiocyanate-labelled anti-parasite monoclonal antibodies, SDS-PAGE and Western blotting techniques as well as ELISA could be employed to detect parasite antigens (Smith, 1998; Ungar, 1990) in a more elaborate study.

The 22 normal samples were confirmed by the study as sero-negative and could therefore be considered as true controls.

In this study, the UAbE and UAgE systems appear to have good prospects as their outcome are consistent with the intentions of development. However, their sensitivity and specificity need to be enhanced, and detailed cross-reactivity studies conducted with respect to infections from other coccidian species, using well-defined and larger study populations, especially, for obstetrics/gynaecology patients.

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The effect of free and polyethylene glycol–liposome-entrapped albendazole on larval mobility and number in *Toxocara canis* infected mice

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Abstract

As part of our exploratory drug research on the larva migrans that causes roundworm in dogs and cats, this study was carried out to clarify the effect of free and liposome-entrapped (LE) albendazole in *Toxocara canis* infected mice. In infected mice, evaluation of mobility and number of larva were examined in detail in the brain, skeletal muscle and liver. Larva mobility was evaluated by using the relative mobility (RM) value. Albendazole was LE as one of the drug delivery systems (DDSs). Polyethylene glycol (PEG) was added to the liposome in order to avoid evoking a response by the reticuloendothelial system (RES). By using the albendazole PEG–LE delivery system, it was possible to target the larvae in the mouse brain and liver resulting in a decrease in the number of larvae. In the skeletal muscle of the infected mice, the intraperitoneal dosages of PEG–LE albendazole did not cause a complete decrease in the number of larvae, even though free albendazole did cause the number to decrease. Therefore, it is necessary to take into consideration the migrating stage of the larvae before the initiation of any drug administration.

Keywords: *Toxocara canis*; Polyethylene glycol–liposome-entrapped; Albendazole; Anthelmintic effect

1. Introduction

Presently there are no effective drugs for larvae migrans such as *Toxocara canis*. Albendazole is currently used in such cases although it is not very effective. Thus, a new formulation for the treatment of *T. canis* larva migrans is urgently needed. The present research was carried out using both in vitro and in vivo experiments in our laboratory with the aim of finding a drug that would be effective in the treatment of *T. canis* larva migrans (Satou et al., 2002,

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2003a,b). It has been reported that isoquinoline alkaloids and crude drug extracts exhibit antinematode activity for *T. canis* larvae. In these previous in vitro experiments, it was documented that the isoquinoline alkaloids of allocryptopine, dehydrocorydaline, papaverine and the crude drug extracts of *Areca catechu*, *Rheum* spp., and *Citrus unshiu* were effective treatment agents. In order to continue to advance the current research, we need to elucidate the most efficacious methods of administration and the correct dosages required to successfully treat this condition.

Recent studies revealed that there is an anthelmintic effect by various liposomes of benzimidazole such as albendazole (Hrckova and Velebny, 2001; Velebny et al., 1997, 2000; Dvoroznakova et al., 1997). This high anthelmintic effect was found to occur when albendazole entrapped in liposome was used. By employing this method, we began a search for new formulations against toxocariasis. In our studies, through the use of the examination of the larvae number and mobility, we have been able to make detailed evaluations of the therapeutic effects that occur for various new compounds after their administration as liposome-entrapped (LE) chemical compounds.

Generally, drugs are recognized as foreign compounds and are metabolized after incorporation into the reticuloendothelial system (RES) by macrophages. Though the absorption to the tissue does increase after liposomizing albendazole, the RES still immediately metabolizes the drug. To avoid RES metabolization, we tried adding polyethylene glycol (PEG) to the liposome as per previously reported methods (Hrckova and Velebny, 2001; Velebny et al., 1997, 2000; Dvoroznakova et al., 1997).

2. Materials and methods

2.1. Animals

For the experiments, we used 5-week-old male BALB/c mice (Nippon Bio-Supp. Center) that weighed 20–22 g. All mice were given a standard commercial diet with free access to water and maintained on a cycle of 14 h light to 10 h dark at a room temperature of 23 °C.

2.2. Parasite and infection

Embryonated eggs of *T. canis* were prepared using the method of Oshima (1961). The eggs were taken out from the pars uterina and portio vaginalis of the *T. canis* imago, and placed in an incubator maintained at 28 °C, where development to larva encapsulated eggs occurred over approximately 10 days. Mice were anesthetized with diethyl ether (Wako Chemical, Japan) and orally infected with 300 embryonated eggs using a glass probe that was coated by silicon. The laboratory animals were handled according to the animal ethics regulations of Tokyo Medical and Dental University.

2.3. Preparation of samples for oral administration

Olive oil was used as a control. After being dissolved in 1% dimethyl sulfoxide (DMSO: Wako Chemical, Japan), albendazole (5 mg/ml) was suspended in olive oil. The suspension solution was administered orally to infected BALB/c mice (100 mg/kg/day).

2.4. Preparation of samples for intraperitoneal dosing

PEG–LE chemical compounds were administered into the peritoneal cavity of the mice. The reagents used for the liposome were 3-sn-phosphatidylcholine (from hydrogenated egg yolk, lecithin (Wako Chemical, Japan)), polyethylene glycol–cholesterol (MW: 5000, PEG–Chol (NOF Corporation, Japan)), and albendazole (Sigma, Japan). Each compound was prepared using the following proportions (weight), lecithin:PEG–Chol:sample = 10:13.6:2.5. After using organic solvent to dissolve these mixtures, a thin film was formed by removing the solvent. Phosphate buffered saline (PBS) was added to the thin film, followed by hydration using an ultrasonic cleaner. Prepared liposomes were given 2 times/day intraperitoneal (50 mg/kg/day).

2.5. Experimental design and necropsy

As seen in Fig. 1, the experimental design followed either plan A ($n = 3$) or B ($n = 5$) and included

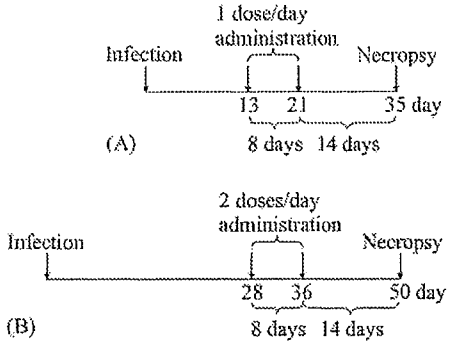

Fig. 1. (A) Oral administration (100 mg/kg/day) and necropsy plan for the infected mice ($n = 3$); (B) intraperitoneal dosage (50 mg/kg/day) and necropsy plan for the infected mice ($n = 5$).

infection, chemical administration, and necropsy of the BALB/c mouse.

The brain of the mice were made even in order to count the larvae. The larvae of the liver and skeletal muscle were collected by artificial digestive juice treatment (2.5 g pepsin (Wako Chemical, Japan), 3.5 ml hydrochloric acid (Wako Chemical, Japan), 500 ml water) and centrifugal treatment (2500 rpm, 5 min).

The observation of the larva was immediately carried out after the necropsy, since there was the possibility that the mobility of the larva would decrease and/or the larva would die with the passage of time.

2.6. Evaluation method for the larval mobility

Larval mobility after chemical administration was evaluated by a scoring system that was expressly designed to examine the effect on the larva in detail. This scoring method was developed by Kiuchi et al. and is generally used only in in vitro experiments (Kiuchi et al., 1987). We were able to modify and improve this scoring method so that it could also be used to evaluate the mobility of larvae in in vivo experiments. We made improvements in the methodology in order to be able to quickly carry out evaluations in the in vivo experiments (Table 1).

Larva that moved using their entire body were classified as 3, and larva that did not move at all or were found to be motionless with a straight form alignment of the body were classified as 0. All other larvae were classified as 1.5. The larvae number of each score was counted and the mobility index (MI) was calculated using the equation, "$\sum nN_n/\sum N_n$" (where N_n equaled the number of larvae with the score of n). Finally, the relative mobility (RM) was calculated using the equation, "$(MI_{sample}/MI_{control}) \times 100$".

Table 1
Criteria for evaluating the effect on larvae

State of larva	Score (n)
Moving using the whole body	3
Moving with only a part of the body during observation or immobile but not dead	1.5
Dead	0

Mobility index (MI) = $\sum nN_n/\sum N_n$, where N_n is the number of larvae with the score of n, relative mobility (RM) = $(MI_{sample}/MI_{control}) \times 100$.

3. Results and discussion

Many researchers have carried out studies on *T. canis* larva migrans. Abo-Shehada and Herbert (1984), Delgado et al. (1989) and Abdel-Hameed (1984) have reported on the effect of drugs during the early infection period. What is required at the present time is a drug that can be used for chronic *T. canis* larva migrans treatment. Fok and Kassai (1998) examined the oral administration of various compounds for treatment of chronic symptoms in *T. canis*, and with the exception of ivermectin, found no remarkable effects. In our study, we primarily carried out examinations on larvae that shifted to different organs due to the effect of the intraabdominal dosage of albendazole. We examined the therapeutic effect of the oral administration of free albendazole and determined both the number of larvae in the mouse brain and their respective mobility.

As shown in Fig. 2, the number of larvae and the RM value for albendazole did not decrease, as compared to the control.

By employing the method of Hrckova and Velebny (2001), we then examined the anthelmintic effect in mice after administration of PEG–LE albendazole. The administration was carried out in the peritoneal cavity with an intraperitoneal dosage that was half of the oral dose. The anthelmintic effect in the infected

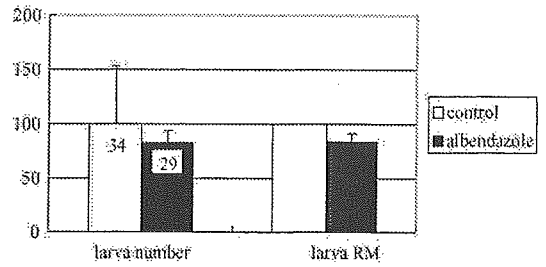

Fig. 2. Results of oral drug administration in *T. canis* infected mice (100 mg/kg/day). The vertical line shows the average number of larvae ± S.D. (%) and average RM value ± S.D. in the brain with the control value set at 100 ($n = 3$). The values within the columns in the graph are the measured values.

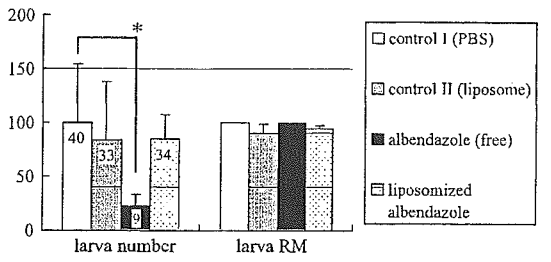

Fig. 4. The anthelmintic effect for intracelial drug administration in the *T. canis* infected mice (50 mg/kg/day). Average number of the larvae (%) ± S.D. and average RM value ± S.D. in the skeletal muscle for which the control I (PBS) value was set at 100 ($n = 5$); $^{*}p < 0.05$. The values within the columns in the graph are the measured values.

mouse was examined by administration of both free albendazole and PEG–LE albendazole. During this procedure we measured not only the number of larvae in the brain, but also the number found in the skeletal muscle and liver.

Though free albendazole did not decrease the larvae number in the brain, PEG–LE albendazole was found to decrease the number of larvae (21 larva/mouse) in the brain (Fig. 3). PEG–LE albendazole was significantly different from the control I (PBS) group (30 larva/mouse) at the 5% level for larval retention rate. It is thought that the decrease of the larvae number with PEG–LE albendazole and control II (liposome) originates from the formulation of the liposome. Thus, in the next step we compared the larval mobility with the number of larvae recovered from the skeletal muscle of the infected mice.

As seen in Fig. 4, free albendazole was found to decrease the number of larvae (9 larva/mouse) in the skeletal muscle. Free albendazole was significantly different from control I (40 larva/mouse) at the 5% level for the larvae number. However, PEG–LE albendazole did not exhibit any effect in the skeletal muscle of the infected mice. The RM value for each of the groups was determined to be approximately 100. However, since in order to count the larvae in the skeletal muscle we had to treat the tissue with an artificial digestive juice, this may have removed the dead larva, thus skewing the actual results.

As shown in Fig. 5, the anthelmintic effect in the liver tended to be almost equal to the anthelmintic effect in the brain. However, PEG–LE albendazole (28 larva/mouse) exhibited a greater anthelmintic effect proportion in the liver as compared to that seen in the brain with regard to larval counting. PEG–LE albendazole was significantly different from the control I (81 larva/mouse) at the 1% level for larvae

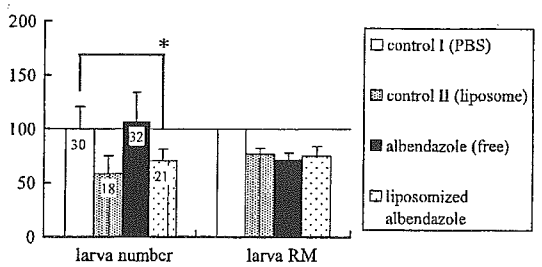

Fig. 3. Anthelmintic effect in the infected mouse after administration of the drug into the peritoneal cavity (50 mg/kg/day). The vertical line shows the average number of larvae (%) ± S.D. and average RM value ± S.D. in the brain for which the control I (PBS) value was set at 100 ($n = 5$); $^{*}p < 0.05$. The values within the columns in the graph are the measured values.

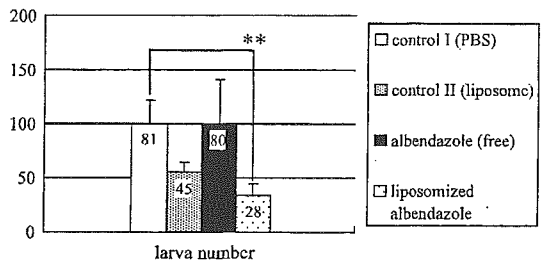

Fig. 5. Anthelmintic effect after intraperitoneal drug administration in the *T. canis* larva infected mouse (50 mg/kg/day). Average number of larvae (%) ± S.D. in the liver for which the control I (PBS) value was set at 100 ($n = 5$); $^{**}p < 0.01$. The values within the columns in the graph are the measured values.

number. In the liver, it was not possible to calculate the RM value since there were mobility problems due to the enzyme reaction of the organ.

In present result, it was not possible that the dead larva was discovered, as only to observe the decrease in the larvae number is. The search of the drug seems to develop more and more, if that point can be improved. The index of the mobility of the larva will be very effective in order to evaluate the condition of the larva in the host tissue.

Overall, these results indicate that there is a greater effectiveness of PEG–LE albendazole in the brain and liver than in the skeletal muscle, and confirm as well the findings of Hrckova and Velebny (2001) on the effectiveness of the drug delivery system in the brain. For the ocular toxocariasis, which we regard as one of targets of the drug, the use of PEG–LE albendazole seems to be effective. The use of free albendazole revealed the activity only in the skeletal muscle. Our results suggest that the decrease of larval number is directly related to the ability of the PEG–LE albendazole formulation to reach the mouse brain and liver. A rise in the accuracy of the liposome seems to be a necessity for its effectiveness. However, in contrast to this, PEG–LE albendazole did not affect the larva in the skeletal muscle. Therefore, when administering the drug it is necessary to take into consideration the migrating stage of the larvae, i.e., at what point do the larvae shift to the skeletal muscle and the brain. Additionally, the migration timing through the liver also needs to be considered. Thus, by identifying the larval stage and subsequently changing the path of administration, it makes it possible to tailor the effects of PEG–LE chemical compounds.

The present results highlight the necessity of carefully taking into consideration the location of the larval parasitic sites when undertaking any search for new drugs for use in the treatment of toxocariasis.

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Fatal *Baylisascaris* Larva Migrans in a Colony of Japanese Macaques Kept by a Safari-Style Zoo in Japan

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ABSTRACT: A colony of Japanese macaques (*Macaca fuscata fuscata*) kept by a safari-style zoo in Japan experienced 9 sporadic cases of fatal neurological diseases, such as epilepsy and posterior paralysis, during the 12 yr from 1989 to 2001. This macaque colony consisted of approximately 30 animals, on average, during this period, and the macaques shared their living space with 11 American black bears (*Ursus americanus*) harboring zoonotic roundworms (*Baylisascaris transfuga*). Close to this enclosure, a cote for 2–3 raccoons (*Procyon lotor*) was placed, and raw sewage from this cote ran into a shallow drain in the area for macaques and bears. However, fecal examinations in recent years did not detect the infection of raccoons with zoonotic roundworms (*Baylisascaris procyonis*). Postmortem histological examination of the latest 2 ill macaques detected multifocal malacia in the brain; 2 ascarid larvae of 60 μm maximum width were encapsulated in the cerebrum and lungs of 1 of the animals. To determine the causative ascarid species of the fatal larva migrans, we analyzed 2 additional encapsulated *Baylisascaris* larvae collected from formalin-fixed lungs by morphological and molecular approaches. This sporadic outbreak is the second record of *Baylisascaris* larva migrans in animals in Japan.

Larva migrans (LM) caused by the raccoon roundworm (*Baylisascaris procyonis*) has been a major public health concern during the last 2 decades in North America, where common raccoons (*Procyon lotor*) are endemic. This ascarid species is reported to cause serious LM in more than 90 species of domestic and wild mammals and birds as well as in humans (Kazacos, 1997, 2001). To date, 12 confirmed human cases of *B. procyonis* LM with severe or fatal neurological symptoms have been documented exclusively from this continent, and the victims were mainly infants of less than 2 yr of age (reviewed by Kazacos, 1997, 2001; Rowley et al., 2000; Gavin et al., 2002; Kazacos et al., 2002). In addition, *B. procyonis* LM caused visual disorders related to diffuse unilateral subacute neuroretinitis (DUSN) in adults (Kazacos et al., 1985; Goldberg et al., 1993; Mets et al., 2003). Raccoons infected with *B. procyonis* have been exported to other continents and become feral. An example is Germany, where a human case of DUSN and serologically suspected human cases of asymptomatic LM caused by *B. procyonis* have been reported (Küchle et al., 1993; Conraths et al., 1996). More than 20,000 raccoons were exported to Japan as personal pets during the 3 decades before the year 2000, and several thousand raccoons currently are naturalized in that country (Kawanaka et al., 2001). Fortunately, for unknown reasons, no ascarid infection except for *Toxocara tanuki*, the roundworm of raccoon dogs (*Nyctereutes procyonoides*), has been documented in feral raccoons in Japan (Miyashita, 1993; Asakawa et al., 1999). However, although a survey carried out in 1992 (Miyashita, 1993) found *B. procyonis* infection in 8% of pet raccoons, and a proportion of them had to be released and settled in the field, Miyashita (1993) also found a high rate of *B. procyonis* infection (40%) in zoo-kept raccoons, and special attention was paid to eradicating the ascarid from raccoons by zoo veterinarians.

Recently, we identified fatal *B. procyonis* LM in rabbits (*Oryctolagus cuniculus*) kept at a small wildlife park where visitors, including infants and children, commonly accessed an egg-contaminated rabbitry and enjoyed close contact with infected rabbits (Sato et al., 2002; Furuoka et al., 2003; Sato, Kamiya, and Furuoka, 2003). Following our report, we were consulted by several groups to identify the species of encapsulated larvae found in histological sections. One example was encapsulated larvae with prominent bilateral alae and 47 μm maximum width in the ileal wall of a 7-mo-old white-fronted marmoset (*Callithrix geoffroyi*). In this case, we isolated several larvae from formalin-fixed tissues, and by morphological examination of the larvae and an epidemiological survey on the faculty, we identified the causative species—that is, accidental encapsulation of the third-stage larvae of *Pterygodermatites nycticebi* (Sato, Matsuo et al., 2003). However, not all cases are as easy to identify. Sometimes, only a few larvae are available for morphological examination, and the epidemiological analyses suggest several possibilities as the causative species.

In a colony of Japanese macaques (*Macaca fuscata fuscata*) kept by a safari-style zoo in Japan, neurological disorders, such as epilepsy and posterior paralysis, were observed sporadically during the 12 yr from 1989 to 2001. The colony consisted of approximately 30 macaques, on average, during this period, and at least 9 macaques developed the disease and died within 1 day to 12 mo of developing signs: 2 macaques in each of the years 1989 and 1991, and 1 macaque in each of the years 1993, 1997, 1998, 2000, and 2001. This macaque colony and 11 American black bears (*Ursus americanus*; 15–27 yr old) were kept freely in the same enclosure (~1 ha), which was bordered by a 4-m-high, strong metal fence. Close to the fence, a cote for 2–3 raccoons was placed, and raw sewage from this cote ran into a soil waste pipe that connected to a shallow drain in the area for macaques and bears (Fig. 1). Use of this cote began in 1980, and cumulatively, 15 raccoons were kept until the year 2001. The latest 2 cases (a 3.7-yr-old male and a 2.9-yr-old male macaque) were submitted for postmortem examination, and his-