in specificity for the test (Morishima et al. 1999, Yimam et al. 2002). The test showed some cross-reactivity with patent *Taenia hydatigena* (Malgor et al. 1997) and *Taenia pisiformis* infections (unpublished data), however, these species are rare in Hokkaido. No cross-reaction was observed with *T. taeniaeformis* and *Taenia crassiceps* infections, which are relatively more prevalent in Hokkaido (Sakashita et al. 1995).

Multiplex PCR system provides a clear contribution to the assessment of field data. In the present survey, faeces with a diameter of less than 2.5 cm were collected and if size were used as the primary criterion for identification, the apparent prevalences of coproantigen and taeniid eggs in foxes were 30.4% and 11.1%, respectively. However, multiplex PCR system indicated that the actual prevalences were 34.0% and 12.9%, respectively. In addition, a rough evaluation of the prevalence of infection in other animals could be determined using multiplex PCR system data. Although other ambiguous criteria such as shape, colour and odour of faeces might be included for more reliable identification of faecal origin, the data from multiplex PCR system provides less equivocal identification and it also enables evaluation of specific carnivore prevalence and identification of their parasites. In addition, multiplex PCR system could be used to evaluate the reliability of previous ambiguous methods for estimating origins of faeces.

Of the 19 samples which origin was determined as cat by multiplex PCR system, five (26.3%) showed positive in coproantigen examination whereas taeniid eggs were not detected from any of those samples (Table 2). Since the chance of *E. multilocularis* to be mature and produce infective eggs in cats is considered to be low, egg examination has less value for assessment of the prevalence in cats. Considering that the necropsy survey of cats (n=108) conducted by the local government showed the prevalence of 5% in Hokkaido (data reported by the Hokkaido government), the present data may indicate the local high prevalence in cats. On the other hand, since the diagnostic reliability of the coproantigen examination has not been specifically determined for cat faeces, the present data may indicate the lower reliability of the method for cat faeces. Further studies are required for clarifying the significance of the observed result.

Faecal DNA did not provide a satisfactory means for evaluating the prevalence of *E. multilocularis*. According to PCR amplification of egg DNA, 27 faecal samples contained *E. multilocularis* eggs and these samples would be expected to contain debris from proglottids. However, only 22 samples gave positive PCR results from faecal DNA. In order to minimise the presence of PCR inhibitors, faecal DNA was extracted from a surface wash of frozen faeces and this procedure may have reduced the chances that *E. multilocularis* DNA would be co-extracted. Al-Sabi' et al. (2007) extracted DNA from whole faeces of foxes experimentally infected with *E. multilocularis*. They observed no PCR amplification in nine of 23 faeces samples containing parasite eggs in the low patent period and suggested that lack of amplification may have been due to PCR inhibitors present in the DNA extracts. Although PCR amplification of faecal DNA has the potential to detect infection more effectively, problems arising from the presence of PCR inhibitors remain to be overcome.

The multiplex PCR system developed will enable precise evaluation of current carnivore prevalence or changes in prevalence of target carnivores over time, thus contribute to epizootiological and control studies of *E. multilocularis*.

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References

Al-Sabi' MNS, Kapel CMO, Deplazes P, Mathis A (2007) Comparative copro-diagnosis of *Echinococcus multilocularis* in experimentally infected foxes. Parasitol Res 101:731–736

Dalen L, Götherström A, Angerbjörn A (2004) Identifying species from pieces of faeces. Conserv Genet 5:109–111

Davison A, Birks JDS, Brookes RC, Braithwaite TC, Messenger E (2002) On the origin of faeces: morphological *versus* molecular methods for surveying rare carnivores from their scats. J Zool 257:141–143

Deuter R, Pietsch S, Hertel S, Müler O (1995) A method for preparation of fecal DNA suitable for PCR. Nucleic Acids Res 23:3800–3801

Eckert J, Deplazes P, Craig PS, Gemmell MA, Gottstein B, Health D, Jenkins DJ, Kamiya M, Lightowlers M (2001) Echinococcosis in animals: clinical aspects, diagnosis and treatment. In: Eckert J, Gemmell MA, Meslin F-X, Pawlowski ZS (eds) WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern. Office International des Epizooties, Paris, pp 72–99

Flagstad Ø, Røed K, Stacy JE, Jakobsen KS (1999) Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with a magnetic bead protocol. Mol Ecol 8:879–883

Foran DR, Crooks KR, Minta SC (1997) Species identification from scat: an unambiguous genetic method. Wildlife Soc Bull 25:835–839

Frantz AC, Pope LC, Carpenter PJ, Roper TJ, Wilson GJ, Delahay RJ, Burke T (2003) Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using faecal DNA. Mol Ecol 12:1649–1661

Frantzen MAJ, Silk JB, Ferguson JWH, Wayne RK, Kohn MH (1998) Empirical evaluation of preservation methods for faecal DNA. Mol Ecol 7:1423–1428

Fraser A, Craig PS (1997) Detection of gastrointestinal helminth infections using coproantigen and molecular diagnostic approaches. J Helminthol 71:103–107

Gerloff U, Hartung B, Fruth B, Hohmann G, Tautz D (1999) Intracommunity relationships, dispersal pattern and paternity success in a wild living community of bonobos (*Pan paniscus*) determined from DNA analysis of faecal samples. Proc Royal Soc London, Series B Biol Sci 266:1189–1195

Hegglin D, Ward PI, Deplazes P (2003) Anthelmintic baiting of foxes against urban contamination with *Echinococcus multilocularis*. Emerg Inf Dis 9:1266–1272

Huber S, Bruns U, Arnold W (2003) Genotyping herbivore feces facilitating their further analyses. Wildlife Soc Bull 31:692–697

Inoue T, Nonaka N, Mizuno A, Morishima Y, Sato H, Katakura K, Oku Y (2007) Mitochondrial DNA phylogeography of the red fox (*Vulpes vuples*) in northern Japan. Zool Sci 24:1178–1186

Kohn MH, Wayne RK (1997) Facts from feces revisited. Trends Ecol Evol 12:223–227

Kovach AI, Litvaitis MK, Litvaitis JA (2003) Evaluation of fecal mtDNA analysis as a method to determine the geographic distribution of a rare lagomorph. Wildlife Soc Bull 31:1061–1065

Malgor R, Nonaka N, Basmadjian I, Sakai H, Carambula B, Oku Y, Carmona C, Kamiya M (1997) Coproantigen detection in dogs experimentally and naturally infected with *Echinococcus granulosus* by a monoclonal antibody-based enzyme-linked immunosorbent assay. Int J Parasitol 27:1605–1612

Mathis A, Deplazes P, Eckert J (1996) Improved test system for PCR-based specific detection of *Echinococcus multilocularis* eggs. J Helminthol 70:219–222

Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, Cabrita J, Mégraud F (1997) Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J Clin Microbiol 35:995–998

Morishima Y, Tsukada H, Nonaka N, Oku Y, Kamiya M (1999) Coproantigen survey for *Echinococcus multilocularis* prevalence of red foxes in Hokkaido, Japan. Parasitol Int 48:121–134

Murphy MA, Waits LP, Kendall KC (2000) Quantitative evaluation of fecal drying methods for brown bear DNA analysis. Wildlife Soc Bull 28:951–957

Murphy MA, Waits LP, Kendall KC, Wasser SK, Higbee JA, Bogden R (2002) An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. Conserv Genet 3:435–440

Nonaka N, Tsukada H, Abe N, Oku Y, Kamiya M (1998) Monitoring of *Echinococcus multilocularis* infection in red foxes in Shiretoko, Japan, by coproantigen detection. Parasitology 117:193–200

Palomares F, Godoy JA, Piriz A, O'Brien SJ, Johnson WE (2002) Faecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx. Mol Ecol 11:2171–2182

Paxinos E, Mcintosh C, Ralls K, Fleischer R (1997) A noninvasive method for distinguishing among canid species: amplification and enzyme restriction of DNA from dung. Mol Ecol 6:483–486

Piggot MP, Taylor AC (2003) Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species. Aust J Zool 51:341–355

Pires AE, Fernandes ML (2003) Last lynxes in Portugal? Molecular approaches in a pre-extinction scenario. Conserv Genet 4:525–532

R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org

Raoul F, Deplazes P, Nonaka N, Piarroux R, Vuitton DA, Giraudoux P (2001) Assessment of the epidemiological status of *Echinococcus multilocularis* in foxes in France using ELISA coprotests on fox feces collected in the field. Int J Parasitol 31:1579–1588

Sakashita M, Sakai H, Kohno H, Ooi H-K, Oku Y, Yagi K, Ito M, Kamiya M (1995) Detection of *Echinococcus multilocularis* coproantigens in experimentally infected dogs using murine monoclonal antibody against adult worms. Jpn J Parasitol 44:413–420

Tsukada H, Morishima Y, Nonaka N, Oku Y, Kamiya M (2000) Preliminary study of the role of red foxes in *Echinococcus multilocularis* transmission in the urban area of Sapporo, Japan. Parasitology 120:423–428

Tsukada H, Hamazaki K, Ganzorig S, Iwaki T, Konno K, Lagapa JT, Matsuo K, Ono A, Shimizu M, Sakai H, Morishima Y, Nonaka N, Oku Y, Kamiya M (2002) Potential remedy against *Echinococcus multilocularis* in wild red foxes using baits with anthelmintic distributed around fox breeding dens in Hokkaido, Japan. Parasitology 125:119–129

Verma SK, Prasad K, Nagesh N, Sultana M, Singh L (2003) Was elusive carnivore a panther? DNA typing of faeces reveals the mystery. Forensic Sci Int 137:16–20

Wasser SK, Houston CS, Koehler GM, Cadd GG, Fain SR (1997) Techniques for application of faecal DNA methods to field studies of Ursids. Mol Ecol 6:1091–1097

Yamauchi K, Hamasaki S, Miyazaki K, Kikusui T, Takeuchi Y, Mori Y (2000) Sex determination based on fecal DNA analysis of the amelogenin gene in Sika deer (*Cervus nippon*). J Vet Med Sci 62:669–671

Yimam AW, Nonaka N, Oku Y, Kamiya M (2002) Prevalence and intensity of *Echinococcus multilocularis* in red foxes (*Vulpes vulpes schrencki*) and raccoon dogs (*Nyctereutes procyonoides albus*) in Otaru city, Hokkaido, Japan. Jpn J Vet Res 49:287–296

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Note

Isolation of Antibabesial Compounds from *Brucea javanica*, *Curcuma xanthorrhiza*, and *Excoecaria cochinchinensis*

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One new curcuminoid, 3′-demethoxycyclocurcumin (1), was isolated from *Curcuma xanthorrhiza* as an antibabesial compound, together with *p*-hydroxybenzaldehyde (2) and cleomiscosin A (3) from *Brucea javanica* and (+)-epiloliolide (4) from *Excoecaria cochinchinensis*. The antibabesial activities were examined *in vitro*, and compounds 1–4, and diminazene aceturate were studied with IC_{50} values of 16.6, 7.6, 15.6, 10.0, and 0.6 μg/ml, respectively.

Key words: *Babesia gibsoni*; antibabesial compound; *Curcuma xanthorrhiza*; *Brucea javanica*; *Excoecaria cochinchinensis*

The infection of dogs with the parasites, *Babesia gibsoni* and *B. canis*, is a worldwide problem, and in recent years, the geographic range of the infection has spread. Canine babesiosis is a tick-borne hemolytic disease of dogs caused by the intraerythrocyte apicomplexan parasites, *B. gibsoni* and *B. canis*. The disease has been found to occur frequently in companion dogs and has become a serious clinical problem in various countries. The drug, diminazene aceturate (Ganaseg), is effective against *B. gibsoni* infection,[1)] but causes side effects such as weakness, irritability, paralysis, non-responsiveness to stimuli, and fatal central nervous system hemorrhage.[2)] There is yet no successful chemotherapy for the disease, because of the limited number of useful drugs, and the side effects and drawbacks of existing medication.[3,4)] To find a new antibabesial drug, the screening of several Indonesian medicinal plants was undertaken.

In Indonesia, *Brucea javanica* Merrill (Simaroubaceae), locally known as "buah makasar," *Curcuma xanthorrhiza* Roxb (Zingiberaceae), and *Excoecaria cochinchinensis* Lour (Euphorbiaceae) are used as drug materials for the traditional folk-medicine, "jamu," to treat malaria, dysentery, and cancer. In our previous articles, some quassinoids from *B. javanica*,[5,6)] and two kinds of xanthorrhizol derivatives from *C. xanthorrhiza*[7)] were reported as antibabesial compounds. However, active fractions remained that have still to be investigated. Furthermore, the isolation of active ingredients against *B. givsoni* has not been done by using an extract obtained from *E. cochinchinensis*. In this article, data on a novel curcuminoid, 3′-demethoxycyclocurcumin (**1**), from *C. xanthorrhiza* together with *p*-hydroxybenzaldehyde (**2**) and cleomiscosin A (**3**) from *B. javanica*, and (+)-epiloliolide (**4**) from *E. cochinchinensis* are presented.

The extracts of *B. javanica* and *C. xanthorrhiza* demonstrated antibabesial activity as reported previously,[5–7)] while the extract of *E. cochinchinensis* was tested for *in vitro* antibabesial activity[8)] by assessing its ability to inhibit *B. gibsoni* growth for the first time, and revealing antibabesial activity as well. The purification procedure, using several silica gel column chromatographic techniques together with high-performance liquid chromatographic (HPLC) methods, yielded 3′-demethoxycyclocurcumin (**1**, 1.4 mg) from the tubers (125 g) of *C. xanthorrhiza*, *p*-hydroxybenzaldehyde (**2**, 2.0 mg) and cleomiscosin A (**3**, 4.0 mg) from the dry fruits (200 g) of *B. javanica*, and (+)-epiloliolide (**4**, 0.5 mg) from the aerial parts (400 g, dry weight) of *E. cochinchinensis*.

Compound **1** was isolated as an amber oil. The field desorption mass spectrometric (FD-MS) data for **1** showed a molecular ion peak at *m/z* 338 $[M]^+$, and electron ionization high resolution mass spectrometry (EI-HR-MS) indicated the molecular formula to be $C_{20}H_{18}O_5$. The ^{1}H- and ^{13}C-NMR, distortionless enhancement by polarization transfer (DEPT), and heteronuclear multiple quantum coherence (HMQC) data showed the presence of 20 carbons of a curcuminoid skeleton, and the ^{1}H-NMR spectrum exhibited resonances ascribable to one methyl (δ 3.82), three olefinic (δ 5.50, 6.74, and 7.28), seven aromatic (δ 6.82, 6.90, 7.11, 7.26, and 7.40) and nonequivalent geminal (δ 2.52 and 2.89) protons, and one proton (δ 5.46) attached to an oxymethine carbon. The connectivity of some protons was revealed by ^{1}H–^{1}H correlated spectroscopy (COSY) as shown in Fig. 1 (bold lines). The correlation of ^{1}H directly attached to the carbon was revealed by an HMQC experiment. The partial structures were con-

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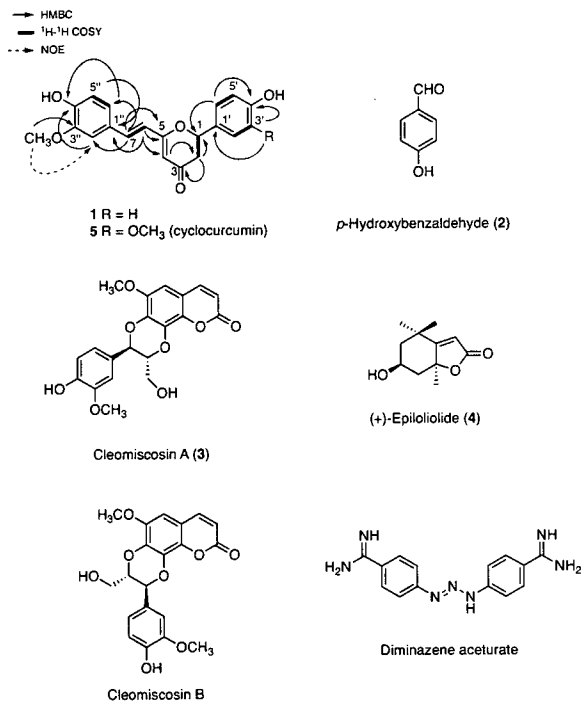

Fig. 1. Chemical Structures of **1**–**5**, Cyclocurcumin B and Diminazene Aceturate, and Important HMBC, ^{1}H–^{1}H COSY, and NOE Correlations for **1**.

nected by a heteronuclear multiple quantum coherence (HMBC, arrows, Fig. 1) experiment to construct the structure of **1** (Fig. 1). Analyses of the ^{1}H-NMR, ^{13}C-NMR, DEPT, HMQC, and HMBC spectral data for **1** gave the total ^{1}H- and ^{13}C-assignments for **1** as given Table 1. The deduced structure of **1** agreed well with that for the reported compound, cyclocurumin (**5**),[9] except for the absence of a resonance of the proton attributed to C3′-OMe (δ 3.91). In order to reconfirm the partial structure of C3″-OMe of **1**, a nuclear overhauser effect (NOE) experiment was carried out. The irradiation of C3″-OMe (δ 3.82) had an effect on H-2″(δ 7.26) (Fig. 1). The reported data for **5** are also given in Table 1 for comparison. Since 3′-demethoxycyclocurcumin (**1**) isolated in this study was racemic, the authors initially assumed that it was an artefact formed from demethoxycurcumin[9] during an isolation process such as silica gel chromatography. To investigate this, multiple reaction monitoring (MRM) was performed by using an ultra-performance liquid chromatography (UPLC)/tandem mass spectrometry (MS/MS) system in the positive-ion mode. The peak of authentic **1** was observed at t_R = 3.15 min, together with daughter ions m/z 56.48 and 272.36 derived from m/z 337.25 $[M + H]^{+1}$ in the MRM analysis (Fig. 2B and C). The crude material of the EtOAc-soluble layer obtained from the tubers of *C. xanthorrhiza* was dissolved with a solution of 80% aq. MeOH and passed through a C18 cartridge column to yield an 80% aq. MeOH eluate. A portion of the eluate was applied to the UPLC/MS/MS system, and the result of the MRM analysis is given in Fig. 2A. Since the corresponding daughter ion peak at m/z 56.48 derived from 3′-demethoxycyclocurcumin (**1**) (m/z 337.25 $[M - H]^{-1}$) was observed at the same retention time (arrow, Fig. 2), this confirmed **1** to be a naturally occurring substance. However, in the case of examining the plant extract, the daughter ion of m/z 272.36 could not be observed, possibly because of low ionization ability of 3′-demethoxycyclocurcumin (**1**).

Compound **2**, *p*-hydroxybenzaldehyde, was determined by direct comparison with an authentic sample. The chemical structures of cleomiscosin A (**3**) and (+)-epiloliolide (**4**) were determined by comparison with the

Table 1. NMR Spectroscopic Data for 3′-Demethoxycyclocurcumin (**1**) and Cyclocurcumin (**5**)[a,b]

Position	3′-Demethoxycyclocurcumin (**1**)		Position	Cyclocurcumin (**5**)[9]	
	δ_H (J in Hz)	δ_C		δ_H (J in Hz)	δ_C
1	5.46, dd (13.7, 3.4)	81.4	1	5.46, dd (13.7, 3.4)	81.7
2α	2.52, dd (16.8, 3.4)	43.5	2α	2.52, ddd (16.6, 3.4, 1.0)	43.8
2β	2.89, dd (16.8, 13.7)		2β	2.93, dd (16.6, 13.7)	
3		192.7	3		192.3
4	5.50, s	105.8	4	5.50, br, s	105.9
5		169.4	5		169.2
6	6.74, d (15.8)	119.8	6	6.74, d (16.1)	119.9
7	7.28, d (15.8)	137.7	7	7.31, d (16.1)	137.7
1′		130.7	1′		131.4
2′, 6′	7.40, d (8.2)	129.0	2′	7.22, d (2.0)	113.2
3′, 5′	6.90, d (8.2)	116.2	3′		148.5
4′		158.8	4′		148.0
			5′	6.90, d (8.3)	115.8
			6′	7.04, dd (8.3, 2.0)	120.6
1″		128.4	1″		128.6
2″	7.26, d (1.5)	112.3	2″	7.28, d (2.0)	113.2
3″		148.8	3″		148.8
4″		149.5	4″		149.4
5″	6.82, d (8.2)	116.2	5″	6.84, d (8.3)	116.2
6″	7.11, dd (8.2, 1.5)	123.0	6″	7.11, dd (8.3, 2.0)	123.0
			3′-OMe	3.91, s	56.39
3″-OMe	3.82, s	56.2	3″-OMe	3.88, s	56.35

[a] ^{1}H-NMR: 500 MHz, acetone d_6
[b] ^{13}C-NMR: 125.8 MHz, acetone d_6

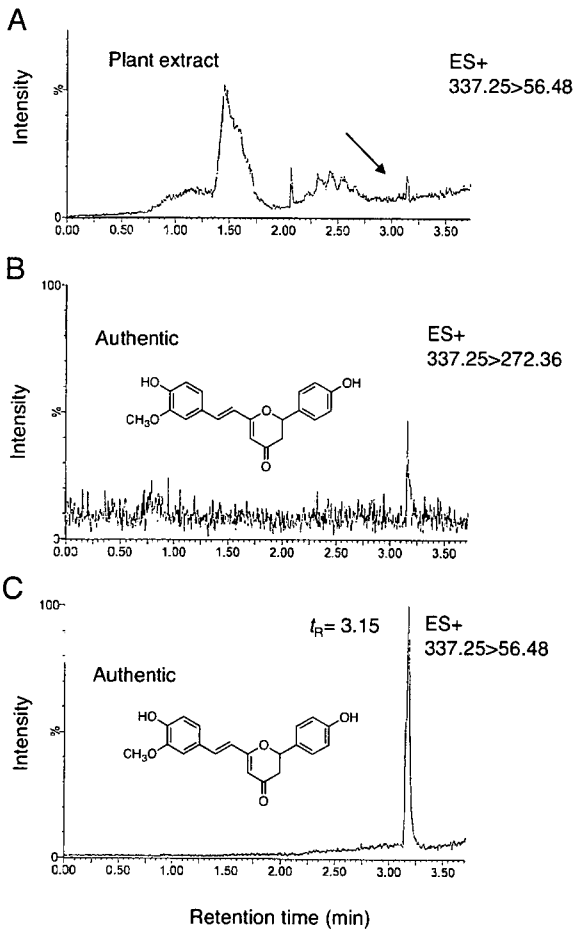

Fig. 2. UPLC/MS/MS MRM Chromatograms for 3′-Demethoxycyclocurcumin (**1**).

A, Chart of MRM monitoring the daughter ion *m/z* 56.48 derived from *m/z* 337.25 $[M + H]^+$ contained in the crude material. B, Authentic MRM chart monitoring the daughter ion *m/z* 272.36 derived from *m/z* 337.25 $[M + H]^+$. C, Authentic MRM chart monitoring the daughter ion *m/z* 56.48 derived from *m/z* 337.25 $[M + H]^+$.

reported data.[10–13] Since there was some ambiguity in the determination of the structure of cleomiscosin A (**3**) due to the presence of cleomiscosin B,[11] which demonstrated similar resonances in the ^{1}H- and ^{13}C-NMR data, the conversion of cleomiscosin A (**3**) to a di-acetate type was carried out to compare the ^{1}H-NMR data with those of the di-acetate type of compound of cleomiscosin B. (+)-Epiloliolide (**4**) was first isolated from *Viburnum dilatatum* Thunb (Caprifoliaceae),[13] but the isolation of **4** from *E. cochinchinensis* was for the first time.

The antibabesial activities for all compounds (**1–4**) isolated in this study were tested against *B. gibsoni*. Diminazene aceturate, a well-known clinical drug against *B. gibsoni*, was used as a positive control. Compound **2**, *p*-hydroxybenzaldehyde, showed the highest activity (7.6 μg/ml) among the isolated compounds, although the activity was almost one-twelfth that of diminazene aceturate (0.6 μg/ml). 3′-Demethoxycyclocurcumin (**1**), cleomiscosin A (**3**), and (+)-epiloliolide (**4**) showed moderate activity of 16.6, 15.6, and 10.0 μg/ml, respectively. The recorded activities in this study were measured only once due to the limited amounts of the isolated compounds. In other studies for measuring the activity, we used diminazene aceturate as a positive control which revealed IC_{50} values of 0.1,[5] 0.07,[6] 0.59,[7] and 0.6[8] μg/ml. We concluded that the values obtained in this study were correct due to the obtained IC_{50} value (0.6 μg/ml) for diminazene aceturate in this experiment. In this study, no compound whose activity could overcome that of diminazene aceturate could be found. However, these findings might provide the basis for further understanding *B. gibsoni* infection and contribute to the development of new and effective treatments against this parasite.

Experimental

General procedure. FD-MS and EI-HR-MS data were obtained with a Jeol JMS-AX500 mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM-500 Fourier transform nuclear magnetic resonance (FT-NMR) instrument. Optical rotation values were determined with a Jasco DIP-370 digital polarimeter. Column chromatography was performed on silica gel 60 (spherical, 70–140 mesh ASTM, Kanto Chemical). Silica gel 60 F_{254} precoated plates (Merck) were used to perform preparative TLC. Ultra performance liquid chromatography (UPLC) was performed with a Waters Acquity UPLC system equipped with a binary solvent delivery manager and a sample manager. MS was performed with a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer. UPLC/MS system control was by MassLynx 4.0.

UPLC conditions. The condition for UPLC separation were as reported in the previous article.[5]

MS/MS conditions. The condition for MS were as reported in the previous article.[5] The MRM transitions for the analyses of 3′-demethoxycyclocurcumin (**1**) were ESI+, 337.25 > 56.48 using cone voltage 42 and collision energy 26; 337.25 > 272.36 using cone voltage 42.00 and collision energy 18.

Plant materials. The plant materials were purchased from Bandar Jaya traditional market, located in Lampung, Indonesia, in April 2005. Voucher specimens have been deposited at the Laboratory of Bioorganic Chemistry, Graduate School of Agriculture, Hokkaido University, Japan.

Extraction and isolation. Extraction and isolation of the active compounds were monitored by an assay of antibabesial activity.[8] The tubers of *C. xanthorrhiza* (125 g, dry weight) were extracted with 3 liters of 70% EtOH (aq.). The extract was filtered, and the volatile components were removed under reduced pressure to give a dark brown oil. To this oil, H_2O (1 liter) was added, and the mixture was extracted with EtOAc (750 ml × 3). The volatile components of the combined EtOAc layers were removed under reduced pressure to give a dark brown oil (19.4 g) which was subjected to silica gel (200 g) column chromatography, eluted with $CHCl_3$ (1 liter), MeOH–$CHCl_3$ (3:97, 1 liter), and MeOH–$CHCl_3$ (1:9, 1 liter) to give fractions A–H. The volatile components of the active fraction (Fr. F) were removed under reduced pressure to give a residue (647 mg) which was subjected to silica gel (60 g) column chromatography, eluted with *n*-hexane–EtOAc (2:1) to give four fractions (I–IV). The volatile

components of the active fraction (Fr. III) were removed under reduced pressure to give a residue (42 mg) which was purified by HPLC (TSK gel ODS-80Ts, Tosoh, ϕ20.0 × 250 mm, H_2O:MeOH = 4:6, 5.0 ml/min flow rate, A_{254nm}), yielding 3′-demethoxycyclocurcumin (**1**, 1.4 mg, t_R = 32.7 min).

The dried fruits of *B. javanica* (200 g, dry weight) were extracted with 3 liters of 70% EtOH (aq.). The extract was filtered, and the volatile components were removed under reduced pressure to give a dark brown oil. To this oil, H_2O (1 liter) was added, and the mixture was extracted with EtOAc (750 ml × 3). The volatile components of the combined EtOAc layer were removed under reduced pressure to give a dark brown oil (4.9 g) which was subjected to silica gel (200 g) column chromatography, eluted with $CHCl_3$ (1 liter), MeOH–$CHCl_3$ (5:95, 1 liter), MeOH–$CHCl_3$ (1:9, 1 liter), and MeOH–$CHCl_3$ (3:7, 1 liter) to give fractions A–G. The volatile components of the active fraction (Fr. B) were removed under reduced pressure to give a residue (1.1 g), which was subjected to silica gel (100 g) column chromatography, eluted with MeOH–$CHCl_3$ (3:97, 500 ml), MeOH–$CHCl_3$ (5:95, 500 ml), and MeOH–$CHCl_3$ (3:7, 500 ml) to give five fractions BI–BV. The volatile components of one active fraction (Fr. BIII) were removed under reduced pressure to give a residue (18.7 mg), which was purified by HPLC (Mightysil RP-18 Aqua, Kanto Chemical, ϕ10.0 × 250 mm, H_2O:MeOH = 7:3, 2.5 ml/min flow rate, A_{254nm}), yielding *p*-hydroxybenzaldehyde (**2**, 2.0 mg, t_R = 19.5 mm). The volatile components of the other active fraction (Fr. BIV) were removed under reduced pressure to give a residue (60 mg), which was purified by HPLC (Inertsill PREP-ODS, ϕ20.0 × 250 mm, GL Sciences, H_2O:MeOH = 6:4, 7 ml/min flow rate, A_{254nm}), yielding cleomincosin (**3**, 4.0 mg, t_R = 54.2 min).

The aerial parts of *E. cochinchinenisis* (400 g, dry weight) were extracted with 3 liters of aq. 70% EtOH. The extract was filtered, and the volatile components were removed under reduced pressure to give a dark brown oil. To this oil, H_2O (1 liter) was added, and the mixture was extracted with EtOAc (750 ml × 3). The volatile components of the combined EtOAc layer were removed under reduced pressure to give a dark brown oil (12.6 g), which was subjected to silica gel (200 g) column chromatography eluted with $CHCl_3$ (1 liter), MeOH–$CHCl_3$ (3:97, 1 liter), MeOH–$CHCl_3$ (1:9, 1 liter), and MeOH–$CHCl_3$ (3:7, 1 liter) to give fractions A–G. The volatile components of the active fraction (Fr. C) were removed under reduced pressure to give a residue (73.2 mg), which was subjected to silica gel (50 g) column chromatography, eluted with MeOH–$CHCl_3$ (7:93) to give fractions I–V. The volatile components of the active fraction (Fr. II) were removed under reduced pressure to give a residue (27 mg), which was purified by HPLC (Capcell Pak C-18, Shiseido, ϕ15.0 × 250 mm, H_2O:MeOH = 55:45, 2.5 ml/min flow rate, A_{254nm}), yielding (+)-epiloliolide (**4**, 0.5 mg, t_R = 17.5 min).

3′-Demethoxycyclocurcumin (1): amber oil, $[\alpha]_D$ ±0° (*c* 0.01, acetone); FD-MS *m/z* (rel. int.) 338 ($[M]^+$); EI-HR-MS *m/z* 338.1173 (calcd. for $C_{20}H_{18}O_5$, 338.1154); ^{1}H-NMR (acetone-d_6, 500 MHz) and ^{13}C-NMR (acetone-d_6, 125 MHz): see Table 1.

Extraction for the MRM experiment. Tubers of *C. xanthorrhiza* (1 g, dry weight) from the market were extracted with 80% aq. MeOH. The resulting 80% aq. MeOH layer was filtered to yield a dark brown solution which was evaporated and extracted with EtOAc to yield aqueous and EtOAc layers. The volatile components of the EtOAc layer were removed under reduced pressure, and the residue was dissolved with 2 ml of 80% aq. MeOH and loaded into a cartridge column of Bond Elut C_{18} (Varian). The column was washed with 80% aq. MeOH (2 ml × 2). The volatile components of the aq. MeOH eluates were removed, and a portion of the residue was subjected to the UPLC/MS/MS experiment.

Antibabesial assay. The *in vitro* assay against *B. gibsoni* has been given in detail in a previous article.[8] In this study, diminazene aceturate (Ganaseg) was used as a positive control.

Acknowledgments

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References

1) Farewell, C. E., LeGrand, E. K., and Cobb, C. C., Clinical observations on *Babesia gibsoni* and *Babesia canis* infections in dogs. *J. Am. Vet. Med. Assoc.*, **180**, 507–511 (1982).
2) Groves, M. G., and Dennis, G. L., *Babesia gibsoni*: field and laboratory studies of canine infections. *Exp. Parasitol.*, **31**, 153–159 (1972).
3) Kuttler, K. L., Zaugg, J. L., and Gipson, C. A., Imidocarb and parvaquone in the treatment of piroplasmosis (*Babesia equi*) in equids. *Am. J. Vet. Res.*, **48**, 1613–1616 (1987).
4) Breitschwerd, E. B., Infectious diseases of dog and cat. In "Babesiosis," ed. Greene, C. E., B. W. Saunders, Philadelphia, pp. 796–803 (1990).
5) Subeki, Matsuura, H., Takahashi, K., Nabeta, K.,Yamasaki, M., Maede, M., and Katakura, K., Screening of Indonesian medicinal plant extracts for anti-babesial activity and isolation of new quassinoids from *Brucea javanica*. *J. Nat. Prod.*, **70**, 1654–1657 (2007).
6) Elkhateeb, A., Yamasaki, M., Maede, Y., Katakura, K., Nabeta, K., and Matsuura, H., Anti-babesial compounds from the fruits of *Brucea javanica*. *Nat. Prod. Commun.*, **3**, 145–148 (2008).
7) Matsuura, H., Nomura, S., Subeki, Yamada, K., Yamasaki, M., Yamato, O., Maede, Y., Katakura, K., Trimurningsih, Chairul, Yoshihara, T., and Nabeta, K., Anti-babesial compounds from *Curcuma xanthorrhiza*. *Nat. Prod. Res.*, **21**, 328–333 (2007).
8) Subeki, Matsuura, H., Takahashi, K., Yamasaki, M., Yamato, O., Maede, Y., Katakura, K., Kobayashi, S., Chairul, and Yoshihara, T., Anti-babesial and anti-plasmodial compounds from *Phyllanthus niruri* L. *J. Nat. Prod.*, **68**, 537–539 (2005).
9) Kiuchi, F., Goto, Y., Sugimoto, Y., Akao, N., Kondo, K., and Tsuda, Y., Studies on crude drugs effective on visceral larva migrans. XVI. Nematocidal activity of turmeric: synergistic

action of curcuminoids. *Chem. Pharm. Bull.*, **41**, 1640–1643 (1993).

10) Arisawa, M., Handa, S., McPherson, D., Lankin, D., Cordell, G., Fong, H., and Farnsworth, N., Plant anticancer agents XXIX. Cleomiscosin A from *Simaba multiflora*, *Soulamea soulameoides*, and *Matayba arborescens*. *J. Nat. Prod.*, **47**, 300–307 (1984).

11) Ray, A. B., Chattopadhyay, S. K., Kumar, S., Konno, C., Kiso, Y., and Hikino, H., Structures of cleomiscosins, coumarinolignoids of *cleome viscosa* seeds. *Tetrahedron*, **41**, 209–214 (1985).

12) Kuba, M., Furuichi, N., and Katsumura, S., Stereocontrolled syntheses of carotenoid oxidative metabolites, (−)-loliolide, (−)-xanthoxin, and their stereoisomers. *Chem. Lett.*, **31**, 1248–1249 (2002).

13) Machida, K., and Kikuchi, M., Norisoprenoids from *Viburnum dilatatum*. *Phytochemistry*, **41**, 1333–1336 (1996).

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CASE REPORT

Case of creeping disease treated with ivermectin

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ABSTRACT

We report a case of creeping disease treated successfully with ivermectin. A 46-year-old man presented with a 1-month history of pruriginous linear erythema on his right thigh after a visit to Indonesia. Although he had no history of eating raw fish or meat, he walked along the river and in the jungle without wearing shoes. Creeping disease caused by animal hookworm was strongly suspected. The presence of parasite larvae was not confirmed in biopsied skin specimens. In enzyme-linked immunosorbent assay, serum samples were negative for binding to hookworm antigens, including *Ancylostoma canium*, *Necator americanus* and *Gnathostoma doloresi*. He was treated with a single 12 mg oral dose (200 μg/kg) of ivermectin. The eruption and pruritus resolved within a few days after the administration and did not relapse.

Key words: ancylostoma, creeping eruption, hookworm, ivermectin, larva migrans.

INTRODUCTION

Creeping disease (cutaneous larva migrans) is a skin disease due to infection by the larval form of nematodes. In Japan, creeping disease caused by *Gnathostoma* spp. is most common in people who eat freshwater fish or the Japanese copperhead snake, whereas larval hookworm infection such as *Ancylosotoma canium* and *Ancylostoma brasilliense* is rare and is usually present as percutaneous infection.[1]

Ivermectin has been commonly used against onchocerciasis and scabies.[2] Recently, some cases have been reported of creeping disease treated successfully by oral administration of ivermectin in European and North American countries.[3–5] However, we are aware of only two patients with *Ancylostoma* spp. infection treated with ivermectin in Japan.[6,7] Herein, we present a case of creeping disease, probably caused by animal hookworms, successfully treated with ivermectin. We also report the results of microplate enzyme-linked immunosorbent assay (ELISA).

CASE REPORT

A 46-year-old-male Japanese office worker consulted our clinic, complaining of an itchy rash elongating by 1 cm per day on his right posterior thigh. He noticed the eruption after travelling to Indonesia. He denied eating any raw fish or meat, but admitted to walking along a river and in the jungle without shoes, and he had a history of leech bite. Creeping disease was suspected because of the typical clinical manifestation.

Serpiginous linear erythema, 2–3 mm wide, was distributed on the back of his right thigh (Fig. 1a). Pinhead-sized papules and vesicles were found at the edge of the erythema. The patient complained of pruritus. Laboratory examination did not reveal eosinophilia or elevation of immunoglobulin (Ig)E levels.

Three days after his first visit, the fresh linear erythema had elongated by approximately 3 cm (Fig. 1b). Ultrasonography did not show presence of the parasite. Histology showed moderate eosinophilic

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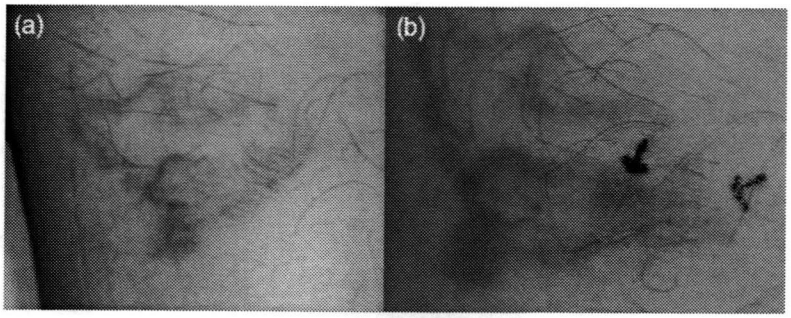

Figure 1. (a) A serpiginous linear erythema on the thigh. (b) A fresh erythema elongating approximately 3 cm long. A skin biopsy was taken from two different locations, indicated by the black arrows.

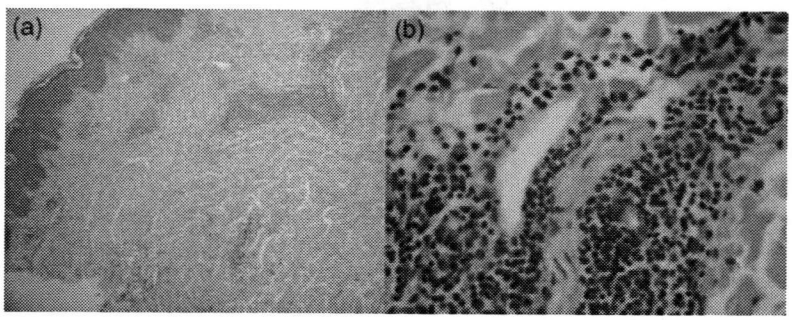

Figure 2. Perivascular eosinophilic infiltration in the dermis is noted. The parasite or the larva tract was not detected (original magnification: [a] ×40; [b] ×400).

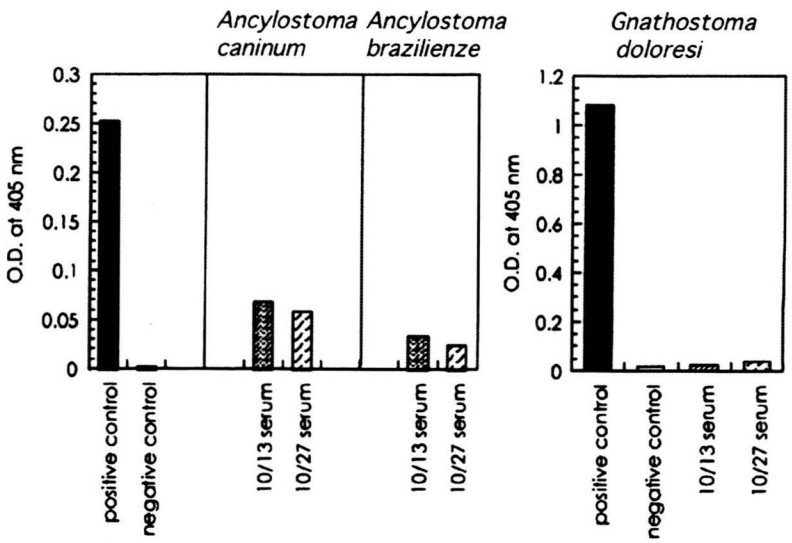

Figure 3. Specific immunoglobulin (Ig)G antibody titers in the sera were measured by microplate enzyme-linked immunosorbent assay using *Necator americanus*, *Ancylostoma caninum* and *Gnathostoma doloresi*. Significant elevation of the titers was not observed. (Positive control was serum from an ancylostomiasis patient in Papua New Guinea. Negative control was sera from healthy volunteers of Miyazaki University.)

infiltration around the small blood vessel. However, neither the body of the parasite nor a cleft indicating the larva tract were detectable (Fig. 2). We investigated the sera from the patient, sampled before and after the treatment. Specific IgG antibody titers in the sera were measured by microplate ELISA using human hookworm (*Necator americanus*), dog hookworm (*Ancylostoma caninum*) and Gnathostoma

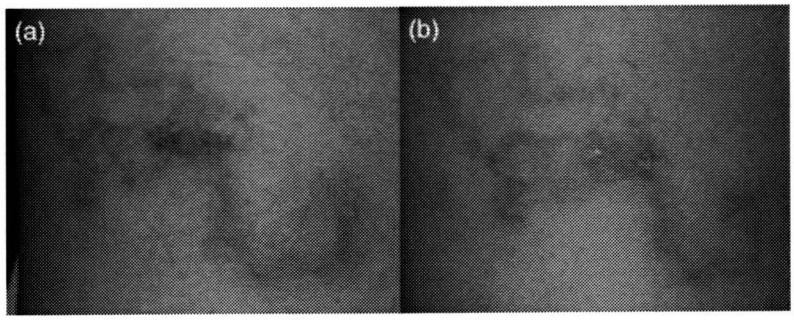

Figure 4. (a) Further elongation of fresh erythema. (b) A brown-colored pigmentation 4 days after taking ivermectin.

(*Gnathostoma doloresi*) (Fig. 3). Examination for Gnathostoma was negative. Antibody titers against *N. americanus* and *A. caninum* were above the negative control, although, compared with the positive control, immunoreactions of the patient's sera were weak. Because the titer was below 0.1 (optical density, 450 nm), we considered that elevation of the titer was not significant.

We diagnosed this case as creeping disease, because of the typical serpiginous erythema and a history of traveling in southeast Asia. Creeping disease caused by percutaneous infection such as *A. canium* and *A. brasilliense* was strongly suspected.

One week after the biopsy, further elongation of the worm tunnel was observed (Fig. 4a). A single oral dose of 200 μg/kg ivermectin between meals was then administered. Four days after taking ivermectin, the erythema faded (Fig. 4b). The pruritus resolved within 2 days of the administration. Neither adverse reaction nor recurrence was observed.

DISCUSSION

Creeping disease is an infectious skin disease caused by the larval form of nematodes. Our case was considered as a percutaneous larval hookworm infection. The patient had traveled to southeast Asia, where the main species is *A. brasilliense*, which is common in tropical areas, but *A. canium* and other canine species may also be the cause of the pathogen. According to Bouchaud *et al.*,[3] in cases of creeping disease with Ancylostoma spp., the incubation period is usually not more than 1 month, and pruritus was observed in all cases. The creeping eruption caused by *A. brasilliense* can resolve spontaneously in most cases, because the larvae are not able to complete their life cycle within humans and die after several months. However, our patient continued to suffer from severe pruritus, and was treated with a single oral dose of 200 μg/kg ivermectin. Previous reports state that the mean interval between ivermectin intake and the disappearance of pruritus is 3 days, and the mean interval between ivermectin therapy and the disappearance of lesions is 9 days.[3]

Ivermectin is a semisynthetic macrolide endectocide which has been often used against onchocerciasis and scabies.[2] Recently, several reports from Europe and North America have reported the efficacy of ivermectin against creeping disease. In a prospective study performed in France, 64 patients with creeping disease were enrolled and treated with a single 200 μg/kg dose of ivermectin taken between meals,[3] and 77% of them were cured. After one or two supplementary doses, the overall cure rate reached 97%. In a previous report by Caumes *et al.*, a single 400 μg/kg dose of ivermectin was effective for all 10 patients.[4] Karavichian *et al.* reported that 17 patients were treated with a single 200 μg/kg dose of ivermectin, and 76% patients were cured.[5] However, we know of only two cases of creeping disease treated with ivermectin published in Japan.[6,7]

To confirm the diagnosis, it is necessary to detect the larvae in the biopsied specimen. But this is difficult because the parasite moves rapidly beyond the obvious lesion. Therefore, we measured the parasite-specific IgG antibody titers in the patient's sera by microplate ELISA. The elevation of the antibody titers, however, was not significant. Immunoserological examination is known to show negative

in most patients with *A. brasilliense* and *A. canium* infection, because hookworms are too small to cause sufficient immunoresponse and produce antibody.[8] According to Uchiyama *et al.*, only three of seven suspected cases of Ancylostomas spp. infection showed seropositive against two hookworm antigens (*N. americanus* and *A. caninum*).[1] In contrast, in cases with *Gnathostoma* and *Ascaris* spp. infection, microplate ELISA is usually positive and has diagnostic significance.[9] Immunoserological examination may be of limited help in the diagnosis of creeping disease by *Ancylostoma* spp. and *Ascaris* spp. because it shows false-negative in many patients.

REFERENCES

1 Nakamura-Uchiyama F, Yamasaki E, Nawa Y. One confirmed and six suspected cases of cutaneous larva migrans caused by overseas infection with dog hookworm larvae. *J Dermatol* 2002; **29**: 104–111.

2 Dourmishev AL, Dourmishev LA, Schwartz RA. Ivermectin: pharmacology and application in dermatology. *Int J Dermatol* 2005; **44**: 981–988.

3 Bouchaud O, Houze S, Schiemann R *et al.* Cutaneous larva migrans in travelers: a prospective study, with assessment of therapy with ivermectin. *Clin Infect Dis* 2000; **31**: 493–498.

4 Caumes E. Treatment of cutaneous larva migrans. *Clin Infet Dis* 2000; **30**: 811–814.

5 Kravichian K, Nuchprayoon S, Sitichalernchai P, Chaicumpa W, Yentakam S. Treatment of cutaneous gnathostomiasis with ivermectin. *Am J Trop Med Hyg* 2004; **71**(5): 623–628.

6 Kinoshita Y, Hara H, Ochiai T, Suzuki H, Morishima K, Nawa Y. [Creeping eruption caused by the larva of *Ancylostoma brasiliiense*.] *Hihukano-rinsyo* 2003; **45**(2): 125–127. (In Japanese.)

7 Murayama J, Ono H, Taniguchi H, Takino C, Ohtaki N, Akao N. Creeping eruption caused by the hookworm larva. *Hihukano-rinsyo* 2006; **48**(5): 671–673 (in Japanese).

8 Nakamura-Uchiyama F. [Cutaneous larva migrans.] *Hihukano-rinsyo* 2004; **46**(11): 1635–1645. (In Japanese.)

9 Maruyama H, Noda S, Choi WY, Ohta N, Nawa Y. Fine binding specificities to Ascaris suum and Ascaris lumbricoides antigens of the sera from patients of probable visceral larva migrans due to Ascaris suum. *Parasitology Int* 1997; **46**: 181–188.

Circ J 2009; **73:** 1344–1348

Fulminant Eosinophilic Myocarditis Associated With Visceral Larva Migrans Caused by *Toxocara Canis* Infection

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A 19-year-old man was transferred to hospital because of myocarditis with cardiogenic shock. Echocardiography showed a left ventricular ejection fraction of 23.8% and an intermediate amount of pericardial effusion. The patient immediately received an intra-aortic balloon pump and percutaneous cardiopulmonary support. Right ventricular endomyocardial biopsy was performed in the acute phase and showed extensive eosinophilic inflammatory cell infiltration, severe interstitial edema and moderate myocardial necrosis. High-dose corticosteroids were administered. Because the patient's antibody titer against *Toxocara canis* was high and his symptoms had appeared after eating raw deer meat, the diagnosis was fulminant eosinophilic myocarditis caused by a hypersensitivity reaction to visceral larval migrans. After starting high-dose corticosteroids, the ejectionfraction dramatically improved, the eosinophilia decreased and the patient made a full recovery. (*Circ J* 2009; **73:** 1344–1348)

Key Words: Corticosteroids; Eosinophilia; Myocarditis; Toxocara canis; Visceral larva migrans

Acute myocarditis occasionally progress to a fulminant course, which can be fatal without mechanical support. Most of these fulminant cases are caused by viral infection for which corticosteroids are not effective![1,2] Visceral larva migrans (VLM), described by Beaver et al in 1952[3] results mainly from infection by the common roundworms of dogs and cats, *Toxocara* (*T.*) *canis* and *T. cati*, respectively. Infection with the parasite usually causes marked eosinophilia and the development of eosinophilic-rich granulomatous lesions in the soft tissues of the body, including the myocardium[4,5] It has been reported that administration of high-dose corticosteroids in the early stage can dramatically improve eosinophilic myocarditis, but fulminant cases are usually diagnosed at autopsy. We report a rare case of fulminant eosinophilic myocarditis caused by a hypersensitivity reaction to VLM.

Case Report

A 19-year-old man presented at a local hospital complaining of chest discomfort and pain. He had been healthy with no significant preceding symptoms, allergic history or past medical history. ECG showed a slight ST elevation in all limb and precordial leads, except for aVR (**Figure 1A**). Chest X-ray showed cardiomegaly, pulmonary congestion

Table 1. Results of Blood Examination on Admission

Complete blood count	
WBC	22,900/μl
Neutrophils	72.2%
Lymphocytes	16.6%
Monocytes	6.6%
Eosinophils	4.6% (1,053/μl)
Basophils	0%
RBC	560×10⁴/μl
Hemoglobin	17.9 g/dl
Hematocrit	54.3%
Platelets	25.8×10⁴/μl
Blood chemistry	
Total bilirubin	3.09 mg/dl
Total protein	5.8 g/dl
Albumin	3.6 g/dl
AST	2,462 IU/L
ALT	2,967 IU/L
LDH	2,126 IU/L
CK	144 IU/L
Troponin T (+)	
BUN	24.7 mg/dl
Cr	1.21 mg/dl
UA	10.2 mg/dl
Na	130 mmol/L
K	4.9 mmol/L
Cl	100 mmol/L
CRP	4.0 mg/dl
ECP	54.9 ng/ml
Infection markers	
HBs-Ag	(–)
HCV-AB	(–)
STS	(–)
TPHA	(–)

WBC, white blood cells; RBC, red blood cells; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; BUN, blood urea nitrogen; Cr, creatinine; UA, uric acid; CRP, C-reactive protein; ECP, eosinophil cationic protein; HBs-Ag, hepatitis B surface antigen; HCV, hepatitis C virus; TPHA, Treponema pallidum hemagglutination.

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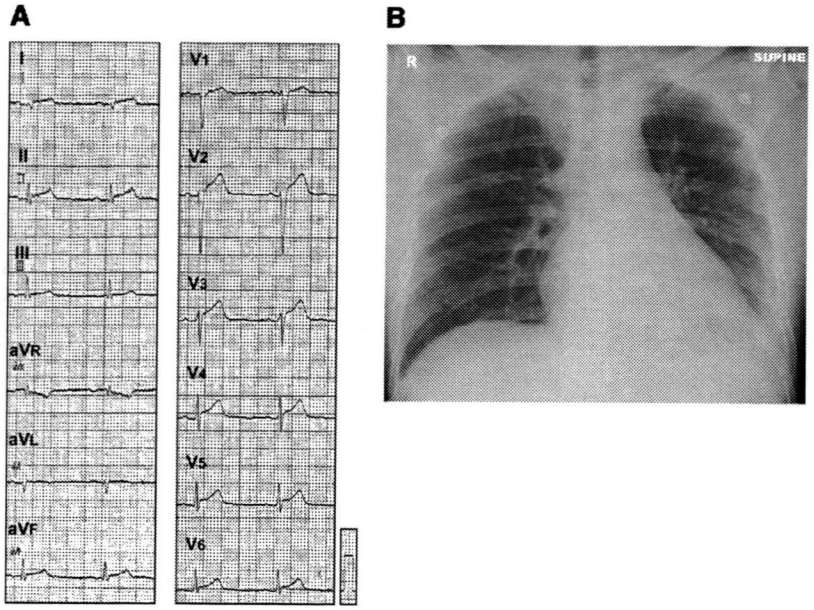

Figure 1. (**A**) Twelve-lead ECG on admission shows ST elevation. (**B**) Chest X-ray on admission shows the enlarged cardiac silhouette with pleural effusion.

Table 2. Viral Antibody Tests

Acute phase (admission)	Chronic phase (day 18)
Positive	Positive
Coxsackie virus A16 (×64)	Coxsackie virus A16 (×64)
Coxsackie virus B4 (×32)	Coxsackie virus B4 (×16)
Parainfluenza 3 (×40)	Parainfluenza 3 (×40)
Herpes simplex (×16)	Herpes simplex (×16)
Echo 12 (×32)	Echo 12 (×16)
Negative	Negative
Echo 3, 6, 7, 11	Echo 3, 6, 7, 11
Coxsakie virus A7	Coxsakie virus A7
Coxsakie virus B1, B2, B3, B5, B6	Coxsakie virus B1, B2, B3, B5, B6
Adenovirus, mumps virus, RS virus	Adenovirus, mumps virus, RS virus

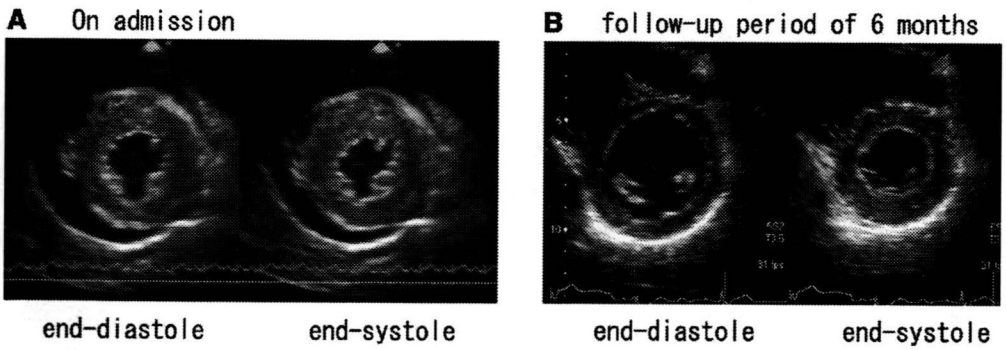

Figure 2. Echocardiography. (**A**) Large amount of pericardial effusion, wall thickening and severely decreased wall motion. (**B**) Six months after discharge, all abnormalities have improved dramatically.

and left pleural effusion (**Figure 1B**). He was admitted as an emergency with the suspicion of acute pericarditis. After admission, his general condition and left ventricular ejection fraction (LVEF) rapidly deteriorated and administration of dopamine was started because of cardiogenic shock. The next day he was transferred to our hospital for treatment of fulminant myo/pericarditis of unknown etiology.

On admission, his systolic blood pressure was 80 mmHg and heart rate was 130 beats/min with paradoxical pulse. A third heart sound was audible and his jugular vein was distended. Hematological and serological examinations (**Table 1**) showed marked increases in the total white blood cell count (22,900/μl) and eosinophil count (1,053/μl). C-reactive protein was also elevated (4.0 mg/dl), as was eosin-

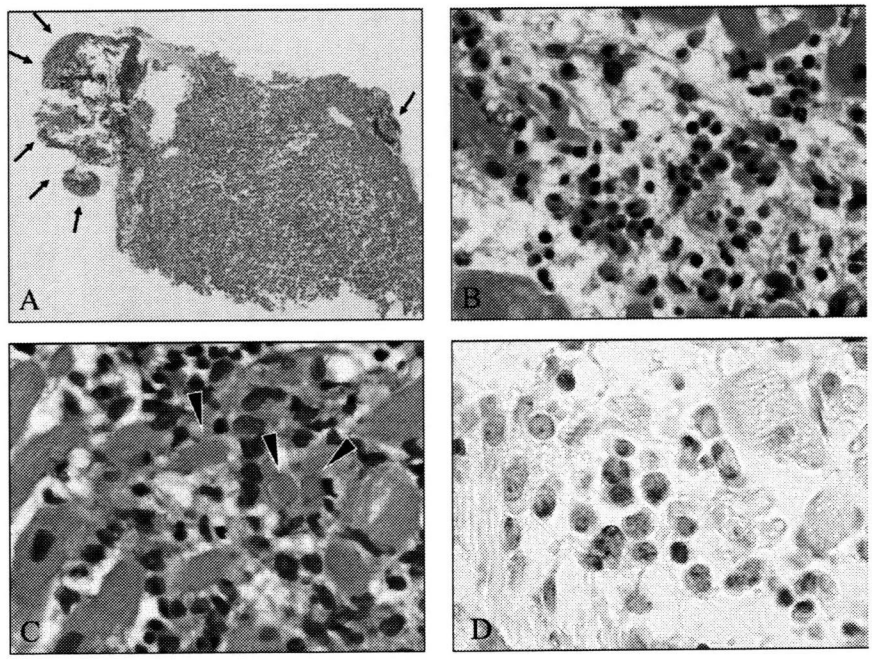

Figure 3. (**A**) Diffuse inflammatory infiltrate of the myocardium and endocardial involvement by mural thrombi containing eosinophils (arrows; H&E, ×25). (**B**) Eosinophil-rich inflammatory infiltrate with associated interstitial edema (H&E, ×200). (**C**) Interstitial Inflammatory infiltrated with associated myocyte necrosis (arrowheads; H&E, ×400). (**D**) Infiltrating eosinophils revealed by immunostaining with major basic protein (×400).

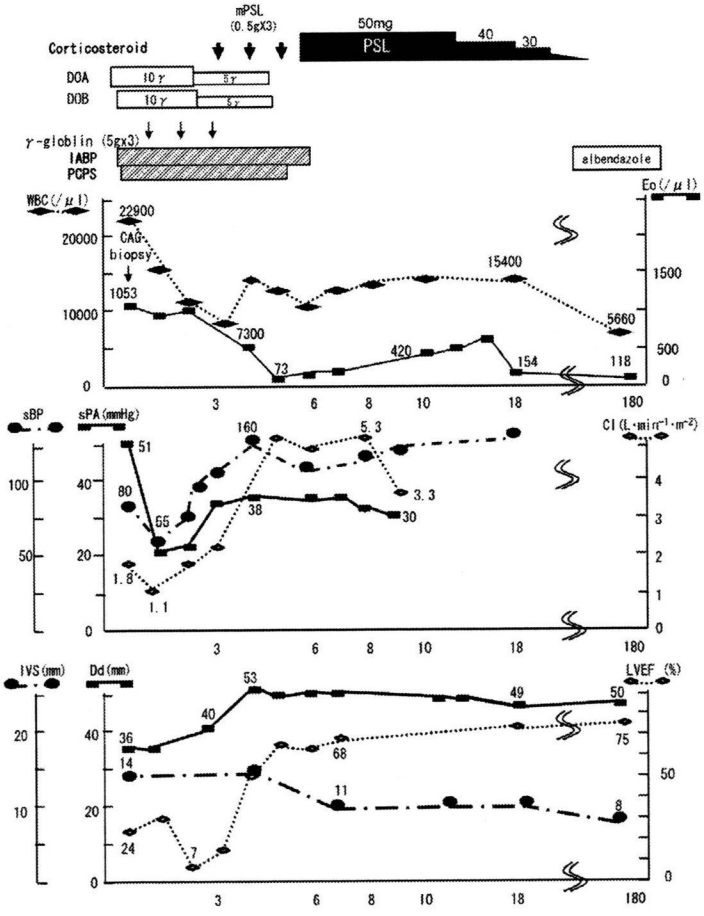

Figure 4. Clinical course. After starting corticosteroid treatment, LVEF and CI dramatically improved, with a decrease in the number of eosinophils. mPSL, methylprednisolone; PSL, prednisolone; DOA, dopamine; DOB, dobutamine; IABP, intra-aortic balloon pump; PCPS, percutaneous cardiopulmonary support; WBC, white blood cell count; Eo, eosinophil; sBP, systolic blood pressure; sPA, systolic pulmonary artery pressure; CI, cardiac index; IVS, interventricular septum; Dd, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction.

ophilic cationic protein (ECP: 54.9 ng/ml; normal range: <14.7 ng/ml). Immunological examination on the first day of admission revealed positive antibody titers for Coxsackie virus A16, Coxsackie virus B4, parainfluenza 3, herpes simplex and echo 12 were positive, and negative titers for adenovirus, mumps, Coxsackie virus, echovirus (except for type 12), herpes simplex, parainfluenza, and RS virus. However, there was no significant change in the antibody titers in the tests performed in the chronic phase, which ruled out the active phase of viral infection (**Table 2**).

Echocardiography showed severe hypokinesis of the left ventricular wall motion (LVEF: 23.8%), with thickening of the left ventricle and a large amount of pericardial effusion (**Figure 2A**). A diagnosis of fulminant myocarditis with cardiogenic shock was made. An intra-aortic balloon pump (IABP) and percutaneous cardiopulmonary support (PCPS) were immediately inserted and cardiac catheterization was then performed to decide the course of treatment.

Coronary angiography revealed no stenotic lesions. Right heart catheterization showed a pulmonary artery systolic pressure of 51 mmHg, pulmonary artery wedge pressure of 20 mmHg, and right atrial pressure 18 mmHg. Right ventricular endomyocardial biopsy was performed and histopathology revealed extensive interstitial edema with diffuse inflammatory interstitial infiltrate and myocardial necrosis. The infiltrating cells were eosinophils that had partially degranulated. These findings were compatible with acute eosinophilic myocarditis (**Figure 3**).

We started intravenous methylprednisolone at 500 mg/day for 3 days, followed by oral administration of prednisolone at 50 mg/day. After starting corticosteroid therapy, his ventricular function dramatically improved and the eosinophil count decreased promptly and normalized. On day 6 (day 1 is date of admission), the IABP and PCPS were removed (**Figure 4**) and prednisolone was tapered over a period of 8 weeks.

The patient has been doing well without any cardiac events since discharge and echocardiographic findings have remained normal during follow-up of 6 months (**Figure 2B**).

Anti-IgG to *T. canis* was detected in his serum by a commercial multiple-dot ELISA kit (SRL, Tokyo, Japan), although we could not find evidence of the parasites in the myocardial biopsy. Therefore, we performed further examination at the Department of Parasitology, Miyazaki Medical University, Miyazaki, Japan. Binding of patient serum to parasite antigens was tested using ELISA. Briefly, wells of microtiter plates were coated with 10 μg/ml of *T. canis* larval excretory–secretory antigen, and incubated with diluted samples (1:900–1:2,700). Binding of antibodies to *T. canis* antigen was detected with horse-radish peroxidase-conjugated anti human IgG and optical densities were read with a microplate reader (BioRAD). The patient had eaten raw deer meat 1 week before admission, so the final diagnosis was fulminant eosinophilic myocarditis caused by a hypersensitivity reaction to VLM after *T. canis* infection. We got the result 4 months after he had been discharged and prescribed albendazole (600 mg/day), which was continued for 4 weeks without any adverse effect.

Discussion

Acute fulminant eosinophilic myocarditis is a rare disorder of unknown etiology, frequently resulting in cardiogenic shock and a fatal clinical course. Predisposing factors include viral infection, history of allergic diathesis, and initiation of new medications[6] It has been suggested[7] that there are 3 clinical stages of eosinophilic myocarditis: acute necrotizing phase, thrombotic phase and endomyocardial fibrosis phase. Loffler's endomyocarditis is considered to correspond to the second stage of eosinophilic endomyocardial disease. The third stage probably corresponds to restrictive myocarditis. Differential diagnoses include other types of myocarditis, Churg-Strauss syndrome, hypersensitivity reaction, malignant diseases, parasitic infection or hypereosinophilic syndrome. Eosinophilic myocarditis associated with hypereosinophilic syndrome is usually less acute and less severe than acute fulminant necrotizing eosinophilic myocarditis[8] In the present case, the pathological findings were compatible with fulminant necrotizing eosinophilic myocarditis.

Because the symptoms of the patient appeared after eating raw deer meat and because the antibody titer against *T. canis* was high, we made a final diagnosis of eosinophilic myocarditis associated with VLM because of *T. canis* infection. *T. canis*, the common dog roundworm, is 1 of the causative agents of VLM. When embryonated eggs of *T. canis* from contaminated meat reach the human gastrointestinal tract, they hatch and enter the portal system, reaching the liver. Some larvae then migrate to the lungs and heart through the systemic circulation[9,10] A previous case of myocarditis associated with VLM has been reported![11] and another in Japan![12] so the present case is a very rare occurrence. Although the Myocarditis Treatment Trial found no statistical advantage of corticosteroid treatment in biopsy-proven myocarditis (Dallas Criteria)[2] it has been suggested that eosinophilic heart disease may be a subset with greater responsiveness[1] to corticosteroids. However, acute fulminant eosinophilic myocarditis is usually fatal and antemortem diagnosis is difficult. Corticosteroid therapy in the early stage can have favorable effect if early diagnosis by endomyocardial biopsy is made. In the present case, endomyocardial biopsy in the acute phase was helpful for diagnosis and therapeutic decision-making. Necrotizing eosinophilic myocarditis associated with VLM is very rare, but it should be taken into consideration.

Albendazole is a benzimidazole anthelmintic and is used as treatment for various parasitic infections. The mechanism of its anthelmintic action is inhibition of tubulin polymerization and microtubule-dependent glucose uptake inhibition. It has been reported that the incidence of adverse effects of albendazole is 23% and that the main adverse effect is liver injury (16%)![13] We were able to continue albendazole treatment of the present patient for 4 weeks without any adverse effects. Prompt anthelmintic treatment for parasite infection is recommended in eosinophilic myocarditis associated with VLM, so we should have administered albendazole earlier in the clinical course.

The patient was thought to be complicated by cardiac tamponade on admission, so PCPS was immediately inserted because of rapid worsening of left ventricular contraction and cardiogenic shock. The patient became hemodynamically stable with the PCPS and pericardial effusion decreased dramatically after starting corticosteroid therapy, so we did not perform pericardiocentesis on admission, which we probably should have done.

References

1. Jones SR, Herskowitz A, Hutchins GM, Bauqhman KL. Effects of immunosuppressive therapy in biopsy-proved myocarditis and borderline myocarditis on left ventricular function. *Am J Cardiol* 1991;

68: 370–376.
2. Mason JW, O'Connell JB, Herskowitz A, Rose NR, McManus BM, Billinqham ME, et al. A clinical trial of immunosuppressive therapy for myocarditis: The Myocarditis Treatment Trial Investigators. *N Engl J Med* 1995; **333:** 269–275.
3. Beaver PC, Snyder CH, Carrera GM, Dent JH, Lafferty JW. Chronic eosinophilia due to visceral larva migrans: Report of three cases. *Pediatrics* 1952; **9:** 7–19.
4. Dent JH, Nichols RL, Beaver PC, Carrera GM, Staggers RJ. Visceral larva migrans with a case report. *Am J Pathol* 1956; **32:** 777–803.
5. Cookston M, Stober M, Kayes SG. Eosinophilic myocarditis in CBA/J mice infected with Toxocara canis. *Am J Pathol* 1990; **136:** 1137–1145.
6. Watanabe N, Nakagawa S, Fukunaga T, Fukuoka S, Hatakeyama K, Hayashi T. Acute necrotizing eosinophilic myocarditis successfully treated by high dose methylprednisolone. *Jpn Circ J* 2001; **65:** 923–926.
7. Brockington IF, Olsen EG. Eosinophilia and endomyocardial fibrosis. *Postgrad Med J* 1972; **48:** 740–741.
8. Cooper LT, Zehr KJ. Biventricular assist device placement and immunosuppression as therapy for necrotizing eosinophilic myocarditis. *Nat Clin Pract Cardiovasc Med* 2005; **2:** 544–548.
9. Mok CH. Visceral larva migrans: A discussion based on review of the literature. *Clin Pediatr (Phil)* 1968; **7:** 565–573.
10. Woodruff AW. Toxocariasis. *BMJ* 1970; **3:** 663–669.
11. Vargo TA, Singer DB, Gillatte PC, Fernbach DJ. Myocarditis due to visceral larva migrans. *J Pediatr* 1977; **90:** 322–323.
12. Abe K, Shimokawa H, Kubota T, Nawa Y, Takeshita A. Myocarditis associated with visceral larva migrans due to Toxocara canis. *Intern Med* 2002; **41:** 706–708.
13. Drugs in Japan Forum. *Drugs in Japan* 2007; 209–210.

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ORIGINAL PAPER

Evaluation of *Babesia bigemina* 200 kDa recombinant antigen in enzyme-linked immunosorbent assay

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Abstract A truncated fragment of the gene encoding the 200-kDa protein (P200) of *Babesia bigemina* was cloned into a plasmid vector, pGEX-4 T-1 and expressed in *Escherichia coli* as a glutathione-*S*-transferase fused protein. An indirect enzyme-linked immunosorbent assay (ELISA) using the rp200/CT detected specific antibodies in cattle experimentally infected with *B. bigemina*. Furthermore, the antigen did not cross-react with antibodies to *Babesia bovis*, a closely related *Babesia* parasite indicating that rp200/CT is a specific antigen for the diagnosis of *B. bigemina* infection. Additionally, ELISA using p200/CT and polymerase chain reaction were conducted on serum and corresponding DNA samples obtained from field cattle to evaluate the diagnostic utility of the p200/CT antigen. Results from the current study suggest that p200/CT ELISA is a sensitive and specific method for improved serodiagnosis of *B. bigemina* infection.

Introduction

Babesia bigemina is a tick-borne hemoprotozoan that causes babesiosis in cattle (Barnett 1974; McCosker 1981). The parasite is widely distributed throughout Africa, Southern Europe, Asia, Australia, Central and South America, coinciding with its main vector, the *Boophilus* tick. Economically, it is the most important cause of heavy losses as a result of infection in susceptible cattle, particularly in imported breeds (Akinboade and Akinboade 1985; Solari et al. 1992). Animals that recover from infection develop lifelong immunity and may become carriers (Böse et al. 1995).

The classical diagnosis of acutely infected animals is by light microscopic demonstration of intra-erythrocytic parasites in Giemsa-stained blood smears (Araujo et al. 1998); however, in subclinical or latent infection, parasites may not always be demonstrable because of low parasitemia (Böse et al. 1995). In epidemiological studies, exposure to *B. bigemina* is best determined by the detection of antibodies in the sera of infected cattle (Molloy et al. 1998). Several serological tests such as the indirect flourescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) have been developed for bovine babesiosis (Boonchit et al. 2006; Goff et al. 2008; Mbati et al. 2002; Ravindran et al. 2007). However, the IFAT has poor specificity because antibodies cross-react between *B. bovis* and *B. bigemina*. Furthermore, this technique is time-consuming, and the interpretation of the results is subjective and depends on individual expertise (Böse et al. 1995). On the other hand, ELISA is quite sensitive and is appropriate for testing large number of samples especially in field surveys (Weiland and Reiter 1988). Previously, ELISAs have been evaluated for detection of antibodies to bovine *Babesia* parasites using native antigens. Although, such tests proved to be powerful tools for serological surveys, the poor quality of the antigens, and sometimes cross-reactions between the *B. bovis* and *B. bigemina* species and with closely related apicomplexan parasites resulting in false positive results have limited their application (Böse et al. 1995).

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Considering these problems associated with *Babesia* infections, it is, therefore, necessary to develop a reliable diagnostic method for this disease. Although some antigens have shown potential for effective diagnosis and putative vaccine candidates in preliminary experiments, a successful antigen to diagnose and prevent this disease has not been found. Several factors have contributed to this lack of proper antigens including a complicated life cycle of the parasite, wide variety of immune responses induced by the parasite, and insufficient information concerning the relationship between the parasite and its hosts Wright et al. 1987. Cross-reactivity with antibodies to *B. bovis* is a complication in regions where the two parasites coexist (Dalrymple et al. 1992; Morzaria et al. 1992). Furthermore, *B. bigemina* immune serum has been shown to cross-react with bovine fibrinogen (El-Ghaysh et al. 1996). Hence, it is essential to develop a suitable serological test to differentiate between *B. bovis* and *B. bigemina*. In a review of serological tests for the diagnosis of babesiosis, Böse et al. (1995) concluded that the existing serological tests for *B. bigemina* were inadequate and that the development of an improved test was a priority. In this study, the gene encoding the 200 kDa protein of *B. bigemina* (Tebele et al. 2000) was expressed as a glutathione-*S*-transferase (GST) fused protein in *Escherichia coli* and used for serological diagnosis of *B. bigemina* infection in enzyme-linked immunosorbent assay (ELISA). In addition, corresponding DNA samples obtained from the same animals were also screened by polymerase chain reaction (PCR) for the detection *B. bigemina*.

Materials and methods

Parasites

B. bigemina (Argentina strain) was continuously cultured with bovine erythrocytes using a microaerophilous stationary-phase culturing system (Vega et al. 1985). When the level of parasitemia reached 5%, the infected erythrocytes were washed thrice with phosphate buffered saline (PBS), and the pellets were stored at −80°C until use.

Bovine sera and DNA

Serum samples used for the evaluation of the ELISA with rP200/CT were as follows: serum samples from cattle experimentally infected with *B. bovis* (n=12) or *B. bigemina* (n=12), negative serum samples (n=12) from healthy cattle with no known past history of *Babesia* infection (Boonchit et al. 2006).

To evaluate the diagnostic utility of p200/CT, field blood samples were collected from cattle in Accra Ghana. Samples were taken to the veterinary services laboratory in Accra for analysis. Blood samples were collected in EDTA tubes from the jugular vein of cattle. For each of the blood samples, Giemsa-stained blood smears were prepared for microscopic examination. Serum samples were separated after centrifugation and were tested by ELISA for detection of antibodies against *B. bigemina*. DNA was also extracted from same samples using a commercial Kit (QIAamp DNA Blood Mini-Kit Madison, WI, USA) according to the manufacturer's instructions.

Microscopic analysis

In the laboratory, blood smears were fixed in methanol for 5 min and stained for 30 min in Giemsa stain diluted with 5% buffer and observed with Bright-field light microscopy at 1,000× for the presence of *Babesia* organisms.

Cloning of the truncated P200 gene

B. bigemina genomic deoxyribonucleic acid (DNA) was extracted from *B. bigemina*-infected erythrocyte pellets with phenol–chloroform as previously described (Boonchit et al. 2002). The genomic DNA was used as the template in the PCR. Oligonucleotide primers were designed based on the DNA sequence of the *B. bigemina* 200 kDa gene (Gene Bank accession no AF142406) with restriction enzyme-compatible ends for subsequent cloning. The nucleotide sequence (nt 3112–3327 nt) encoding the 200 kDa gene was obtained by PCR using primers Big-P200-F-5′-CCGGAATTCATGGTAAAACATGCATCC-3′ and Big-P200-R-5′-CCCTGCGAGCTAAGAGTCATCACC-3′ containing *Eco*RI and *Xho*I sites, respectively. The PCR conditions were 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min. Each of the PCR-amplified DNA fragments was digested with restriction enzymes, and *Eco*RI and *Xho*I were then ligated to a similarly digested pGEX-4T-1 expression plasmid (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), resulting in plasmid pGEX-P200/CT containing the truncated P200 gene fragment.

Expression and purification of *B. bigemina* rP200/CT recombinant protein

The recombinant plasmid pGEX-P200/CT was transformed in *E. coli* (BL21). Each transformed colony was cultured in LB medium (1% bacto tryptone, 0.5% yeast extract, 1% NaCl, 0.1% 5-*N* NaCl) with ampicillin sodium (50 μg/mL) at 37°C. At optical density (OD; 600 nm) 0.5, the plasmids were induced to synthesize the recombinant fused proteins by the addition of 1-M isopropyl-*b*-*d*-thiogalactopylanoside (IPTG), (Wako, Tokyo. Japan) and incubated for 4 h. The bacterial cultures were harvested by low centrifugation and

sonicated with STE buffer (50-mM Tris–HCl at pH9.5, 100 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA)) containing 50-μg/mL lysozyme and 1% Triton X-100. The supernatant containing the recombinant fused proteins (rP200/CT) was removed by centrifugation and then purified with glutathione sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia Biotech, little Chalfont, UK).

SDS-PAGE and Western blotting

The expression and purification of the recombinant proteins were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide gel according to Laemmli's method (1970). Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, USA), by the method of Towbin et al. (1979). Thereafter, the membranes were treated as described by Boonchit et al. (2006).

ELISA

ELISA was performed according to the method followed by Xuan et al. (2001) with some modifications. Briefly, the optimal working conditions for the rP200/CT conjugates were determined in a checkerboard assay by using serial dilutions. Pre-infection (negative control) and post-infection (positive control) cattle sera were diluted 1:100 in blocking solution before use. The conditions rendering the highest difference in OD measured for positive and negative sera were chosen for screening all the sera. Purified rP200/CT antigen was diluted to 7 μg/mL in 0.05 M carbonate–bicarbonate buffer (pH9.6), and 50 μL of this solution was added to each well of a flat-bottomed microtiter Maxisorp plate (Nalgen Nunc, Denmark). Control wells for each of the sera were coated with 7 μg/mL purified GST. Duplicate serum samples were titered by doubling the serial dilutions in a blocking solution containing 3% skimmed milk, and the mixture was incubated at 37°C for 1 h. After blocking, the plates were washed once with PBS-T and then 50 μL of diluted sera was added to each well. The plates were incubated at 37°C for 1 h and then washed six times using PBS-T. Bovine antibodies were detected by using HRPO conjugated sheep anti-bovine immunoglobin G (IgG; 1:5,000). The plates were incubated at 37°C for 1 h and then washed six times with washing buffer. To each well, 100 μL of the substrate (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, 0.5 mg 2,2′-azino-di-3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemicals, USA) was added. The plates were further incubated at room temperature for 1 h, and the OD (415 nm) was measured by using an automatic ELISA plate reader (Corona, Japan). The net OD values were obtained by subtracting the OD for the GST control from that of the test sera. This control procedure ensured that the net OD values were due to the presence or absence of *B. bigemina* antibodies and not due to nonspecific binding. The cutoff point of OD>0.25 was used as the mean OD for the negative sera plus three standard deviations. *B. bigemina* RAP-1CT ELISA was performed as previously described by Boonchit et al. 2006.

PCR amplification and sequence analysis

A PCR was performed to detect *B. bigemina* DNA using previously described method by (Boonchit et al. 2006). Amplification was performed using 50-μl reactions. Each reaction contained a 1× concentration of PCR buffer (Roche), 2.5 U of Taq polymerase, 5 μl of DNA template, 10 pmol of each primer, 2 mM concentration of each deoxynucleoside triphosphate. A forward primer pair (BigF-CGAAGCAGCTGTAGAGGAAA and reverse primer BigR-TTTCTGCATCTGGAAGCTGC) were designed to amplify approximately 195 bp fragment from *B. bigemina* DNA. The PCR conditions were as follows; initial denaturation at 95°C for 5 min, followed by 35 amplification cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 1 min), and a final extension step at 72°C for 7 min. The PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV-transillumination. Positive *B. bigemina* amplified DNA products were gel purified by a GENECLEAN kit (BIO 101, Vista, CA, USA). The nucleotide sequence of DNA fragments were directly sequenced by an ABI PRISMTM 3100 Genetic Analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) with ABI PRISMTM BigDyeTM terminator cycle-sequencing ready-reaction kit (Applied Biosystems Japan Ltd).

Results

Expression of the rP200/CT in *E. coli*

To establish an effective and improved method for the diagnosis of *B. bigemina* infection, the DNA sequence encoding a truncated 200 kDa gene (1,038–1,108 aa) was amplified by PCR and inserted into the pGEX-4 T-1 plasmid vector, resulting in the recombinant plasmid pGEX-rP200/CT. The recombinant plasmids were transformed and expressed in *E. coli* as GST fused proteins. The resultant rP200/CT was purified and observed by SDS–PAGE (Fig. 1). The protein had a molecular size of approximately 33.9 kDa. In the Western blot analysis, rP200/CT reacted strongly with the sera from cattle infected by *B. bigemina* but not with sera from *B. bovis*-infected cattle (Fig. 2).

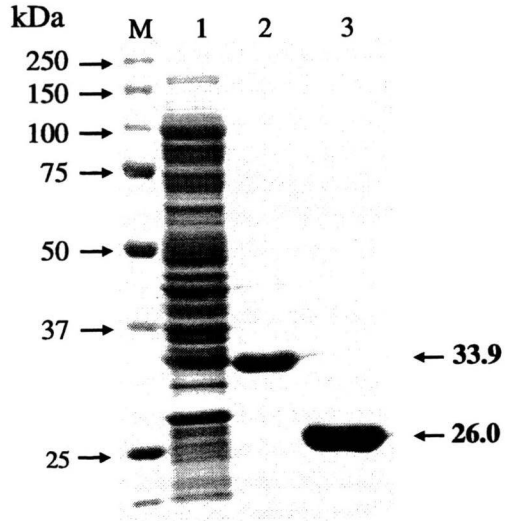

Fig. 1 Expression and purification of the GST fusion with *B. bigemina* P200/CT protein expressed in *E. coli*. *Lane 1* proteins in soluble fraction; *Lane 2* purified protein; *Lane 3* GST. Proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue

Reactivity and specificity of rP200/CT in ELISA

ELISA was used to evaluate the rP200/CT expressed in *E. coli* to ascertain whether it can be a useful antigen for the serodiagnosis of babesiosis. The OD_{415} cutoff point was set as the mean value of the negative samples plus three standard deviations.

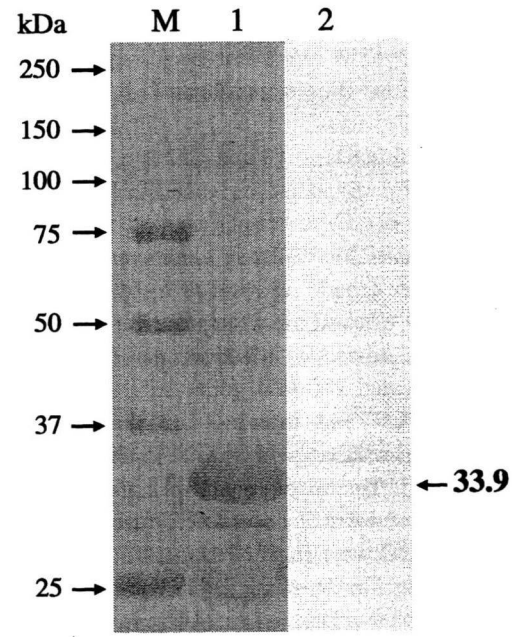

Fig. 2 Western blot analysis of *B. bigemina* P200/CT protein. The membrane was incubated with anti-*B. bigemina* polyclonal antibodies (*Lane 1*) and *B. bovis*-infected sera (*Lane 2*)

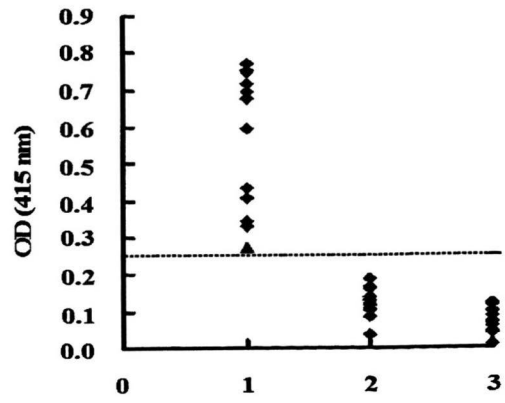

Fig. 3 Reactivity of the bovine sera with the *B. bigemina* rP200/CT in enzyme-linked immunosorbent assay (ELISA). *Column 1*: *B. bigemina*-infected bovine sera (n=12), *Column 2 B. bovis*-infected bovine sera (n=12); *Column 3* noninfected bovine sera. *Dotted line* cutoff point

The ELISA detected antibodies (OD>0.25) in the sera of *B. bigemina*-infected cattle but was negative (OD<0.25) for *B. bovis*-infected cattle sera and uninfected bovine sera (Fig. 3). Similarly, when the OD values of rP200/CT were compared with those of the *B. bigemina* rhoptry-associated protein 1 (rRAP-1/CT) ELISA, the absorbance values of the rRAP-1/CT ELISA were higher than those of the rP200/CT ELISA. In addition, the absorbance values of the negative serum samples were less than the cutoff point in both ELISAs (Fig. 4).

Diagnosis of *B. bigemina* infection in cattle by the ELISA with rP200/CT and PCR

A total of 108 blood samples collected from cattle Ghana were tested for the detection of antibodies to *B. bigemina*

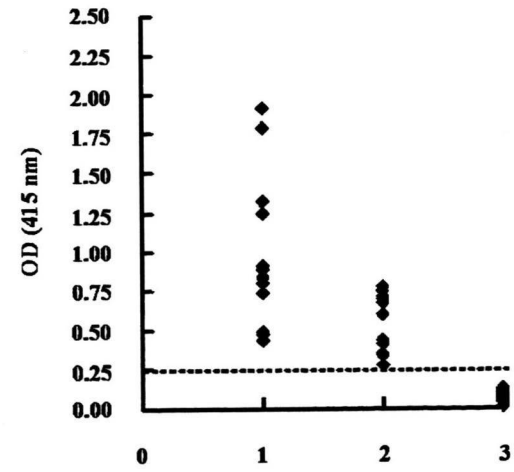

Fig. 4 Reactivity of rhoptry-associated protein 1 (rRAP-1/CT) antigen (*1*) and the *B. bigemina* rP200/CT (*2*) with *B. bigemina*-infected sera and noninfected bovine sera (*3*) in the enzyme-linked immunosorbent assay (ELISA). The *dotted line* represents the cutoff point