sents intracellular growth and the epimastigote number that reflects extracellular growth in the insect vector were also lowered in the presence of these extracts, indicating the existence of potential inhibitors of the parasite growth. In the present study, the crude extracts of various marine algae were used, and therefore, we need to verify whether anti-DHOD compound(s) coincide with anti-*T. cruzi* compound(s). Identification of such an inhibitory compound with both activities from *F. evanescens* and *P. babingtonii* and further screening, using these two assay systems, of other various marine algae may facilitate the discovery of a new, anti-trypanosomal lead compound.

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References

[1] Urbina JA. Chemotherapy of chagas disease. Curr Pharm Des 2002;8:287–95.
[2] Urbina JA, Docampo R. Specific chemotherapy of chagas disease: controversies and advances. Trends Parasitol 2003;19:495–501.
[3] Gutteridge WE, Gaborak M. A re-examination of purine and pyrimidine synthesis in the three main forms of *Trypanosoma cruzi*. Int J Biochem 1979;10:415–22.
[4] Gao G, Nara T, Nakajima-Shimada J, Aoki T. Novel organization and sequences of five genes encoding all six enzymes for de novo pyrimidine biosynthesis in *Trypanosoma cruzi*. J Mol Biol 1999;285: 149–61.
[5] Nara T, Hshimoto T, Aoki T. Evolutionary implications of the mosaic pyrimidine-biosynthetic pathway in eukaryotes. Gene 2000; 257:209–22.
[6] Takashima E, Inaoka DK, Osanai A, Nara T, Odaka M, Aoki T, et al. Characterization of the dihydroorotate dehydrogenase as a soluble fumarate reductase in *Trypanosoma cruzi*. Mol Biochem Parasitol 2002;122:189–200.
[7] Annoura T, Nara, T, Makiuchi T, Hashimoto T, Aoki T. The origin of dihydroorotate dehydrogenase genes of kinetoplastids, with special reference to their biological significance and adaptation to anaerobic, parasitic conditions. J Mol Evol [in press].
[8] Harada H, Noro T, Kamei Y. Selective antitumor activity in vitro from marine algae from Japan coasts. Biol Pharm Bull 1997;20: 541–6.
[9] Isnansetyo A, Horikawa M, Kamei Y. In vitro anti-methicillin-resistant *Staphylococcus aureus* activity of 2,4-diacetylphloroglucinol produced by *Pseudomonas* sp. AMSN isolated from a marine alga. J Antimicrob Chemother 2001;47:724–5.
[10] Nakano T, Noro T, Kamei Y. In vitro promoting activity of human interferon β production by extracts of marine algae from Japan. Cytotechnology 1997;25:237–9.
[11] Sims JJ, Donnell MS, Leary JV, Lacy GH. Antimicrobial agents from marine algae. Antimicrob Agents Chemother 1975;7:320–1.
[12] Kaneda Y, Nagakura K, Goutsu T. Lipid composition of three morphological stages of *Trypanosoma cruzi*. Comp Biochem Physiol, B 1986;83:533–6.
[13] Nakajima-Shimada J, Hirota Y, Aoki T. Inhibition of *Trypanosoma cruzi* growth in mammalian cells by purine and pyrimidine analogs. Antimicrob Agents Chemother 1996;40:2455–8.
[14] Harada H, Kamei Y. Dose-dependent selective cytotoxicity of extracts from marine green alga, *Cladophoropsis vaucheriaeformis*, against mouse leukemia L1210 cells. Biol Pharm Bull 1998;21:386–9.
[15] Nara T, Hirayama-Noguchi Y, Gao G, Murai E, Annoura T, Aoki T. Diversity of aspartate carbamoyltransferase genes of *Trypanosoma cruzi*. Int J Parasitol 2003;33:845–52.
[16] Karibian D. Dihydroorotate dehydrogenase (*Escherichia coli*). Methods Enzymol 1978;51:58–63.
[17] Chen SF, Ruben RL, Dexter DL. Mechanism of action of the novel anticancer agent 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt (NSC 368390): inhibition of de novo pyrimidine nucleotide biosynthesis. Cancer Res 1986;46: 5014–9.
[18] Ittarat I, Asawamahasakda W, Bartlett MS, Smith JW, Meshnick SR. Effects of atovaquone and other inhibitors on *Pneumocystis carinii* dihydroorotate dehydrogenase. Antimicrob Agents Chemother 1995;39:325–8.
[19] Rückemann K, Fairbanks LD, Carrey EA, Hawrylowicz CM, Richards DF, Kirschbaum B, Simmonds HA. Leflunomide inhibits pyrimidine de novo synthesis in mitogen-stimulated T-lymphocytes from healthy humans. J Biol Chem 1998;273:21682–91.
[20] Sanders S, Harisdangkul V. Leflunomide for the treatment of rheumatoid arthritis and autoimmunity. Am J Med Sci 2002;323: 190–3.
[21] McLean JE, Neidhardt EA, Grossman TH, Hedstrom L. Multiple inhibitor analysis of the brequinar and leflunomide binding sites on human dihydroorotate dehydrogenase. Biochemistry 2001;40: 2194–200.
[22] Baldwin J, Farajallah AM, Malmquist NA, Rathod PK, Phillips MA. Malarial dihydroorotate dehydrogenase. Substrate and inhibitor specificity. J Biol Chem 2002;277:41827–34.

表1　エキノコックス感染犬の届け出基準

1. 病原体の検出
虫体またはその一部（片節）の確認
2. 病原体の遺伝子の検出※
PCR 法による遺伝子の検出
3. 病原体の抗原の検出
ELISA 法による成虫由来抗原の検出
（駆虫治療の結果、成虫由来抗原が不検出になったものに限る）

※虫卵はテニア科条虫では形態上区別できないので遺伝子の検出を試みる。

記調査期間で3例，2005年1月の届出制施行後の1例，1997年酪農学園大学で報告された1例の計5例が確認されており，これら感染機会の少ない室内飼育犬の感染例は飼い犬への感染圧の高まりを示している。同時に，室内飼育犬は人との接触がより密接なため，人への感染源としての危険度が高いことは言うまでもない。ただし，室外飼育犬の感染例も放し飼いと密接に関連しており，犬の感染は飼育場所ではなくあくまでもネズミを食べる機会に依存することを理解してほしい。また，15頭の感染確定例のうち6頭は札幌市など市部から検出されており，都市部においても感染例があることも認識する必要がある。1997年以降の飼い犬の感染については，上記以外に，2000年の有珠山噴火時に避難住民が放逐した犬から2頭の抗原陽性例が，北海道行政がキツネの剖検調査（感染率の定点観測）時に同時に行っている犬の剖検（毎年10～20頭程度；主に捕獲犬）で1997年に1頭の感染例が報告されている。

上記調査では本州からも感染犬が検出されており，虫卵のDNA検査で1頭の感染が確定している。この犬は北海道から移動したもので，北海道で感染したものと思われる。さらに，2003～4年に国立感染症研究所が行った，フェリーによって北海道から移動した犬の調査では，検査希望者の飼い犬69頭中2頭で抗原陽性例が報告されている。犬以外では青森県の豚で感染例が報告されているが，野生動物からは報告がなく，本州にエキノコックスが定着している証拠は今のところ得られていない。

2．飼い犬のエキノコックス診断と届け出基準

エキノコックス感染犬の届け出制に対して厚生労働省が作成したガイドライン（http://www.mhlw.go.jp/topics/2004/10/tp1001-4.html）では3つの届け出基準を設けている（表1）。第一は病原体の検出で，虫体または片節を確認した場合，届け出の対象となる。虫卵の検出は，エキノコックスと他のテニア科条虫（猫条虫，胞状条虫や豆状条虫など）の虫卵が形態的に区別できないため，虫卵の検出のみでは届け出対象とならない（遺伝子診断が必要）。第二は遺伝子の検出であるが，現在エキノコックスの遺伝子検査をルーチンで行っている検査機関はなく，研究あるいは公共サービスの一環として専門機関が請け負っているのが現状である。したがって，遺伝子検査は，虫体や虫卵が検出されるなどエキノコックス感染が強く疑われる場合にかぎり確定診断のために行われている。第三は抗原の検出で，エキノコックス成虫の排泄分泌物が犬の糞便中に出てくるものを捉える方法である。本法は虫卵排出前（エキノコックスは幼虫が犬に感染してから虫卵を排出するまで約1か月を要する）の検査でも感染を検出できるため有用性の高い診断法であるが，一定の確率で偽陽性反応が検出される。北海道大学が開発した抗原検出法（環境動物フォーラムにより実施）では，0.2～0.5％の確率で偽陽性反応が検出されている。ところが，飼い犬の感染率も0.4～0.7%（偽陽性率のほぼ2倍程度）と低いため，抗原検査における陽性反応のほぼ2/3は真陽性（感染），1/3は偽陽性（非感染）となる。したがって，抗原の検出のみ（虫卵陰性）では確定診断することはできず，駆虫治療の結果、抗原が不検出になったものに限って届け出義務が発生することになる。

おわりに

北海道の登録犬は約23万頭であり，未登録犬を含めると推定30～50万頭の犬が北海道で飼育されている。したがって，相当数の飼い犬がエキノコックスに感染していると考えられ，その中には北海道外へ移動する犬も含まれるであろう。北海道で人の感染リスクを減らすために，さらには本州への拡散を防止するために，飼い主，獣医師および行政がこれらの状況を十分に認識して、ペットの適切な飼育管理と感染予防にあたらなければならない。

飼い犬のエキノコックス感染とその診断

野中成晃

要　約

2004年10月から獣医師によるエキノコックス感染犬の届け出が義務づけられた。これまでの調査の結果（検査希望者を対象），北海道では飼い犬の0.4～0.7%がエキノコックスに感染していることがわかり，また北海道から本州へ移動した犬からも感染例が見つかった。この状況を考えると，獣医師，特に北海道の臨床家がエキノコックス感染犬に遭遇する機会は少なくなく，本稿では，日本における飼い犬のエキノコックス感染状況とその診断および届け出基準について概説する。

はじめに

北海道に蔓延する人獣共通寄生虫，エキノコックス（多包条虫）は成虫がキツネや犬などの犬科動物（終宿主）に，幼虫が齧歯類（中間宿主）に寄生する（図1）。人への感染は終宿主糞便中に排泄される虫卵の摂取によって起こり，放置すると死に至る。北海道では，過去10年間のキツネの感染率は40%前後を推移し，人とキツネの行動圏の重なりにより人とペットへの感染リスクが増している（図1）。人と密接に関係する飼い犬の感染は人の感染源として無視できない。このような状況の中，感染症法の改正に伴って2004年10月から獣医師によるエキノコックス感染犬の届け出制が施行され，2005年1月には早くも初の届け出がなされた。本稿では，日本における飼い犬の感染状況およびその診断と届け出基準について概説する。

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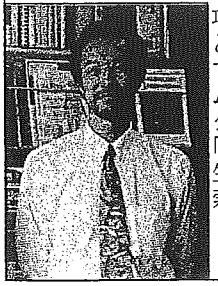

巧みに生きている「寄生虫」に人生を学べ！とこの世界へ入り，エキノコックスと関わって早20年。北大・獣医・寄生虫学教室のチームプロジェクトとして進んできたエキノコックス対策ですが，まだまだ課題は山積みです。「昨日より今日，今日より明日」の精神で，寄生虫の専門家として貢献できることを日々模索しています。

1．飼い犬のエキノコックス感染状況

北海道大学と環境動物フォーラム（http://www.k3.dion.ne.jp/~fea/）では1997年より獣医師を通して飼い犬のエキノコックス検査を実施してきた。検査は糞便を材料とし，虫卵および糞便内抗原検査をまず行い，陽性または擬陽性となったものについては飼い主に駆虫を依頼し，駆虫前後の糞便を再検査する；また，虫卵が検出された場合にはさらに虫卵DNA検査（PCR）を行うというものである。

2004年6月までに北海道の飼い犬3,688頭および本州の飼い犬152頭の検査を実施し，それぞれ15～24頭（感染率0.4～0.7%）および1～2頭の感染例が検出された。感染頭数に幅があるのは，感染していた可能性が強いが確定判定ができなかったものを含めたためである。これらの犬の飼育方法を分析すると，普段放し飼いの犬や散歩時に放される犬での感染率が高く，ネズミを食べる機会に依存している。記憶に新しいところでは，2002年12月に新聞等で「札幌市の室内飼育犬の感染」が報道され各所で議論された。この犬は外出時に放されることがよくあり，そこでネズミを食べて感染したものと思われる。1997年以降，このように普段室内で飼育されている犬の感染例が上

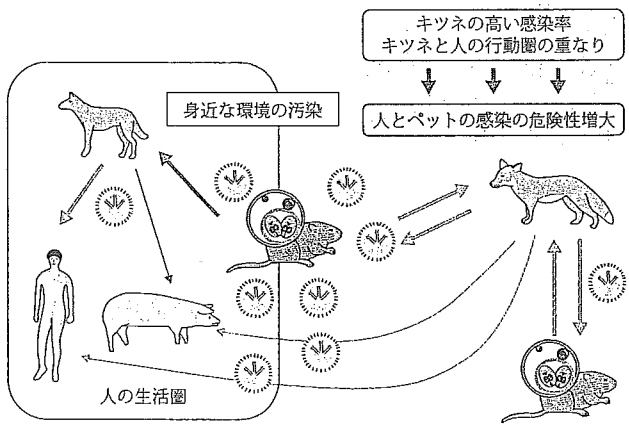

図1　人の生活圏の虫卵汚染

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Towards the control of *Echinococcus multilocularis* in the definitive host in Japan

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Abstract

Echinococcus multilocularis is distributed all over Hokkaido, the northern island of Japan. The prevalence in foxes has been around 40% in the last decade. Three trials of anthelmintic bait distribution have been conducted in Hokkaido to reduce the prevalence in foxes. In those trials, bait distribution was done along roads in the study area using cars and/or around fox breeding dens by hand. Changes in the prevalence in foxes were evaluated either by necropsy of captured foxes or by coproantigen and egg detection of faeces collected in field. All of the trials showed bait distribution was effective for the reduction of the prevalence in foxes; however, it was also suggested that a frequent and continuous baiting program is necessary for effective and stable control of the prevalence in foxes. As observed in some cities in Europe, urban foxes infected with the parasite were also recognized in Sapporo. A survey of pet dogs showed that 0.4% of surveyed dogs were determined infected. In addition, a dog which was transported from Hokkaido to the main island of Japan was found excreting *E. multilocularis* eggs. The results raised the public recognition of canine infections, which in turn lead to the modification of a Japanese law for infectious diseases and to the enforcement of a national reporting system of dogs infected with *E. multilocularis* by veterinarians.

Keywords: *Echinococcus multilocularis*; Foxes; Dogs; Control; Bait distribution; Japan

1. Introduction

High prevalence in foxes with *Echinococcus multilocularis* infection is now recognized in Hokkaido (78,500 km^2, 5,660,000 inhabitants), Japan. To monitor the change of prevalence, the Hokkaido government has performed necropsy surveys of foxes captured in winter at various sites, showing an overall prevalence of 19.1% in 23,852 foxes surveyed during 1966–2003. However, since the mid-1980s, the prevalence in foxes has tended to increase and has been around 40% in the last decade. Our recent necropsy surveys conducted at the suburbs of the city of Sapporo showed similar high prevalences in foxes [1,2]. In those surveys, 6 raccoon dogs (*Procyonoides necteuretes*) 1 live raccoon dog was found excreting taeniid eggs should be intact taeniid eggs were found in the rectal faeces in one of the raccoon dogs.

Infections in domestic dogs were also found in Hokkaido. According to the necropsy survey conducted by Hokkaido government, 99 dogs (1.0%) were found infected out of 9,907 dogs during the period 1966–2003. However, most of the dogs were examined before 1990 and only <15 dogs on average per year were examined over the last decade. Therefore, the data did not provide up-to-date infection status for dogs in Hokkaido.

Accordingly, effective counter-measures against high prevalence in foxes and precise evaluation of infection status of pet dogs are necessary in Hokkaido. In this paper, recent trials and their achievements toward the control of the infection in foxes and pet dogs in Hokkaido are reviewed.

2. Baiting against *E. multilocularis* in foxes

The first deworming trial against *E. multilocularis* infection in foxes was conducted in Germany in a study area of 566 km^2 [3]. Baits containing 50 mg of praziquantel were repeatedly distributed in the study area. The deworming effect was most pronounced in the central part of the baiting area; however, the reduction of the prevalence was moderate in marginal areas. In that campaign, high hunting pressure for foxes (2.2 foxes/km^2)

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for the prevalence evaluation created free niches in the baiting area to which foxes residing outside the baiting area migrated. The observed higher prevalence in the marginal part was, therefore, due to the effect of those migrating foxes. This deworming trial showed that a bait distribution is effective for reducing the prevalence in foxes; however, it also suggested that the scale of operation must be large enough to have the core area covered, in order to evaluate a true effect of bait distribution if the prevalence in foxes is evaluated by necropsy.

Use of coproantigen detection techniques in field studies enables surveys with minimal disturbance in the local ecology because the prevalence in foxes could be estimated from faeces [4–6]. In our laboratory, a monoclonal antibody (EmA9) based sandwich ELISA was developed for coproantigen detection [7]. Using this method, a deworming trial was conducted in Koshimizu [8]. After a preliminary survey of the prevalence in foxes from 1997 to 1998 [9], the study area (200 km^2) was divided into two parts (Fig. 1), one (occupied by 18 fox families) with bait distribution and the other (20 fox families) without bait distribution. Baits containing 25 to 50 mg of praziquantel were repeatedly distributed around each fox breeding den. The changes of the prevalence in foxes were evaluated by the coproantigen and fecal egg examination of field collected fox faeces. It was observed that egg containing faeces was rapidly reduced in the bait distributed area. In contrast, coproantigen positive faeces was not dramatically reduced in the first year, indicating that foxes were readily re-infected by ingesting the intermediate hosts, which had been infected before the bait distribution. However, obvious reduction in the number of coproantigen positive faeces was recognized from the second year probably due to the decrease of infected rodents. The results suggested that longer-term strategic bait distribution would be required for the efficient control of *Echinococcus* infection in foxes.

Based on these results, a new deworming program was conducted in 2001 to 2002 for covering the whole study area in Koshimizu. In the program, baits were distributed along roads in the town (20 baits/km) or at the cross sections of roads and wind-shielding forests (20 baits/km^2). This 2-year bait distribution resulted in the successful reduction of environmental egg contamination. However, the proportion of egg containing faeces has been gradually increasing after completion of the campaign.

In the evaluation of prevalence in foxes using faeces collected in field, identification of fox faeces were critically important. Most of the studies have been conducted with ambiguous identification of the origins of faeces, which was based on the size, shape, color and odor of the faeces. Accordingly, none of the criteria alone or combinations could perfectly distinguish fox faeces from other carnivore faeces. Therefore, the survey results were always accompanied with a certain level of bias. Recent advancement of molecular techniques have enabled the identification of the origin of faeces from fecal DNA, which is derived from sloughed intestinal mucosal cells excreted with faeces [10]. In our laboratory, a multiplex PCR system for the identification of carnivore animals should be for the identification of foxes and other carnivore animals in Hokkaido (raccoons, raccoon dogs, weasels, dogs and cats), which excrete faeces resembling fox faeces has now been developed and applied to field study. With this kind of technique, it will be feasible to evaluate more precisely the prevalence in individual carnivore species.

Another approach for evaluation of baiting has been conducted since 2001 in Otaru. In this trial, tetracycline (TC)

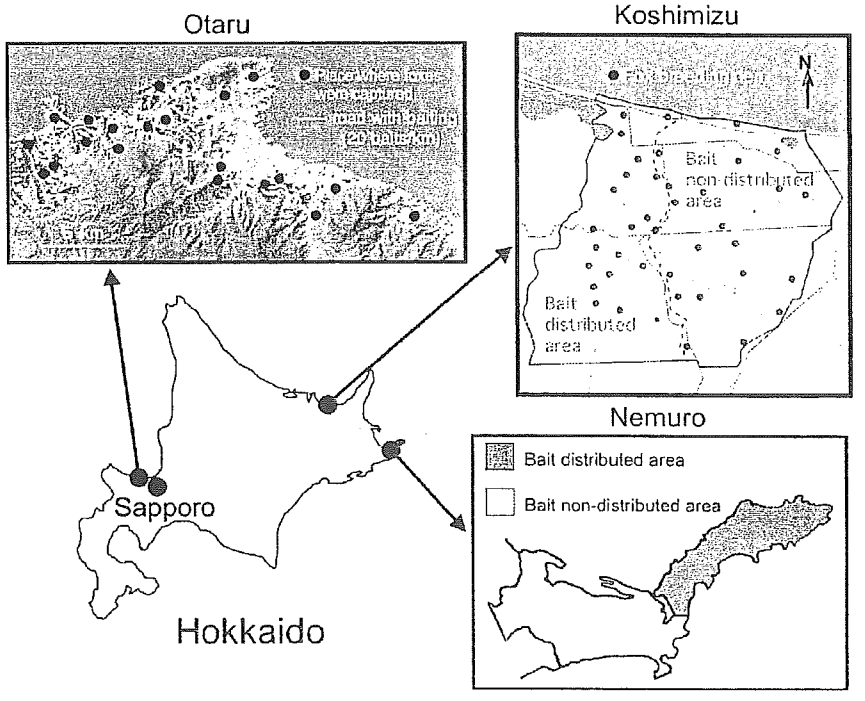

Fig. 1. Places where anthelmintic bait distribution trials were conducted in Hokkaido.

was put into the anthelmintic baits as a biomarker so that consumption of the baits by foxes could be detected by the examination of the TC line in the canine teeth. The baits were distributed along roads in the study area (120 km^2, 20 baits/km) (Fig. 1). The prevalence in foxes was evaluated by the necropsy of the animals captured by local hunters. Majority of the foxes having a TC line on teeth did not harbor the parasites, indicating foxes consuming the baits were effectively dewormed. Detailed report of this study is now being prepared.

In Nemuro, another baiting trial has also been conducted by Hokkaido Institute of Public Health since 1999 to 2004 [11]. The study area has a geographical advantage for the trial because the bait distributed area (135 km^2) is a protruding peninsula with a lake almost crossing its base part so that animal movement to and from the bait distributed area is restricted to minimum (Fig. 1). In this trial, single to multiple bait distributions along roads (15 baits/km^2) and around fox breeding dens were conducted annually and the prevalence of foxes were evaluated by necropsy. According to the preliminary data up to March 2002, a tendency of the reduction in the prevalence in foxes was observed in the bait distributed area.

The bait distribution trials in Hokkaido seemed to be effective for reduction of *E. multilocularis* prevalence in foxes. In Koshimizu, bait distribution has started again in 2004 and a new campaign is also planned in Kucchan, both of which are conducted by local residents with support by our laboratory and Forum of Environmental Animals. Although the scales of the campaigns are small (local town base), participation of local residents in the control measure against *E. multilocularis* infection in foxes is a new movement in the control strategy. There are many hurdles to clear, however, we hope this movement will be a driving force to a large scale control program in near future.

3. A possible urban cycle in Sapporo

There are increasing observations that foxes inhabit urban areas [12]. Infected foxes with *E. multilocularis* have been reported in several European cities and a risk of urban residents to get the infection has been increasing. An active urban cycle of the parasite was recognized in Zurich where counter-measures against the infected foxes were conducted [6].

In Sapporo, coproantigen positive fox faeces were found in the parks or woodlands of the urban area where foxes had their dens [13]. Infected foxes with *E. multilocularis* were found by necropsy in four out of six fox families inhabiting in northeastern region of Sapporo (unpublished data). In those studies, arvicolid rodents were captured at the urban fringe, although none were infected with *E. multilocularis*, arvicolid rodents were captured at the urban fringe. Those studies suggested that the urban fringe offers a potential condition for the maintenance of *E. multilocularis* life cycle.

For preparing a future bait distribution in Sapporo, bait uptake by foxes in Sapporo were evaluated by a photo-trap system [14]. Foxes were photo-trapped at seven out of eight baiting sites where foxes were previously observed by local residents or where the inhabitance of foxes could be suspected from the local environment. The result suggested that, by collecting local information (witness) of foxes, bait distribution to foxes could be efficiently conducted in the urban area of Sapporo.

Table 1
Criteria for diagnosis in the national reporting system for dogs infected with *E. multilocularis*

(1) Finding the parasite body, which can be morphologically identified
(2) Detecting the parasite DNA from eggs or a part of the parasite body
(3) Detecting the parasite coproantigen, which should turn to be negative due to deworming treatment

4. Infections in pet animals and enforcement of national reporting system of infected dogs

In central Europe, domestic dogs were infected with *E. multilocularis* with prevalence between 0.3% and 7% in endemic regions [12]. It was estimated that more than 10% of dogs would be infected at least once in their life even in the low prevalent regions [15]. In some endemic areas such as in Gansu, China and in St. Lawrence Island, USA, dogs play important roles both in the maintenance and in the transmission of echinococcosis to humans [12].

In Hokkaido, little studies have been done on the recent prevalence of pet dogs (number of registered dogs in 2003 was 248,149). In our laboratory, a survey of pet dogs has been conducted since 1997 using a diagnostic system composed of coproantigen, faecal egg and faecal egg DNA examinations. The detailed survey report is now prepared for publication, but briefly, up to June in 2004, 15 (0.4%) dogs were determined infected, among which 8 dogs were confirmed excreting *E. multilocularis* eggs by PCR. In addition, a dog which was transported from Hokkaido to the main island of Japan (Honshu) was found excreting *E. multilocularis* eggs.

The survey result raised the public recognition of canine infections, which in turn lead to the modification of a Japanese law for infectious diseases and a national reporting system of dogs infected with *E. multilocularis* by veterinarians has been enforced from October in 2004 to monitor the occurrence of the canine infections by the country base (Table 1).

Finding of an infected dog which moved from Hokkaido to the main island of Japan raised attention for the risk of animal movement in disease introduction to non-endemic areas. The issue was emphasized in Europe [12] and some countries such as UK and Norway have actually enforced some regulations for transporting animals to prevent introduction or spreading of the disease. In near future, if the prevalence in foxes in Hokkaido will continue to be high, establishment of similar regulation may be necessary in Japan.

5. Conclusion

As represented by the appearance of urban foxes, human–fox contacts have been frequently observed in the human living environment. Accordingly, infection pressures to pet animals are increasing. Considering the high prevalence in foxes and

potential risk for pet animal infections with *E. multilocularis*, management of the disease in wildlife and pet animals is now ultimately required. At present, a large-scale efficient control measure for wildlife has not been completely established. Nevertheless, risk control by individuals with baiting foxes coming in contact to each individual or with proper management of their pet animals could be immediately started. Such an effort for the disease control with individual or small scale applications would reduce the local risk of echinococcosis and moreover lead to a regional and national program for the risk control of alveolar echinococcosis.

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References

[1] Morishima Y, Tsukada H, Nonaka N, Oku Y, Kamiya M. Evaluation of coproantigen diagnosis for natural *Echinococcus multilocularis* infection in red foxes. Jpn J Vet Res 1999;46:185–9.

[2] Yimam AW, Nonaka N, Oku Y, Kamiya M. 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 2002;49:287–96.

[3] Schelling U, Frank W, Will R, Romig T, Lucius R. Chemotherapy with praziquantel has the potential to reduce the prevalence of *Echinococcus multilocularis* in wild foxes (*Vulpes vulpes*). Ann Trop Med Parasitol 1997;91:79–86.

[4] Raoul F, Deplazes P, Nonaka N, Piarroux R, Vuitton DA, Giraudoux P. Assessment of the epidemiological status of *Echinococcus multilocularis* in foxes in France using ELISA coprotests on fox faeces collected in the field. Int J Parasitol 2001;31:1579–88.

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

[6] Hegglin D, Ward PI, Deplazes P. Anthelmintic baiting of foxes against urban contamination with *Echinococcus multilocularis*. Emerg Infect Dis 2003;9:1266–72.

[7] Nonaka N, Iida M, Yagi K, Ito T, Ooi HK, Oku Y, et al. Time course of coproantigen excretion in *Echinococcus multilocularis* infections in foxes and an alternative definitive host, golden hamsters. Int J Parasitol 1996;26:1271–8.

[8] Tsukada H, Hamazaki K, Ganzorig S, Iwaki T, Konno K, Lagapa JT, et al. Potential remedy against *Echinococcus multilocularis* in wild red foxes using baits with anthelmintic distributed around fox breeding dens in Hokkaido, Japan. Parasitology 2002;125:119–29.

[9] Morishima Y, Tsukada H, Nonaka N, Oku Y, Kamiya M. Coproantigen survey for *Echinococcus multilocularis* prevalence of red foxes in Hokkaido, Japan. Parasitol Int 1999;48:121–34.

[10] Kohn MH, Wayne RK. Facts from feces revisited. Trends Ecol Rev 1997;12:223–7.

[11] Takahashi K, Uraguchi K, Romig T, Hatakeyama H, Tamura M. Preliminary report on *Echinococcus multilocularis* control by fox baiting with praziquantel. Rep Hokkaido Inst Publ Health 2002;52:61–3 [in Japanese].

[12] Deplazes P, Eckert J. Veterinary aspects of alveolar echinococcosis—a zoonosis of public health significance. Vet Parasitol 2001;98:65–87.

[13] Tsukada H, Morishima Y, Nonaka N, Oku Y, Kamiya M. Preliminary study of the role of red foxes in *Echinococcus multilocularis* transmission in the urban area of Sapporo, Japan. Parasitology 2000;120:423–8.

[14] Takahashi K, Uraguchi K, Hatakeyama H. Observation on acceptance of fox-baits in an urban area using photo-trap system. Rep Hokkaido Inst Public Health 2003;53:95–101 [in Japanese].

[15] Deplazes P, Hegglin D, Gloor S, Romig T. Wilderness in the city: the urbanization of *Echinococcus multilocularis*. Trends Parasitol 2004;20:77–84.

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Epidemiological study of equine piroplasmosis in Mongolia

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Abstract

The purpose of this study was to demonstrate the occurrence of equine piroplasmosis in Mongolia, a country in which the disease occurs epidemically in different climatic conditions. Antibodies to *Babesia equi* and *B. caballi* were determined in serum samples of 254 pastured horses in different locations of Mongolia using an enzyme-linked immunosorbent assay with recombinant antigens. One hundred and eighty-five (72.8%) and 102 (40.1%) of all serum samples were positive for *B. equi* and *B. caballi* infections, respectively. In addition, 78 (30.7%) samples were positive for both *B. equi* and *B. caballi* infections. These results indicate that equine piroplasmosis is widespread in Mongolia. To our knowledge, this is the first report describing an epidemiological study on equine piroplasmosis in different geographic regions in Mongolia.

Keywords: *Babesia equi*; *Babesia caballi*; ELISA; EMA-1; P48

Equine piroplasmosis, caused by *Babesia equi* and *B. caballi*, is considered to be the most important tick-borne disease of horses in tropical and subtropical areas (Schein, 1988). Babesiosis is generally characterized by fever, anemia, jaundice, and edema. In some cases, it causes the death (Freidhoff, 1982; Bruning et al., 1997; Schein, 1988; De Waal, 1992). Mongolia, a country located in a landlocked plateau of Central Asia, covering an area of 1,566,500 km^2, has a human population of 2.5 million and a horse population of 2.2 million and is known for its pasture animal husbandry, which raises thousands of livestock species, including horses. The country is mountainous, with an average altitude of 1580 m a.s.l. The geography of the country is characterized by great diversity, such as a mountain-forest steppe, a mountain steppe, a semi-desert, and a desert. The climate is continental, with long cold, dry winters and short warm summers. The average temperature ranges from 20 to 25 °C in summer (July) and −20 to −32 °C in winter (January). In Mongolia, the provinces of Tuv, Sukhbaatar, Selenge, Khovd, Uvs, and Umnugobi are

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situated in different climatic zones. Using the conventional thin blood smear examination, indirect fluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) babesiosis has been documented as widespread in horses of Central Mongolia (Dash, 1966; Avarzed et al., 1997; Xuan et al., 1998; Ikadai et al., 2000). Tick infestation with *Dermacentor nuttalli*, *Dermacentor silvarum*, and *Hyalomma dromedari* is common in Mongolian horses (Dash, 1966; Byambaa et al., 1994). Amplification of specific equine *Babesia* gene fragments in field-collected blood samples, *D. nuttalli* adult ticks (i.e., unfed), and *D. nuttalli* ticks (i.e., partially engorged) on horses in Mongolia has been reported. These reports suggest the role of tick vector in *Babesia* transmission. Furthermore, the detection of parasite DNA in eggs and larvae is suggestive of transovarial parasite transmission in this species (Battsetseg et al., 2001, 2002). In endemic countries, the control of equine piroplasmosis is important to keep international markets open to the horse industry (Freidhoff, 1988). *Babesia* seroprevalence in horses is a good indicator of tick distribution (Tenter and Friedhoff, 1986). In the present study, we performed a preliminary epidemiological study on equine piroplasmosis in different environmental areas of Mongolia using recombinant ELISA antigens.

ELISA with recombinant *B. equi* merozoite antigen-1 (EMA-1) expressed by baculovirus in insect cells for the diagnosis of *B. equi* infection in horses was performed as described elsewhere (Xuan et al., 2001a, 2001b). EMA-1 was purified from a recombinant baculovirus AcEMA-1-infected Sf9 cell culture and used as an ELISA antigen for detecting antibodies to *B. equi* in horses. The ELISA using recombinant P48 protein expressed in *Escherichia coli* by the pGEX vector for the diagnosis of *B. caballi* infection in horses was carried out as described elsewhere (Ikadai et al., 1999). To evaluate whether the recombinant EMA-1 expressed by baculovirus and recombinant P48 expressed by *E. coli* can be suitable antigens for use in the diagnosis of *B. equi* and *B. caballi* infections in horses, the related antibodies were tested in an ELISA. Serum samples from horses, experimentally infected with *B. equi* and *B. caballi*, and healthy horses in Japan were used as positive and negative controls, respectively. The serum samples from horses experimentally infected with *B. equi* reacted positively to recombinant EMA-1 antigen (optical densities >0.1), while serum samples from 10 normal horses and 10 horses experimentally infected with *B. caballi* were negative (optical densities >0.1). The serum samples from horses experimentally infected with *B. caballi* reacted positive to recombinant P48 antigen (optical densities >0.1), while serum samples from 10 normal horses and 10 horses experimentally infected with *B. equi* were negative (optical densities >0.1). The ELISA titer was expressed as the reciprocal of the maximum dilution that showed an ELISA value equal to or greater than 0.1, which is the difference in absorbance between that for the recombinant EMA-1 antigen or for the control lacZ antigen well and recombinant P48 antigen or for the control GST antigen well, respectively.

A total of 254 serum samples were collected from pastured horses in the Tuv, Sukhbaatar, Selenge, Khovd, Uvs, and Umnugobi provinces in Mongolia (Table 1). Although no parasites were detected in the Giemsa-stained blood smears from any of 254 horses, 185 (72.8%) and 102 (40.1%) were ELISA positive for *B. equi* and *B. caballi* infections, respectively (Table 1). Seventy-eight (30.7%) samples were positive and 45 (17.7) samples were negative for both *B. equi* and *B. caballi* infections (Table 2). The ages of the positive horses varied from months to 20 years. *B. equi* and

Table 1
Prevalence of equine piroplasmosis in Mongolia[a]

Province	No. of examined	No. of positive (%)	
		B. equi[b]	*B. caballi*[c]
Tuv	46	36 (78.2)	29 (63.0)
Selenge	39	22 (56.4)	7 (17.9)
Sukhbaatar	47	39 (82.9)	19 (40.4)
Khovd	48	19 (39.5)	10 (20.8)
Uvs	23	20 (86.9)	6 (26.1)
Umnugobi	51	49 (96.0)	31 (60.7)
Total	254	185 (72.8)	102 (40.1)

[a] Values in parentheses are in percentage.

[b] Antibodies to *B. equi* were detected by ELISA using the recombinant EMA-1 expressed in insect cells. The ELISA was considered positive when an optical density at 415 nm equal to or greater than 0.1 was observed at dilutions of 1:100 and above.

[c] Antibodies to *B. caballi* were detected by ELISA using the recombinant P48 expressed in *E. coli*. The ELISA was considered positive when an optical density at 415 nm equal to or greater than 0.1 was observed at dilutions of 1:100 and above.

Table 2
Mixed infection of *B. equi* and *B. caballi* in horses in Mongolia[a]

	B. equi +	*B. equi* −	Total
B. caballi +	78 (30.7)	24 (9.5)	102 (40.2)
B. caballi −	107 (42.1)	45 (17.7)	152 (59.8)
Total	185 (72.8)	69 (27.2)	254 (100)

[a] Values in parentheses are in percentage.

B. caballi antibodies were detected in all age groups, except for 1–3-month-old foals (data not shown).

An estimate of the prevalence of a parasitic infection in a population can be made by identifying the agent in animals or through serologic studies. Seroprevalence of *B. equi* and *B. caballi* have been performed in horses, using the ELISA, PCR, IFAT, and latex agglutination tests. Prevalence studies of *B. equi* and *B. caballi* infections in Central Mongolia showed that 88.2 and 84.5% of horse serum samples were positive by IFAT, respectively (Avarzed et al., 1997); 20 and 69% were positive for *B. caballi* by PCR and IFAT, respectively (Xuan et al., 1998); 46.4% were positive for *B. caballi* by ELISA (Ikadai et al., 2000); 90% were positive for *B. equi* by LAT (Xuan et al., 2001a, 2001b); and 89% were positive for *B. equi* by ELISA (Xuan et al., 2001a, 2001b). In the present study, the examination of field samples from different geographical regions of Mongolia shows that the prevalence of *Babesia* in horses ranges mostly 17.9–96.0% and that the prevalence was higher for *B. equi* than for *B. caballi* infection. The results strongly suggest that *Babesia* is widely spread in Mongolia. There was a lower prevalence of equine babesiosis in subtropical areas as opposed to tropical areas. In this study, we demonstrated a higher prevalence of equine babesiosis in the semi-desert southern region. This is probably related to the optimal development of the tick population (Kerber et al., 1999).

B. caballi is almost always diagnosed as a mixed infection with *B. equi* in Mongolian horses (Avarzed et al., 1997; Battsetseg et al., 2001, 2002). Battsetseg et al. (2001) used PCR to demonstrate mixed infection *B. equi* and *B. caballi* in unfed female *D. nuttalli*. Considering these ticks might be play an important role in initiating mixed infection of horses in Mongolia. Equine husbandry in Mongolia has a nomadic pasture system, which includes seasonal migration and rotations of migration routes. In the last several years, the equine industry was forced to move a long distance away because of the winter disaster in Mongolia. Movement through endemic areas may contribute to infestation of horses with tick vectors and infection with *B. equi* and *B. caballi*.

Data, obtained in this study, contributes to understanding the prevalence of equine piroplasmosis in Mongolia. The prevalence of *Babesia* infection of horses in Mongolia suggests that endemic stability is present in the areas studied. Such data should be valuable to the Mongolian authorities that are in charge of determining national tick control policies.

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References

Avarzed, A., De Waal, D., Igarashi, I., Saito, A., Oyamada, T., Toyoda, Y., Suzuki, N., 1997. Prevalence of equine piroplasmosis in central Mongolia. Onderstepoort J. Vet. Res. 64, 141–145.

Battsetseg, B., Xuan, X., Ikadai, H., Jose, R.B.L., Byambaa, B., Boldbaatar, D., Battur, B., Battsetseg, G., Batsukh, Z., Igarashi, I., Nagasawa, H., Mikami, T., Fujisaki, K., 2001. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. Int. J. Parasitol. 31, 384–386.

Battsetseg, B., Susana, L., Xuan, X., Claveria, F., Byambaa, B., Battur, B., Boldbaatar, D., Batsukh, Z., Khaliunaa, T., Battsetseg, G., Igarashi, I., Nagasawa, H., Fujisaki, K., 2002. Detection of equine *Babesia* spp. gene fragments in *Dermacentor nuttalli* Olenev 1929 infesting Mongolian horses, and their amplification in egg and larval progenies. J. Vet. Med. Sci. 64, 727–730.

Bruning, A., Phipps, P., Posnett, E., Canning, E.U., 1997. Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. Vet. Parasitol. 68, 11–26.

Byambaa, B., Dash, M., Tarasevich, I.V., 1994. Newly Diagnosed Rickettsiosis. Esen erdene, Ulaanbaatar, pp. 10–13 (in Mongolian).

Dash, M., 1966. Haemosporidioses of Horses. Khevlekh uildver, Ulaanbaatar, pp. 7–9 (in Russian).

De Waal, D.T., 1992. Equine piroplasmosis: a review. Br. Vet. J. 148, 6–14.

Freidhoff, K.T., 1982. The piroplasms of equidae. Significance for the international equine trade. Berl. Muench. Teiraertzl. Wonchenschr. 95, 368–374.

Freidhoff, K.T., 1988. Transmission of babesia. In: Ristic, M. (Ed.), Babesiosis of Domestic Animals and Man. CRC Press, Boca Raton, FL, pp. 23–52.

Ikadai, H., Xuan, X., Igarashi, I., Tanaka, S., Kanemaru, T., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 1999. Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry protein and potential use of the recombinant antigen in an enzyme-linked immmunosorbent assay. J. Clin. Microbiol. 37, 3475–3480.

Ikadai, H., Osorio, C.R., Xuan, X., Igarashi, I., Kanemaru, T., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2000. Detection of *Babesia caballi* infection by enzyme-linked immmunosorbent assay using recombinant 48-kDa merozoite rhoptry protein. Int. J. Parasitol. 30, 633–635.

Kerber, C.E., Ferreira, F., Pereira, M.C., 1999. Control of equine piroplasmosis in Brazil. Onderstepoort J. Vet. Res. 66, 123–127.

Schein, E., 1988. Equine babesiosis. In: Ristic, M. (Ed.), Babesiosis of Domestic Animals and Man. CRC Press, Boca Raton, FL, pp. 197–209.

Tenter, A.M., Friedhoff, K.T., 1986. Serodiagnosis of experimental and natural *Babesia equi* and *Babesia caballi* infections. Vet. Parasitol. 20, 49–61.

Xuan, X., Igarashi, I., Avarzed, A., Ikadai, H., Inoue, N., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 1998. Diagnosis of *Babesia caballi* infection in horses by polymerase chain reaction. J. Protozool. Res. 8, 85–89.

Xuan, X., Larsen, A., Ikadai, H., Tanaka, T., Igarashi, I., Nagasawa, H., Fujisaki, K., Toyoda, Y., Suzuki, N., Mikami, T., 2001a. Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 39, 705–709.

Xuan, X., Igarashi, I., Tanaka, T., Fukumoto, S., Nagasawa, H., Fujisaki, K., Mikami, T., 2001b. Detection of antibodies to *Babesia equi* in horses by a latex agglutination test using recombinant EMA-1. Clin. Diagn. Lab. Immunol. 8, 645–646.

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Cloning of a Novel *Babesia equi* Gene Encoding a 158-Kilodalton Protein Useful for Serological Diagnosis

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In this study, we characterized a *Babesia equi* Be158 gene obtained by immunoscreening a *B. equi* cDNA expression phage library with *B. equi*-infected horse serum. The Be158 gene consists of an open reading frame of 3,510 nucleotides. The recombinant Be158 gene product was produced in *Escherichia coli* and used for the immunization of mice. In Western blot analysis, mouse immune serum against the Be158 gene product recognized 75- and 158-kDa proteins from the lysate of *B. equi*-infected erythrocytes. In an indirect fluorescent-antibody test with the mouse immune serum, the Be158 antigen appeared in the cytoplasm of Maltese cross-forming parasites (which consist of four merozoites) and was located mainly in the extraerythrocytic merozoite body. When the recombinant Be158 gene product was used in an enzyme-linked immunosorbent assay as a serological antigen, it was found to react to *B. equi*-infected horse sera, indicating that the Be158 gene product is useful as a serologically diagnostic antigen for *B. equi* infection.

Babesiosis, a well-recognized disease of veterinary importance in horses, cattle, and dogs, is gaining attention as an emerging zoonotic disease (15). It has been found in a wide variety of mammals but is perhaps most prevalent in rodents, carnivores, and cattle (25). Two species of *Babesia* parasites, *Babesia equi* and *Babesia caballi*, infect equids (22). Acute equine babesiosis is characterized by fever, anemia, icterus, hepatosplenomegaly, lethargy, and in some cases death (4, 5, 22), leading to great economic losses in the horse industry (14). The infected horses often remain carriers of the parasites for a long period and are known to act as sources for subsequent infections for other horses via tick vectors (11). Therefore, the development of a high-quality system for the serological diagnosis of babesial infection is necessary. In Japan, no clinical cases of equine babesiosis have been reported up to now (12), but there has been a long-term increase in the number of horses imported from foreign countries, including those from areas where equine babesiosis is endemic. The existence of two tick vectors, *Dermacentor reticulates* and *Rhipicephalus sanguineus*, has also been reported in Japan (28). These conditions indicate that Japan is facing the risk of the introduction of infected or carrier horses.

Recently, we reported an enzyme-linked immunosorbent assay (ELISA) that is specific for the detection of equine anti-*B. equi* antibodies by using a recombinant Be82/236–381 gene product as the antigen (9, 10). The serodiagnostic ELISA could clearly distinguish the *B. equi*-infected horse sera from noninfected or *B. caballi*-infected horse sera (9, 10). However, in order to analyze all sera infected with various types of field strains, further study was necessary to search for other serological antigens applicable in epidemiological surveys. These studies might lead to a more practical usage of ELISA worldwide.

In this study, we identified a novel Be158 gene by immunoscreening a cDNA library with *B. equi*-infected horse serum and characterized the gene product immunologically in *B. equi*-infected equine erythrocytes. Subsequently, the recombinant gene product was subjected to ELISA and evaluated for its serologically diagnostic utility against *B. equi* infection.

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MATERIALS AND METHODS

Parasites. U.S. Department of Agriculture strains of *B. equi* and *B. caballi*, which had been kindly provided previouslyby the Equine Research Institute of the Japan Racing Association, were grown in equine erythrocytes in vitro as described by Avarzed et al. (1, 2). The parasite development was monitored by microscopic observation of Giemsa-stained thin smears.

Immunoscreening of a *B. equi* cDNA expression phage library and DNA sequencing. The immunoscreening and DNA sequencing were performed as described previously (9). Open reading frame (ORF) and protein homology searches were performed using the Mac Vector program (Oxford Molecular Ltd., Oxford, United Kingdom) and the National Center for Biotechnology Information database, respectively.

Expression and purification of the recombinant Be158 gene product in *Escherichia coli*. Two oligonucleotide primers, 5′-acgtcgacAAATGAGGTTACGCACGCAGA-3′ and 5′-acgcggccgcTTAAACATTGCTAGA-3′ (lowercase letters form SalI and NotI restriction site linkers, respectively), were used to amplify the Be158 gene from the cDNA clone by PCR (17). The amplified DNA was digested with SalI and NotI and then ligated into the SalI and NotI sites of a pGEX-4T *E. coli* expression plasmid vector (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom). The resulting plasmid, designated pGEX/Be158, was used to transform the *E. coli* BL21 strain (Stratagene, La Jolla, Calif.) and express the recombinant Be158 gene product fused with glutathione *S*-transferase (GST), designated GST/Be158 protein, by standard techniques (21). The GST/Be158 protein was purified from the soluble fraction with glutathione-Sepharose 4B (Amersham Pharmacia Biotech), as described previously (9, 14, 23).

Preparation of mouse anti-Be158 protein immune serum. Six-week-old female ddY mice (CLEA, Tokyo, Japan), which are often used for obtaining the specific immune serum in Japan, were intraperitoneally immunized with 0.2 ml of the purified GST/Be158 protein (0.1 mg/ml) emulsified with the same volume of

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MDGEPKEEQQ QPAVVPQPQD HNKTLQEAQK LAQEAKNKEE QLARERADLE RRKAELELLV KNAQSAEERA    70
KAEQRTLEEA MEQQKTQHEQ EVKRLEDLRR AQKSEHDQQQ DLILRAKEEL KRKEDAHKLK EEAAEAKRIQ   140
DEADRLAKIK AEEKARLDKI EQEDQERKDK IAAEEERLKQ AREAEQQRLA EERRALEKER EEELAKRKAH   210
EEDIVKRRRD ANQALEDLQA TRSEVAKTLS HNKEAKAALE KERAAFDAAV AKLREQEKSV EQSAEDAKKA   280
LERATAAQED YERRLKDVQD RESAVQKRED EVKTKSDTVD SKEITVNAKD EDLKIKQKSL EERAVTLAAD   350
EKKVRDSENA VSNRERAANE RDVELTKKEK LLNDKEANLN AKEKDLEKKE KELEERRTAV ELGEKELKAK   420
VAAAEETDRN LAEKDTRLKT READAAKKEA KNLEESVKLE EETKALKTKT EEHNEESRKL IKKEGELKAL   490
EQTLEERKTR VAASERASDK RVKDLDAREA QINADEAKVK EGLEARRLAV VSSEQSVKTQ LENLLEAQKG   560
HQTKSAELLA FEAQLKNQQT QLDATKQQLD AKEKELKNNQ EQLNSKKKEL EDAVAKSKEL EEKQKEMKQQ   630
AEKDAENLSA AKNELTTAKA DNAALENRKK ELETELEKYK ADLEDSKNTV TTKESELNKL KSDLESKADQ   700
LQQKTQEAIE KQKVIETKTK ELEIKSEQLS SKDSELEAKK KELSDKNDEL LMKSKELDSK EKDLLAKQVQ   770
IMKGDEERTK LSNDIVALKK SRDEITVELG KAKLACSGAR QVEDHTPAAH EEEDVQDSEW DLEQPNFQRE   840
EQDHVDWEGD VEHDEETEEH EQGEEEQHEQ EEHVESEEEQ NARDEQAWLH DEQAKELAEH IREKRAKEGT   910
SSTKKKTVTS NVATASIVAG VFLLVAGAAY GFKRRNTEYL EFCDDVDADG YADESDDEQV DDSETKIRIE   980
EGEFWSKSSN V*                                                                  991
```

FIG. 1. Putative amino acid sequence of the Be158 gene product. The bold letters and underlining show the glutamic acid-rich region and the conserved region of apical membrane antigen 1 signature, respectively.

TiterMax Gold (Bio Scientific Pty., Ltd., Sydney, Australia) three times every 2 weeks. Sera were collected from the mice 10 days after the last booster.

Western blot analysis and indirect fluorescent-antibody test. Infected or non-infected erythrocytes were boiled for 5 min in a sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) and subjected to SDS-polyacrylamide gel electrophoresis with a 5 to 20% gradient gel (ATTO Corp., Tokyo, Japan) (24). The proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, Mass.). The blots were incubated with mouse anti-Be158 protein immune serum (1:100) for 1 h and then washed three times with phosphate-buffered saline (PBS). The membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (1:2,000; ICN Biochemicals, Aurora, Ohio) for 1 h. After three washes with PBS, the membranes were exposed to a substrate solution containing 0.5 mg of diaminobenzidine/ml and 0.03% H_2O_2 to develop the color. An indirect fluorescent-antibody test was performed as described previously (27). In brief, smears of *B. equi*- or *B. caballi*-infected erythrocytes were prepared on slides and fixed in methanol for 1 min at −20°C. The mouse anti-Be158 protein immune serum (1:100) was applied as the first antibody on the fixed smear and incubated for 30 min at 37°C. After three washes with PBS, an Alexa Fluor 488 goat anti-mouse IgG conjugate (1:2,000; Molecular Probes, Eugene, Ore.) was used as a secondary antibody and incubated for 30 min at 37°C. The slides were washed three times with PBS, incubated with 25 μg of propidium iodide per ml (Molecular Probes) and 50 μg of RNase A (Roche, Basel, Switzerland) per ml for 10 min at 37°C, and then mounted in 50% glycerol–PBS with a coverslip. The slides were observed with a confocal laser scanning microscope (TCS NT; Leica, Heidelberg, Germany) (original magnification, ×4,000).

ELISA. ELISA was performed in 96-well microplates (Nunc, Roskilde, Denmark) (26). Each well was coated with 0.25 μg of the antigen at 4°C overnight. After the unabsorbed antigen was discarded, the wells were blocked with 3% skim milk in PBS (blocking solution) at 37°C for 1 h. Then the blocking solution was discarded, and 50 μl of serum sample diluted in blocking solution (1:80) was added to each well. After 1 h of incubation at 37°C, the wells were washed for six cycles with a wash solution (PBS containing 0.05% Tween 20) and then incubated with horseradish peroxidase-conjugated goat anti-horse IgG antibody (ICN Biochemicals) diluted in the blocking solution at 37°C for 1 h (50 μl per well). After six cycles of washing, 100 μl of the substrate [0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, 0.3 mg of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) per ml] was added per well. The optical density at 415 nm (OD_{415}) was read after 1 h by means of an MTP-120 ELISA reader (Corona Electric, Ibaraki, Japan). GST was used as a control antigen for the GST/Be158 protein. The ELISA result was determined for each sample by subtracting the mean OD value of two readings with GST protein from the mean OD value of two readings with the GST/Be158.

Serum samples. Ten serum samples from 25 uninfected horses, 13 from horses experimentally infected with *B. equi*, and 9 from horses experimentally infected with *B. caballi* were used for the ELISA. These horses were infected with both protozoan parasites by intravenous inoculation of the infected erythrocytes or by infected ticks. All experimental horse sera were collected 30 days to 2 years after infection without significant hemolysis at the Equine Research Institute of the Japan Racing Association in Japan. Student's *t* test was used to determine the significant difference of anti-*B. equi* titers in the three groups. A *P* value of <0.05 was considered a significant difference. Four additional sequential horse serum samples were collected on days 6, 12, 18, 25, 30, and 36 after the experimental infection with either *B. equi* (E3 and E4) or *B. caballi* (C3 and C4) to further examine the specificity and sensitivity of the ELISA using the GST/Be158 protein. All serum samples were kept at −80°C until use in the ELISA.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are available in the GenBank, EMBL, and DDBJ databases under accession number AB159602.

RESULTS AND DISCUSSION

Cloning of the Be158 gene. A cDNA clone was isolated from a *B. equi* cDNA expression phage library by immunoscreening with *B. equi*-infected horse serum. The cDNA had a total of 3,942 nucleotides (GenBank accession number AB159602) and showed an ORF of 3,510 nucleotides, which was designated the Be158 gene. The ORF encodes a polypeptide of 1,169 amino acid residues with a putative size of 134.2 kDa, as shown in Fig. 1. The amino acid sequence showed a broad glutamic acid-rich region from positions 34 to 908 and also contained an apical membrane antigen 1 (AMA-1) signature of *Plasmodium falciparum* (19) from positions 894 to 918. The AMA-1 is located in the microneme of *Plasmodium* merozoite and is anticipated to be a vaccine candidate to prevent merozoite invasion into host erythrocytes (8). In the homology search using the National Center for Biotechnology Information database, the Be158 amino acid sequence showed high similarity to the *P. falciparum* liver stage antigen (LSA-1; 28%) (GenBank accession number AE014834-50) (7), the p200 antigen located in the merozoite cytoplasm of *Babesia bigemina* (P200; 27%) (GenBank accession number AF142406) (24), and the *P. falciparum* erythrocyte-binding protein (MAEBL) (26%) (GenBank accession number AY042084-2) (3). The LSA-1 plays an important role in hepatic cell invasion of sporozoites as well as erythrocyte invasion of merozoites (6, 20). The MAEBL is an erythrocyte-binding protein located in the rhoptries and on the surface of mature merozoites; it is expressed at the beginning of schizogony (3, 18). P200 was previously identified as a diagnostic antigen for the serological detection of *B. bigemina* infection and also has a glutamic acid-rich region, as does the Be158 protein (23). Taken together, these findings indicate that the Be158 gene product might be a novel candidate for a vaccine molecule as well as a diagnostic antigen for *B. equi* infection.

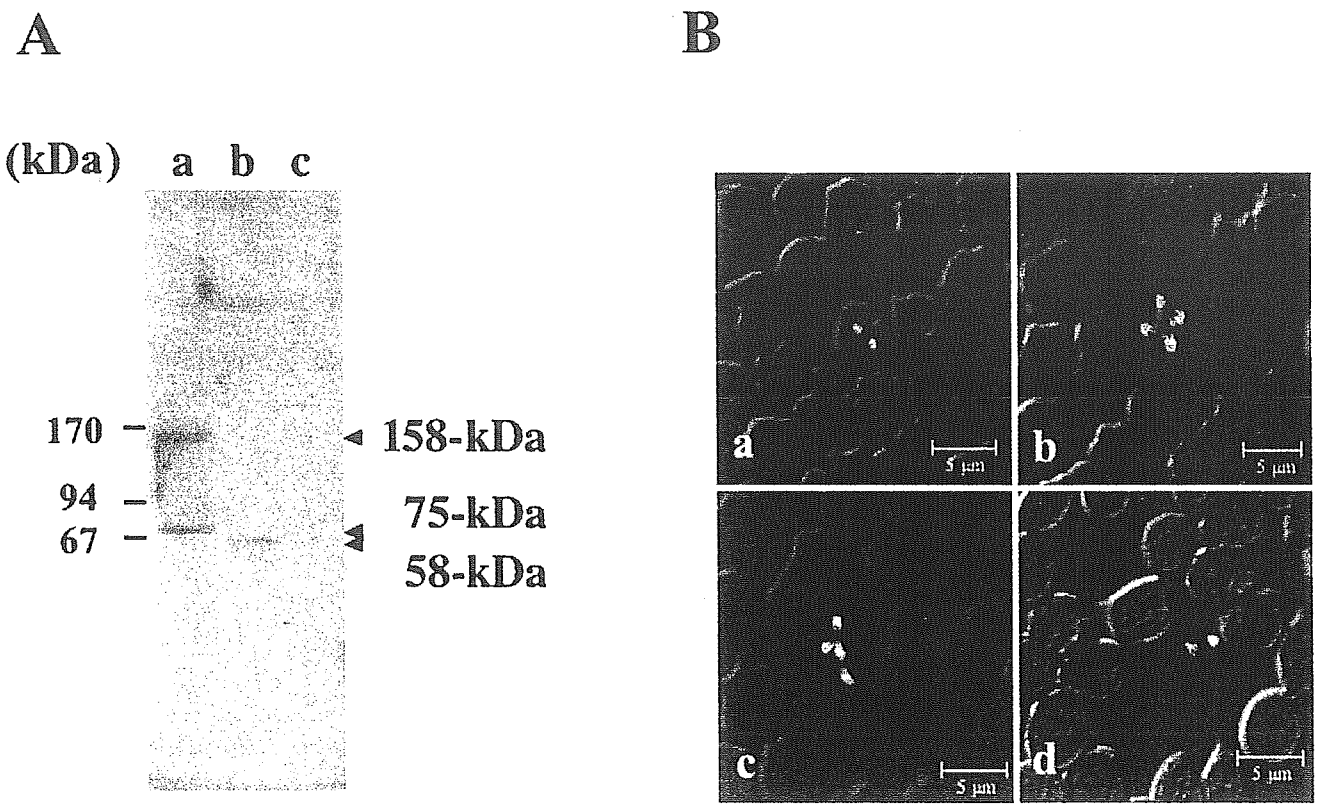

FIG. 2. (A) Western blot analysis of the lysates of *B. equi* (lane a)- and *B. caballi* (lane b)-infected and noninfected (lane c) equine erythrocytes with the mouse anti-Be158 protein immune serum. The positions of the standard molecular mass markers are indicated on the left side of the panel. (B) Methanol-fixed smears of *B. equi*-infected erythrocytes (panels a to c) and *B. caballi*-infected erythrocyte (panel d) were incubated with the mouse anti-Be158 protein immune serum. All samples were observed with confocal laser scanning microscopy (magnification, ×2,964). The immune reaction (green) and nucleus (red) were visualized with an Alexa Fluor 488 goat anti-mouse IgG-conjugated secondary antibody and propidium iodide staining, respectively. Bar, 5 μm.

Immunological characterization of native Be158 antigen. One hundred ninety kilodaltons of GST/Be158 gene product was expressed in *E. coli* and, after purification (data not shown), used for the immunization of mice to produce the anti-Be158 protein serum. In Western blot analysis, the immune serum against the GST/Be158 gene product recognized 75- and 158-kDa proteins from the lysate of *B. equi*-infected erythrocytes as well as the 58-kDa protein from the lysate of *B. caballi*-infected erythrocytes (Fig. 2A). No reaction with anti-GST protein immune serum was observed in these lysates (data not shown). These results suggested that the 158-kDa protein might be a precursor of the 75-kDa protein in *B. equi* and also that an antigenically similar antigen of the Be158 protein might exist in *B. caballi*. In an indirect fluorescent-antibody test with the immune serum (Fig. 2B), the Be158 antigen appeared in the cytoplasm of Maltese cross-forming parasites (Fig. 2B, panel a) and was mainly located in an extraerythrocytic merozoite body (Fig. 2B, panels b and c) in *B. equi*. However, the Be158 antigen was not detected in the ring stage of *B. equi* (Fig. 2B, upper middle part of panel a). The anti-Be158 protein immune serum was also found to react with the extraerythrocytic merozoites of *B. caballi* but did not recognize the intraerythrocytic parasites in the stages of the ring-shaped and subsequent pear-shaped forms (Fig. 2B, panel d). The control immune serum against GST was not reactive in any of the developmental stages of *B. equi* and *B. caballi* (data not shown). These results indicated that the Be158 protein of *B. equi* is expressed at a late stage of the asexual cycle of development and suggested the presence of an antigenically similar antigen in *B. caballi*. Further study would contribute to a broader understanding of the biological function of the Be158 protein in the asexual growth cycle.

Detection of anti-Be158 protein antibodies from *B. equi*-infected horse sera in ELISA. To evaluate the utility of GST/Be158 as a diagnostic antigen, the GST/Be158 antigen or control GST antigen was subjected to ELISA. None of the horse sera showed any reaction to the GST antigen (data not shown). The anti-Be158 protein antibodies were detected in all 13 *B. equi*-infected horse sera at an OD_{415} of >0.4, whereas all 9 *B. caballi*-infected and all 25 uninfected serum samples had an OD_{415} of <0.4 (Fig. 3A). The ELISA using the GST/Be158 antigen was able to differentiate clearly between the sera of *B. equi*-infected horses (OD_{415}, 1.06 ± 0.5 [mean ± standard deviation]) and those of either *B. caballi*-infected (OD_{415}, 0.13 ± 0.12) or uninfected horses (OD_{415}, 0.04 ± 0.04) at an OD_{415} of 0.4 ($P < 0.05$), which was considered the cutoff. Next, to confirm the sensitivity and specificity of the ELISA, we further examined the reactivity of sequential sera obtained from horses experimentally infected with *B. equi* or *B. caballi* that had been shown to have specific antibodies to *B. equi* and *B.*

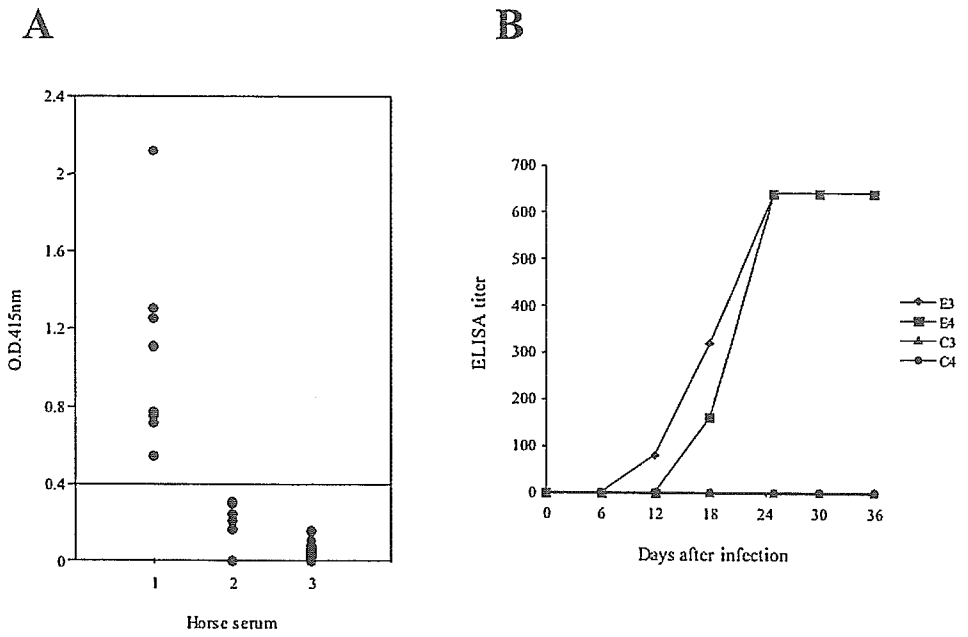

FIG. 3. (A) ELISA showing the reactivity of GST/Be158 antigen to horse sera. Filled circles represent the OD_{415}s of *B. equi*-infected (column 1), *B. caballi*-infected (column 2), and noninfected (column 3) horse sera. The significant difference between the mean OD_{415} ± standard deviation for *B. equi*-infected serum samples and those for serum samples from either *B. caballi*-infected or uninfected horses was observed by Student's *t* test ($P < 0.05$). (B) Antibody responses of sequential serum samples from *B. equi*-infected (E3 and E4) and *B. caballi*-infected (C3 and C4) horses to the Be158 antigen in ELISA. Antibody titers were expressed as the highest serum dilutions which showed an OD_{415} of >0.4.

caballi by the complement fixation test (13, 26). As shown in Fig. 3B, sequential sera from the two *B. equi*-infected horses recognized the GST/Be158 antigen as early as days 12 and 18 after infection. On the other hand, sequential sera from two *B. caballi*-infected horses failed to recognize the antigen. These results demonstrated that the GST/Be158 antigen specifically recognizes the sera of *B. equi*-infected horses at an OD_{415} of 0.4 or higher in an ELISA and is useful for the detection of *B. equi*-infected horses.

For *B. equi* infection, several diagnostic ELISAs have been developed by using recombinant EMA-1, Be82/236-381, Be82, and EMA-2 gene products (9, 10, 16). The ELISA described here, which uses the GST/Be158 protein, also proved to have high specificity and sensitivity for the detection of *B. equi*-specific antibodies. In order to analyze all sera infected with various types of field strains, it is important to use ELISAs with various antigens. In conclusion, we have provided convincing data demonstrating the utility of a Be158 gene product specific for the serological detection of *B. equi* infection in horses.

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REFERENCES

1. Avarzed, A., D. T. De Waal, I. Igarashi, A. Saito, T. Oyamada, Y. Toyoda, and N. Suzuki. 1997. Prevalence of equine piroplasmosis in Central Mongolia. Onderstepoort J. Vet. Res. 64:141–145.
2. Avarzed, A., I. Igarashi, T. Kanemaru, K. Hirumi, Y. Omata, A. Saito, T. Oyamada, H. Nagasawa, Y. Toyoda, and N. Suzuki. 1997. Improved *in vitro* cultivation of *Babesia caballi*. J. Vet. Med. Sci. 59:479–481.
3. Blair, P. L., S. H. Kappe, J. E. Maciel, B. Balu, and J. H. Adams. 2002. *Plasmodium falciparum* MAEBL is a unique member of the *ebl* family. Mol. Biochem. Parasitol. 122:35–44.
4. Bruning, A., P. Phipps, E. Posnett, and E. U. Canning. 1997. Monoclonal antibodies against *Babesia caballi* and *Babesia equi* and their application in serodiagnosis. Vet. Parasitol. 68:11–26.
5. Friedhoff, K. T. 1982. Piroplasmas of horses–impact on the international horse trade. Berl. Muench. Tieraerztl. Wochenschr. 95:368–374.
6. Garcia, J. E., A. Puentes, R. Lopez, R. Vera, J. Suarez, L. Rodriguez, H. Curtidor, M. Ocampo, D. Tovar, M. Forero, A. Bermudez, J. Cortes, M. Urquiza, and M. E. Patarroyo. 2003. Peptides of the liver stage antigen-1 (LSA-1) of *Plasmodium falciparum* bind to human hepatocytes. Peptides 24:647–657.
7. Gardner, M. J., N. Hall, E. Fung, O. White, M. Berriman, R. W. Hyman, J. M. Carlton, A. Pain, K. E. Nelson, S. Bowman, I. T. Paulsen, K. James, J. A. Eisen, K. Rutherford, S. L. Salzberg, A. Craig, S. Kyes, M. S. Chan, V. Nene, S. J. Shallom, B. Suh, J. Peterson, S. Angiuoli, M. Pertea, J. Allen, J. Selengut, D. Haft, M. W. Mather, A. B. Vaidya, D. M. Martin, A. H. Fairlamb, M. J. Fraunholz, D. S. Roos, S. A. Ralph, G. I. McFadden, L. M. Cummings, G. M. Subramanian, C. Mungall, J. C. Venter, D. J. Carucci, S. L. Hoffman, C. Newbold, R. W. Davis, C. M. Fraser, and B. Barrell. 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature 419:498–511.
8. Healer, J., S. Crawford, S. Ralph, G. McFadden, and A. F. Cowman. 2002. Independent translocation of two micronemal proteins in developing *Plasmodium falciparum* merozoites. Infect. Immun. 70:5751–5758.
9. Hirata, H., H. Ikadai, N. Yokoyama, X. Xuan, K. Fujisaki, N. Suzuki, T. Mikami, and I. Igarashi. 2002. Cloning of a truncated *Babesia equi* gene encoding an 82-kilodalton protein and its potential use in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. 40:1470–1474.
10. Hirata, H., X. Xuan, N. Yokoyama, Y. Nishikawa, K. Fujisaki, N. Suzuki, and I. Igarashi. 2003. Identification of a specific antigenic region of the P82 protein of *Babesia equi* and its potential use in serodiagnosis. J. Clin. Microbiol. 41:547–551.
11. Holbrook, A. A. 1969. Biology of equine piroplasmosis. J. Am. Vet. Med. Assoc. 155:453–454.
12. Ikadai, H., A. Nagai, X. Xuan, I. Igarashi, K. Tsugihiko, N. Tsuji, T. Oyamada, N. Suzuki, and K. Fujisaki. 2002. Seroepidemiologic studies on *Babesia caballi* and *Babesia equi* infections in Japan. J. Vet. Med. Sci. 64: 325–328.
13. Ikadai, H., C. R. Osorio, X. Xuan, I. Igarashi, T. Kanemaru, H. Nagasawa, K. Fujisaki, N. Suzuki, and T. Mikami. 2000. Detection of *Babesia caballi*

infection by enzyme-linked immunosorbent assay using recombinant 48-kDa merozoite rhoptry protein. Int. J. Parasitol. **30**:633–635.

14. **Ikadai, H., X. Xuan, I. Igarashi, S. Tanaka, T. Kanemaru, H. Nagasawa, K. Fujisaki, N. Suzuki, and T. Mikami.** 1999. Cloning and expression of a 48-kilodalton *Babesia caballi* merozoite rhoptry protein and potential use of the recombinant antigen in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **37**:3475–3480.
15. **Kjemtrup, A. M., and P. A. Conrad.** 2000. Human babesiosis: an emerging tick-borne disease. Int. J. Parasitol. **30**:1323–1337.
16. **Knowles, D. P., Jr., L. S. Kappmeyer, D. Stiller, S. G. Hennager, and L. E. Perryman.** 1992. Antibody to a recombinant merozoite protein epitope identifies horses infected with *Babesia equi*. J. Clin. Microbiol. **30**:3122–3126.
17. **Nishisaka, M., N. Yokoyama, X. Xuan, N. Inoue, H. Nagasawa, K. Fujisaki, T. Mikami, and I. Igarashi.** 2001. Characterization of the gene encoding a protective antigen from *Babesia microti* identified it as η subunit of chaperonin containing T-complex protein 1. Int. J. Parasitol. **31**:1673–1679.
18. **Ocampo, M., H. Curtidor, R. Vera, J. J. Valbuena, L. E. Rodriguez, A. Puentes, R. López, J. E. Garcia, D. Tovar, P. Pacheco, M. A. Navarro, and M. E. Patarroyo.** 2004. MAEBL *Plasmodium falciparum* protein peptides bind specifically to erythrocytes and inhibit in vitro merozoite invasion. Biochem. Biophys. Res. Commun. **315**:319–329.
19. **Polley, S. D., W. Chokejindachai, and D. J. Conway.** 2003. Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen. Genetics **165**:555–561.
20. **Puentes, A., J. Garcia, R. Vera, R. Lopez, J. Suarez, L. Rodriguez, H. Curtidor, M. Ocampo, D. Tovar, M. Forero, A. Bermudez, J. Cortes, M. Urquiza, and M. E. Patarroyo.** 2004. Sporozoite and liver-stage antigen *Plasmodium falciparum* peptides bind specifically to human hepatocytes. Vaccine **22**:1150–1156.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Schein, E.** 1988. Equine babesiosis, p. 197–209. *In* M. Ristic (ed.), Babesiosis of domestic animals and man. CRC Press, Inc., Boca Raton, Fla.
23. **Tamaki, Y., H. Hirata, N. Takabatake, S. Bork, N. Yokoyama, X. Xuan, K. Fujisaki, and I. Igarashi.** 2004. Molecular cloning of a *Babesia caballi* gene encoding the 134-kilodalton protein and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. **11**:211–215.
24. **Tebele, N., R. A. Skilton, J. Katende, C. W. Wells, V. Nene, T. McElwain, S. P. Morzaria, and A. J. Musoke.** 2000. Cloning, characterization, and expression of a 200-kilodalton diagnostic antigen of *Babesia bigemina*. J. Clin. Microbiol. **38**:2240–2247.
25. **Telford, S. R., III, A. Gorenflot, P. Brasseur, and A. Spielman.** 1993. Babesial infections in humans and wildlife, p. 1–47. *In* J. P. Kreier (ed.), Parasitic protozoa, vol. 5. Academic Press, Inc., New York, N.Y.
26. **Xuan, X., A. Larsen, H. Ikadai, T. Tanaka, I. Igarashi, H. Nagasawa, K. Fujisaki, Y. Toyoda, N. Suzuki, and T. Mikami.** 2001. Expression of *Babesia equi* merozoite antigen 1 in insect cells by recombinant baculovirus and evaluation of its diagnostic potential in an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **39**:705–709.
27. **Yokoyama, N., B. Suthisak, H. Hirata, T. Matsuo, N. Inoue, C. Sugimoto, and I. Igarashi.** 2002. Cellular localization of *Babesia bovis* merozoite rhoptry-associated protein 1 and its erythrocyte-binding activity. Infect. Immun. **70**:5822–5826.
28. **Yoshihara, T.** 1997. Equine piroplasmosis. J. Anim. Protozool. **11**:1–7. (In Japanese with English summary.)

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Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood

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Abstract

With the aim of developing more simple diagnostic alternatives, a differential single-round and multiplex polymerase chain reaction (PCR) method was designed for the simultaneous detection of *Babesia caballi* and *Babesia equi*, by targeting 18S ribosomal RNA genes. The multiplex PCR amplified DNA fragments of 540 and 392 bp from *B. caballi* and *B. equi*, respectively, in one reaction. The PCR method evaluated on 39 blood samples collected from domestic horses in Mongolia yielded similar results to those obtained from confirmative PCR methods that had been established earlier. Thus, the single-round and multiplex PCR method offers a simple tool for the differential diagnosis of *B. caballi* and *B. equi* infections in routine diagnostic laboratory settings as well as in epidemiological studies.

Keywords: *Babesia caballi*; *Babesia equi*; Multiplex PCR; Diagnosis

1. Introduction

Babesia parasites are Ixodid tick-transmitted protozoa of many wild and domestic animals; they have a unique life cycle involving asexual and sexual developmental stages within the erythrocytes of vertebrates and the tissue of ticks (Ristic et al., 1988). Two species of *Babesia* parasites, *Babesia caballi* and *Babesia equi*, are known to infect horses, and the disease is endemic in most tropical and subtropical regions of the world (Avarzed et al., 1997a; Schein, 1988). Acute equine babesiosis is usually characterized by fever, anemia, icterus, lethargy, and, in some cases, death (Knowles, 1996). Horses that recover from an acute infection often continue to function as reservoirs for subsequent infecting ticks (Holbrook, 1969).

Babesial infection is usually diagnosed by microscopic examination, which enables the detection of

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parasites from Giemsa or Wright-stained blood films (Saal, 1964). Although this method is simple, it is insufficient for the accurate identification of *B. caballi* and *B. equi* during mixed infections and low parasitemias (Quintao-Silva and Ribeiro, 2003; Krause, 2003). Serological methods have also been developed for the diagnosis of equine babesiosis (Hirata et al., 2003; Ikadai et al., 2000; Kappmeyer et al., 1999; Bruning et al., 1997; Bose et al., 1995). However, these assays are generally restricted by antibody detection limits and cross-reactivity (Allred, 2003; Tenter and Friedhoff, 1986; Weiland, 1986; McGuire et al., 1971).

Recently, the polymerase chain reaction (PCR) method has been applied for the detection of *Babesia* parasites (Bashiruddin et al., 1999; Calder et al., 1996; Figueroa et al., 1993; Conrad et al., 1992; Fahrimal et al., 1992). The sensitivity of these PCR methods for detecting equine babesiosis has been shown to be higher than that of microscopic detection methods (Rampersad et al., 2003; Nicolaiewsky et al., 2001; Bashiruddin et al., 1999). However, these methods are still relatively time-consuming and require complex procedures such as nested PCR and or hybridization to achieve higher sensitivity. Therefore, there is a need to develop simpler PCR-based systems suitable for routine diagnosis. In this study, we developed a single-round and multiplex PCR method for the simultaneous detection of *B. caballi* and *B. equi* with improved rapidity and sensitivity, based on the 18S ribosomal RNA genes, which are present in multiple copies through the genome, and evaluated it on field blood samples.

2. Materials and methods

2.1. In vitro cultures of parasites

United State Department of Agriculture (USDA) strains of *B. caballi* and *B. equi* were grown in equine erythrocytes with a microaerophilous stationary-phase culture system as described previously (Zweygarth et al., 2002; Ikadai et al., 2001; Holman et al., 1998; Avarzed et al., 1997b).

2.2. DNA extraction

The parasites were harvested from the cultures in order to extract the parasitic DNA as described by Battsetseg et al. (2002). Briefly, 50 μl of each *B. caballi*- and *B. equi*-infected erythrocytes were washed three times with cold phosphate-buffered saline (PBS) by centrifuging at 1000 × *g* for 5 min at 4 °C and resuspended in a DNA extraction buffer (0.1 mM Tris–HCl [pH 8.0], 0.1% sodium dodecyl sulfate (SDS), 100 mM NaCl, and 10 mM EDTA). The mixture was digested with 100 μg/ml proteinase K (Invitrogen, Carlsbad, CA, USA) for 2 h at 55 °C. The parasitic DNA was extracted with phenol-chloroform and precipitated with ethanol. A mixed infection study was simulated by simultaneously extracting DNA from a mixture of *B. caballi* and *B. equi*-infected erythrocytes at a ratio of 1:1. The purified DNA was used as a template for subsequent PCR amplifications. Normal horse blood DNA was also purified and used as a negative control.

2.3. Primer design and PCR amplification

The 18S ribosomal RNA gene sequences of *B. caballi* and *B. equi* (Criado-Fornelio et al., 2003; Allsopp et al., 1994) were used to design suitable diagnostic primers.

The accession numbers used in this study are Z15104 for *B. caballi* and Z15105, AY150062, and AY150063 for *B. equi*. By aligning these sequences using a Mac Vector (Oxford Molecular, Ltd., Oxford, UK), a universal screening primer pair common for *B. caballi* and *B. equi*, Bec-UF1 and Bec-UR, was designed to amplify the DNA of both parasites in one reaction. Additionally, a set of primer combinations including Bec-UF2 as a universal forward primer and Cab-R and Equi-R as reverse primers specific for *B. caballi* and *B. equi*, respectively, was also designed for the species-specific detection. Furthermore, species-specific primer pairs were designed based on the genes of *Babesia equi* Merozoite Antigen 1 (EMA-1) and *Babesia caballi* 48-kDa antigen (BC48), and used to confirm the accuracy of the results obtained by the multiplex PCR. The EMA-1 is encoded by a single copy gene of *B. equi* (Knowles et al., 1997; Kappmeyer et al., 1993) and BC48 is present as a multi-copy gene encoding the 48-kDa rhoptry protein of *B. caballi* (Ikadai et al., 1999). The primer pairs designed from these genes have so far been used for the single detection of these parasites in horse blood

Table 1
List of PCR primers used in the present study

Primers	Sequences
Bec-UF1	5′-GTTGATCCTGCCAGTAGTCA-3′
Bec-UR	5′-CGGTATCTGATCGTCTTCGA-3′
Bec-UF2	5′-TCGAAGACGATCAGATACCGTCG-3′
Cab-R	5′-CTCGTTCATGATTTAGAATTGCT-3′
Equi-R	5′-TGCCTTAAACTTCCTTGCGAT-3′
BC48-F	5′-GGCTCCCAGCGACTCTGTGG-3′
BC48-R	5′-CTTAAGTGCCCTCTTGATGC-3′
EMA-1F	5′-GCATCCATTGCCATTTCGAG-3′
EMA-1R	5′-TGCGCCATAGACGGAGAAGC-3′

Bec-UF1 and Bec-UF2: universal forward primers; Bec-UR: universal reverse primers; Cab-R: *B. caballi*-specific reverse primer, Equi-R: *B. equi*-specific reverse primer. BC48-F: BC48-specific forward primer, BC48-R: BC48-specific reverse primer, EMA-1 F: EMA-1-specific forward primer, EMA-1 R: EMA-1-specific reverse primer.

and in ticks (Rampersad et al., 2003; Battsetseg et al., 2001; Nicolaiewsky et al., 2001).

The nucleotide sequences of the primers used in this study are shown in Table 1. PCR was performed in 50 μl of a mixture (10 mM Tris–HCl [pH 8.3], 50 mM KCl, and 1.5 mM $MgCl_2$) containing 3 μl of the template DNA, 2.5 pmol of each of the primers, 0.2 mM dNTP mixture, and 2.5U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Tokyo, Japan). The mixture was heated for 10 min at 96 °C to activate the AmpliTaq Gold DNA polymerase, and 40 cycles of the following conditions were repeated: denaturation for 1 min at 96 °C, annealing for 1 min at 60.5 °C, extension for 1 min at 72 °C; and a final extension for 10 min at 72 °C. The amplified DNA samples were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light (Battsetseg et al., 2002).

2.4. Sensitivity of the single-round and multiplex PCR

In order to determine the detection limit of the single-round and multiplex PCR method, both *B. caballi* and *B. equi* DNAs were 10-fold diluted serially and subjected to the amplification condition as described above. Additionally, the PCR sensitivity for the mixed infection was also evaluated using a mixture of *B. caballi* and *B. equi* DNAs. The approximate number of parasites corresponding to each DNA dilution was calculated as described by Birkenhuer et al. (2003).

2.5. Evaluation of the single-round and multiplex PCR on field blood samples

Thirty-nine horse blood samples were collected from individual horses in Mongolia, an endemic area for equine babesiosis (Battsetseg et al., 2002, 2001; Avarzed et al., 1997a), and subsequently used to evaluate the utility of the PCR method. To confirm the results obtained by the multiplex PCR, an additional PCR method was performed using the specific BC48 and EMA-1 primers, respectively. Control blood samples were also taken from six healthy individual horses with no history of equine babesiosis in Japan (Avarzed et al., 1997b). The DNAs of these field samples were prepared as described above.

2.6. DNA sequencing

The amplified DNA products were extracted from 1.5% agarose gel using a commercial kit (QIAquick Gel Extraction Kit; Qiagen K. K., Tokyo, Japan) and cloned into a plasmid vector (PCR 2.1®, Invitrogen, Carlsbad, CA, USA). The inserted DNAs were sequenced using an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosytems Japan Ltd., Tokyo, Japan).

3. Results

3.1. Development of the single-round and multiplex PCR method

For the first screening of equine babesiosis, a universal primer pair, Bec-UF1 and Bec-UR, was designed to simultaneously amplify DNA fragments of *B. caballi* and *B. equi* (Table 1). The PCR amplification revealed positive bands of 867 or 913 bp for *B. caballi* and *B. equi* (Fig. 1A). In contrast, no amplification was observed when normal horse blood DNA was used as the template. The limit of detection was estimated as 0.18 and 0.018 parasites for *B. caballi* and *B. equi*, respectively (data not shown). Next, a single-round and multiplex PCR was constructed for the simultaneous identification of *B.*

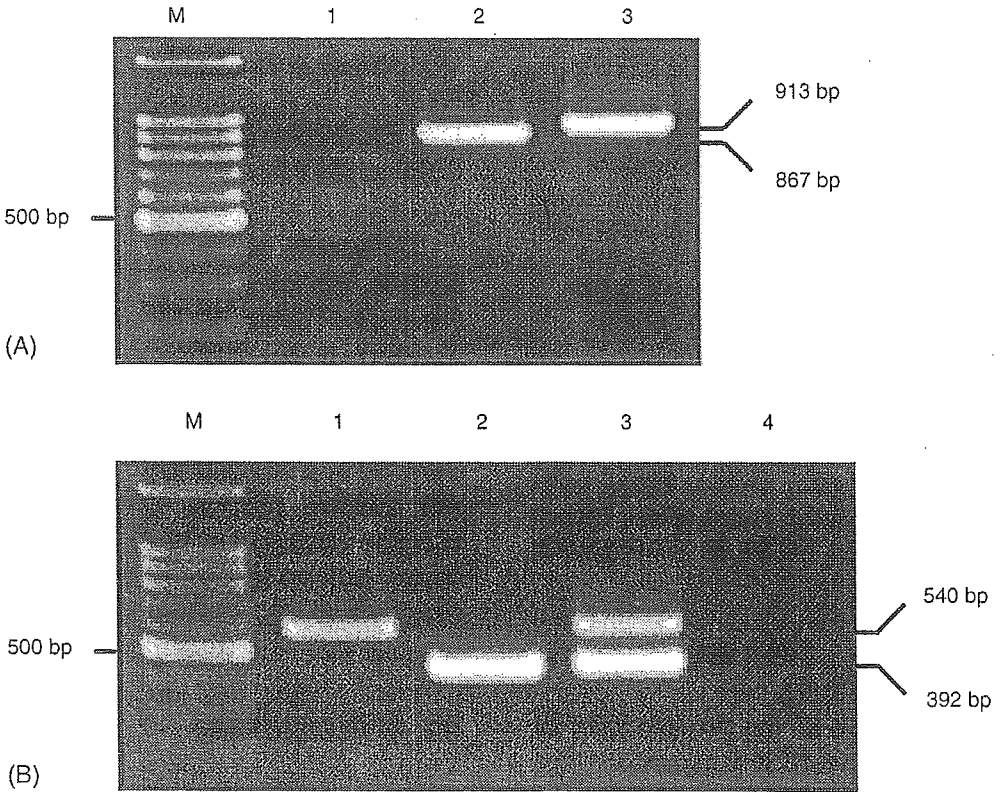

Fig. 1. PCR detection of *B. caballi* and *B. equi* with a pair of universal screening primers (Bec-UF1 and Bec-UR) (A) and a set of primer combinations (Bec-UF2, Cab-R, and Equi-R) (B). Panel A: M: 100 bp ladder DNA marker; lane 1: equine whole blood DNA; lane 2: *B. caballi* DNA; lane 3: *B. equi* DNA. Panel B: M: 100 bp ladder DNA marker; lane 1: *B. caballi* DNA; lane 2: *B. equi* DNA; lane 3: a mixture of *B. caballi* and *B. equi* DNAs; lane 4: equine whole blood DNA. The band of 500 bp determined from the 100 bp ladder DNA marker is indicated on the left. The size of the positive bands is indicated on the right.

caballi and *B. equi*. This approach used a set of primer combinations in which a forward primer (Bec-UF2) was common for both parasites while two other reverse primers (Cab-R and Equi-R) were species-specific for *B. caballi* and *B. equi*, respectively (Table 1). These primers amplified DNA fragments of 392 and 540 bp for *B. equi* and *B. caballi*, respectively (Fig. 1B: lanes 1 and 2). Additionally, the multiplex PCR could simultaneously detect both parasites from a mixture of *B. caballi* and *B. equi* DNAs (Fig. 1B: lane 3). Furthermore, the sensitivity of the single-round and multiplex PCR was evaluated using a series of 10-fold serially diluted DNA samples containing known numbers of *B. caballi* and *B. equi*. As shown in Fig. 2A and B, the multiplex PCR was able to individually detect the parasites at levels as low as 0.18 and 0.018 cells of *B. caballi* and *B. equi*, respectively. Besides, the limit of detection for the mixed infection decreased slightly to 1.8 and 0.18 cells of *B. caballi* and *B. equi*, respectively (Fig. 2C). Sequences of the amplified DNA fragments in the screening and multiplex PCR methods were identical to the original sequences reported for *B. caballi* and *B. equi* (GenBank: Z15104, Z15105, AY150062 and AY150063) (data not shown).

3.2. PCR analyses of field blood samples

Thirty-nine blood samples from domestic horses in Mongolia were examined for the infections with *B. caballi* and *B. equi* using both of the screening and the multiplex PCR methods, as shown in Table 2. Firstly, the screening primers revealed positive reactions in 14 (35.9%) out of the 39 samples for babesial infection. With the multiplex PCR, 7 (17.9%) and 10 (25.6%) out of the 14 positive samples showed species-specific reactions for *B. caballi* and *B. equi*, respectively. Interestingly, 3 (7.7%) blood samples showed simul-