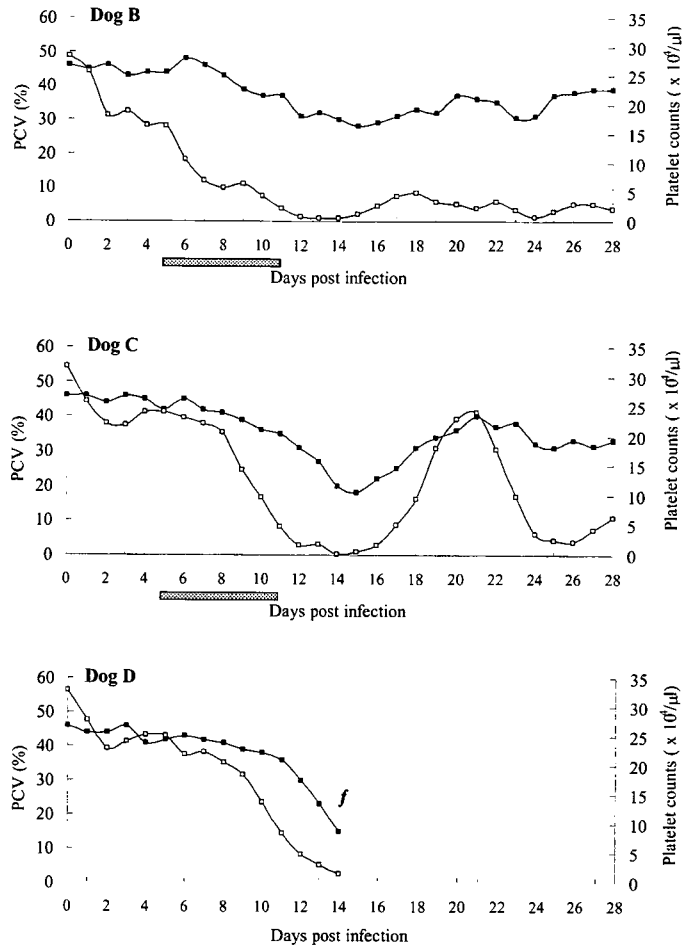

Fig. 3. Changes in packed cell volume (PCV) (-■-) and platelet counts (-□-) in the dogs (B, C, and D) infected with *Babesia gibsoni*. Dogs B and C were administered bruceine A (6.4 mg/kg/day) orally from day 5 post-infection for 6 days. The dark grey bars indicate the period of bruceine A administration. Humane endpoint is indicated by *f*.

tissues and prevent the manifestation of clinical signs. Another possible explanation is that metabolized-compounds derived from bruceine A exhibited reduced antiparasitic activities. In fact, a slight structural change and modification of quassinoid compounds have resulted in a variety of antibabesial activities [24]. Further studies including curative dosage, effective route, and period of administration of bruceine A as well as a rational design of combination therapy are required.

Bruceine A was found to have some toxic effects on treated animals [2, 23]. The risk of side-effects is likely to increase with higher dosages of the drug; therefore, reduction of the risk needs to be considered. In fact, liposomal encapsulation of another antibabesial drug, imidocarb, resulted in the reduction of its *in vivo* toxicity by 50-fold compared to the unencapsulated drug [27]. Hence, a similar approach may be useful for the development of more rational treatment regimens with bruceine A.

Brucea javanica, a plant species of the family Simaroubaceae, is widely distributed throughout South East Asian countries and its fruits have been used as a source of traditional medicine against malaria, dysentery, and cancer. The bitter principles of this plant are quassinoids, some of which have been investigated for their biological properties including antitumor [6, 12, 15], antiamoebic [31], antimalarial [7, 23], and antibabesial activities [24]. Kirby *et al.* [13] showed that seven quassinoids such as ailanthinone, bruceantin, bruceine B, glaucarubinone, holacanthone, chaparrin, and glaucarubol were proved to inhibit protein synthesis in malaria parasites. Inhibition of nucleic acid synthesis was also detected as a subsequent reaction. There are reports on the *in vitro* activity of bruceine A against protozoan parasites, including *Plasmodium falciparum*, *Entamoeba histolytica*, and *Giardia intestinalis* [23, 31, 32].

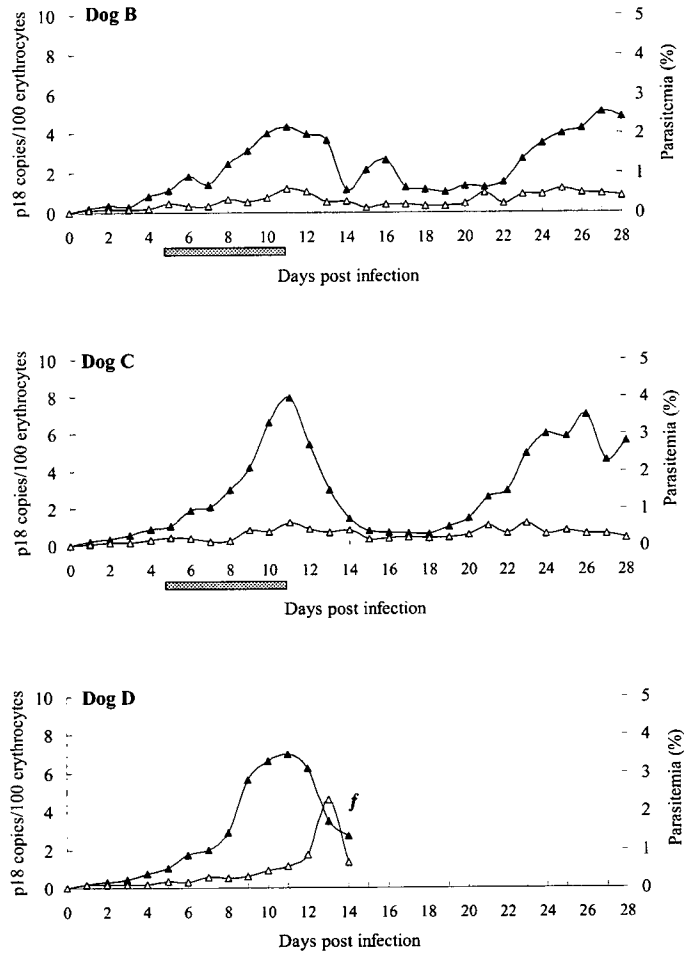

Fig. 4. Changes in copy number of *Babesia gibsoni* p18 gene (-▲-) and parasitemia (-△-) in the dogs (B, C, and D) infected with *Babesia gibsoni*. Dogs B and C were administered bruceine A (6.4 mg/kg/day) orally from day 5 post-infection for 6 days. The dark grey bars indicate the period of bruceine A administration. Humane endpoint is indicated by *f*.

Although the mode of action of bruceine A against *Babesia* species is unknown, it appears to be different from those of the currently available antibabesial drugs [25, 30]. Further studies are required to elucidate the mechanism by which bruceine A displays its potent antibabesial activity for the further development of novel combination chemotherapies with different antibabesial compounds.

Quassinoid compounds have anti-inflammatory activities *in vitro* and *in vivo* [8, 14, 28]. Hall *et al.* [8] reported that brusatol, another analogous quassinoid to bruceine A, reduced inflammation and arthritis in rodents. They found that the structures of 3-hydroxy-delta3-2-oxo moiety, a C-15 ester-bearing delta-lactone ring, and C-11 and C-12 free hydroxyl groups are important for the anti-inflammatory activity of brusatol. Bruceine A possesses a similar structure with potent active sites. Investigation of the anti-inflammatory properties of bruceine A may explain the mechanisms of the reduction in disease severity in dogs infected with *B. gibsoni*.

The standard method for quantification of babesial parasites is microscopic examination of a blood smear specimen. However, it is often difficult to quantify the low levels of parasites in the peripheral blood during acute phase of infection, asymptomatic infection and chemical treatment of infected animals. In the present study a real-time PCR method using the *B. gibsoni* p18 gene was applied to monitor the growth of *B. gibsoni in vitro* and a good correlation was found between p18 gene copy numbers and parasitemia levels in the bruceine A-treated cultures (unpublished). However, in this *in vivo* study, the copy number was not parallel to the level of parasitemia. In particular, the copy numbers were smaller than those expected from the data of

parasitemia on days 11–14 in dog D, and higher than those on days 23–28 in dogs B and C. Since multi-divided forms of *B. gibsoni* were not commonly detected in the peripheral blood of infected dogs, extra-erythrocytic merozoites existing in the blood or circulating macrophages may take the parasite DNA. In fact, *Plasmodium chabaudi* DNA could be detected in mouse peripheral blood by PCR at least until 24 hr after the injection of dead parasites [22]. This real-time PCR method will be useful not only for antibabesial chemotherapeutic studies but also for understanding the mechanisms of circulation and hiding/sequestration of *B. gibsoni* parasites in the host.

In conclusion, bruceine A is a potent antibabesial compound. Further pharmacokinetic and pharmacodynamic studies could contribute for novel information on the efficacy of bruceine A for canine babesiosis and its rational administration schedule in dogs.

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Molecular epidemiology of leishmaniasis in Asia (focus on cutaneous infections)

Ken Katakura

Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

Correspondence to Dr Ken Katakura, Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18 Nishi 9, Kita-ku, Sapporo 060-0818, Japan
Tel: +81 11 706 5198; fax: +81 11 706 5196;
e-mail: kenkata@vetmed.hokudai.ac.jp

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Purpose of review

Leishmaniasis has a wider geographical distribution than before. This review focuses on molecular epidemiological studies of new foci of cutaneous leishmaniasis in South and Southeast Asia.

Recent findings

In Pakistan, the main causative agent in southern dry areas was determined as *Leishmania major* followed by *Leishmania tropica*. There was no correlation between the skin lesion types and the causative species. In the western Indian Himalayas, *L. tropica* and *Leishmania donovani* have been reported as the causative pathogens. In Nepal, molecular methods revealed the first case of *L. major* cutaneous leishmaniasis. Microsatellite analysis of Sri Lankan strains isolated from cutaneous leishmaniasis patients identified *L. donovani* strains that were closely related to a group of visceral *L. donovani* isolates from India, Bangladesh, and Nepal. In Taiwan, a third case of indigenous cutaneous leishmaniasis was reported, and the parasite was *L. tropica*.

Summary

The distribution of cutaneous leishmaniasis has expanded definitively in South and Southeast Asia. Because the possible sandfly vector species are distributed widely over the disease endemic areas, further epidemiological studies are required. Microsatellite analysis of the parasites will be a powerful tool for population genetic and epidemiological studies of *Leishmania* species in Asia.

Keywords

cutaneous leishmaniasis, epidemiology, microsatellite, sandfly, South and Southeast Asia

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Introduction

Leishmaniasis is a worldwide vector-borne zoonotic disease caused by several species of the genus *Leishmania*. By clinical symptoms, the disease is mainly classified into cutaneous leishmaniasis and visceral leishmaniasis. Cutaneous leishmaniasis is usually caused by *Leishmania major*, *Leishmania tropica*, and *Leishmania aetiopica* complex in the Old World, and in the New World by *Leishmania mexicana* and *Leishmania braziliensis* complex. The annual incidence is estimated at 1–1.5 million cases of cutaneous leishmaniasis, with 90% of cases occurring in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru (http://www.cdc.gov/ncidod/dpd/parasites/leishmania/factsht_leishmania.htm). However, the distribution of cutaneous leishmaniasis is expanding to South and Southeast Asia. This review focuses on molecular epidemiological studies of cutaneous leishmaniasis in new endemic foci in South and Southeast Asian countries, with particular reference to Pakistan, India, Nepal, Sri Lanka, and Taiwan. Molecular epidemiology approaches can contribute to identification of the species in outbreak areas and determination of the population structure and the extent of migration of pathogens or vectors [1••].

Pakistan

Pakistan is divided into four provinces, namely North-West Frontier Province (NWFP), Punjab, Sindh, and Balochistan. Visceral leishmaniasis caused by *Leishmania infantum* has been reported in the Himalayan region of Pakistan, and cutaneous leishmaniasis by *L. tropica* has been reported in NWFP, Punjab, and Balochistan. Recently, more than 1200 cases of cutaneous leishmaniasis were newly found in the lowland of Sindh province, the southern part of Pakistan. On the basis of multilocus enzyme electrophoresis (MLEE) of the isolated parasites, all 11 isolates from the lowland areas of Sindh province were assigned to *L. major* and all six isolates from the highland areas of Balochistan province were identified as *L. tropica* [2]. Intraspecific polymorphisms were found by MLEE among these *L. major* isolates. Furthermore, skin biopsy samples taken from 69

cutaneous leishmaniasis patients, which were subjected to PCR and sequencing analysis of the cytochrome *b* (cyt *b*) genes, showed 52 cases of *L. major* and 17 cases of *L. tropica*. However, no correlation was observed between clinical presentation (wet, dry, mixed, or all types of cutaneous lesions) and causative *Leishmania* species [3••]. Three types of cyt *b* polymorphisms of *L. major* were detected. This highlighted the presence of *L. major* in the central part of Sindh province, although the reservoir animals are still unknown in this region. At least 29 species of the genus *Phlebotomus*, including *P. papatasi*, *P. alexandri*, *P. sergenti*, *P. major*, *P. longiductus*, and *P. argentipes*, have been recorded in Pakistan.

India

The Indian subcontinent (Bangladesh, India, and Nepal) has highly endemic visceral leishmaniasis and these countries have experienced a resurgence of this lethal disease, accounting for an estimated 300 000 cases annually. The mode of transmission is anthroponotic, and patients with visceral leishmaniasis provide the major reservoir for transmission of post-kala azar dermal leishmaniasis (PKDL) [4]. However, cutaneous leishmaniasis has been reported primarily in some areas of the Thar Desert of Rajasthan state, which is located in the western part of India and borders Pakistan. Recently, a comprehensive study [5] for the identification and characterization of isolates from patients revealed that the causative species of cutaneous leishmaniasis was *L. tropica*. DNA samples of 14 isolates from patients were subjected to PCR–restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer 1 (ITS-1) region. All the parasite isolates displayed a similar banding pattern, which was identical to that obtained from *L. tropica* WHO reference strains but not from *L. donovani* and *L. major* reference strains.

A similar PCR–RFLP analysis of the ITS-1 region was used on four parasite isolates and 10 biopsy samples from cutaneous leishmaniasis patients in Himachal Pradesh in the western Indian Himalayas [6]. Three of the isolated strains and eight skin samples were assigned to *L. donovani*, whereas a fourth isolate and two skin samples were *L. tropica*. Thus, *L. donovani* seems to be the predominant strain in this new focus, although more detailed genomic analysis of the isolates will be required. Because a possible vector, *P. longiductus*, was collected in 1970 in this region, further vectorial studies are required.

Nepal

In Nepal, visceral leishmaniasis is common mainly in southern parts of the Terai region, which borders the visceral leishmaniasis endemic districts of Bihar state in India. The first analysis of natural infection of phlebotomine sandflies was performed using PCR amplification of kinetoplast DNA (kDNA) in Nepal [7]. A total of 674 female sandfly specimens were collected between 2004 and 2006 in endemic areas of Dhanusa district. Morphologically identified species were *P. argentipes* (60%), *P. papatasi* (30%), and *Sergentomyia* spp. (10%). At least 6.7% of *P. argentipes* were positive for PCR amplification of *L. donovani*, but parasite DNA was not detected from *P. papatasi*.

Recently, a case of cutaneous leishmaniasis was confirmed [8•]. A 60-year-old Nepali woman with a crusted lesion on the right side of the upper face was initially referred as a case of basal cell carcinoma. Histological examination, culture, PCR analysis of kDNA, and PCR–RFLP analysis of ITS-1 revealed that the agent of cutaneous leishmaniasis was *L. major*. Because the patient had not traveled outside her native country of Nepal, this appears to be the first report of *L. major* causing cutaneous leishmaniasis in the Indian subcontinent.

Sri Lanka

Over 600 cutaneous leishmaniasis cases have been reported in Sri Lanka since 2001. The majority of patients were soldiers or farmers from northern to central provinces [9]. In addition, a significant increase in the number of cases was reported recently from southern provinces during 2004. There were no military camps in the new endemic areas and almost all those who were affected were civilians living in moderately built brick houses. The areas are residential, though rural, with no jungles in the immediate vicinity, and the wild reservoir fauna is thus restricted. A total of 113 cases were confirmed as cutaneous leishmaniasis by microscopic examination of smears, cultures, or both from the lesions [10•]. The highest number of cases was within the 10–19-year age group. Men and women were affected equally. All lesions were seen on exposed body surfaces, and the majority of lesions were on the face (41%) and upper limbs (33%). The majority of lesions were ulcers (36%) and nodules (28%). Single lesions were the most prevalent (74%) and multiple lesions were rare. During the study period a high number of cases were observed during February–March and July–September.

Previous MLEE characterization of five Sri Lankan isolates led to the surprising conclusion that the strains were *L. donovani* and typed as zymodeme MON-37, which is close to zymodeme MON-2, the most common zymodeme in India. The two types of zymodeme were differentiated by a single enzyme, 6-phosphogluconate dehydrogenase (6PGDH). The partial 6PGDH sequence of 11 Sri Lankan isolates revealed that all these isolates were *L. donovani* or *L infantum* [11•]. Multilocus

microsatellite typing (MLMT) was then performed on five strains. The Sri Lankan isolates formed one, or possibly two, distinct groups, but they clustered together and were close to a group of *L. donovani* isolates from India, Bangladesh, and Nepal. The *L. donovani* isolates from Sudan and Kenya and the *L. infantum* isolates formed distinct, respective clusters [11•]. Although *P. argentipes*, a well know vector of *L. donovani*, exists in Sri Lanka, little information on possible vectors of cutaneous leishmaniasis is available.

Taiwan

Taiwan is not considered to be an endemic area of leishmaniasis, but about 100 imported cases of visceral leishmaniasis were observed in civilians and military personnel from mainland China during and after World War II. In 1985, two native born aboriginal Taiwanese, who were from the same village in northern Taiwan and had never traveled far from home, were diagnosed with cutaneous leishmaniasis. In 2005, a 57-year-old woman with an erythematous plaque on the nasal bridge and a plaque on the right thumb was diagnosed as the third case of indigenous cutaneous leishmaniasis in Taiwan because she had not traveled outside the country [12•]. Histological examination of both skin biopsy materials revealed numerous Leishman–Donovan bodies in histiocytes. Western blot analysis of the patient's serum showed strong reactivity to antigens from *L. tropica* and *L. infantum*, but not to *L. donovani* and *L. major*. The sequence of the PCR-amplified ITS-1 was comparable with that of *L. tropica* in GenBank. The infection route in this case was unclear, but unrecognized autochthonous factors, such as wild animal reservoirs, may exist at the remote mountain areas in Taiwan. A human-biting sandfly species of *Phlebotomus kiangsuensis* had been identified in Taiwan, but only *Phlebotomus iyengari taiwanensis* was identified to feed on animals in the area.

Other countries

In East Timor, 46 cases of cutaneous leishmaniasis were reported in 1999 for the first time in this country, although no molecular data were presented [13].

In Thailand, the first nonimported visceral leishmaniasis case was reported in a 3-year-old girl in southern Thailand in 1996, and another patient was diagnosed as a second case of autochthonous visceral leishmaniasis in 2005 [14]. The patient was addicted to drugs and alcohol, but had no history of injecting drugs, blood transfusion, or travel abroad. From bone marrow biopsy materials, *Leishmania* spp. amastigotes and *L. donovani* DNA were identified. More than 5000 sandflies were collected in a cave in Saraburi Province from 2005 to 2006. Thirteen *Sergentomyia* species and five *Phlebotomus* species were identified morphologically. The most common cave dweller was *P. argentipes* followed by *P. major major* [15].

Finally, Japan is not endemic for leishmaniasis. All leishmaniasis patients have been imported cases, including over 300 visceral leishmaniasis, seven PKDL, and about 60 cutaneous leishmaniasis cases [16]. In addition, several imported canine leishmaniasis cases have been recently recognized. Few studies on the distribution of sandfly have been conducted in Japan, but a new species of *Phlebotomus squamirostris* Newstead, 1923, was found to be distributed in various parts of Japan.

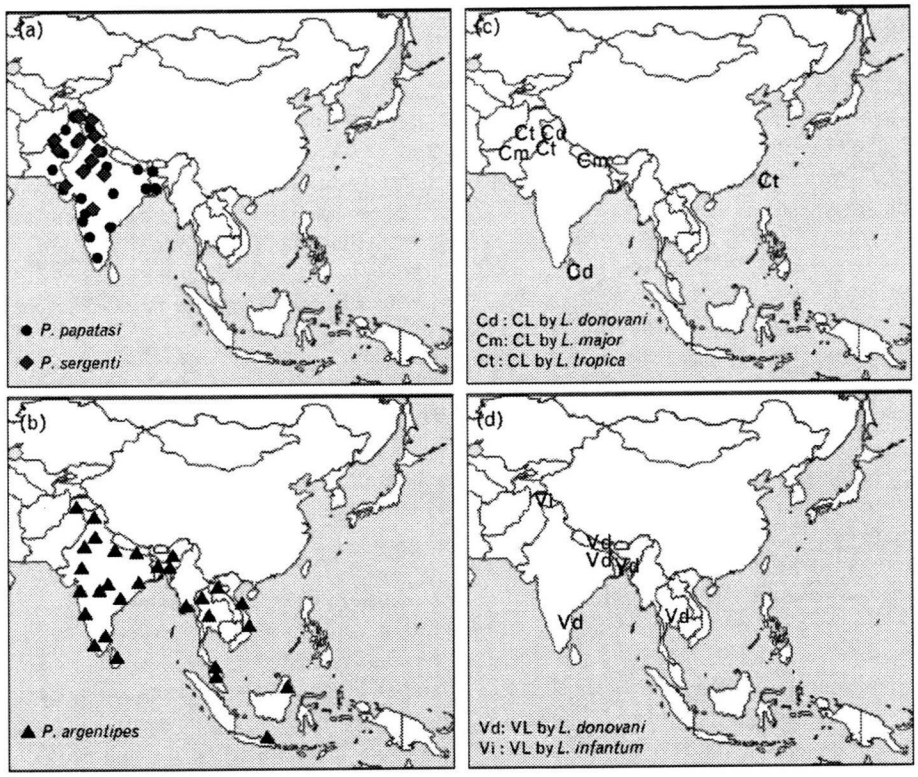
Distribution of sandfly vectors and leishmaniasis

Various species of the genus *Phlebotomus* are widely distributed in Asia, including some islands [17]. The distribution of *P. sergenti*, *P. papatasi*, and *P. argentipes*, which are the main vectors for *L. tropica*, *L. major*, and *L. donovani*, respectively, is shown in Fig. 1. The distribution of *P. sergenti* appears to be restricted to western India, whereas *P. papatasi* has expanded to Nepal and Bangladesh. Species of *P. argentipes* are also distributed across the Indochinese Peninsula. Currently, the distribution of cutaneous or visceral leishmaniasis does not overlap completely with each distribution of known vector species (Fig. 1). Recent global warming may cause the spread of these species to further eastern Asian countries, leading the establishment of new foci of cutaneous or visceral leishmaniasis.

Multilocus microsatellite typing

Isolation of the parasite is the most important aspect for epidemiological studies, but molecular methods should be included in the diagnosis of leishmaniasis. Microsatellites are becoming one of the principal genetic marker systems in phylogenetic, population genetic, and molecular epidemiological studies. The leishmanial genome is relatively rich in microsatellite sequences; about 600 $(CA)_n$ loci per haploid genome. MLMT based on more than 10 different microsatellite markers have been used successfully for characterization and detecting genetic variation in various *Leishmania* species, including *L. donovani* [18], *L. infantum* [19], *L. tropica* [20], and *L. major* [21••]. MLMT revealed that 106 strains of *L. major* from different regions separated into three main populations: Central Asia, the Middle East, and Africa. Each of these populations separated further into two subpopulations [21••]. *Leishmania* strains discovered from South and Southeast Asian regions, including Pakistan, India, Nepal, and Taiwan, remain to be investigated by MLMT.

Figure 1 Distribution of three major sandfly vectors compared with known and new foci for leishmaniasis in South and Southeast Asia



Phlebotomus papatasi, *P. sergenti* (a), *P. argentipes* (b), cutaneous leishmaniasis (c), and visceral leishmaniasis (d). CL, cutaneous leishmaniasis; VL, visceral leishmaniasis. Adapted from [2,3••,4,5,7,8•,11•,12•,14].

Conclusion

There are many risk factors for *Leishmania* infections. Suburbanization, new settlements, migration, deforestation, and irrigation projects may cause a major change in the environmental balance and aid the spread of the disease. Another important contributory factor could be the breeding of domesticated animals. Cattle, buffalo, and other animals provide a ready warm-blood meal for sandflies to lay their eggs. Because it is sometimes difficult to differentiate between a real and an artificial increase in incidence because of better awareness and detection, epidemiological studies using advanced molecular tools should be carried out carefully. A molecular survey for the detection of patients, sandfly vectors, and reservoir animals should be continued in collaboration with experts from different fields for better understanding of the transmission mechanisms and control of this neglected tropical disease.

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Cutaneous leishmaniasis caused by *Leishmania* (*L.*) *major* infection in Sindh province, Pakistan

Abdul Manan Bhutto[a,*], Farooq Rahman Soomro[b], Javed Hassain Baloch[b], Jun Matsumoto[c], Hiroshi Uezato[d], Yoshihisa Hashiguchi[e], Ken Katakura[c]

[a] *Department of Dermatology, Chandka Medical College, Doctors Colony, Bunglow No. 14, VIP Road, Larkana, Pakistan*
[b] *Leprosy Centre, Larkana, Pakistan*
[c] *Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan*
[d] *Division of Dermatology, Department of Organ-oriented Medicine, School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan*
[e] *Department of Parasitology, Kochi Medical School, Kochi University, Kochi 783-8505, Japan*

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ABSTRACT

Leishmaniasis is endemic in Pakistan and is wide-spread throughout the country. Polymerase chain reaction (PCR) was performed to identify the *Leishmania* species present in cutaneous leishmaniasis (CL) patients from new endemic areas of the central part of Sindh province, Pakistan. The PCR primers used were designed for the identification and differentiation of *Leishmania* (*Leishmania*) *major* and *Leishmania* (*Leishmania*) *tropica* species, and PCR bands at 620 and 830 bp of the parasite-specific kinetoplast DNA sequences was identified for *L.* (*L.*) *major* and *L.* (*L.*) *tropica*, respectively. Among a total of 144 DNA samples purified from the skin biopsies of clinically suspected CL patients, 108 (75%) were positive for PCR amplification. Out of the 108 cases, 105 (97.2%) were determined to be positive for *L.* (*L.*) *major* infection, and 3 (2.8%) were positive for *L.* (*L.*) *tropica* infection. It was concluded that CL caused by *L.* (*L.*) *major* is the main source of infection in the central part of Sindh province in Pakistan. This rapid screening technique could be used for the diagnosis of a large number of samples from skin lesions, which commonly contain other bacterial and fungal infections.

1. Introduction

Leishmaniases are a group of parasitic diseases caused by several species of the genus *Leishmania*. On epidemiological grounds, classically cutaneous leishmaniasis (CL) of the Old World can be broadly separated into two categories: (1) anthroponotic cutaneous leishmaniasis (ACL); caused mainly by *Leishmania* (*Leishmania*) *tropica*, found in urban towns and cities, and clinically characterized by dry-type lesions, and (2) zoonotic cutaneous leishmaniasis (ZCL); caused mainly by *Leishmania* (*Leishmania*) *major*, found in rural or semi-urban areas, and clinically characterized by wet-type lesions. Pakistan is divided into four provinces, namely North-West Frontier Province (NWFP), Punjab, Sindh, and Balochistan. Various epidemiological studies showed the presence of *L.* (*L.*) *tropica* in each province (Noyes et al., 1998; Marco et al., 2006) and the presence of anthroponotic cutaneous leishmaniasis in Afghan refuge camps has been reported in northwest of country (Kolaczinski et al., 2004). In Pakistan, CL had been differentiated on the basis of clinical presentations (dry- and wet-type lesions) (Mujtaba and Khalid, 1998). However, recent molecular studies have determined that dry and wet lesions do not automatically indicate that infections are caused by *L.* (*L.*) *tropica* and *L.* (*L.*) *major*, respectively, demonstrating that the manifestations of the skin lesions do not indicate the etiological *Leishmania* species (Myint et al., 2008). The differentiation of *Leishmania* species, which is important for eco-epidemiology, clinical diagnosis, and management of patients, must be done based on molecular approaches. Recently, a total of 1210 clinically suspected cases of CL were found in the lowland of Sindh province and the disease is becoming endemic in the whole province including the Larkana, Dadu, and Jacobabad districts (Soomro et al., 2002; Bhutto et al., 2003).

The present study reports on PCR analysis and the detection of parasite-specific kinetoplast DNA (kDNA) sequences from skin biopsies of suspected CL patients from new endemic areas of the central part of Sindh province.

2. Materials and methods

2.1. Patients

The skin biopsy samples were taken from clinically suspected CL patients at the outpatient clinic of the Department of Dermatology, Chandka Medical College (CMC), Larkana, Sindh province, Pakistan

* Corresponding author. Tel.: +92 74 4046853/92 300 341 6775; fax: +92 74 4046853.
E-mail address: bhutto_manan1@yahoo.com (A.M. Bhutto).

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Fig. 1. Map showing the distribution of *Leishmania* species reported previously in Kashmir, North-West Frontier Province, Punjab, and Balochistan (Baluchistan) provinces, and the study area (shaded) around Larkana city of Sindh (Sind) province in Pakistan for *L.* (*L.*) *major* and *L.* (*L.*) *tropica*.

from 2003 to 2004. All the cases were diagnosed on the basis of clinical examination. The patients resided in different villages and cities of Larkana, Jacobabad, and Dadu districts of Sindh province or part of Balochistan province (Fig. 1). The biopsy sites were cleaned carefully using the sterile gauze and physiologic saline and 6 mm punch biopsies were taken from the ulcerative lesions. A total of 144 biopsies were taken from suspected CL patients and fixed in 100% ethanol until analysis. Each patient gave informed consent for surgical biopsies to be taken.

2.2. Parasites

Three *L.* (*L.*) *major* and three *L.* (*L.*) *tropica* strains were used as positive controls in this study. Of these, two WHO reference strains, *L.* (*L.*) *major* (MHOM/SU/73/5ASKH) and *L.* (*L.*) *tropica* (MHOM/SU/58/strain OD) were used. The remaining four Pakistani strains were *L.* (*L.*) *major* (MHOM/PK/03/SK2), *L.* (*L.*) *major* (MHOM/PK/03/SHD7), *L.* (*L.*) *tropica* (MHOM/PK/03/QH4), and *L.* (*L.*) *tropica* (MHOM/PK/03/MA14), which were isolated from Pakistani patients (Marco et al., 2006). Promastigotes of these parasites were first cultured on USAMRU medium ('Difco' blood agar medium) and later transferred to medium 199 (Gibco) enriched with 15% heat-inactivated fetal calf serum (Gibco, BRL). Biopsies from the healthy skin of Japanese persons, who had not visited endemic countries of CL and no skin ulcers, were used as negative control.

2.3. DNA extraction control and PCR amplification

Genomic DNA was extracted from the skin biopsy specimens using GenomicPrep™ Cell and a Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). In brief, the tissues were placed in 600 μl of cell lysis solution plus 5 μl of proteinase K (Wako Pure Chemical Industries, Ltd., Japan) solution (20 mg/ml) and incubated at 56 °C overnight. The lysate was treated with RNAase at 37 °C for 1 h and add 200 μl of protein precipitation solution, followed by centrifugation at 15,000 rpm for 3 min. The supernatant was mixed with 600 μl of 100% isopropanol followed by centrifugation, and the pellet was washed with 70% ethanol followed by a further centrifugation. Finally, 100 μl of DNA hydration solution was added and the DNA was kept at 4 °C till use. Total DNA was also extracted from parasite cultures of identified *Leishmania* strains. Total DNA concentration of each sample was measured by spectrophotometer at a wavelength of 260 nm and adjusted to 100 ng/μl with distilled water.

PCR amplification detecting the parasite-specific kDNA was carried out in a reaction mixture of 20 μl, containing followings (shown in final concentrations): $MgCl_2$ (1.5 mM), four dNTPs (0.2 mM each), forward (5′-TCG CAG AAC GCC CCT ACC-3′) and reverse (5′-AGG GGT TGG TGT AAA ATA GGC-3′) primers (1 μM each), DNA template (10 ng/μl), and Taq DNA polymerase (0.025 U/μl) (Qiagen). The PCR primers used were designed for the identification and differentiation of *L.* (*L.*) *major* and *L.* (*L.*) *tropica* species. Two bands at 620 and 850 bp or one band at 620 bp was identified for *L.* (*L.*) *major*, and only one band at 820 bp was identified for *L.* (*L.*) *tropica* (Mahboudi et al., 2001). The reaction mixtures were subjected to PCR including initiation step at 93 °C for 4 min, followed by 36 cycles of denaturation at 93 °C for 0.5 min, annealing at 64 °C for 1 min, and extension at 72 °C for 1.5 min in a thermal cycler, GeneAmp® PCR System 9700 (Applied Biosystems). The reaction was terminated by treating the samples at 72 °C for 5 min before they were preserved at 4 °C. The PCR products were run on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light.

3. Results

Among a total of 144 DNA samples purified from the skin biopsies of suspected CL patients, 108 (75%) were positive for PCR amplification. The remaining samples might contain no or undetectable leishmanial DNA or may be due to bacterial or fungal infections. Out of the 108 samples, 105 (97.2%) from different geographical origins near Larkana city were determined to be positive for *L.* (*L.*) *major* infection (Table 1). All of these PCR products showed a single band of approximately 620 bp and the size was coincident

Table 1
Number of *Leishmania*-infected cases in several areas of Larkana district, Pakistan, as determined by PCR amplification of parasite-specific kinetoplast DNA.

Residential place	No. of samples examined	No. of positive samples	
		L. (*L.*) *major*	*L.* (*L.*) *tropica*
Ghaibi Dero	22	18	0
Warah	20	15	0
Shahdadkot	18	12	1
Miro Khan	19	13	0
Larkana	19	12	0
Dokri	10	8	1
Balochistan	4	4	0
Mehar	5	5	0
Others	27	18	1
Total	144	105	3

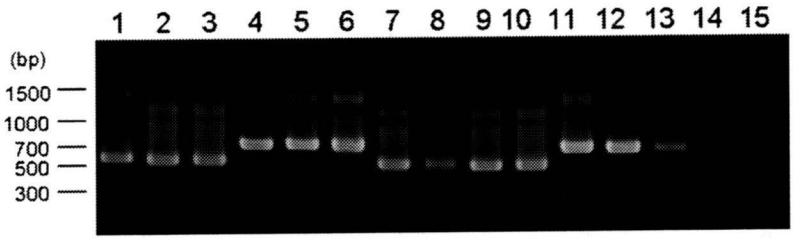

Fig. 2. PCR amplification of kinetoplast DNA using DNA samples from skin biopsies of cutaneous leishmaniasis patients in Pakistan and parasite cultures. Lane 1, *Leishmania* (*L.*) *major* (MHOM/SU/73/5 ASKH); lanes 2 and 3, *L.* (*L.*) *major* from Pakistani isolates (MHOM/PK/03/SK2 and MHOM/PK/03/SHD7); lane 4, *L.* (*L.*) *tropica* (MHOM/SU/58/Strain OD); lanes 5 and 6, *L.* (*L.*) *tropica* from Pakistani isolates (MHOM/PK/03/QH4 and MHOM/PK/03/MA14); lanes 7–13, skin biopsy samples collected from ulcerative lesions of patients; lanes 14 and 15, healthy human skin samples used as negative controls. Numbers on the left show the size of PCR products as indicated by a 100 bp ladder DNA molecular marker.

with those observed with DNA samples from the three identified *L.* (*L.*) *major* strains (Fig. 2). Only three samples (2.8%) from Shahdadkot, Dokri, and an unidentified place were positive for *L.* (*L.*) *tropica* infection (Table 1). All three samples showed a specific band of 830 bp and the three *L.* (*L.*) *tropica* strains also produced the same sized PCR product (Fig. 2).

4. Discussion

The present results showed the presence of CL due to *L.* (*L.*) *major* infection in Sindh Province (Fig. 1). The identified parasites from the majority of patients (105 of 108 positive cases) were *L.* (*L.*) *major*. These results were consistent with the previous observations, in which six strains from patients in highland areas in Balochistan province and eleven from lowland areas in Sindh province were assigned as *L.* (*L.*) *tropica* and *L.* (*L.*) *major*, respectively, by multilocus enzyme electrophoresis (Marco et al., 2006). In another study, PCR amplification and sequencing of the cytochrome *b* gene (cyt *b*) from skin biopsies of CL patients also revealed that *L.* (*L.*) *tropica* is predominant in highland areas and *L.* (*L.*) *major* was abundant in lowland areas (Myint et al., 2008). Furthermore, three skin biopsy samples, which were positive for kDNA of *L.* (*L.*) *tropica*, were also positive for cyt *b* of *L.* (*L.*) *tropica* (data not shown).

Geographically, Pakistan is comprised of widely distributed in highland and lowland areas. The current situation of the distribution of *Leishmania* species in Pakistan is shown in Fig. 1. Among the highland areas, Baltistan forms the northern areas of Pakistan, east of the Gilgit, and north of the Himalayas in the Karakoram ranges of high mountains with an average altitude of 20,000 ft, and there is permanent snow cover in parts of this region. There are several valleys in the Indus river valley at an altitude of 6000–10,000 feet above sea level. The central and southern parts of Pakistan represent lowlands areas and the weather is extremely hot. Rab and Evans (1995) reported the existence of *L.* (*L.*) *infantum* in the Himalayas region, but the present species of *Leishmania* in northern areas including NWFP and bordering Afghanistan is *L.* (*L.*) *tropica* (Reithinger et al., 2003; Brooker et al., 2004). A high incidence of CL has also been reported from various cities of Punjab province (Raja et al., 1998). In Balochistan province, which is also a high-altitude region, and in the areas bordering Afghanistan and Iran, there have been reports of high incidences of both cutaneous and visceral leishmaniasis, and the strains were determined as *L.* (*L.*) *tropica* by an electrophoresis technique (Ayub et al., 2001). Moreover, reports from the southern part of Balochistan (Bhutto et al., 2003) and bordering Iran showed the prevalence of CL due to *L.* (*L.*) *major* (Yaghoobi-Ershadi et al., 2001; Parvizi et al., 2004). Hence, on the basis of the above results the disease invasion may have originated from the area bordering Iran to the Southern part of Sindh province via Balochistan province but not from Afghanistan. Since intraspecific polymorphisms were found among these Pakistani *L.* (*L.*) *major* populations (Marco et al., 2006) as well as among *L.* (*L.*) *major* populations in Central Asia, the Middle East, and Africa (Parvizi et al., 2004; Elfari et al., 2005), further microsatellite analysis may elucidate the origin of Pakistani *L.* (*L.*) *major* populations.

In conclusion, CL caused by *L.* (*L.*) *major* infection is prevalent in this region of Sindh province. In addition, this rapid screening technique could be used for the diagnosis of a large number of samples from skin lesions, even if they are contaminated with bacterial or fungal infections. This study highlights the presence of ZCL in the central part of Sindh province, although the reservoir animals are still unknown in this region. Further studies, including demonstration of the natural infection of reservoir animals and sandfly vectors, will be carried out for better understanding of the transmission mechanisms of *L.* (*L.*) *major* as well as *L.* (*L.*) *tropica* parasites in Sindh province.

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ORIGINAL PAPER

Multiplex PCR system for identifying the carnivore origins of faeces for an epidemiological study on *Echinococcus multilocularis* in Hokkaido, Japan

Nariaki Nonaka · Takafumi Sano · Takashi Inoue · Maria Teresa Armua · Daisuke Fukui · Ken Katakura · Yuzaburo Oku

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Abstract A multiplex PCR system was developed to identify the carnivore origins of faeces collected in Hokkaido, Japan, for epidemiological studies on *Echinococcus multilocularis*. Primers were designed against the D-loop region of mitochondrial DNA. Two separate primer mixtures (mix 1, specific forward primers to fox, raccoon dog and dog, and a universal reverse primer [prH]; and mix 2, specific forward primers to cat, raccoon and weasels and prH) were used so that the PCR products (160 bp, fox and cat; 240 bp, raccoon dog and raccoon; and 330 bp, dog and weasel) were distinguished by size. The multiplex PCR exhibited no cross-reactivity between carnivore species and did not amplify DNA from rodent prey. When 270 field-collected faeces were examined, 250 showed single PCR products belonging to specific target sizes, suggesting successful carnivore identification for 92.6% of samples. Taeniid eggs were detected in 11.1% of samples and coproantigen in 30.4%; whereas the prevalences of taeniid eggs and coproantigen were 12.9% and 34.0% in fox faeces, and 0% and 26.3% in cat faeces, respectively. These results suggest that the prevalence in different target animals can be evaluated individually and precisely using multiplex PCR system.

N. Nonaka (✉)
Laboratory of Veterinary Parasitic Diseases,
Department of Veterinary Sciences, Faculty of Agriculture,
University of Miyazaki,
Gakuen-Kihanadai Nishi 1-1,
Miyazaki 889-2192, Japan
e-mail: nnonaka@cc.miyazaki-u.ac.jp

T. Sano · T. Inoue · M. Teresa Armua · K. Katakura · Y. Oku
Laboratory of Parasitology, Department of Disease Control,
Graduate School of Veterinary Medicine, Hokkaido University,
Kita-ku Kita 18 Nishi 9,
Sapporo, Hokkaido 060-0818, Japan

D. Fukui
Asahikawa Municipal Asahiyama Zoological Park & Wildlife Conservation Center, Kuranuma, Higashi Asahikawa-cho,
Asahikawa, Hokkaido 078-8205, Japan

Introduction

Field-collected faeces can provide valuable information about the animals in an area and it can be used for ecological studies in conservation biology and wildlife management (Foran et al. 1997; Kohn and Wayne 1997). Such information can also be used for epizootiological studies in which aetiologic agents or their derivatives are excreted in the faeces, enabling identification of infection from these samples (Fraser and Craig 1997; Morishima et al. 1999).

Echinococcus multilocularis is one of the most important zoonotic parasites and in humans it causes the lethal disease alveolar echinococcosis. The parasites are basically maintained in wildlife; foxes, other wild carnivores and occasionally domestic carnivores playing as the definitive host and voles playing as the intermediate host. The prevalence in foxes, which may be directly related to the risk of human infections, has been evaluated by the necropsy of captured or hunted animals (Eckert et al. 2001). In the last two decades, a new approach for evaluating infection has been developed using faeces collected in the field, in which parasite prevalence was estimated from the proportion of faeces containing parasite eggs or antigens (Nonaka et al. 1998; Raoul et al. 2001; Tsukada et al. 2000, 2002; Hegglin et al. 2003). This approach is far less disturbing to the local ecology than necropsy surveys, in which a certain proportion of animals have to be removed from the local ecosystem.

Even though studies with faeces represent an ecologically preferable approach, their reliability as an assessment of parasite prevalence remains controversial since faecal origin remains difficult to determine. For example, the criteria used for the identification of fox faeces include size, shape, colour and odour, as well as any traces such as tracks around the sample. Since none of these criteria are sufficient for unequivocal discrimination of fox faeces from that of other carnivore faeces, a certain level of bias always accompanied such survey results.

Recently, molecular techniques have been developed that enable faecal origin to be identified from faecal DNA, since faeces contain sloughed intestinal mucosal cells. Foran et al. (1997) and Paxinos et al. (1997) have developed a method to distinguish between various canid and felid species, including domestic dogs and cats, using restriction fragment length polymorphism of PCR products (PCR-RFLP). In general, PCR-RFLP requires relatively long PCR products for sequential digestion with restriction enzymes and thus, fresh faecal samples are preferred for this type of analysis, since the DNA must be in good condition (Foran et al. 1997). Long-range PCR tends to be unsuccessful for faeces collected in field, since these samples are rarely fresh and the DNA is often fragmented (Wasser et al. 1997; Frantzen et al. 1998; Murphy et al. 2000). Accordingly, for field studies on faecal samples of varied age, it is more appropriate to use target-animal-specific primers for PCR amplification of short products than to use PCR-RFLP. Moreover, this technique is also better suited to the examination of a large number of faecal samples.

Faeces contain a variety of components that are known to inhibit PCR and these can have a significant effect on the outcome of a PCR reaction (Monteiro et al. 1997). A number of techniques have been developed to improve extraction of faecal DNA and removal of PCR inhibitors. However, their efficiency varies depending upon the target animal and technique used (Huber et al. 2003; Palomares et al. 2002; Piggot and Taylor 2003; Pires and Fernandes 2003), and as yet there is no technique that is both applicable and reliable for all species.

In order to perform epizootiological studies of echinococcosis in Hokkaido, Japan, we selected a faecal DNA extraction method that results in minimal inclusion of PCR inhibitors. We then performed multiplex PCR system to identify the origins of carnivore faeces and used this technique for a field study.

Materials and methods

Faecal DNA samples

Faeces were collected from silver fox (*Vulpes vulpes fulvus*) at a fox fur farm (Kaji Mink Farm, Hokkaido, Japan) and from northern red fox (*V. v. schrencki*), raccoon dog (*Nyctereutes procyonoides*), raccoon (*Procyon lotor*), sable (*Martes zibellina*) and mink (*Mustela vison*) at Asahikawa Municipal Asahiyama Zoological Park and Wildlife Conservation Centre (Hokkaido, Japan). All faeces samples were stored individually in plastic bags at −20°C. In order to examine the efficiency of excluding PCR inhibitors in faeces, faecal DNA from silver foxes was extracted using the following four methods, QIAamp DNA Mini Kit (Qiagen; A-1, 2) and QIAamp DNA Stool Mini Kit (Qiagen; B-1, 2). Faecal DNA samples used in other experiments were extracted using method B-2.

Method A-1 was used for DNA extraction from whole faeces. Each faecal sample (200 mg) was placed in a microcentrifuge tube (2.0 mL) and mixed thoroughly in 1.6 mL SLP buffer (0.5 M Tris–HCl [pH 9.0], 0.05 M EDTA [pH 8.0] and 0.01 M NaCl), as described by Piggot and Taylor (2003). Following incubation at 70°C for 10 min, the mixture was centrifuged at 20,000×*g* for 1 min and then the supernatant (1.4 mL) was transferred to a fresh tube and recentrifuged at 20,000×*g* for 3 min. The supernatant (600 μL) was transferred to a new tube and then 15 mAU of Proteinase K (Qiagen) was added. We then added 600 μL AL buffer and incubated the mixture at 70°C for 10 min. The remaining extraction procedures were followed according to the manufacturer's instructions and DNA was extracted in 50 μL AE buffer.

Method A-2 was used for DNA extraction from a surface wash of faeces. About 1.5 cm of an intact faecal sample was placed in a plastic bag and frozen at −20°C. An appropriate amount of SLP buffer was added directly to the frozen samples so that approximately 1.4 mL of wash could be collected after removal of the faeces. Immediately after addition of the SLP buffer, the plastic bag was shaken vigorously 50 times and then the faecal sample was removed. The buffer in the plastic bag was transferred into a tube and incubated at 70°C for 10 min. The tube was centrifuged at 20,000×*g* for 3 min and then the supernatant (600 μL) was transferred to a new tube, to which 15 mAU of Proteinase K was added. The remaining procedures were performed as described for method A-1.

Method B-1 was used for DNA extraction from whole faeces. Each faecal sample (200 mg) was placed in a microcentrifuge tube (2.0 mL) and mixed thoroughly with 1.6 mL ASL buffer (provided in the QIAamp DNA Stool Mini Kit). The mixture was centrifuged at 20,000×*g* for 1 min and then 1.4 mL supernatant was transferred to a fresh tube (2.0 mL), to which an InhibitEX tablet was added. The mixture was mixed vigorously for 1 min and then incubated at room temperature for 1 min. The sample was centrifuged at 20,000×*g* for 3 min and 600 μL of the supernatant transferred to a fresh tube to which 15 mAU of Proteinase K was added. The remaining extraction procedures

were followed according to the manufacturer's instructions and DNA was extracted with 50 μL AE buffer.

Method B-2 was used for DNA extraction from a surface wash of faeces. The surface wash was performed as described in method A-2, except that ASL buffer was used instead of SLP buffer. The wash (1.4 mL) was then transferred into a fresh tube (2.0 mL), to which an InhibitEX tablet was added. The remaining procedures were performed as described for method B-1.

DNA concentrations were determined using a UV spectrophotometer (UV mini 1240, Shimadzu) with a DNA/protein software programme (Shimadzu).

Muscle, mucus and liver DNA samples

Muscle samples were collected from a fox and seven raccoon dogs captured during an effort to reduce agricultural loss due to foraging and trampling, from a marten (*Martes melampus*) and a grey-sided vole (*Clethrionomys rufocanus*) captured for this study, from a mouse and a rat purchased from a commercial breeder (SLC, Shizuoka), and from a cotton rat (*Sigmodon hispidus*) raised in our laboratory. All samples were stored at −20°C prior to DNA extraction using the QIAamp DNA Mini Kit Tissue Protocol. Mucus samples were obtained from a dog and a cat by swabbing the inside of the cheek with cotton-wool swabs. DNA was extracted using the QIAamp DNA Mini Kit Buccal Swab Spin Protocol. All animal experiments were conducted under the Guidelines for Animal Experiments of the Graduate School of Veterinary Medicine in Hokkaido University. In addition, DNA samples were extracted from the livers of various field rodents including *Clethrionomys rex*, *Clethrionomys rutilus*, *Apodemus argenteus*, *Apodemus speciosus* and *Apodemus penninsulae*, which were kindly provided by Dr. Hitoshi Suzuki, Graduate School of Environmental Science, Hokkaido University. DNA concentrations were determined as described above.

PCR for comparison of the efficiency of PCR inhibitor removal

In order to compare the efficiency of PCR inhibitor removal, we performed PCR amplifications of silver fox faecal DNA prepared by each of the four extraction methods. The primers prL (5′-CACCATTAGCACCCAAAGCT-3′) and prH (5′-CCTGAAGTAGGAACCAGATG-3′) were modified from primers L15997 and H16498, described by Gerloff et al. (1999), and designed to amplify part of the D-loop region present in all carnivores in this study. The reaction mixtures (20 μL) were prepared using a Taq PCR Core Kit (Qiagen) and comprised 2 μL template DNA, 0.8 μL of each primer (25 μM in Tris–EDTA [TE] buffer), 0.08 μL Taq polymerase (5 U/μL), 0.4 μL dNTP (10 mM), 2 μL 10× PCR buffer, 4 μL Q solution and 9.92 μL distilled water. Amplifications were performed in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) using the following conditions: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, and a final incubation at 72°C for 5 min. The amplicons were examined by agarose gel electrophoresis.

If no amplification product was obtained, DNA extracted from fox muscle was added to the samples (1.2 ng of DNA in 2 μL of template DNA solution), using a concentration within the limits of detection by this method. In order to evaluate the effect of PCR inhibitors that may have been included in the faecal DNA solutions, the PCR reaction was then performed again using the mixed template. All amplifications included a positive control containing 1.2 ng fox muscle DNA as template, and a negative control containing no DNA.

DNA sequencing

Sequencing was performed on PCR products amplified from muscle samples of seven raccoon dogs using primers prL and prH. Amplicons were sequenced with a CEQ 8000 (Beckman Coulter) using Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter).

Design of forward primers specific for target animals

Target animals included foxes, raccoon dogs, dogs, cats, raccoons and members of the weasel family (*M. melampus*, *M. zibellina*, *Mustela itatsi* and *M. vison*), which excrete faeces that are analogous to that of foxes. The respective DNA sequences for the mitochondrial D-loop region were obtained from GenBank (fox, AF09815; dog, AF008145; cat, U20753; raccoon, AF080182; *M. melampus*, AB152721; *M. zibellina*, AF336970; *M. itatsi*, AB052718; and *M. vison*, AB052720) and from this study (raccoon dog, AB292740). These sequences were aligned using Genetyx-Win ver. 4.0 (Software Development Co.), in order to design target-animal-specific forward primers that satisfied the following conditions: (1) target animals could be distinguished by the size of their amplification products; (2) there was a high level of variation between the sequences of different target animals; (3) primer lengths were between 20 and 30 bp; and (4) melting temperature (T_m) values were 56–58°C, temperatures equivalent to that of the reverse primer prH. The primers were modified further using more DNA sequences from target animals registered in GenBank, so that each primer sequence contained the lowest possible divergence for each target animal. In particular, absolute conservation of at least 3 bp, was maintained at the 3′ end of the primer (Table 1).

Table 1 Forward primers designed for target carnivores and diversity in the sequences within the same target carnivores

Carnivore	Forward primer	No. bp	T_m value	Size (bp) of products[a]	Max no. mismatches[b]	Conservation at 3′ end[c]
Fox	spFox (5′-GGAGCATATATGACTGCACG-3′)	20	56°C	165	4	8
Raccoon dog	spRdg (5′-GCAGGTACATATCCATGTATTGTC-3′)	24	56°C	232	0	24
Dog	spDog (5′-TTCCCTGACACCCCTACATTC-3′)	21	58°C	355	2	6
Cat	spCat (5′-CGATCTTCTATGGACCTCAACTAT-3′)	24	56°C	160	3	15
Raccoon	spRcn (5′-CCCCATATATAACCTTTAAACTACCC-3′)	26	57°C	245	1	16
Weasel	spWsl (5′-GACATTCTAACTAAACTATTCCCTGATTT-3′)	29	56°C	323-334	1	13

[a] Expected size of amplification products when PCR was performed with a corresponding forward primer and prH

[b] Maximum number of mismatched base pairs in sequence within target animals

[c] Number of consecutive base pairs that are conserved at the 3′ end of the sequence among the same target animals

Construction of multiplex PCR system for identifying carnivores

To use faecal DNA for the identification of target carnivores in Hokkaido, we designed a multiplex PCR system using two sets of primers (primer mix 1 contained spFox, spRdg, spDog and prH, and primer mix 2 contained spCat, spRcn, spWsl and prH). The concentration of each primer in each mix was 12.5 μM, in TE buffer. Reaction mixtures (20 μL) were prepared using a HotStarTaq Master Mix Kit (Qiagen) and comprised 2 μL template DNA, 1.6 μL primer mix, 10 μL PCR master mix, and 6.4 μL distilled water. Amplification was performed in a thermal cycler (GeneAmp PCR system 9700) using the following conditions: 95°C for 15 min, followed by five cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, after which there were 30 cycles of 94°C for 1 min, 56°C for 30 s and 72°C for 1 min, and finally 72°C for 10 min. Amplification products were examined by agarose gel electrophoresis.

Detection of DNA in aged faeces by multiplex PCR

To evaluate the ability of the multiplex PCR to detect DNA in aged faeces, we performed multiplex PCR using DNA extracted from fox faeces that had been left to age for 1, 2, 4 and 8 weeks. The ageing process was performed on grass (natural conditions) during the summer (from June to August).

Field survey

Animal faeces with diameters <2.5 cm were collected on roads at Otaru City and Yoichi Town, Hokkaido, between May and November, 2004. In order to kill *E. multilocularis* eggs, the faeces samples were packed individually and stored at −80°C for at least 10 days. Samples were then stored at −20°C until use.

In order to determine the carnivore origins of each faeces sample, faecal DNA was extracted using method B-2, as described above, and then multiplex PCR system was performed. For some samples showing single-amplification products in the multiplex PCR system, PCR with primers PrL and PrH was further performed. For samples showing products with a size of a cat, since multiple products were obtained in PCR with PrL and PrH, PCR with spCat and PrH was performed. The products were sequenced with a 3130 Genetic Analyzer (Applied Biosystems) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and subjected to BLAST sequence similarity searching (National Centre for Biotechnology Information) in order to confirm the results of the multiplex PCR system.

Then, faeces were subjected to taeniid egg and coproantigen examinations, as reported previously (Morishima et al. 1999). Briefly, 0.5 g of faeces were weighed and put in plastic tubes. Then 1% formalin containing 0.3% Tween 20 were added to a total volume of 15 ml. The tubes were centrifuged at 1,000×*g* for 10 min. The resultant sediments were used for taeniid egg examination with centrifugal sucrose (specific gravity=1.27) flotation method, and the supernatants were used for coproantigen examination (sandwich enzyme-linked immunosorbent assay). Optical density (OD) values with more than the mean plus five standard deviations of negative controls (OD=0.289) were considered to be positive. The negative controls used were faecal samples from 605 companion dogs raised and kept only on the main island of Japan which is free from *Echinococcus* infection.

When taeniid eggs were detected, the eggs were isolated by a sieving/flotation technique (Mathis et al. 1996). DNA was then extracted from the eggs using a QIAamp DNA mini kit (Qiagen), followed by PCR amplification of the egg DNA using the primers EmSP1-A' (5′-GTCA TATTTGTTTAAGTATAAGTGG-3′) and EmSP1- B' (5′-CACTCTTATTTACACTAGAATTAAG-3′). These primers

were designed to amplify a partial fragment (243 bp) of the *E. multilocularis* cytochrome *c* oxidase subunit I (COI) gene. In a preliminary experiment, we found that the PCR-amplified DNA from five isolates of *E. multilocularis*, and it showed no cross-reactivity in in silico analysis with COI gene sequences from taeniid cestodes registered in GenBank, or in assays with DNA extracted from *Echinococcus granulosus* (G1 and G6), *Echinococcus vogeli*, *Taenia ovis*, *Taenia pisiformis*, *Taenia hydatigena*, *Taenia crassiceps* or *Taenia taeniaeformis*. The PCR amplification was performed in a GeneAmp PCR System 9700 using a HotStarTaq Master Mix Kit. The reaction mixture (20 μl) comprised the PCR mixture provided in the kit (10 μL), water (6.4 μL), each primer (0.8 μL; final concentration was 1 μM) and template DNA (2.0 μL). The PCR conditions were pre-incubation at 95°C for 15 min, followed by 40 cycles of 94°C for 60 s, 50°C for 90 s and 72°C for 60 s, and a final incubation at 72°C for 10 min. Amplicons were examined by agarose gel electrophoresis. A negative control containing no DNA was included in all tests.

For the samples positive for taeniid eggs, the same PCR reaction was also performed on the faecal DNA solutions described above.

Statistical analyses

Ninety-five percent confidence intervals (C.I.) of prevalences were calculated on the basis of binomial distributions using the software programme R (R Development Core Team, 2008).

Results

Comparison of four methods of faecal DNA extraction for excluding PCR inhibitors

Using primers prL and prH, PCR reactions were performed on faecal DNA extracted using the methods A-1, A-2, B-1 and B-2. These faeces samples were obtained from ten to 12 silver foxes. No amplification products were obtained from DNA extracted by method A-1. When 1.2 ng of fox muscle DNA was added to reactions containing DNA prepared by method A-1, only one sample showed an amplification product. In contrast, 11 of 12 samples showed amplification products when PCR was performed on DNA extracted by method A-2. DNA extracted by method B-1 showed an amplification product in five of ten samples and no product could be obtained in the five negative samples, even after the addition of 1.2 ng of fox muscle DNA. In contrast, all ten samples showed amplification products for DNA extracted by method B-2.

Variation of sequence in forward primer region within target animals

Variation of sequence in the forward primer region was evaluated using sequence data obtained from GenBank (37 foxes, one raccoon dog, 490 dogs, 27 cats, six raccoons, five *M. melampus*, eight *M. zibellina*, one *M. itatsi* and four *M. vison*) and from foxes in Hokkaido, which were registered in our recent study (25 foxes with accession numbers from AB292741–AB292765, including 17 haplotypes; Inoue et al. 2007; Table 1). Although some intra-species (intra-group for weasels) variations were observed within the sequences corresponding to primer regions, the mismatches were less than 4 bp. Sequences for the 3′ end of primers were well conserved within the corresponding target animals.

Multiplex PCR system on DNA from carnivores and their prey

Using primer mixes 1 and 2, we performed multiplex PCR amplifications on DNA extracted from target animals (Fig. 1). Single-amplification products were observed at the expected sizes using a combination of fox, raccoon dog or dog DNA with primer mix 1, and a combination of cat, raccoon or weasel DNA with primer mix 2. No cross-reactions were observed in other combinations. The same results were obtained regardless of the material (muscle, mucus or faeces) used for DNA extraction.

To evaluate detection sensitivity, we performed multiplex PCR on serial dilutions of DNA extracted from the muscles of a fox, raccoon dog and marten, as well as from the mucus of a dog and cat. The detection sensitivity of the multiplex PCR was 1–10 pg of DNA. Since carnivore faeces would likely contain the undigested debris of prey animals, we evaluated

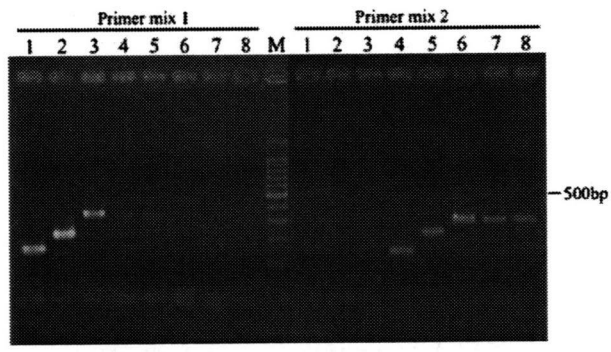

Fig. 1 Multiplex PCR amplification of the D-loop region of carnivore animals. Amplifications were performed with primer mix 1 (spFox, spRdg, spDog and prH) and primer mix 2 (spCat, spRcn, spWsl and prH). *Lane 1* fox (extracted from muscle), *2* raccoon dog (muscle), *3* dog (mucus), *4* cat (mucus), *5* raccoon (faeces), *6* mink (faeces), *7* sable (faeces) and *8* marten (muscle). *M* 100 bp DNA ladder

cross-reactivity of the multiplex PCR system to DNA from potential prey, including voles (*C. rufocanus*, *C. rex*, *C. rutilus*, *A. argenteus*, *A. speciosus* and *A. penninsulae*), mouse, rat, gerbil and cotton rat. Amplification was not observed with any of the rodent DNA.

Multiplex PCR on DNA extracted from aged samples

Multiplex PCR was performed with primer mix 1 on DNA extracted from fox faeces that had been left on grass under natural conditions for 8 weeks during the summer. During the experimental period, the minimum and maximum temperatures ranged between 9–24°C and 18–33°C, respectively. There were 15 days with >1 mm precipitation and the maximum precipitation was 26.5 mm.

Ten faeces samples were selected after each period of exposure (1, 2, 4 and 8 weeks). DNA was extracted from each sample and multiplex PCR performed. The amount of DNA obtained from faeces samples decreased gradually with ageing (starting at ca. 371.5 ng/sample and reaching ca. <65 ng/sample after 8 weeks of exposure). Nevertheless, amplification products were obtained from all samples including those collected after 8 weeks of exposure.

Field survey with multiplex PCR system

A total of 270 faeces samples were collected in the field. These samples were subjected to multiplex PCR system, in order to identify their carnivore origins. Single-amplification products of expected sizes were obtained from 250 samples (92.6%). The carnivore origins of these faeces samples are shown in Table 2. The origin of 20 samples could not be identified since four of these produced multiple PCR products and the remaining 16 did not result in amplification products. Five, three, four, two and four samples showing single products with sizes of fox, dog, cat, raccoon dog and weasel, respectively, were selected and the PCRs and sequencing were performed for subjecting to BLAST sequence similarity searching. All of samples showing single products with sizes of fox, dog, cat and raccoon dog showed 100% sequence identities with the respective species registered in GenBank. All of the samples showing single products with a size of weasel hit best to *M. melampus* with 97–98% sequence identities.

Results of parasite examinations are also shown in Table 2. In total, 30.4% (82/270, C.I., 24.9–36.2%) of faeces were positive for coproantigen. However, when individual species were considered, 34.0% (71/209, C.I., 27.6–40.8%) of fox faeces and 26.3% (5/19, C.I., 9.1–51.2%) of cat faeces were positive. Although six positives were found in samples in which the carnivore origin remained unknown, no positives were detected in any of the other animals identified by multiplex PCR system. In the taeniid egg examination, 11.1% (30/270, C.I., 7.6–15.5%) of all faeces tested were positive. However, 12.9% (27/209, C.I., 8.7–18.2%) of fox faeces contained eggs and three positives were found in samples in which the carnivore origin remained unknown. No positives were detected in any of the other animals identified by multiplex PCR system. Among the 30-taeniid egg-positive faeces, 27 were also positive for coproantigen (25 of 27 samples identified as being of fox origin).

In an *E. multilocularis*-specific PCR performed on egg and faecal DNA, all 27 faeces identified as being of fox origin produced PCR products from egg DNA, whereas only 22 generated products from faecal DNA (Table 3).

Discussion

Faeces are known to contain various PCR inhibitors that may not be completely excluded during DNA extraction and thus, these inhibitors can interfere with subsequent PCR reactions. Substances such as polysaccharides (Monteiro et al. 1997) and bile salts (Deuter et al. 1995) have been reported to act as potential PCR inhibitors. However, since faeces is supposed to contain rectum-derived cells on its surface, sampling methods such as scraping or swabbing of the faecal surface (Yamauchi et al. 2000; Davison et al. 2002; Pires and Fernandes 2003) or washing of faeces (Flagstad et al. 1999; Palomares et al. 2002; Piggot and Taylor 2003; Verma et al. 2003), have been proposed for collecting rectum-derived cells with the minimum inclusion of faecal materials. On the other hand, potato flour (Deuter et al. 1995) and magnetic beads (Flagstad et al. 1999) have

Table 2 Carnivore origins of field-collected faeces identified by multiplex PCR and results of coproantigen and taeniid egg examinations

	Origin							Total
	Fox	Cat	Weasel	Raccoon dog	Dog	Raccoon	Unidentified	
No. samples	209	19	16	3	3	0	20	270
No. positives in coproantigen examination	71	5	0	0	0	0	6	82
No. positives in taeniid egg examination	27	0	0	0	0	0	3	30

Table 3 Results of the *Echinococcus multilocularis*-specific PCR performed on egg and faecal DNA

Origin identified by multiplex PCR	Fox	Unidentified	Total
No. examined	27	3	30
No. positives in PCR on egg DNA	27	2	29
No. positives in PCR on faecal DNA	22	0	22

been reported to absorb inhibitory substances effectively. Guanidine thiocyanate has been also used as a deactivator of inhibitory substances because of its strong protein denaturation activity (Frantzen et al. 1998; Huber et al. 2003; Frantz et al. 2003). Commercial kits such as the QIAamp DNA Stool Mini Kit (Qiagen), use a combination of absorption and deactivation of these inhibitory substances.

In this study, we compared four methods for their ability to exclude PCR-inhibitory substances. No PCR products were generated from fox faecal DNA for which no attempts had been made to exclude inhibitory substances. Since products could not be obtained even after the addition of control fox DNA, it was suggested that PCR inhibiters played a role in the amplification failure. Although the QIAamp DNA Stool Mini Kit was somewhat effective at the exclusion of PCR-inhibitory substances, extraction of DNA from a surface wash of frozen faeces was more effective. However, surface wash alone was insufficient to completely eliminate inhibitory substances. Subsequently, we found that a combination of the QIAamp DNA Stool Mini Kit and surface washing of frozen faeces was the most effective method for excluding inhibitory substances.

For constructing the multiplex PCR system, we designed forward primers specific for the mitochondrial DNA of individual target animals. Faecal DNA obtained from field samples is likely to be fragmented and limited in abundance. Therefore, we chose to amplify mitochondrial DNA, since it represents a more robust target than nuclear DNA. In addition to being present in multiple copies per cell, mitochondrial DNA is circular, which renders it relatively resistant to DNase. Furthermore, PCR amplification of mitochondrial DNA is known to be more successful than that of nuclear DNA (Frantzen et al. 1998; Murphy et al. 2000, 2002) and amplification of a 150–400 bp product is more successful than that of a 700 bp product (Wasser et al. 1997; Murphy et al. 2000). Accordingly, primers were designed to amplify products of less than 400 bp.

Dalen et al. (2004) developed a multiplex PCR that can distinguish between the DNA of foxes, arctic foxes and wolverines. They performed the reactions using animal-specific forward primers designed against the mitochondrial D-loop region and the mammalian-common reverse primer (H16498) described by Gerloff et al. (1999). Following Dalen et al. (2004), we designed animal-specific forward primers against the mitochondrial D-loop region (Table 1). However, it was difficult to construct a single multiplex PCR that could distinguish six different animal groups simultaneously. Therefore, the forward primers were divided into two groups for multiplex PCR system with a modified mammalian-common reverse primer (prH). This method provided a similar sensitivity to all target animals, since the same region of DNA was being amplified. Moreover, PCR amplification with a mammalian-common D-loop primer set (prL and prH) makes it possible to determine whether a negative result is due to a sample being derived from a non-target animal (such as hares, deer or human) or due to a failure in DNA extraction or PCR inhibition. In fact, we found one multiplex PCR system-negative sample that did exhibit an amplification product using the prL and prH primers (data not shown).

Under natural conditions, faecal DNA may degrade gradually due to DNase activity or ultraviolet rays, and rectum-derived cells on the surface of faeces may be washed off by rain. DNA loss and degradation remain significant considerations for field studies dependent upon faecal DNA. Kovach et al. (2003) observed a significant reduction in successful PCR amplification of DNA from ageing faeces, and the degree of reduction varied among three species of lagomorphs. In contrast, Palomares et al. (2002) reported that ageing of faeces did not have a significant effect upon the successful PCR amplification of faecal DNA from Iberian lynx. In the present study, the total amount of DNA obtained from fox faeces was reduced by ageing under natural summer conditions for 8 weeks. However, this DNA was amplified successfully and the carnivore origins of 92.6% of faeces collected in the field were identified successfully.

Another consideration for field studies is potential cross-reactivity with the DNA from prey animals that may be present in carnivore faeces. Therefore, DNA was extracted from various rodents including field and experimental animals, and then examined with the multiplex PCR system. No cross-reactivity was detected with any rodent DNA. Relating to this consideration, Foran et al. (1997) reported that the digestive tract adequately degraded ingested animal matter and that cross-contamination did not occur in carnivore faecal DNA analysis.

In assessment of the prevalence of *E. multilocularis* in foxes using field-collected-faeces, coproantigen examination has been widely used (Raoul et al. 2001; Tsukada et al. 2002) because the method is considered as most appropriate for mass-screening purpose. The sensitivity and specificity of the coproantigen test used in this study had been evaluated using fox samples. The combined data from two studies comparing the test results of rectum faeces and necropsy results of wild foxes in Hokkaido (77 infected and 59 uninfected foxes) measured 92.2% in sensitivity and 96.6%