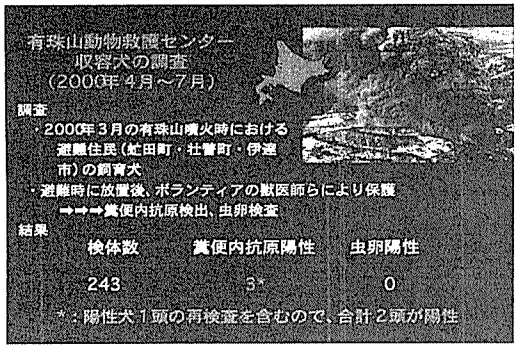

図20　環境動物フォーラムの業務例「有珠山噴火の際、救護した犬から陽性犬を発見」

小動物獣医師会の有志との共同で実施したプロジェクトであったが、リスク検出・分析・除去の重要性が参加したボランティアー獣医師の間で認識され、その後のペット（感染源動物）検査が普及することとなった。北海道小動物獣医師会（会長・川又哲、当時）は「小動物臨床家のためのエキノコックス症対応マニュアル2003」を作成し日常の診療業務の中でペットであるイヌからエキノコックス症を疑う症例に遭遇した場合に、小動物臨床家がエキノコックス症に対して診断、検査、駆虫および予防をどのようにすればよいのか、また動物病院スタッフや飼主を感染から防御する方法を示した（http://vpcserv.vetmed.hokudai.ac.jp/echinomanual/echinomanual.html）。後日、北海道ならびに㈳北海道獣医師会から有珠山噴火被災動物の救護活動について報告書がまとめられたが、その中にエキノコックス・リスク管理についての記録はない。

環境動物フォーラムは、その後、2004年度から検査を有料化にして自立を図った。2005年度には、「北海道大学人獣共通感染症センター」が設置され、その仮研究室を手当てするため、この国内唯一のエキノコックス・リスク対策専門機関は、先端研から立ち退きを要求された。2006年5月、会社法施行に伴い日本版LLC合同会社「環境動物フォーラム」として認可され、完全に民間ベースで再スタートすることとなった。業務内容は下記のとおりである。

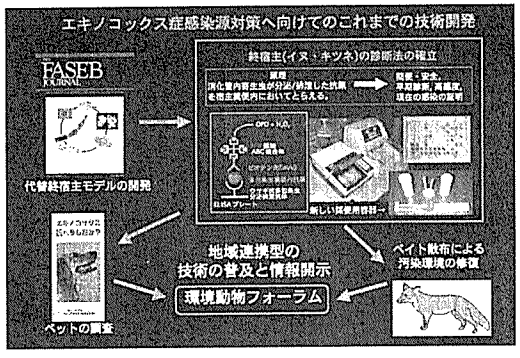

図21　環境動物フォーラムの業務例「リスク動物診断診断技術を中心とした調査・研究・対策」

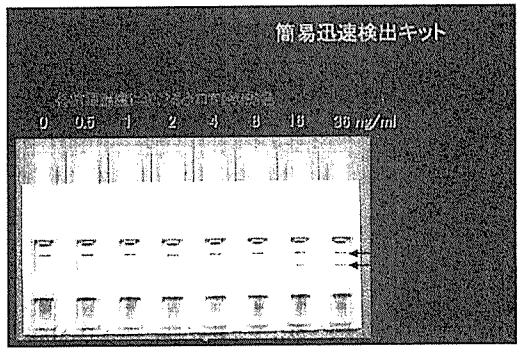

図22　環境動物フォーラムの業務例「リスク動物しんだん法の改良：簡易迅速キットの開発」

A．人獣共通感染症（特に寄生虫と寄生虫症）に関する情報提供・教育啓蒙活動～講演会、ホームページなど

B．人獣共通感染症の調査・研究・対策に関する活動（図21、22）

1）伴侶動物（イヌ・ネコ）の検査

2）駆虫薬散布によるエキノコックス感染源対策のサポート

C．エキノコックス浄化関連～環境教育教材の開発・普及

1）エキビン（感染源動物の糞便採取・輸送容器：特許申請中）

2）エキコロ（ベイト：商標登録）

3）エキット（診断キット：商標登録）

〈連絡先：〒065-0020　札幌市東区北20条東2丁目2－29

合同会社　環境動物フォーラム
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電話：011-731-6767
E-mail：fea@yahoogroups.jp

6-4 その他

2005年5月、OIE（国際獣疫事務局、本部パリ）の総会で酪農学園大学環境システム学部環境動物学研究室がエキノコックス症研究拠点機関（リファレンス・ラボラトリー）に指定され活動を開始した。この拠点機関からの最新の業績として、OIE技術シリーズ『日本のエキノコックス感染源対策』を作成[8]、米国疾病制御センター（CDC）感染症専門誌（Emerging Infectious Diseases：EID）に『日本におけるペット由来エキノコックス症リスク』[9]をまとめた。

奥　祐三郎（北海道大学COEエキノコックス研究推進者）グループは、多包条虫（幼虫）の全長cDNAライブラリー・データベースを公表した[10]。今のところ、部分的なシークエンスであるが、関連する遺伝がわかり、その後のシークエンスも可能となる。今後、この遺伝子を解析することにより、今まで不明であった、多くの酵素、蛋白成分の機能解析ができ、病原体の代謝、分化、増殖等の生理・生化学的な解明が進むと思われる。さまざまな研究者がこの全長cDNAライブラリー・データベースを利用して研究を進めることが可能になる。培養シストを用いてRNA干渉により、その遺伝子の機能も直接解析できる。機能のわからない遺伝子についても、網羅的に調べることが可能である。特に、発現量の多い蛋白をコードするmRNAは、診断用抗原の解析に有用と考えられる。たとえば、幼虫ではAntigen Bグループが多く発現しており、人（中間宿主）の血清診断にかかわる抗原の詳細な解析が可能となる。

7… おわりに

「寄生虫だらけの世界（This wormy world）」と題した論説がある（Stoll, 1947）。20世紀のなかば、人体寄生虫病の蔓延を説明したものである。回虫、鉤虫などの感染者総数が、当時の世界の人口23億人を超えることから1人が1種以上の寄生虫を保有している状況を示したものである。我が国においても戦後は、まさしく「寄生虫だらけの世界」であった。しかし、回虫などは駆虫薬「虫下し」の普及や衛生環境の改善などでほとんど見られなくなった。その後、半世紀の人口爆発で60億人を超え、感染症をめぐる情況は大きく変わった。人間活動による環境の改変――野生動物の餌となる厨芥，農水産廃棄物の増加、人、動物、物の移動によって分布が拡大する動物由来寄生虫症が目立つようになった。

D.ムレール博士は、米国寄生虫学会長就任講演「寄生虫だらけの世界、再訪――無視されている動物病」で寄生虫による家畜の損耗と公衆衛生面の被害の両面から世界的な動物寄生虫病対策の必要性を唱えている（Murrell, 1994）。また、WHOは、1990年代半ば、「我々は、今や世界規模で感染症の危機に瀕している。もはや、どの国も安全ではない」としている。そのことはSARSの出現で現実のものとなった。また、1995年、「新たに出現する感染症：Emerging Communicable Disease」部門（EMC）を創設し21世紀へ向けて世界戦略を発表している。重点的に取り組む19の疾患を挙げているが、そのなかにエキノコックス症が含まれている。

エキノコックスによる被害は健康被害のみならず、経済問題にまで波及する。食品に病原体が混入していれば、その商品は売れなくなり、環境に毒が撒かれていれば、その地域の農業や観光業は成り立たなくなるように、寄生虫・エキノコックスは、我が国の生物リスクとしては、きわめて大きな問題をかかえており、代表的な動物

由来感染症である。しかしながら、エキノコックス症の重大さが、一般に認識されることもなく、その感染源（リスク）へ向けた対策も、長い間、欠けていた「無視されすぎた動物由来感染症」である。

北海道では、1930年代から発生したヒトのエキノコックス症研究の中心は医学であった。その対策も医療が中心であった。初期の対応は仕方がないにしても、現在もその方針が継続されている。環境や野生動物が関与する人獣共通感染症に対して医学という狭い領域にとどまったために問題解決を遅らせている。

この問題に対峙するそれぞれの関係者（ステークホルダー）の貢献が期待されているが、これまでのシステムの限界から十分機能しているとは言い難い。国レベルでも、厚生労働省ではヒトの病気という視点を重視するので、野生動物であるキツネを担当する部局がない。野生動物領域は環境省の管轄であるが、ヒトの感染症となると対応する部局がない。北海道レベルでも同じで、総合的に「担当する部署がない」のが現状である。したがって、エキノコックス感染源（リスク）については公的機関での対応は十分であるとはいえない。学会、研究機関でも反省する点は多い。たとえば、関連する学会として日本寄生虫学会が、この感染源リスクに対峙するためのプロジェクトを立案することもなかった。このエキノコックス研究領域を医学会の一分科会の狭い領域に留めたことにも反省すべきことは多い。

2005年度に文部科学省の委託事業として発足した「感染症研究と国際貢献の新しいかたち：新興・再興感染症研究拠点形成プログラム」[11]は、国内4拠点――①大阪大学／感染症国際研究拠点、②長崎大学／新興・再興感染症臨床疫学研究拠点、③東京大学／医科学研究所アジア感染症研究拠点、④北海道大学／人獣共通感染症の研究・教育中核拠点と海外拠点が併設され、実質、8拠点をつなぐ壮大なプログラムである。そのすべての拠点機関に日本寄生虫学会を中心にして活躍する多くの専門家が参加しながら、エキノコック

ス症の専門家が配置されていない。特に、④の「人獣共通感染症リサーチ・センター」は北海道に位置し、エキノコックス研究は、地域ならびに海外から期待されている特色ある分野と期待されているが、教授スタッフ10名、研究員を合わせると30名を超える陣容にエキノコックス感染源（リスク）の専門家は1名も手当てされていない。

このプログラムでは、「医学・獣医学連携を図る」としながら、従来の学問の枠組みや学閥を超えられるのか？　国内の感染症一般の対策には、二つの太いパイプがすでにあった。WHO→厚生労働省とOIE（国際獣疫事務局）、FAO（国連食糧農業機関）→農林水産省の流れにある研究拠点機関である。これらと文部科学省主導の本プログラムとの連携は十分なされるのか？　はなはだ心もとない。対峙するのは省庁ではなく生物リスクであることを銘記する必要がある。「100年の計の出発点」としているが、注意深く、国費の行方、その成果を見守りたい。

エキノコックス問題は、国内的にも国際的にも十分大きな関心事であると冒頭にも述べたとおり、北海道で始まったローカルな感染源対策ではあるが、地域の住民・研究者が共同して研究し、成果を地域に還す作業は海外の流行地へも通用すると考える。この流れは「内発的開発論」を唱えた故鶴見和子教授[12]や、公害と環境を経済学に採り入れ、その成果を市民運動に応用し問題解決を図った宮本憲一教授の仕事[13]の基礎となる部分に通じている。人間は自然環境の一部であるという視点から水俣病などの「わざわい」を、より広い世界から論じている。さらにはアマルティア・セン教授と緒方貞子博士の「人間の安全保障」や、故橋本龍太郎首相が1998年にバーミンガムでの主要国首脳会議で提案した「国際寄生虫病対策」やその後の「水問題」にかけたパトスにも通じるものがある。

人間を含めて生き物の世界は、現実の他生物個体との関係（＝web of life：網目状に繋がる生活）で成り立っている。エキノコッ

クスのリスク・マネジメント業務は、そのネットワーク上で無償のボランティアー活動などによって産み出される新たな資源や地域にすでに存在する資源、人材、文化をも含めた「環境力」に依拠した自然環境再生事業と考える。「学会ごっこ」や「会社ごっこ」で終わらせるわけにはいかない。

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Short communication

Trichinella nativa and *Trichinella* T9 in the Hokkaido island, Japan☆

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Abstract

Trichinella sp. muscle larvae were isolated from the thigh muscle of two red foxes (*Vulpes vulpes*) captured in Sapporo and Otofuke, Hokkaido, Japan, in 2003. Multiplex PCR designed for genotyping the genus *Trichinella* revealed that the Sapporo isolate showed a specific pattern to *T. britovi* complex (*T. britovi*, *Trichinella* T8 and *Trichinella* T9) and the Otofuke isolate showed that to *T. nativa*. Nucleotide sequences of a part of the mitochondrial cytochrome oxidase subunit I (COI) gene and internal transcribed spacer 2 (ITS2) of the Sapporo isolate showed the highest similarity to those of *Trichinella* T9, a species detected in the mainland of Japan. This study shows that both *T. nativa* and *Trichinella* T9 are circulating in wildlife of the Hokkaido island.

Keywords: Zoonosis; *Trichinella nativa*; *Trichinella* T9; Epidemiology; Fox; Japan

Trichinellosis is one of the important helminthic zoonosis caused by eating raw or undercooked meat containing *Trichinella* sp. muscle larvae. Currently, the genus *Trichinella* is classified into eight species (*T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murrelli*, *T. nelsoni*, *T. papuae* and *T. zimbabwensis*) and three unclassified genotypes (*Trichinella* T6, *Trichinella* T8 and *Trichinella* T9) [1]. All known *Trichinella* species were morphologically indistinguishable except for *T. pseudospiralis*, which species is smaller in size than others.

In Japan, the first case of *Trichinella* sp. infection was found in a domestic dog in 1957 [2]. The first human outbreak occurred in 1974 in Aomori prefecture [3], and then in 1979 in Hokkaido prefecture [4] and in 1981 in Mie prefecture [5]. Following the human outbreaks, intensive surveys for *Trichinella* infection in wild mammals have been carried out, and two black bears, *Ursus thibetanus* [6], one red fox, *Vulpes vulpes* [7] and one raccoon dog, *Nyctereutes procyonoides* [8] in the northern part of mainland Japan, and five red foxes in Hokkaido [9] were found to be infected. The story of the parasite identification was troubled. *Trichinella* sp. isolated from a black bear (International *Trichinella* Reference Center (ITRC) isolation code ISS 408) and raccoon dog (code ISS 409) in mainland Japan were first analyzed by RAPD and once identified as *T. britovi* [10]. These isolates were, however, reanalyzed by DNA sequencing and PCR-RFLP, and then classified as a new genotype, *Trichinella* T9 [11]. In 2001, *Trichinella* sp. (code ISS 1028) were detected in foxes of the Otaru city, Hokkaido [9], and one isolate was sent to ITRC for its identification. This isolate (ISS1028) was first identified as *T. nativa* by multiplex PCR. However, when larvae of the same sample were tested again by multiplex PCR and PCR-RFLP in the same laboratory, they were identified as belonging to the *Trichinella* T9 genotype; consequently, so far, *T. nativa* was never documented in Japan. The aim of the present work is to describe the first identification of both *T. nativa* and *Trichinella* T9, in the Hokkaido island, Japan.

Two fresh carcasses of red foxes shot by hunters in Sapporo and Otofuke, Hokkaido, Japan in 2003 were sent to our laboratory. A part of the thigh muscle was investigated for *Trichinella* sp. muscle larvae by an artificial digestive method. Briefly, a part of the thigh muscle (approximately 20 g) was minced by scissors and incubated in artificial gastric juice (NaCl saline containing 0.75% pepsin and 0.75% HCl) for 2 h at 37 °C. After digestion, larvae were detected in muscles of both foxes with a worm burden of 7.1 larvae per gram (LPG) from the fox in Sapporo and 31.1 LPG from the fox in Otofuke. The collected

☆ Nucleotide sequences data reported in this paper are available in the EMBL, GenBank and DDBJ data bases under the accession numbers: AB255885, AB255886.

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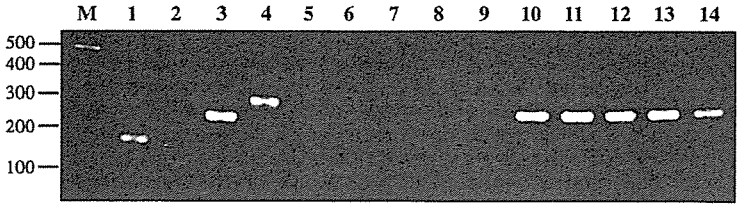

Fig. 1. Agarose gel separation of multiplex PCR products. Amplification was carried out with 35 cycles as follows: 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min. M, 100 bp ladder. Lane 1–4 reference straines, Lane 1, *Trichinella spiralis* (code ISS413). Lane 2, *T. nativa* (code ISS 10). Lane 3, *Trichinella* T9 (code ISS408). Lane 4, *T. pseudospiralis* (code ISS 13). Lane 5–9, five individual larval DNA samples from a red fox (*V. vulpes*) of Otofuke, showing a single band specific for *T. nativa* Lane 10–14, five individual larval DNA samples from a red fox in Sapporo, showing two bands specific to *Trichinella* T9.

muscle larvae were washed with saline and 100 larvae of each isolate were orally inoculated into gerbils. After 3 months, muscle larvae were collected from the gerbils and stored in 70% ethanol until use. All of the experimental infections were carried out according to the Guidelines for Animal Experiments of the Graduate School of Veterinary Medicine in Hokkaido University.

For PCR amplification, DNA from single larvae was prepared by digesting larvae using proteinase K (Takara, Ohtsu, Japan). Five single larvae of each isolate were analyzed individually. Four reference strains; *T. spiralis* (code ISS 413), *T. nativa* (code ISS 10), *T. pseudospiralis* (code ISS 13) and *Trichinella* T9 (code ISS 408) were used as standards for multiplex PCR using five primer sets according to a previous published protocol [12]. Agarose gel electrophoresis of the multiplex PCR products of the Otofuke isolate showed a single band of 127 bp (Fig. 1, lanes 5–9), which was the pattern specific for *T. nativa* (Fig. 1, lane 2). In the Sapporo isolate, two bands of 127 bp and 253 bp (Fig. 1, lane 10–14) were observed. This banding pattern was specific for the *T. britovi* complex (*T. britovi*, *Trichinella* T8 and *Trichinella* T9) (Fig. 1, lane 3) [13]. For a further identification of the Sapporo isolate, part of the mitochondrial cytochrome oxidase subunit I (COI) gene (379 bp) and part of the internal transcribed spacer 2 (ITS2) (395 bp) were amplified and sequenced. Total DNA extracted using QIAamp® DNA Mini Kit (QIAGEN) from 200 larvae of Sapporo isolate was used for DNA sequencing. Two sets of primers (5′-CACCCAGAAGTATACATCC-3′ and 5′-GTAATAATAG GTCTAGGGAGG-3′ for COI and 5′-CAATTGAAAACCGG TGAG-3′ and 5′-ATCACTCAACATTAACCG-3′ for ITS2) for PCR and DNA sequencing were designed based on the sequences of *T. nativa* (accession no. DQ007891 and AY851267) and *Trichinella* T9 (DQ007898 and AY851274). The PCR product of COI was purified, cloned into the plasmid vector (pCR®4-TOPO®, Invitrogen). Three clones were sequenced using capillary sequencer (CEQ8000, Beckman Coulter) and these three clones of COI gene were identical (accession number AB255885). The PCR product of ITS2 was sequenced directly (AB255886). Phylogenetic analysis of COI and ITS2 sequences of Sapporo isolate were performed using software MEGA version 3.1 [14] with those of all known *Trichinella* species. A total of 11 COI sequences (DQ007890–DQ007900) and 11 ITS2 sequences (AY851266–AY851276) were collected from GenBank. The result revealed that the Sapporo isolate showed the highest similarity to the *Trichinella* T9 COI gene (DQ007898) and ITS2 region (AY851274). Taken together with the results of multiplex PCR and phylogenetic analysis, the Otofuke isolate was identified as *T. nativa* and the Sapporo isolate as *Trichinella* T9.

Trichinella nativa has been reported from the arctic and subarctic zones of the Holarctic region including eastern continental countries, such as China [15] and eastern Russia [16]. The life cycle of *T. nativa* is mainly maintained in wild animals and muscle larvae survive in frozen meat; *e.g.* 4 years in arctic foxes at −18 °C [17] and 5 years in polar bears at −18 °C [18]. From an epidemiological aspect, *T. nativa* is thought to be one of the important species among all *Trichinella* nematodes because of its resistance against low temperature. To prevent *T. nativa* infection, adequate cooking is strongly recommended. Freezing is one of the methods to inactivate *T. spiralis* larvae in muscles of pigs, but it is not a suitable method to inactivate *T. nativa* larvae in muscles of carnivores [19]. *Trichinella* T9 has only been found in Japan [1]. Based on a recent phylogenetic study, *Trichinella* T9 seems to be more related to *T. murrelli* of the Nearctic region than to *T. britovi*, and may represent a peripheral isolate on the islands of Japan derived from a widespread ancestral population that occupied a Holarctic distribution [20]. The Japanese islands remained isolated for a prolonged period from the continent. In consequence, various animals evolved indigenously. *Trichinella* T9 might be speciated in the similar way.

Acknowledgments

The authors are grateful to Dr. Edoardo Pozio (International *Trichinella* reference center, Rome, Italy) for supplying the *Trichinella* reference larvae (*T. nativa* and *Trichinella* T9), the late Mr. Takeshi Ohde and Mr. Kazuyoshi Tamori (Hokkaido Hunters Association) for supplying the fox samples. This research was supported in part by a grant for Research on Emerging and Re-emerging Infectious Diseases (H15-Shinkou-7), Ministry of Health, Labor and Welfare, Japan.

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多包条虫疫学調査への応用を目的とした野外採取糞便の排泄動物鑑別法の検討

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Idenitification of animals excreting feces collected in field for epizootiological studies of echinococcosis

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これまで我々は野外で採取した糞便を材料として多包条虫の動物疫学調査を実施してきたが、糞便の排泄動物の判定は、大きさ、形、内容物や匂い等に頼っていた。今回、糞便排泄動物の区別をより明確にするため、糞便内DNAを利用した糞便排泄動物鑑別法の開発を試みた。まず、糞便DNA抽出法の改善として、糞便中に含まれるPCR阻害物質の混入を最小限にし、かつ、糞便表面に付着する腸粘膜細胞を効率的に回収するために、凍結糞便表面の洗浄液を抽出材料として、QIAmp DNA Stool Mini Kitにより DNA抽出を行った。次に、北海道において多包条虫の終宿主となるキツネ、タヌキ、イヌ、ネコおよびこれらの動物と類似の糞便を排泄するアライグマ、イタチ類のミトコンドリアDNA D-loop領域について、増幅産物の大きさの違いで鑑別が可能となるようにそれぞれ特異的な6種類のプライマーを設計しmultiplex-PCRを行ったところ、本法は食肉目間で交差反応を起こさず、糞便排泄動物の鑑別が可能であることが示された（図1）。糞便に含まれる餌動物の影響についても検討したが、北海道に生息する野鼠のDNAとの交差反応は認められなかった。実験的に屋外環境で8週間放置したキツネ糞便からも10個全てのサンプルで明瞭な増幅バンドが認められ、本法の安定性が示された。2004年5～8月に小樽市および余市町で採取した147個の糞便に本法を適用したところ、140個（95%）について糞便排泄動物を鑑別することができた。以上の結果、野外採取糞便を材料とした多包条虫の動物疫学調査において、本法を併用することにより、宿主動物種を区別したより精度の高い感染率調査が実施可能となることが示された。

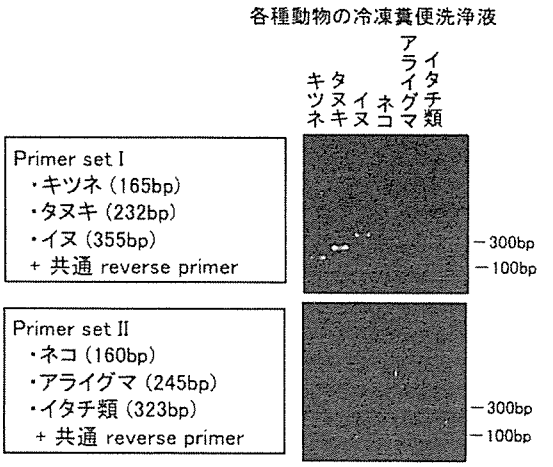

図1　Multiplex PCRの糞便由来DNAへの適用

Keywords: *Echinococcus*, feces, PCR, epizootiology

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駆虫を組み合わせたプレパテント期における多包条虫感染のcopro-DNA診断

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Copro-DNA diagnosis of prepatent infection with *Echinococcus multilocularis* in combination with anthelmintic treatment

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Prevalence of *Echinococcus multilocularis* in foxes in Hokkaido has been around 40%, increasing the risk of infections to domestic dogs and cats. At present, standard diagnostic procedure for dogs and cats is detection of coproantigen and fecal taeniid eggs, followed by the confirmative DNA detection from the eggs. However, during the prepatent period, eggs cannot be detected while coproantigen is already positive. Unfortunately, positive results of coproantigen alone can not be definitive indication of the infection because of its cross reactivity with other *Taenia* infection and occasional false positive result. To correctly evaluate the risk of *E. multilocularis* and to take effective preventive measures, it is essential to detect DNA from the feces even during the prepatent period.

Five dogs were orally given 1,000 (1 dog), 150,000 (3) and 1,000,000 (1) protoscoleces of *E. multilocularis*. Feces were collected daily until 21 days after infection and DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen) and tested by specific PCR [1]. As a result, DNA was detected only sporadically, indicating that it is difficult to detect DNA during the prepatent period.

In the next experiment, two dogs were orally given 10,000 and 100,000 protoscoleces respectively. Fourteen days after infection, they were treated with praziquantel.

Feces were collected until 21 days post infection and DNA was extracted and tested by the PCR. Before the praziquantel treatment, only one sample was positive for the PCR. But after the treatment, the PCR was positive in feces of both dogs. Detected DNA is probably derived from the worms that were killed and excreted together with feces. These results suggest that DNA detection from feces in combination with the praziquantel treatment can be a new option for the diagnosis of *E. multilocularis* infection in the definitive hosts.

Key words: *Echinococcus multilocularis*, Diagnosis, copro-DNA

Reference

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FULL PAPER *Parasitology*

Genetic Uniformity of *Echinococcus multilocularis* Collected from Different Intermediate Host Species in Hokkaido, Japan

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ABSTRACT. DNA from several isolates of *Taenia taeniaeformis* and *Echinococcus multilocularis* were digested with restriction enzymes and hybridized with digoxigenated oligonucleotide probe $(CAC)_5$. Within the six wild isolates of *Taenia taeniaeformis* from Norway rats in Hokkaido, although several bands were common among isolates, fingerprinting patterns were specific to each isolate. In the case of *E. multilocularis*, regardless of hosts from which each isolate has been isolated, the five isolates collected from Hokkaido, showed the same fingerprinting pattern. These results indicate that there was very little genetic difference among these isolates. Although the fingerprinting pattern of *E. multilocularis* from St. Lawrence Is. was similar to that of the Hokkaido isolates, some bands were different from those in the Hokkaido isolates. *Echinococcus multilocularis* in Hokkaido seems to be closely-related genetically to that from St. Lawrence Is.

KEY WORDS: different intermediate host species, DNA fingerprinting, *Echinococcus multilocularis*, genetic uniformity, Hokkaido.

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Intraspecific variation has been described from different geographic areas or host species in all major groups of parasites by applying a number of differential criteria [25]. Traditionally, the morphology was used as the sole differential criterion. However, more recently several intrinsic or extrinsic characteristics have been used [26]. *Echinococcus granulosus*, which is responsible for cystic hydatid disease in humans and animals, is known to exist as biologically and genetically distinct subspecific variants or strains. However, few intraspecific variations of *E. multilocularis* have been reported [2, 4].

Echinococcus multilocularis, which is responsible for alveolar hydatid disease in humans, is one of the most medically important cestodes in the holarctic region. The natural intermediate hosts of *E. multilocularis* are arvicoline rodents. However, in Hokkaido Japan, natural infections of *E. multilocularis* in swine and horse, which have been scarcely reported in other geographical areas, have been frequently observed [12, 21]. Moreover, natural infection of *E. multilocularis* in a Norway rat, *Rattus norvegicus*, has been found in southern Hokkaido [16]. Although many species of mammals have been reported as possible natural or experimental intermediate hosts of *E. multilocularis*, there are few reports on infection in the Norway rat, swine, or horse outside Hokkaido [15, 20, 23]. Therefore, the possibility that a different strain or population exists in the Hokkaido district from those reported in other geographical areas is considered.

Sequencing of mitochondrial or nuclear genes is a useful tool to infer the phylogenetic relationships of organisms. So we have examined the partial sequences of mitochondrial CO1 gene for Hokkaido isolates of *E. multilocularis*. However, all isolates shared the same sequence of CO1 gene [19].

DNA fingerprinting has proved to be powerful in resolving genetic identity or relationships and is applied in many diverse areas of biological sciences including forensic science, paternity testing, animal breeding and population genetics [6, 8, 9]. One attractive DNA fingerprinting method is the detection of hypervariable simple repetitive DNA by means of oligonucleotide probes, which make it possible to establish highly informative DNA fingerprints for any eucaryotic organisms. Oligonucleotide probe $(CAC)_5$ is multilocus and a very informative probe to identify human individuals [28]. It is also useful for verifying genetic relationships in domestic animals and wild birds [5]. Okamoto *et al.* [18] applied DNA fingerprinting with $(CAC)_5$ to analysis of genetic variation within *Taenia taeniaeformis* and reported that $(CAC)_5$ was a highly resolvable and informative probe for cestodes.

Taenia taeniaeformis is a common parasite of cats in Japan, and its intermediate hosts are Norway rat (*Rattus norvegicus*), small Japanese field mouse (*Apodemus argenteus*) and gray red-backed vole (*Clethrionomys rufocanus bedfordiae*) so far reported. From these intermediate hosts, all isolates of *T. taeniaeformis* from Norway rats shared the same sequences for CO1 gene so far examined [19]. However, DNA fingerprinting patterns of those isolates constructed with the oligonucleotide probe $(CAC)_5$ were different from each other [18]

In this study, we examined the genetic variability of *Echinococcus multilocularis* collected from different intermediate host species using DNA fingerprinting and discussed the genetic features of *E. multilocularis* population in Hokkaido comparing with the case of *T. taeniaeformis*.

Table 1. Hosts and geographical origins of cestodes examined in this study

Species	Isolate	Host	Geographical origin
Echinococcus multilocularis	EmHok	Gray red-backed vole	Higashimokoto, Hokkaido, Japan
	EmTob	Gray red-backed vole	Tobetsu, Hokkaido, Japan
	EmYak	Gray red-backed vole	Yakumo, Hokkaido, Japan
	Empig	Pig	Kitami, Hokkaido, Japan
	Emrat	Norway rat	Kamiiso, Hokkaido, Japan
	EmStL	Tundra vole	St. Lawrence Is., U.S.A.
Taenia taeniaeformis	TtSap1	Norway rat	Sapporo, Hokkaido, Japan
wild isolates	TtSap2	Norway rat	Sapporo, Hokkaido, Japan
	TtSap3	Norway rat	Sapporo, Hokkaido, Japan
	TtTom	Norway rat	Tomikawa, Hokkaido, Japan
	TtKam	Norway rat	Kamiiso, Hokkaido, Japan
	TtEbe	Norway rat	Ebetsu, Hokkaido, Japan
	TtCat	Cat	Sapporo, Hokkaido, Japan

Gray red-backed vole: *Clethrionomys rufocanus bedfordiae*, Norway rat: *Rattus norvegicus*, Tundra vole: *Microtus oeconomus*.

MATERIALS AND METHODS

Parasites: Five Hokkaido isolates and one Alaskan isolate of *E. multilocularis* were examined. Each isolate was passaged by intra-peritoneal injection in Mongolian gerbils (*Meriones unguiculatus*) in Hokkaido University. As for isolates of *Taenia taeniaeformis*, 6 wild isolates were used. These taeniid samples were stored in liquid nitrogen, at –80°C or in 70% ethanol until required for DNA extraction. The sample list and locality map are shown in Table 1 and Fig. 1, respectively.

Preparation of DNA: DNA fingerprinting requires comparably high-molecular weight DNA, so we have prepared the genomic DNA using the extraction with phenol. The details of the method have been given in a previous report [18].

Sequencing for CO1 gene: A partial fragment of CO1 gene was amplified from the total DNA by PCR using the primer pair pr-a and pr-b [19]. Direct sequencing of the PCR amplification product was performed with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, U.S.A.) using the pr-a and pr-b.

DNA fingerprinting with oligonucleotide probe $(CAC)_5$: Approximately 1 μg of taeniid DNA was digested with 30–50 units of restriction endonuclease *Pst* I or *Pvu* II (Nippon Gene), using the buffer and reaction conditions as recommended by the manufacturer of the respective enzymes. Reaction was stopped by the addition of 1/10 volume of 50% glycerol, 10 mM $NaHPO_4$, 100 mM EDTA and 0.4% bromophenol blue. Digested DNA was loaded on 0.8% agarose gel and run in TBE buffer (89 mM boric acid, 89 mM Tris, 2 mM EDTA).

After electrophoresis, DNA was denatured by soaking the gel for 30 min in 0.5 M NaOH, 1.5 M NaCl with constant gentle agitation and then neutralized by soaking for 30 min in 0.5 M Tris-HCl (pH 7.8), 1.5 M NaCl. Thereafter, DNA was blotted with 20 × SSC(3 M NaCl, 0.3 M Nacitrate, pH7.0) on to positively charged nylon membranes (Hybond N+, Amersham, U.S.A.) for 6 hr.

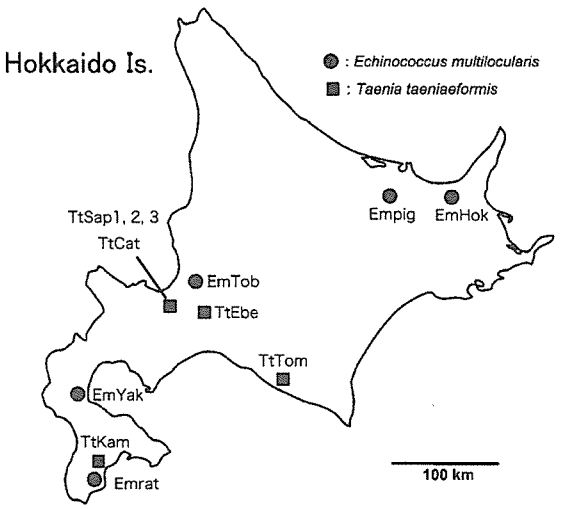

Fig. 1. Collection localities of *Echinococcus multilocularis* and *Taenia taeniaeformis* in Hokkaido Island. For explanations of abbreviations of sample names, see Table 1. Four isolates of *T. taeniaeformis* from Sapporo were collected from distant localities in the same city.

The oligonucleotide $(CAC)_5$ was chemically synthesized and purified by reversed phase HPLC. Labeling with digoxigenated dUTP was done in terminal deoxynucleotidyl transferase using a DNA Tailing Kit (Roche Diagnostics, Germany). Membranes were baked at 80°C for 2 hr. Hybridization with digoxigenated probe and immunological detection were performed by using a DIG Nucleic Acid Detection Kit (Roche Diagnostics). Prehybridization and hybridization were done at 42°C for 1 hr and for at least 10 hr respectively. Filters were then washed twice in 2 × SSC containing 0.1% SDS at room temperature for 5 min and for 15 min, respectively. These were washed twice in 2 × SSC containing 0.1% SDS at 45°C for 30 min. Subsequent immunological detections were performed according to the manufacturer's instructions.

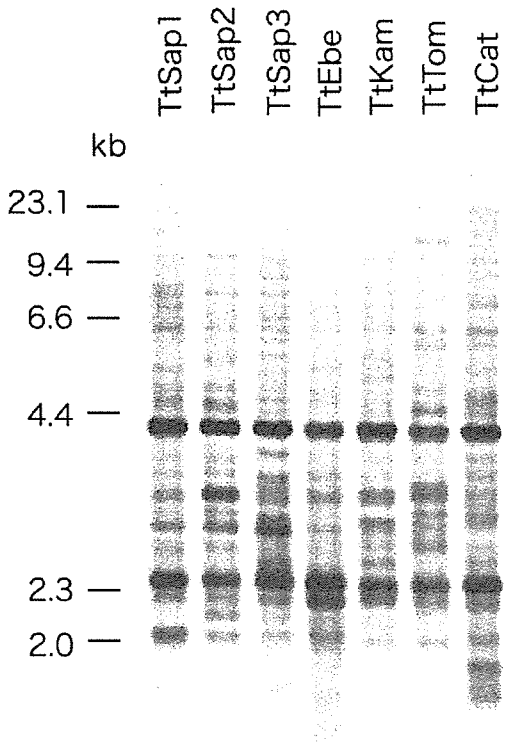

Fig. 2. DNA fingerprinting of wild isolates of *T. taeniaeformis* with digoxigenated oligonucleotide probe $(CAC)_5$. DNA was digested with *Pst* I and electrophoresed in 0.8% agarose gel at 30 V 15 hr. Molecular weight markers are given on the left in kilobases.

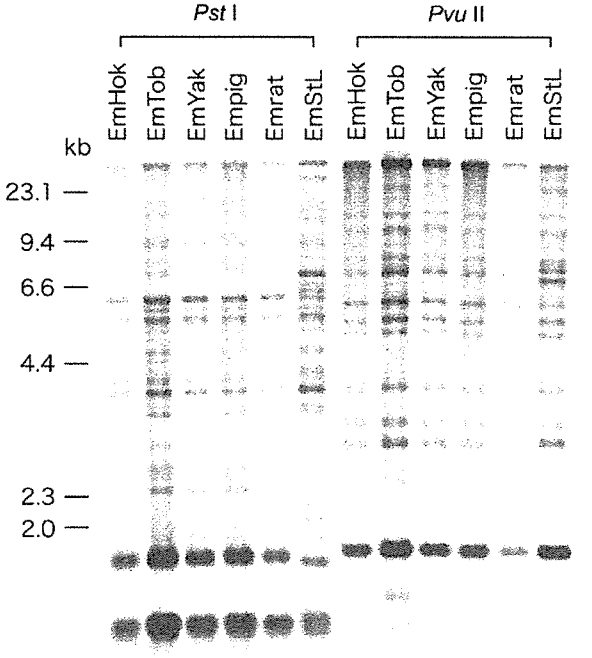

Fig. 3. DNA fingerprinting of six isolates of *Echinococcus multilocularis* with digoxigenated oligonucleotide probe $(CAC)_5$. DNA was digested with *Pst* I or *Pvu* II and electrophoresed in 0.8% agarose gel at 20 V 24 hr. Molecular weight markers are given on the left in kilobases.

RESULTS

Sequencing of the CO1 gene: Partial sequences of the mitochondrial CO1 gene from some samples had been examined [19]. In this study, therefore, all wild isolates of *T. taeniaeformis* except TtTom were examined. All wild isolates of *T. taeniaeformis*, including TtCat, shared the same sequence as that from TtSRN (GenBank/EMBL/DDBJ accession no. AB221484) [19]. No sequence variation was observed in *E. multilocularis*, regardless of the hosts or geographical areas from which each metacestode had been isolated [19]. Phylogenetic relationships of taeniid cestodes, including some isolates examined in this study, inferred from the CO1 gene have been reported [19].

DNA fingerprinting: DNA from *T. taeniaeformis* were digested with *Pst* I and hybridized with digoxigenated oligonucleotide probe $(CAC)_5$. All isolates showed clear multibanding patterns, which were characteristic of multilocus DNA fingerprinting (Fig. 2). Fingerprinting patterns of wild isolates of *T. taeniaeformis* resembled each other. However, several bands were specific to some isolates, so isolate was easily distinguishable each other by fingerprinting patterns.

Fingerprinting patterns of 6 isolates of *E. multilocularis* when digested with *Pst* I and *Pvu* II are shown in Fig. 3. Regardless of hosts from which each isolate had been isolated, the five isolates from Hokkaido showed completely the same fingerprinting pattern. Fingerprinting pattern of EmStL was similar to that of the Hokkaido isolates. However, some bands seen in EmStL were either different in molecular size, or missing in Hokkaido isolates. Moreover additional bands were seen in EmStL.

DISCUSSION

The DNA fingerprinting pattern often is specific to an individual, except in extreme case of inbreeding or in monozygotic twins, or clones. It was reported that $(CAC)_5$ represented the informative fingerprints for genetic analysis

of *T. taeniaeformis*, when digested with *Hinf* I [18]. In the case of *E. multilocularis*, digestion with either *Pst* I or *Pvu* II was effective in preliminary study (data not shown). In this study, therefore, the digestion with *Pst* I was applied.

One of the major problems with applying multilocus DNA fingerprinting to population analysis is that it is impossible to identify which bands are derived from the same locus, especially between distant organisms. Although wild populations usually are highly polymorphic, with many alleles at a single locus, bands that appear to be shared by individuals are not always identical alleles at the same locus [3]. On the contrary, among closely-related, especially blood-related organisms, many identical alleles are derived from the same locus, and the fingerprinting patterns resemble each other. In the present study, it seemed that six wild isolates of *T. taeniaeformis* were genetically related to each other. However, no isolate had a fingerprinting pattern that was same as that of any other isolate.

As contrasted with *T. taeniaeformis*, five isolates of *E. multilocularis* from Hokkaido showed the identical fingerprinting pattern. The main intermediate host of *E. multilocularis* in Hokkaido is a gray red-backed vole. However, several reports have been published on natural infections of *E. multilocularis* in uncommon intermediate hosts in Hokkaido Island, namely, swine [21], horses [12] and Norway rats [16]. There have been few reports of natural infection of *E. multilocularis* in these intermediate hosts in other endemic areas. In the present study, although we compared isolates derived from gray red-backed voles, swine and Norway rats, no difference was detected in their fingerprints. These results indicate that there was very little genetic difference among these isolates. It seemed that infections with *E. multilocularis* to the unusual animals in Hokkaido were not responsible for the variation in *E. multilocularis*.

Generally, wild populations are highly polymorphic, with many alleles at a single locus. It is very rare that the same fingerprinting pattern is obtained from two individuals, except in the case of monozygotic twins. Nevertheless, why were fingerprints from all Hokkaido isolates of *E. multilocularis* identical?

Although all isolates from Hokkaido Island showed an identical fingerprinting pattern, this did not mean that all isolates were as uniform as clones. Actually, Nakao *et al.* [13] reported that polymorphism of microsatellite DNA was detected in Hokkaido's population of *E. multilocularis*. Because the multilocus fingerprint is the technique used to detect RFLPs of genome DNA, sometimes it cannot detect a slight difference in DNA such as several base indels in microsatellites. Although it is known that self-insemination occurs in *Echinococcus* [10, 24], the heterozygosity of microsatellite alleles indicates that cross-fertilization also occurs in *E. multilocularis*. However, the heterozygosity observed was low in Hokkaido's population of *E. multilocularis* [13].

Since asexual proliferation occurs in the larval stage of *Echinococcus*, a large number of clonal protoscoleces are produced in intermediate hosts. When foxes prey on the rodent infected, the majority of adult worms, therefore, are clonal. An increase in homozygosity within a population of *Echinococcus* is well explained by self-fertilization [13], which can be achieved by a sperm of the same individual (autogamy) or of another clonal individual (geitonogamy) [11].

Even though the population of *Echinococcus* has such properties, if *E. multilocularis* is native to Hokkaido and has inhabited that area for a long time, geographical variation in fingerprinting should be detected, because the mutation rate of fingerprinting is very high. In humans, the spontaneous mutation rate of fingerprinting with $(CAC)_5$ has been estimated to be approximately 0.001 per DNA fragment and gamete [14]. *Echinococcus multilocularis* in Hokkaido seems to be a recent population that invaded from another endemic area. Actually, endemic areas of *E. multilocularis* on Hokkaido Island were restricted to its eastern part before 1975 [7]. In addition, if the *E. multilocularis* that invaded Hokkaido had been polymorphic genetically, variation in its fingerprinting should still have remained. The origin of *E. multilocularis* endemic in Hokkaido seems to be a single or very uniform population.

Yamashita [27] assumed that the *E. multilocularis* prevalent in Hokkaido Island was introduced from St. Lawrence Island via Komandorskie and Kuril Islands. It was reported that a sequence of CO1 gene of *E. multilocularis* from Kunashiri Island, which was the southern Island of Kuril Islands, was same as that from Hokkaido [22]. The fingerprinting pattern of EmStL resembled that from Hokkaido isolates with slight differences in several bands. Comparing with the case of *Taenia taeniaeformis*, it appears that Hokkaido's *E. multilocularis* is not identical to EmStL, but is closely-related genetically.

A partial sequence of the mitochondrial CO1 gene of EmStL examined is shared with that from Hokkaido's isolates. However, it was recently revealed that *E. multilocularis*, which had a different type of the mitochondrial CO1 gene, also inhabits St. Lawrence Island (data not shown). Thus, *E. multilocularis* endemic to St. Lawrence Island may be polymorphic. In the present study, only one isolate from St. Lawrence Island was examined. Therefore, it is possible that population, which is identical to *E. multilocularis* in Hokkaido, may inhabit St. Lawrence Island. In order to determine the origin of *E. multilocularis* in Hokkaido, additional investigations are needed, including isolates from St. Lawrence Island, Komandorskie and Kuril Islands.

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FULL PAPER

Epizootiological survey of *Trichinella* spp. infection in carnivores, rodents and insectivores in Hokkaido, Japan

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Abstract

In order to evaluate the present epidemiological situation of *Trichinella* infection in wild animals in Hokkaido, Japan, red foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*), brown bears (*Ursus arctos*), martens (*Martes melampus*), rodents and insectivores captured in Hokkaido were examined for muscle larvae by the artificial digestion method from 2000 to 2006. Foxes (44/319, 13.8%), raccoon dogs (6/77, 7.8%) and brown bears (4/126, 3.2%) were found to be infected with *Trichinella* larvae and all other animal species evaluated were negative. Multiplex PCR and DNA sequencing revealed that larvae from a fox captured in Otofuke, in south-eastern Hokkaido, were *T. nativa*, and larvae from 27 animals including 21 foxes, 2 raccoon dogs and 4 brown bears captured in western Hokkaido were *Trichinella* T9.

Key Words : Epizootiology wild animals, Japan, *Trichinella nativa, Trichinella* T9, Zoonosis

Introduction

Trichinellosis is a zoonotic disease caused by nematodes of the genus *Trichinella*. Numerous mammals as well as birds and reptiles are known to harbor this parasite in their muscles. So far, the genus *Trichinella* is classified into eight species (*T. spiralis, T. nativa, T. britovi, T. pseudospiralis, T. murrelli, T. nelsoni, T. papuae* and *T. zimbabwensis*) and three genotypes (*Trichinella* T6, *Trichinella* T8 and *Trichinella* T9)[14]. Since the discovery

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of *T. spiralis* in 1835, human trichinellosis had been considered to be associated with the consumption of pork[2]. However, the recent advances in the molecular techniques could reveal the presence of sylvatic sycles and sylvatic *Trichinella* containing *Trichinella* spp. other than *T. spiralis*. Today, it is known that *T. spiralis* is maintained mainly in domestic swine and the sylvatic *Trichinella* are maintained in the wild animals[17].

In Japan, the first case of trichinellosis was reported in a domestic dog in Hokkaido prefecture in 1957[11], thereafter, there have been three human outbreaks. The first occurred among local hunters in Aomori prefecture in 1974[22], caused by the consumption of black bear (*Ursus thibetanus*) meat, and the second and third outbreaks occurred at the restaurant in Hokkaido prefecture in 1979 and in Mie prefecture in 1981, respectively, caused by the consumption of brown bear (*U. arctos*) and black bear meat[22]. Since 1974, many wild animals have been examined to disclose the epizootiology of *Trichinella* infection in Japan ; however, by 1998, only two black bears[22], one red fox (*Vulpes vulpes*)[9] and one raccoon dog (*Nyctereutes procyonoides*)[20] in the northern part of mainland Japan, were found to harbor *Trichinella* muscle larvae.

In Hokkaido, the northern island of Japan, several kinds of mammals, including 198 foxes and 89 brown bears, were examined for *Trichinella* infection before 1999 but no animals were found to be infected[22,23]. In 1999, 5 of 43 (11.6%) red foxes examined were found to be infected with *Trichinella* larvae in Otaru, Hokkaido[24]. In addition, the presence of *T. nativa* and *Trichinella* T9 in Hokkaido was reported in 2006[6]. However, before the present study, only the six cases of *Trichinella* T9 from foxes in Otaru and Sapporo and one case of *T. nativa* from fox in Otofuke were reported in the limited area of Hokkaido. In this study, we investigated the prevalence of *Trichinella* infection in wild animals on a large scale and discussed the distributional pattern of *T. nativa* and *Trichinella* T9 in Hokkaido.

Materials and Methods

Animals and parasitological examination

From 2000-2006, 525 carnivores, including 319 red foxes (*V. vulpes*), 77 raccoon dogs (*N. procyonoides*), 126 brown bears (*U. arctos*) and 4 martens (*Martes melampus*) were shot or trapped by local hunters to prevent agricultural losses or for academic surveys in Hokkaido prefecture, Japan (Fig. 1). A total of 344 rodents and 27 insectivores were also trapped in this study (Table 1). Most of the foxes, rodents and insectivores and all of the raccoon dogs were captured in Otaru and Sapporo ; other animals were captured elsewhere in Hokkaido. Foxes, raccoon dogs and martens captured in Otaru were frozen at −80℃ for at least one week prior to muscle sampling in order to sterilize the eggs of *Echinococcus multilocularis* that are prevalent in Hokkaido. Some brown bears were frozen at around −30℃ for preservation prior to transportation to our laboratory. The other foxes and brown bears were delivered to the laboratory at low temperature but not frozen. Rodents and insectivores were examined as fresh samples.

Muscles were collected from the hind legs or tongue of all the animals, except for rodents and insectivores, from which the whole diaphragm, tongue and masseter were collected. At least 10 g of the muscles of carnivores or all of the collected muscles of rodents and insectivores (approximately 1-2g) were digested with artificial digestion fluid (200 ml of NaCl saline containing 1% pepsin and 1% HCl) at 37℃ for 2 hours according to a standard procedure[3]. Motile larvae detected were

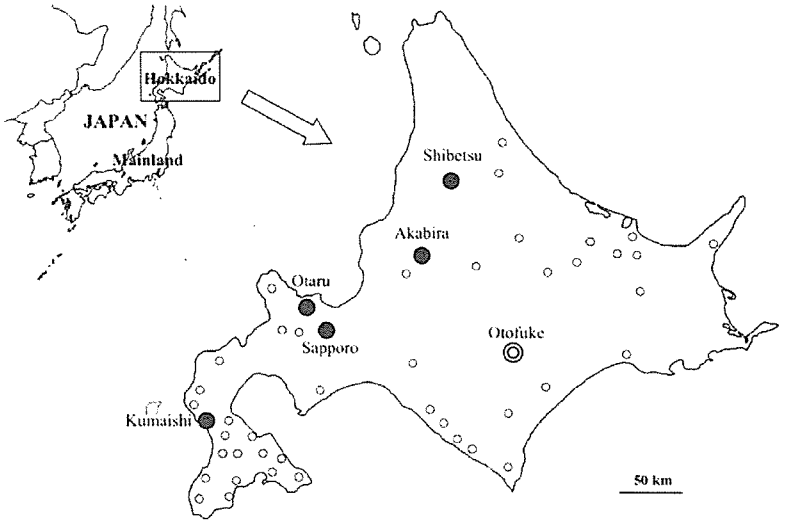

Fig. 1. Geographical distribution pattern of *Trichinella nativa* and *Trichinella* T9 in Hokkaido, Japan. The localities where foxes, raccoon dogs or brown bears were examined for *Trichinella* spp. are indicated by filled symbols (*Trichinella* T9 positive), double circle (*T. nativa positive*) or open circles (*Trichinella* not detected).

Table 1 Prevalences of *Trichinella* spp. infection in wild animals in Hokkaido, Japan from 2000-2006.

Host animals	Sampling places (no. positive / no. examined)			
	Otaru city	Sapporo city	Other areas	Total (%)
Carnivora				
Red fox (*Vulpes vulpes*)	41 / 254	1 / 39*	2 / 26*	44 / 319 (13.8)
Raccoon dog (*Nyctereutes procyonoides*)	6 / 77			6 / 77 (7.8)
Brown bear (*Ursus arctos*)		0 / 1	4 / 125	4 / 126 (3.2)
Japanese marten (*Martes melampus*)	0 / 4			0 / 4
Rodentia				
Gray red-backed vole (*Clethrionomys rufocanus*)	0 / 62	0 / 72		0 / 134
Northern red-backed vole (*Clethrionomys rutilus*)	0 / 3	0 / 4		0 / 7
Large japanese field mouse (*Apodemus speciosus*)	0 / 114	0 / 43		0 / 157
Small japanese field mouse (*Apodemus argenteus*)	0 / 28	0 / 13		0 / 41
Brown rat (*Rattus norvegicus*)		0 / 3		0 / 3
Black rat (*Rattus rattus*)		0 / 2		0 / 2
Insectivora				
Long-clawed shrew (*Sorex unguiculatus*)	0 / 12	0 / 14		0 / 26
Shrewmouse (*Sorex caecutiens*)	0 / 1			0 / 1

* One fox each captured in Sapporo and Otofuke[6] were included.

inoculated orally into gerbils for serial passage. Gerbils were kept in our laboratory under the Guidelines for Animal Experiments of the Graduate School of Veterinary Medicine in Hokkaido University.

Host age determination

The ages of foxes captured in Otaru were determined by counting the number of canine cementum annuli[10]. Extracted canines were cut into 3 mm pieces using a microcutter (Maruto, MC-201) and decalcified with Plank-Rychro solution (8.5% hydrochloric acid, 7% alumini chloridum and 5% formic acid in distilled water) for 48 hours. The canines were then deacidified with 5% sodium sulfate for 24 hours and washed in tap water for 24 hours. The decalcified canines were cut into 45 μm pieces using a freezing microtome. Sections were stained with Delafield's hematoxylin, mounted in Canada balsam and the number of annuli were counted under a microscope.

The raccoon dogs captured in Otaru and the foxes captured in Sapporo were divided into juveniles (<1 year) and adults (≥1 year) by the dental formula method as previously described[5,21].

Worm preparation and DNA extraction

Since our preliminary examination demonstrated that it was difficult to yield the PCR amplicon from DNA obtained from dead larvae after the artificial digestion of frozen muscles, worms were collected directly from muscles to avoid artificial digestion in this study. The presence of *Trichinella* spp. muscle cysts was confirmed by pressing *Trichinella*-infected muscles using Petri dishes. Muscle larvae were then collected with forceps and needles under a dissection microscope. Collected larvae were individually preserved in Tris-EDTA buffer at −30℃ until use. DNA was extracted from single larvae according to the previously described method[1]. Briefly, individual larvae were placed in a 200 μl tube containing 2 μl of 5mM Tris-HCl, overlaid by mineral oil and heated at 90℃ for 10 minutes. To the tube was added 1 μl of proteinase K (1 μg/μl, Takara) and 2 μl of water, followed by incubation at 37℃ for 2 hours. After incubation, the tube was heated at 90℃ for 10 minutes to inactivate the enzyme and preserved at −30℃ until use.

Trichinella larvae from 28 infected animals were subjected to molecular identification. Four individual larvae from each animal were analyzed separately by Multiplex PCR and DNA sequencing of the mitochondrial cytochrome oxydase subunit I (COI) gene as described previously[6,25].

Statistical analysis

Among the 254 foxes captured in Otaru in 2000, 2001 and 2004, 206 of predetermined sex and age were analyzed for the risk factors of *Trichinella* infection by logistic regression model. Sex and age of host and the year of capture were set as independent variables. Statistical analyses were performed using StatView® 5.0 (SAS Institute Inc.).

The prevalence of *Trichinella* larvae in adult foxes in Otaru and Sapporo was statistically analyzed by Fisher's exact test using the R software package version 2.0.1 (http://www.r-project.org/). These two cities are located next to each other in Hokkaido. Foxes in Sapporo were captured on a plain where farms, houses and factories were scattered. Foxes in Otaru were mainly captured on cropland at the foot of wooded hills.

P-values ≤0.05 were considered statistically significant.

Results

A total of 44 foxes (infection rate = 13.8%), 6 raccoon dogs (7.8%) and 4 brown

bears (3.2%) were found to be infected with *Trichinella* spp. and all other animal species examined were negative (Table 1). Among the 54 infected animals, 47 were found in Otaru. Although no motile larvae were obtained from frozen samples, motile larvae were collected from non-frozen samples of three red foxes captured in Sapporo, Shibetsu and Otofuke, and two brown bears in Akabira. Gerbils inoculated with motile larvae were sacrificed a few months later and *Trichinella* larvae were collected from the muscle by artificial digestion.

Among the 254 samples of foxes captured in Otaru, 206 were of predetermined sex and age. Of these 206 foxes, none of the 60 juveniles were infected with *Trichinella* larvae, whereas 21.2% (31/146) of adult foxes were infected. The logistic regression model showed that age was the only significant variable associated with the prevalence and prevalence increased along with host age (odds ratio=2.006, 95% CI=1.501-2.681, $p<0.001$).

The prevalence of *Trichinella* infection in adult foxes in Otaru (20.9%, 31/148) was significantly higher than that in Sapporo (4.5%, 1/22) (Fisher's exact test, $p<0.05$).

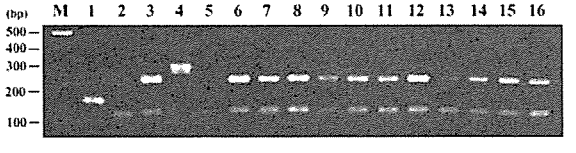

Fig. 2. Multiplex PCR products of *Trichinella* muscle larvae from various animals in Hokkaido, Japan. Lane 1: *T. spiralis* reference larva (ISS 413), lane 2: *T. nativa* reference larva (ISS 10), lane 3: *Trichinella* T9 reference larva (ISS 408), lane 4: *T. pseudospiralis* reference larva (ISS 13), lane 5: *T. nativa* from red fox (*), lane 6-12. *Trichinella* T9 from fox, lane 13-14: *Trichinella* T9 from raccoon dog, lane 15-16: *Trichinella* T9 from brown bear, M: 100bp DNA ladder.
*The result of *T. nativa* was reported previously[6].

On agarose gel electrophoresis of multiplex PCR amplicons, the muscle larvae detected from 21 foxes, 2 raccoon dogs and 4 brown bears showed two bands of 127 bp and 253 bp (Fig. 2, lanes 6-16), a specific pattern of the *T. britovi* complex (*T. britovi, Trichinella* T8 and *Trichinella* T9)[15]. The nucleotide sequence of part of the COI gene of larvae belonging to the *T. britovi* complex showed the highest similarity to *Trichinella* T9. As reported previously, the muscle larvae from a fox in eastern Hokkaido identified as *T. nativa*[6], but all of the present muscle larvae detected from 27 animals in western Hokkaido were identified as *Trichinella* T9 (Fig. 1). Among these 27 *Trichinella* T9 muscle larvae DNA sequences of 26 muscle larvae were identical while the other sample showed a single nucleotide difference. The former was completely identical to previously reported sequences of *Trichinella* T9 (DQ007898, AB091477) isolated in mainland Japan.

Discussion

The present study demonstrated that *Trichinella* infections were prevalent among foxes, raccoon dogs and bears in Hokkaido and *Trichinella* T9 distributed widely in the western part of Hokkaido.

Until 1999 when a relatively high prevalence of *Trichinella* infection among the fox population in Otaru city (11.6%, 5 out of 43 foxes) was reported[24], the prevalence of *Trichinella* infection in wild animals in Japan was considered low[22]. The present work demonstrated that the report in Otaru in 1999[24] was not a temporal phenomenon but a high prevalence was maintained in foxes and raccoon dogs in Hokkaido. In studies carried out about 20 years ago, *Trichinella* infections were not detected among 198 foxes and 88 brown bears examined in Hokkaido[22,23]. The previous researchers examined small portions

(approximately 5 g) of masseter muscles that were considered the common muscle site for *Trichinella* detection based on the study of pigs and rodents ; however, the site was later shown not to be a preferable site for *Trichinella* detection in carnivores[7]. Above mentioned defects of sampling in the previous studies might cause the underestimation of *Trichinella* infections in Hokkaido. The 198 foxes in the previous study were captured in areas different from in the present study, only the 3 foxes were captured in Otaru, in which most of the foxes were investigated in this study. The present result indicated that the prevalence differed in each sampling area (Table 1). Therefore, it is difficult to compare directly the present result with previous studies.

Statistical analysis by a logistic regression model showed that the prevalence of *Trichinella* infection in foxes increased with host age. Similar observations were reported in polar bears and lynxes and were assumed to be related with the increase of opportunities for acquiring *Trichinella* infection, and long survival of the larvae in the muscle[12, 18, 26].

The difference in the prevalence of *Trichinella* infection among foxes in Otaru and Sapporo is related to their food differences. The foxes captured in Sapporo were nesting in the anthropogenic structures, such as farms, barns or houses, and were considered to depend on more human products for their food compared with the foxes captured in Otaru, which were nesting near cropland[4]. In the Otaru area, there are more wild animals and animal cycles of *Trichinella*, such as fox-fox, -raccoon dog, -bear, or-vole transmission may maintain stably.

Besides foxes and raccoon dogs, four brown bears were infected with *Trichinella* T9. Before this study, only one case of *Trichinella* sp. infection in brown bear had been reported in Japan[22]. So far, bear meat has been the exclusive means of transmission for human trichinellosis in Japan (excluding suspected or imported cases). Recently, the cases of human trichinellosis associated with non-pork products increased in the United States and during 1997-2001, 51% of the cases were associated with bear meat[19]. Although the observed prevalence in brown bears was lower than that of foxes, bear meat seemed to be a more important source of human trichinellosis when considering Japanese dietary habits. In Japan, hunters and their relatives tend to eat bear meat[22, 23], but, as to the foxes, Japanese do not have a traditional culture for eating fox meat, although the human trichinellosis caused by consumption of fox meat was reported in Italy[16]. In addition, the large mass of brown bears may serve as a infectious source of infection for large numbers of humans even if the actual prevalence of the disease is low. Game meats, such as bears and deer which are possibly harboring the *Trichinella* larvae must be cooked well, since the freezing is not efficient to inactivate the *T. nativa* larvae, which was reported to survive at −18℃ for 4 years[8].

Trichinella nematodes from 23 red foxes, 2 raccoon dogs and 4 brown bears in 5 localities in western Hokkaido were identified as *Trichinella* T9, whereas the nematode from the south-eastern part was identified as *T. nativa* (Fig. 1). The distribution pattern of *Trichinella* spp. in the northern hemisphere is known to be separated according to climate zones[13]. Since the number of samples examined in this study was limited, it could not show whether the distribution of *T. nativa* and *Trichinella* T9 were separate or overlapped. Further survey, especially in eastern districts would elucidate the distribution pattern of *Trichinella* spp. in Hokkaido.