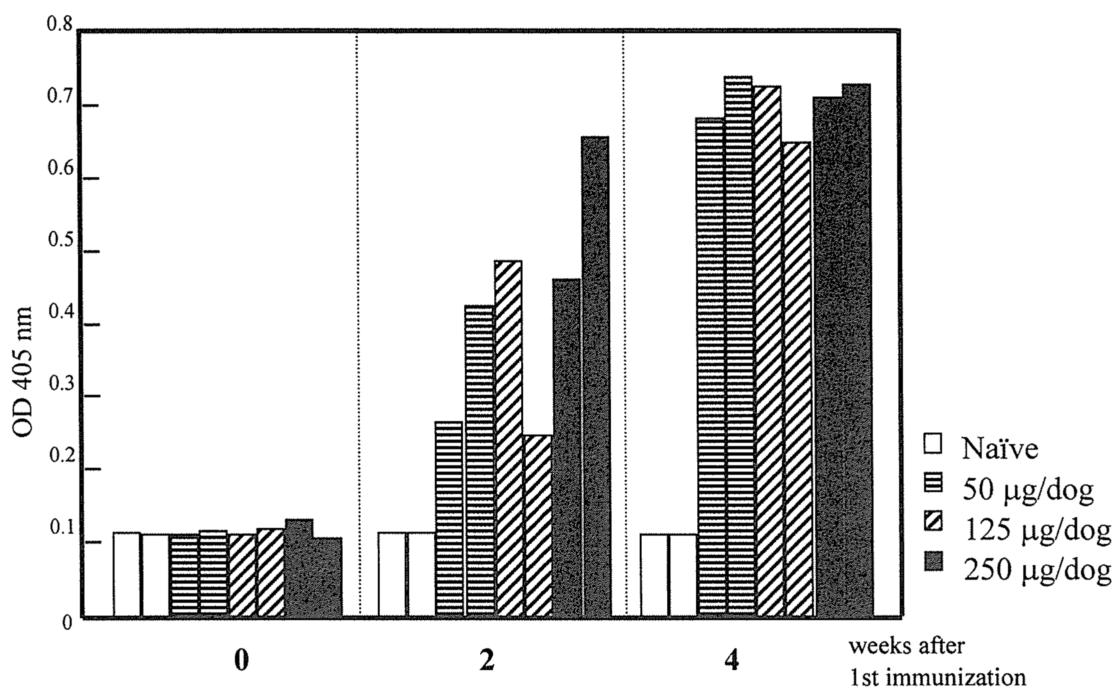


図1. LigA-m免疫イヌのIgG抗体産生

表 1. 宮崎県北部で発生したレプトスピラ症確定事例

番号	性別	年齢	職業	農業種	発生地	推定感染血清型	ネズミ分離株との反応性	
							061129-7	061201-3
1	女性	61	遺跡発掘	田・畑	延岡	Hebdomadis	＋	－
2	男性	64	農業	畑・山	日之影	Hebdomadis	＋	－
3	女性	59	農業	田・畑	延岡	Hebdomadis	＋	－
4	男性	77	農業	田・畑	延岡	Hebdomadis	未実施	未実施
5	男性	72	農業	田・畑・山	高千穂	Autumnalis	－	＋
6	男性	66	農業	田・畑	日向	Australis	－	－
7	女性	53	ホテル従業員	畑	延岡	Poi	未実施	未実施

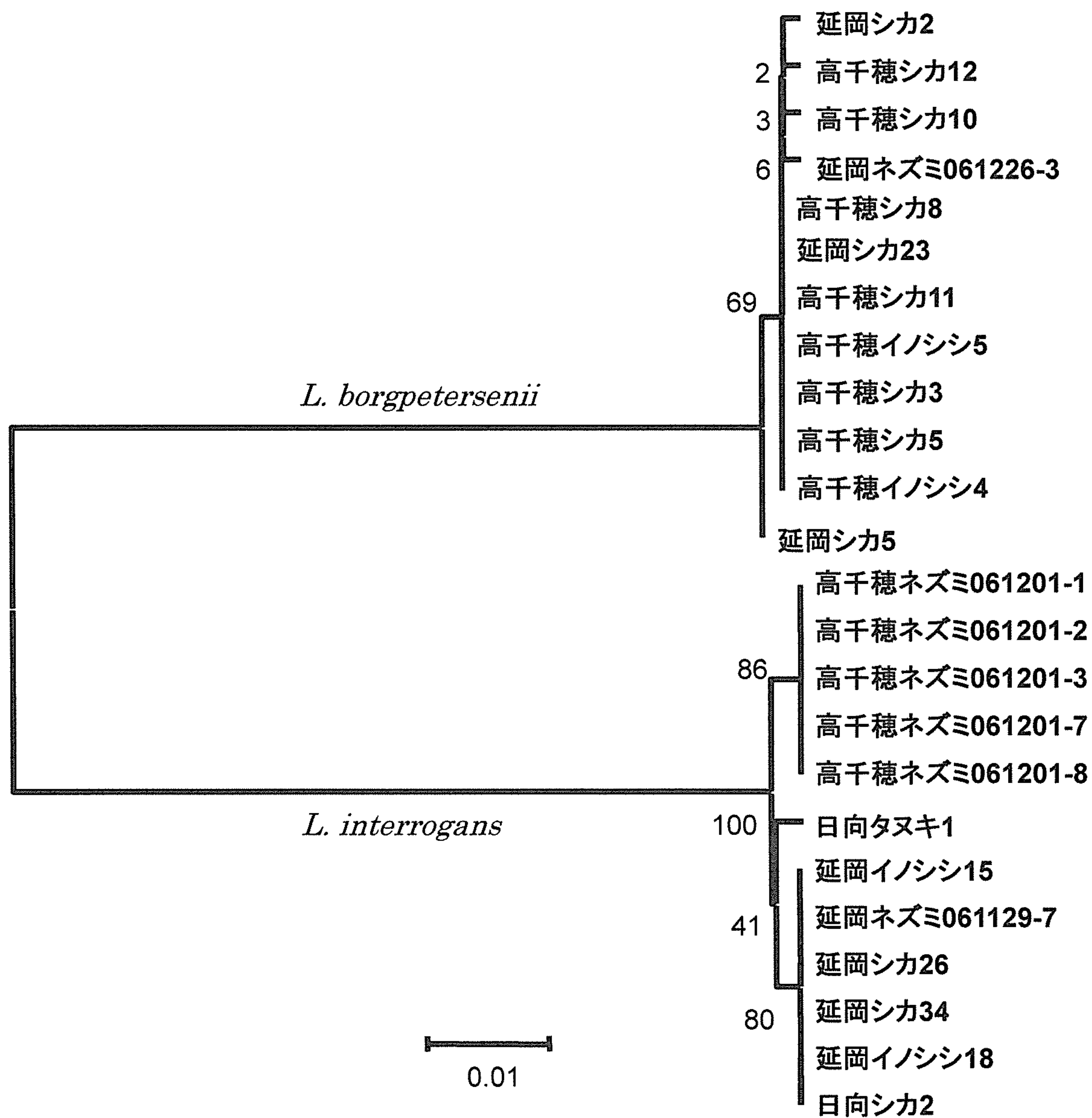
表2. イノシシ，シカ、タヌキ腎臓からのレプトスピラ *flaB* 遺伝子の検出

捕獲地域	動物種	陽性数／捕獲数	陽性率（%）
延岡市	イノシシ	2／22	9.1
延岡市	シカ	5／36	13.9
延岡市	タヌキ	1／1	100
高千穂町	イノシシ	2／14	14.3
高千穂町	シカ	6／14	42.9
日向市	イノシシ	0／3	0
日向市	シカ	1／2	50

表3. 延岡市Y獣医科病院レプトスピラ疑いイヌの血清診断結果

No	初診日	採血年月日	主要症状	飼養場所	飼養目的	ワクチン	MAT
1	不明	不明	発熱・黄疸・粘膜の充出血	延岡市	猟犬	無	陰性
2	2004/9/15	2004/9/15	発熱・黄疸・粘膜の充出血	延岡市	猟犬	無	陰性
3	2004/8/2	2004/8/2	黄疸・粘膜の充出血	延岡市	猟犬	無	Hebdomdis, Kremastos
4	2004/9/13	2004/9/13	黄疸・粘膜の充出血	北川町	猟犬	無	Hebdomdis, Kremastos, Castellonis
5	2002/2/12	2002/2/12	記録なし	延岡市	猟犬	無	Australis
6	2000/9/18	2000/9/18	記録なし	北方町	猟犬	無	Castellonis
7	2000/9/18	2000/9/18	記録なし	北方町	猟犬	無	Hebdomadis
8	2000/9/18	2000/9/18	記録なし	北方町	猟犬	無	Hebdomadis

図 2. *flaB* 塩基配列(691 bp)に基づく系統樹



厚生労働科学研究費補助金（新興・再興感染症研究事業）
総括研究報告書

輸入動物、並びに野生動物におけるレプトスピラ保有実態の解析

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要旨

輸入齧歯類10種151頭からレプトスピラの検出を行った。腎臓培養からはレプトスピラは検出されなかった。一方、鞭毛遺伝子を標的としたPCRによりステップレミング1頭の膀胱抽出DNAからレプトスピラ特異的遺伝子が検出された(検出率 0.7%)。検出された増幅DNAは遺伝子のシークエンス解析、ならびに遺伝系統解析により*Leptospira alexsanderii*と同定された。日本に存在しない遺伝種のレプトスピラが輸入齧歯類を通じて侵入していることが示された。宮城県内、長野県内、名古屋市内のマンホール、西宮市内のマンホールにおいて野鼠の捕獲調査を行いレプトスピラの分離を試みた。名古屋市内(保有率 11.1%)および西宮市内(保有率 3.1%)のドブネズミからレプトスピラの分離に成功し、これらはいずれも*L. interrogans*に属するが、血清型を同定できない未同定血清型であることが明らかとなった。一方、宮城県内(保有率 7.7%)および長野県内(保有率 4.4%)の山間部や田園地帯において捕獲されたアカネズミからレプトスピラが分離された。これらは*gyrB*遺伝子解析により*L.interrogans*と同定され、さらには免疫抗血清を用いた交差凝集試験により血清型Autumnalisであると同定された。

以上の結果より、都市部の下水道などに生息するドブネズミは今日でもレプトスピラの重要な保有体動物であることが確認され、下水道などの管理従事者などは感染のリスクがあることが明らかとなた。さらに、古来よりレプトスピラ症の1大発生地であった宮城県においては今日においても、野生齧歯類のレプトスピラ保有が確認され、農業従事者などの感染リスクがあることが明らかとなった。

研究協力者

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A. 研究目的

本研究班では主にげっ歯類を保有動物とするレプトスピラ症の輸入動物、ならびに国内野鼠の保有状況を調査した。すでに、我々は過去の調査から国内では全く予想しない血清型レプトスピラを見いだしている。また、2005年にはアメリカ産のアメリカモモンガを感染源とするレプトスピラ症の診断、並びに感染源の特定を行い、輸入動物を介した感染症の侵入があることを明解に示した。本年度は、輸入げっ歯類並びにかつてのレプトスピラ症の大発生地であった宮城県、さらにこれまで定点調査を実

施してきた名古屋市内、さらに新たに西宮市内、長野県、石川県白山で、調査を実施した。他に北海道でも調査を行ったが、これについては、現在培養を観察中である。

B. 研究方法

1. 野鼠の捕獲

1. 輸入げっ歯類からのレプトスピラの分離

日本で輸入したげっ歯類の腎臓及び膀胱は宇根有美助教授（麻布大学獣医学部）より分与を受けた。動物を安楽死させ、腎臓を注射器を用いて、ホモジェナイズし 0.1%アガロース、2.5%ウサギ血清を含むEMJH培地に注入した。翌日、上清約 0.1ml を同じ別の培地に接種し、これを30℃で3カ月間培養した。培養は2週間ごとに増殖の有無を暗視野顕微鏡で観察した。

2. PCR による膀胱からのレプトスピラ *flaB*遺伝子の検出

輸入げっ歯類の膀胱を滅菌したカッターナイフで米粒大に切断し、Quick gene 800 DNA 抽出機を用いて、DNA を抽出した。これを PCR 試料とした。First PCR には、表 1 に示した L-flaB-F1 と L-flaB-R1 を用いた。PCR は熱変性 94℃で 30 秒、アニー リング反応 62℃で 30 秒間、伸長反応 72℃で1分間のサイクルを 40 サイクル繰り返し、最後に最終伸長反応を72℃で 10 分間行った。nested PCR は first PCR 産物を鋳型とし、プライマーL-flaB-F2 と L-flaB-R2 のセットを用いて行った。PCR 反応は、94℃で3分間前熱変性を行った。さらに、熱変性 94℃で 15 秒、アニー リング反応 60℃で 30 秒間、伸長反応 72℃で 60 秒、または 15 秒間のサイクルを 30 サイクル繰り返し、最後に最終伸長反応を 72℃で 10 分間行った。

3. 野鼠からのレプトスピラの分離

野鼠は金網カゴ、またはシャーマントラップで捕獲した。捕獲後、エーテルで安楽死させ腎臓を注射器を用いてホモジェナイズして、0.1%アガロース、2.5%ウサギ血清を含むEMJH培地に注入した。翌日、上清約 0.1ml を同じ別の培地に接種し、これを30℃で3カ月間培養した。培養は2週間ごとに増殖の有無を暗視野顕微鏡で観察した。

4. DNA ジャイレース B サブユニット遺伝子（*gyrB*）のシークエンス解析

培養した菌液 1mL をより InstaGene ™ Matrix（Bio Rad）を使用して鋳型 DNA を調製した。PCR は、表1に示した *gyrB*に特異的なプライマーUP1TL、UP2rTL を用い、94℃で 5 分間前熱変性、さらに熱変性 94℃で 30 秒、アニー リング反応 48℃で 1 分間、伸長反応 72℃で 90 秒間のサイクルを 35 サイクル繰り返し、最後に最終伸長反応を 72℃で 10 分間行った。増副産物を Montage ™ PCR Centrifugal Filter Devices（Millipore）を用いて精製し、これを鋳型として、BigDye Terminator v3.1 Cycle Sequencing Kit（Applied Biosystems）を用いてサイクルシークエンス反応を行った。反応後 Montage ™ SEQ96 シークエンスクリーンナップキット（Millipore）を用い、マニュアルに従って精製した。シークエンスにはプライマーUP1TL、UP2rTL、GyrF1、GyrR1 を使用した。シークエンス解析には、ポリマーは ABI Prism POP-7™（Applied Biosystems）、80cm のキャピラリーを用い ABI PRISM 3110-Avant（Applied Biosystems）により 4 時間泳動し解析した。得られたシークエンスはシークエンス連結ソフト ACGT（ゼネティクス）を用いて行った。

5. 遺伝系統解析

得られた配列をこれまで国内で分離できたレプトスピラ株の配列、ならびに日本に存在が予想される参照株の配列と比較した。解析は MegAlign（DNA star）を使用し、ClustalW ア

ルゴリズムにより配列を整列し、さらに近接結合法（NJ法）により系統樹を作製した。系統解析結果よりレプトスピラ遺伝種の決定を行った。

6. 顕微鏡凝集試験（microscopic agglutination test, MAT）

96穴丸底プレート（Iwaki）にリン酸緩衝生理食塩水（PBS）、及びPBSで25倍希釈した参照株に対するウサギ抗血清をそれぞれ25µL加え、段階希釈液を調製した。各穴に30℃で1×10^{8} cells/mLまで培養した菌液25µLを加えた。したがって最終希釈倍率は100－102,400倍となる。室温で2時間インキュベートした後、暗視野顕微鏡で観察し、抗血清を加えていないコントロールと比較し菌体の50%以上に凝集が見られた時、陽性と判定した。参照株の凝集抗体価と比較して、分離株の凝集が見られる抗血清を明らかにし、血清型の同定を行った。

C. 結果と考察

1. 輸入げっ歯類からのレプトスピラの検出

輸入齧歯類からのレプトスピラの検出を行った。10種151匹について腎臓のレプトスピラ培養を調製した。今回培養に供した151頭中でレプトスピラが培養されたものは無かった。一方、採取した膀胱よりDNAを調製して、鞭毛遺伝子*flaB*によるPCRによる検出を行ったところ、ステップレミング1頭から特異的増幅産物が検出された。増幅産物のダイレクトシークエンス反応により遺伝子配列を解析し、遺伝系統解析を行ったところ、比較に用いた参照株のうち*L. alexanderi*とクラスターを形成した。

すでに我々はこれまでにも、アメリカモモンガを介したヒト感染事例の報告、さらにはアフリカヤマネからのレプトスピラの分離、さらには何種かの輸入齧歯類からのPCRによるレプトスピラの検出について報告しているが、今回も151頭中1頭ではあるが、レプトスピラの検出に成功した。今回検出された遺伝種は*L. alexanderi*であり、この種はこれまで日本に存在することは知られていない。この事実は日本に存在しない血清型、あるいは遺伝種のレプトスピラが輸入齧歯類を通じて侵入していることをする可能があることを示している。

2. 国内の野鼠からのレプトスピラの検出

西宮市内、ならびに名古屋市内のマンホール内での野鼠の捕獲調査を行った。それぞれ97頭、並びに7頭中、それぞれ3頭からレプトスピラの分離に成功した。これらの分離株はいずれも*gyrB*のシークエンス解析により*L. interrogans*と同定された。またその配列はこれまでに日本全国で分離されている血清型の同定できないタイプ（仮に未同定血清型とする）レプトスピラと同一であった。これら分離株について、免疫抗血清を用いた交差凝集試験を行ったが、いずれも凝集は見られなかった。また、これらの分離株はいずれもドブネズミより分離されている。以上の結果からこれらの分離株が日本全国幅広く分離している未同定血清型レプトスピラであると推定された。なおこの血清型株の培養については、これまで様々な試行錯誤を繰り返して培養を試みてきたが、十分に増殖させることができず、そのために血清学的検討が実施できなかった。今年度からEMJH培地に0.1%のアガロースを添加する改良培地を用いたところ、この株群がきわめてよく増殖することが明らかになった。今後は改良培地を用いて大量培養することで、血清学的検討が可能となる。

一方、宮城県内の各地、長野県内各地、および石川県の白山で捕獲された野鼠についてレプトスピラの分離を行った。宮城県はかつてレプトスピラ症の一大発生地であったが、

従来より患者の発生が見られていた岩木山周辺地域でレプトスピラが分離された。39頭のうち3頭が培養陽性となり、gyrBのシークエンス解析から*L. interoganan*血清型Hebodmadisと同一の配列を示した。一方、血清学的検討からは、血清型Autumnalisと同定された。同じく、長野県内で分離された株も、*gyrB*シークエンス解析からは血清型Hebdomadisと同一の配列を示したが、抗Hebodmadis抗血清とは反応せず、一方抗Autumnalis抗血清とは強く反応し、血清型Autumnalisと同定された。これらはすでに日本で分離されている血清型であり、その存在は確定しているが、今回はいずれもアカネズミから分離された。

今回の結果からは、都市部のドブネズミからは血清型未同定のレプトスピラを、また農村部、山間部ではアカネズミから血清型Autumnalis が分離されるという結果が得られた。都市部のドブネズミから分離された血清型未同定株については、その培養方法を確立することができたので、今後免疫学的な検討を実施することはできる。

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E. 知的財産権の出願・登録状況

なし

Leptospirosis in Squirrels Imported from United States to Japan

Toshiyuki Masuzawa,* Yoshihiro Okamoto,* Yumi Une,† Takahiro Takeuchi,‡ Keiko Tsukagoshi,‡ Nobuo Koizumi,§ Hiroki Kawabata,§ Shuji Ohta,¶ and Yasuhiro Yoshikawa#

We diagnosed leptospirosis in 2 patients exposed to southern flying squirrels imported from the United States to Japan. Patients worked with exotic animals in their company. *Leptospira* isolates from 1 patient and 5 of 10 squirrels at the company were genetically and serologically identical and were identified as *Leptospira kirschneri*.

Leptospirosis is a worldwide zoonosis caused by infection with *Leptospira interrogans* sensu lato species. *Leptospira* is mostly transmitted to humans through contaminated water or soil and by direct contact with a variety of infected animals (*1–3*). To date, a variety of wild animals have been imported from foreign countries to Japan. In this study, 2 men working at an animal trading company were infected with *Leptospira* spp. To determine the source of infection, *Leptospira* spp. were isolated from animals in their company and sequenced.

The Cases

An animal trading company in Shizuoka, Japan, imported 106 southern flying squirrels from Miami, Florida, on March 27, 2005. Three workers handled these animals, which were housed 10 animals to a cage. Before patient 1 became ill, the workers dressed casually and touched the animals with bare hands in their routine work. Wild rats (such as *Rattus norvegicus* or *R. rattus*) had not invaded the animal house.

On April 22, 2005, patient 1, a 29-year-old man who handled a variety of exotic animals at the company, was hospitalized in Shizuoka Saisei-kai General Hospital with fever (temperature 40°C), headache, chills, nausea, vomiting, jaundice, and uremia, symptoms similar to those of locally acquired leptospirosis. Leptospirosis was diagnosed by polymerase chain reaction (PCR) targeted to the flagellin gene (*flaB*) and confirmed serologically with convalescent-phase serum by microscopic agglutination test. The patient was seronegative and PCR-negative for hantavirus, which causes symptoms similar to those observed in the patient. He was treated with an intramuscular injection of streptomycin (2 mg/day) for 7 days, which is the recommended treatment for leptospirosis in Japan (*4*); he consequently recovered.

On June 1, 2005, patient 2, a 28-year-old man who worked at the same company, was hospitalized in Shizuoka Saisei-kai General Hospital with fever (temperature 39°C), headache, chills, nausea, vomiting, jaundice, and uremia. The patient had been in contact with imported animals. He recovered with intramuscular injections of streptomycin (2 mg/day) for 3 days, followed by treatment with oral amoxicillin for 3 days.

Leptospira DNA was detected in serum samples from patient 1 and whole blood from patient 2 by *flaB* PCR (*5*). Sequences were determined by Prism 3130-avant DNA Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences of *flaB* detected from both patients were identical and showed a high degree of similarity to *L. kirschneri*.

Diagnosis was performed serologically by microscopic agglutination test with a panel of *Leptospira* reference strains (*3*). Convalescent-phase serum samples from both patients reacted to *L. kirschneri* strain Moskva V and strains isolated from southern flying squirrels, although serum collected on the day of hospitalization was negative in both patients (Table 1). To cultivate *Leptospira*, a few drops of blood from patient 2 were placed in several tubes of Ellinghausen-McCullough-Johnson-Harris medium supplemented with 2.5% rabbit serum. After 7 days of incubation at 30°C, *Leptospira* was detected from the culture (isolates P5.4, P10.1, P10.2).

To determine the validity of the association between animals held by the company and the illness, exotic animals (75 animals, 7 species) housed in the company were tested. *Leptospira* was isolated from 5 of 10 kidney cultures (isolates AM1, AM2, AM3, AM7, AM8) from southern flying squirrels. DNA from the urinary bladders, including the animals' urine, was extracted by using proprietary DNA extraction kits (Quick gene, Fuji Film Co., Tokyo, Japan). Five of 10 southern flying squirrels were *flaB* PCR-positive (Table 2). Species of the isolates were identified by using *flaB* and DNA gyrase B subunit gene (*gyrB*) sequencing analysis. We amplified 1.2-kb partial sequences of *gyrB* by using primers UP1TL (5′-CAyGCnGGnGGnAArTTyGA-3′; n: A, G, T, or C; r: A or G; y: C or T) and UP2rTL (5′-TCnACrTCnGCrTCnGTCAT-3′; n: A, G, T, or C; r: A or G) (*6*). The isolates obtained from patient 2 and southern flying squirrels had identical

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Table 1. Microscopic agglutination titer of patients' sera collected while hospitalized and during the convalescent phase

Patient	*Leptospira* strain used as antigen*	Patient serum	
		Hospitalized	Convalescent-phase
1	Serovar Grippotyphosa Moskva V	<50	100
	Animal isolate AM3	<50	800
	Animal isolate AM1	<50	800
2	Serovar Grippotyphosa Moskva V	<50	200
	Animal isolate AM3	<50	200

*Samples were not reactive to a panel of representative serovars, Australis, Autumnalis, Carlos, Bataviae, Cynopteri, Hebdomadis, Copenhageni, Icterohemorrhagiae, Javanica, Pomona, Pyrogenes, Hardjo, Sejroe, Wolffi, and Tarassovi; serovars Canalzonae, Huanuco, Muelleri, and Valbuzzi belong to serogroup Grippotyphosa.

flaB (data not shown) and *gyrB* (Figure 1) DNA sequences and were identified as *L. kirschneri*. The *flaB* sequences from the serum of patient 1 and whole blood of patient 2 were identical to those of isolates from patient 2 and animals. Additionally, restriction fragment length polymorphism (RFLP) analysis based on pulse-field gel electrophoresis was conducted (*7*). These isolates showed identical RFLP patterns (Figure 2), which suggests that patients were infected with *L. kirschneri* from southern flying squirrels.

To determine serovar of the isolates, a cross-agglutination test was performed with a panel of hyperimmune rabbit serum raised to representative serovars Icterohemorrhagiae, Copenhageni, Autumnalis, Hebdomadis, Australia, Grippotyphosa, Javanica, and Castellonis, which are present in Japan. These isolates reacted with anti-Grippotyphosa serum but not with the others (data not shown). Convalescent-phase sera from patients reacted with *Leptospira* isolates from the squirrels and also with serovar Grippotyphosa strain Moskva V (Table 1).

On April 24, the local health government prohibited the company from trading animals and directed them to use protection, such as latex gloves and disinfection of the floor with sodium hypochlorite, against infection. On June 2, all southern flying squirrels were euthanized by carbon dioxide, and the animal house was disinfected by the local health government. PCR detected *flaB* DNA on the surface of the squirrels' bodies and in urine on the soaked paper in the cages; the sequences were identical to those of the isolates. Before the first case was detected, 27 southern flying squirrels had been distributed to retail pet shops. Sixteen were returned, 2 died, 7 remained at pet shops, and 2 had been sold. The 2 sold animals and 7 remaining at the pet shops were recovered and euthanized. No illness was reported among persons in contact with these animals.

Table 2. Detection and isolation of *Leptospira* from imported animals in the company

Animal	No. samples positive/ no. samples tested	
	Kidney culture	*flab* PCR
Spiny mouse (*Acomys cahirinus*)	0/9	0/9
House mouse (species unknown)	0/4	0/4
Golden spiny mouse (*Acomys russatus*)	0/13	0/13
Mongolian gerbil (*Meriones unguiculatus*)	0/9	0/9
Southern flying squirrel (*Graucomys volans*)	5/10*	5/10*
Baluchistan pygmy jerboa (*Salpingotulus michaelis*)	0/20	0/20
Siberian chipmunk (*Tamias sibiricus*)	0/10	0/10

*Four of 5 culture-positive animals were positive by polymerase chain reaction (PCR). Remaining culture-positive animal was PCR negative, whereas 1 culture-negative animal was PCR positive.

Conclusions

Serovar Grippotyphosa commonly causes canine leptospirosis (*8*,*9*) and infects a variety of domestic and wild animals in the United States (*10*–*13*). In Japan, serovar Grippotyphosa is distributed in the southernmost islands, the Okinawa archipelago (*14*), but not on Honshu Island, the main island. Patients did not travel to Okinawa or foreign countries before disease onset. Our findings support the conclusion that the patients were infected with *L. kirschneri* serovar Grippotyphosa by contact with southern flying squirrels. Similarly, in the United States, humans have acquired monkeypox infection from pet prairie dogs, which had themselves been infected by exotic African

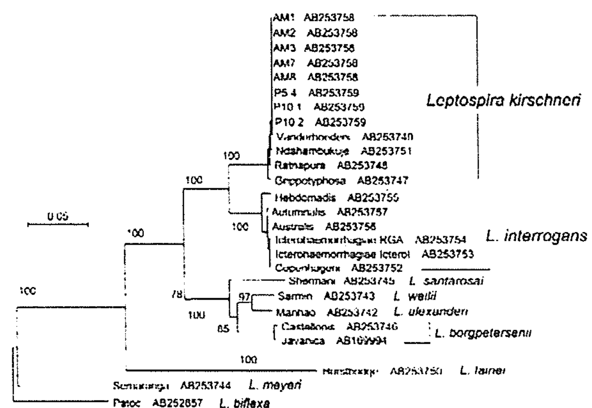

Figure 1. Phylogenetic tree based on the *Leptospira* DNA gyrase B subunit gene (*gyr*B) sequence. The sequences obtained have been deposited in DDBJ/GenBank/EMBL with accession numbers indicated.

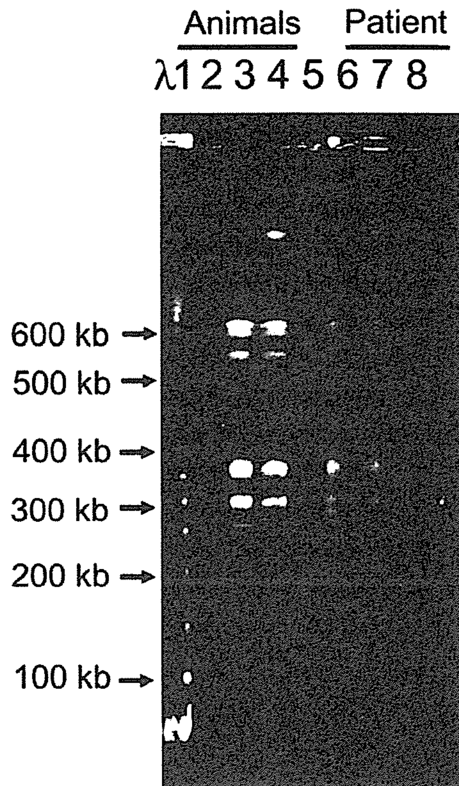

Figure 2. Pulsed-field gel electrophoresis analysis of *NotI* restriction fragment of *Leptospira* isolates from patient 2 and southern flying squirrels. *Leptospira* cells were lysed, and DNA was digested with restriction enzyme *NotI* in agarose gels. DNA in the gel was electrophoresed with 1% pulsed-field certified agarose in 0.5x Tris-borate-EDTA buffer under a pulse time of 10 s for 5 h and 30 s for 12 h, followed by 60 s for 7 h at 200 V. Lane 1, AM1; lane 2, AM2; lane 3, AM3; lane 4, AM7; lane 5, serovar Grippotyphosa strain Moskva V; lane 6, P10.2; lane 7, P10.1; lane 8, P5.4; λ phage DNA concatemer is used as a DNA size marker. Isolate AM8 showed an identical restriction fragment length polymorphism pattern to that of others.

rodents (*15*); these findings show that exotic pets represent a substantial hazard. The outbreak demonstrated how new infectious diseases could be emerging because of importation from overseas. If, during shipping and housing of the animals, the infection were to have expanded among southern flying squirrels, the infection rates and risk for humans would have increased. The leptospirosis cases reported here warn against importing exotic animals.

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表 1. ***gyrB*** および ***flaB*** の **PCR,** ならびにシークエンス解析に使用したプライマー

Primer	**Sequence (5'-3')**	**Position**	**Origin**
UP1TL	CAYGCNGGNAARTTYGA	301-320	Ictero No. 1
UP2rTL	TCNACRTCNGCRTCNGCTAT	1520-1502	Ictero No. 1
LgyrF	GGTCTTTCCGGAGAAGATG	940-958	Ictero No. 1
LgyrF4	AAAGAAAAATTAGTGAACGC	1024-1043	Ictero No. 1
LgyrR	GAATTGAATTGAGGTTGAGG	1016-997	Ictero No. 1
LgyrR3	TTMCCNGGAAGVCCDCCHCC	1232-1213	Ictero No. 1
L-flaB-F1	CTCACCGTTCTCTAAAGTTCAAC	35-57	Akiyami A
L-flaB-F2	TGTGCACAAGACGATGAAAGC	66-86	Akiyami A
FlaB-710F	GAATCTAGAATTCGAGACGCCG	730-709	Akiyami A
L-flaB-R1	TGAATTCGGTTTCATATTTGCC	825-804	Akiyami A
L-flaB-R2	AACATTGCCGTACCACTCTG	797-778	Akiyami A
M13F	TGTAAAACGACGGCCAGT		
M13R	CAGGAAACAGCTATGAC		

(M= A or C, R=A or G, Y=C or T, V=A or C or G , N=A or C or G or T)

表2 輸入げっ歯類からのレプトスピラの検出(2006年度)

動物種	被検頭数	培養	*flaB*－PCR	*flaB* Sequecnes
デグー	20	0	0	
ﾌﾄｵｱﾚﾁﾈｽﾞﾐ	10	0	0	
ｼﾞｬﾝｶﾞﾘｱﾝﾊﾑｽﾀｰ	20	0	0	
ｽﾃｯﾌﾟﾚﾐﾝｸﾞ	10	0	1	*L. alexanderi*
ｼﾏﾘｽ	20	0	0	
ピグミージェルボア	10	0	0	
シマリス	10	0	0	
ゴールデンハムスター	20	0	0	
ロボロフスキーハムスタ	20	0	0	
ジャンガリアンハムスタ	10	0	0	
ヨツユビハリネズミ	1	0	0	
計	151	0	1	

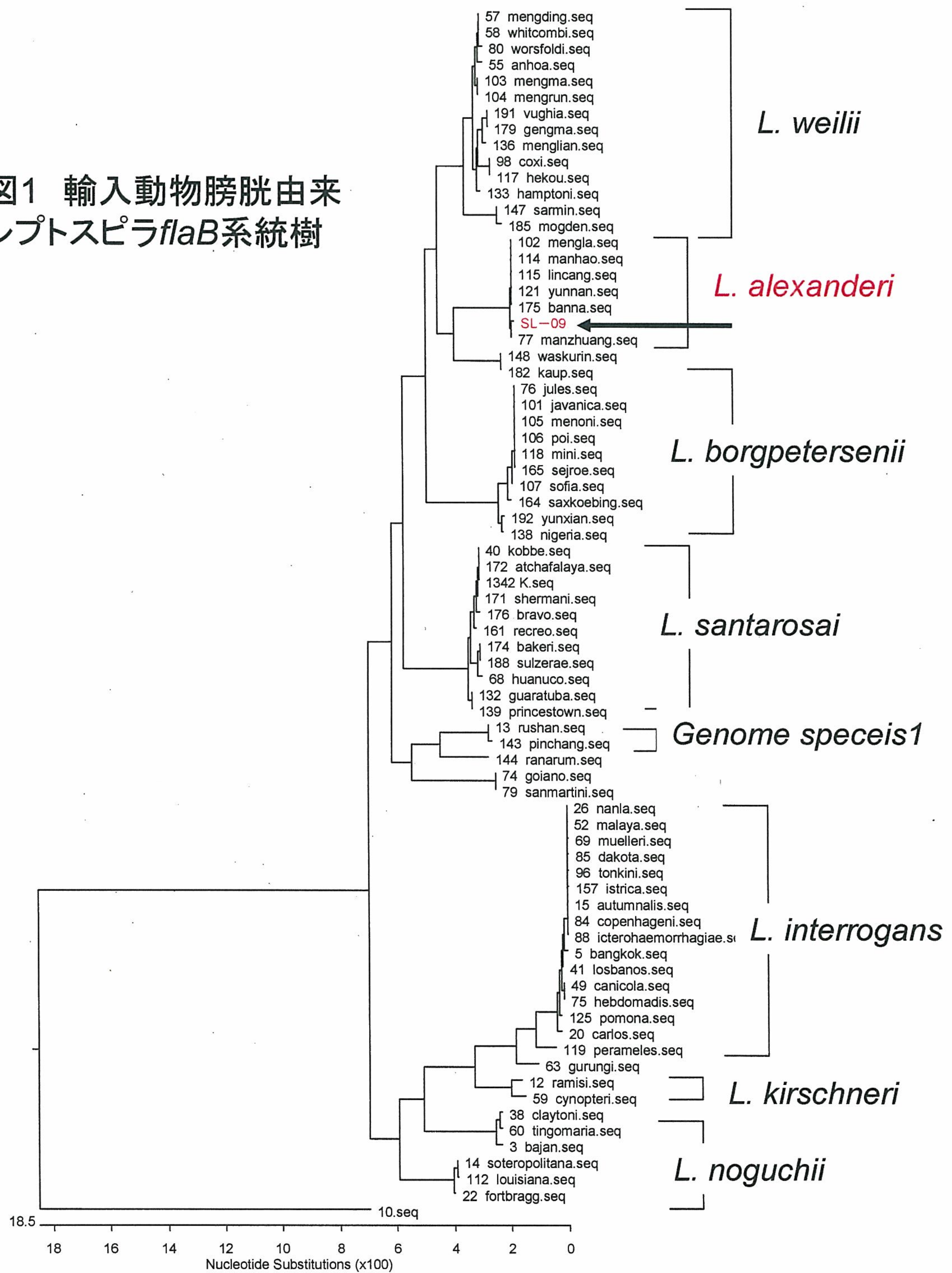

図1　輸入動物膀胱由来
レプトスピラ*flaB*系統樹

表3 2006年度野鼠からのレプトスピラ分離調査結果

調査地域	捕獲頭数	レプトスピラ培養陽性	陽性率	遺伝種	血清型	株名	由来野鼠種
西宮	97	3	3.1	*L. interrogans*	未同定血清型	Ni86-06, Ni88-06, Ni89-06	*Rattus norvegicus*
名古屋	27	3	11.1	*L. interrogans*	未同定血清型	AC4-06, AC21-06, AC24-06	*Rattus norvegicus*
宮城県内	39	3	7.7	*L. interrogans*	Autumnalis	M28-06, M32-06, M35-06	*Apodemus speciosus*
長野県	45	2	4.4	*L. interrogans*	Autumnalis(推定)	N29-06, N35-06(コンタミ)	*Apodemus speciosus*
白山(石川県)	8	0	0.0				
合計	216	11	5.1				

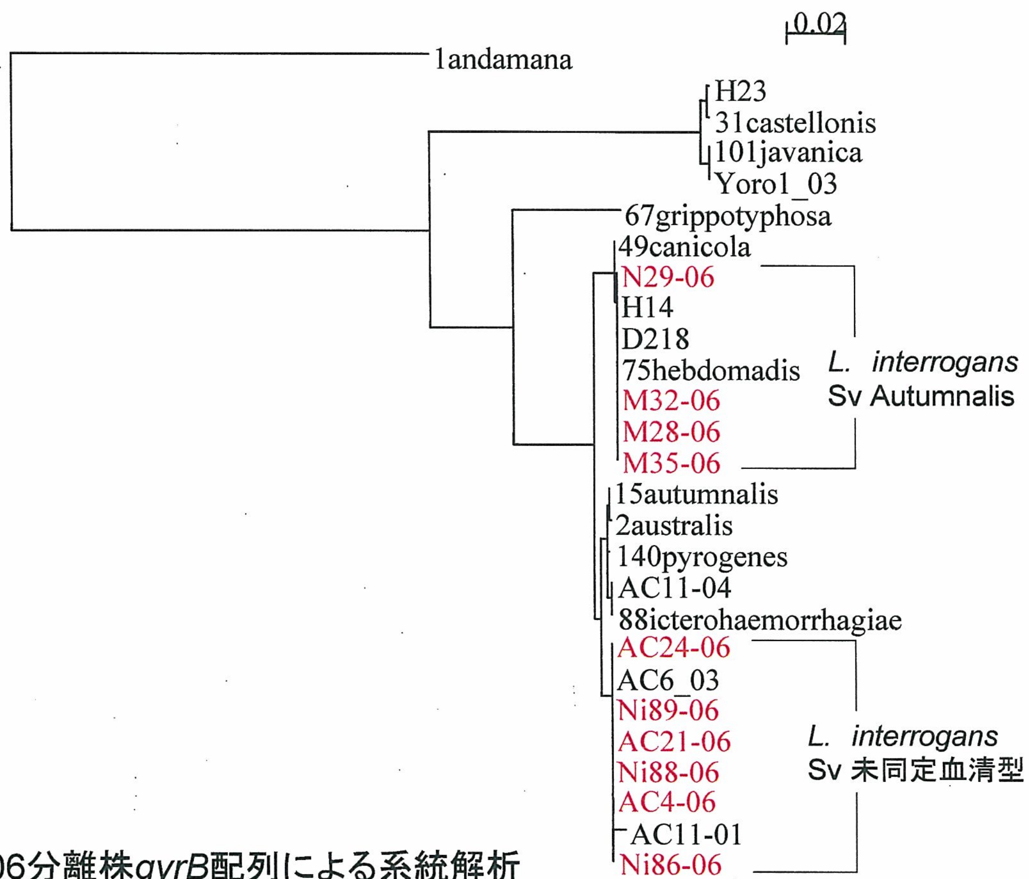

図2 2006分離株*gyrB*配列による系統解析

資料・業績

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Development of *Taenia asiatica* cysticerci to infective stage and adult stage in Mongolian gerbils

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Abstract

The development of metacestodes and adult worms of *Taenia asiatica* in Mongolian gerbils (*Meriones unguiculatus*) were observed. Cysticerci were recovered from gerbils subcutaneously injected with hatched oncospheres. The recovery rate ranged from 0.1 to 3.2%. No cysticerci were recovered from the orally inoculated gerbils. The infectivity of the cysticerci recovered at 48 weeks post-infection was evaluated. Tapeworms were recovered on day 14 post-infection from the small intestine of 5 of 11 gerbils, with a recovery rate of 27% (6 worms recovered/22 worms inoculated). Three and four adult worms were recovered from two human volunteers who ingested five cysticerci after 4 months post-infection. In worms recovered from gerbils, segmentation and genital primordia in the posterior proglottids and hooklets in the residual rostellum were observed. The results indicate that gerbils can serve as an alternative intermediate host and that partial development of the adult worm stage occurs in gerbils.

Introduction

Human taeniasis caused by *Taenia asiatica* (Bowles & McManus, 1994; Fan *et al.*, 1995) has been reported in Taiwan, China, Korea, Indonesia, Thailand, Philippines, Malaysia and Myanmar (Fan *et al.*, 1990a, 1992; Bowles & McManus, 1994; Simanjuntak *et al.*, 1997; Zhang *et al.*, 1999; Fan, 2000; Eom & Rim, 2001; Ito *et al.*, 2003). Adult worms of *T. asiatica* develop only in the human intestine but its metacestodes can develop in a wide range of intermediate hosts, such as pigs, wild boars, cattle, goats and monkeys (Fan *et al.*, 1990c). *Taenia asiatica* has been proposed to be a new species (Eom *et al.*, 2002; Ito *et al.*, 2003).

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Recently, *T. asiatica* metacestodes have been reported to develop in SCID mice (Ito *et al.*, 1997; Ito & Ito, 1999). Compared to SCID mice, Mongolian gerbils do not require any special facility, and they have been used as animal model for many parasites. For human *Taenia* species, only *T. saginata* metacestodes (Belgian isolate and African isolate) had been reported to develop in gerbils subcutaneously inoculated with hatched oncospheres (Geerts *et al.*, 1981/1982; Wouters *et al.*, 1988), and no study has been done with *T. asiatica*. Thus, in the first part of this study, Mongolian gerbils were orally inoculated with eggs or subcutaneously injected with hatched oncospheres to examine whether the animal can serve as an alternative intermediate host.

Mongolian gerbils have been reported to serve as alternative definitive hosts for *T. solium* and *T. saginata* (African isolate) after oral inoculation with cysticerci derived from their natural intermediate hosts (Kamiya *et al.*, 1990; Maravilla *et al.*, 1998). Moreover, golden

hamsters have been reported to serve as alternative definitive hosts for *T. solium* after oral inoculation with cysticerci derived from SCID mice (Wang *et al.*, 1999). However, gerbils have not been reported to be alternative definitive hosts for human *Taenia* by inoculation with the cysticerci derived from rodent alternative intermediate hosts. Thus, in the present study, the development of *T. asiatica* cysticerci in gerbils and the infectivity of cysticerci and the partial development of the adult stage in gerbils were examined.

Materials and methods

Eggs of *T. asiatica* were collected from gravid segments that were excreted in the faeces of Taiwanese aborigine patients after deworming with atabrine (Quinacrine) (Fan *et al.*, 1990b).

Development of metacestodes

Embryophores were removed by 20 min incubation of eggs in sodium hypochlorite solution, which was a 10 times dilution with physiological saline of stock solution (containing at least 5% of active chlorite, Kanto, Tokyo, Japan) (Lightowlers *et al.*, 1984). The released oncospheres were washed 5 times with sterile physiological saline containing antibiotics (penicillin G: 400 IU s ml^{-1}, streptomycin: 1 mg ml^{-1}). Fourteen female Mongolian gerbils, 7–13 weeks old, raised in our laboratory were used. Five were orally inoculated with eggs, nine were subcutaneously inoculated with oncospheres. Gerbils were fed commercial pellet food (CLEA, Tokyo, Japan) and given water *ad libitum*. Gerbils were then divided into three groups, namely, G1, G2 and G3 (Table 1). Each animal of group G1 and G2 was subcutaneously injected with 2.5 mg prednisolone acetate (Tong Yong Pharmaceutical, Shanghai, China) once a week from 4 days before inoculation to 38 days post-inoculation (DPI) (a total of seven times). Each animal of group G3 was subcutaneously injected with 2.5 mg prednisolone acetate once a week from 7 days before inoculation to 21 DPI (a total of five times). Animals of group G1 and G2 were all killed at 9 weeks after inoculation. Animals of group G3 were killed at 45 to 48 weeks after inoculation. For recovering the parasite, the inoculation sites, livers and peritoneal cavities of the gerbils were examined at necropsy. For morphological observations, cysticerci were relaxed in physiological saline at 4°C overnight after evagination, fixed in 70% alcohol and cleared in glycerin. The animal experiments in this study complied with the Guidelines for Animal Experiments of the Graduate School of Veterinary Medicine in Hokkaido University according to Japanese law.

Development of adult worms in gerbils and humans

Eleven male gerbils, 11–14 weeks old, raised in our laboratory were used in this study. Each gerbil was orally inoculated with two 48-week-old cysticerci recovered from the group G3 gerbils, fed with commercial pellet food (CLEA, Tokyo, Japan) and given water *ad libitum*. Each gerbil was subcutaneously injected with 2.5 – 5 mg prednisolone acetate once a week from 5 days before inoculation to 9 DPI with the cysticerci (a total of three times). All gerbils were killed under ethyl ether anaesthesia at 14 DPI and adult worms were recovered from their small intestine. The worms were relaxed in distilled water, fixed in 70% ethanol followed by observation under light microscope after staining with acid carmine. In addition, two human volunteers (volunteer A: female, 24 years old; volunteer B: male, 42 years old) ingested five of the 48-week-old cysticerci individually. After ingesting the cysticerci, the human volunteers checked their faeces daily for the presence of proglottids.

Statistical analysis

The morphometrics of the 9 and 45–48-week-old cysticerci were assessed using a non-parametric Mann-Whitney's U test. Statistical analyses were performed using the Stat View 4.0 for Macintosh.

Results

Development of metacestodes

Metacestodes were recovered from the inoculation sites of all nine gerbils (group G2 and G3) subcutaneously inoculated with oncospheres, but not from the liver or peritoneal cavity. The recovery rate of cysticerci in gerbils of group G2 ranged from 1.2 to 3.2%, while those in gerbils of group G3 ranged from 0.1 to 0.8%. None were found in five gerbils (group G1) orally inoculated with intact eggs (fig. 1 and table 1).

The total length, width of the scolex and diameter of the suckers of the 9- and 45-48-week-old cysticerci after

Table 1. Recovery of metacestodes from gerbils subcutaneously inoculated with oncospheres and those orally inoculated with eggs of *Taenia asiatica*.

Gerbils			Inoculation		Metacestode recovery	
Group	Number	Weeks post-infection	Inoculation route	Inoculation dose*	Number	Rate (%)
G1	5	9	Oral	12,000	0	0
G2	5	9	Subcutaneous	10,000	122–315	1.2–3.2
G3	4	45–48	Subcutaneous	24,000	30–183	0.1–0.8

*Inoculation of group G1 was done by intact eggs and those of other groups by hatched oncospheres.

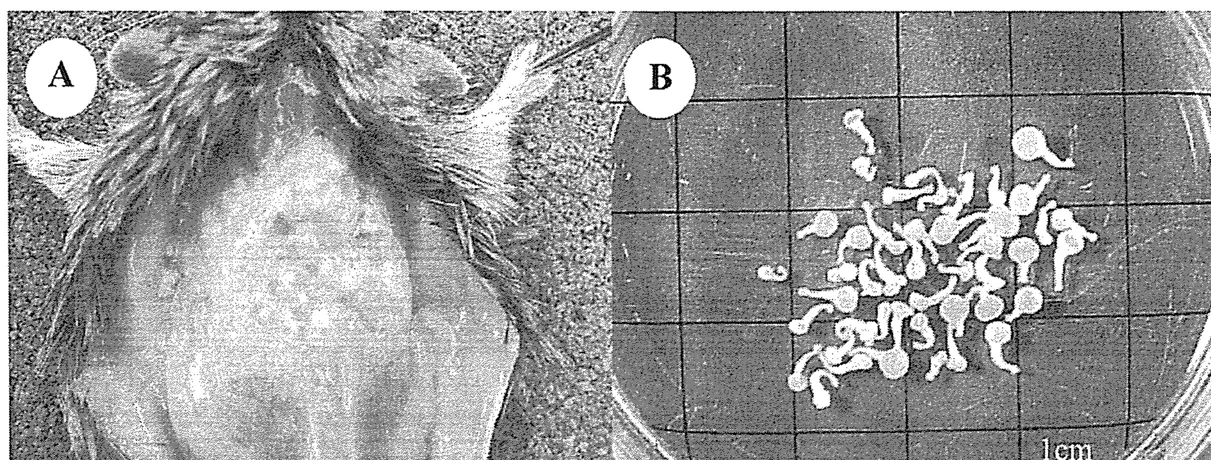

Fig. 1. Cysticerci of *Taenia asiatica* in gerbils at 48 weeks post-infection. (A) Cysticerci seen in the subcutaneous tissue at the inoculated site. (B) The evaginated 48-week-old cysticerci.

evagination are shown in Table 2. The 45-48-week-old cysticerci were significantly larger than the 9-week-old cysticerci in the total length, width of the scolex and diameter of the suckers (Mann-Whitney's U test, $P < 0.0001$).

Microscopic observations revealed that calcareous corpuscles were few in 9 week-old cysticerci but abundant in 45–48 week-old cysticerci. Many small-granule aggregates could be seen at the rostellar region of 9-week-old cysticerci, but only a few aggregates were observed in the 45–48 week-old cysticerci.

Development of adult worms

Six adult worms of *T. asiatica* were recovered from 5 of 11 inoculated gerbils (designated gerbil A, B, C, D and E). All tapeworms were recovered from the anterior part of the small intestine. The length of the tapeworms varied from 3 to 40 mm. Genital primordia were observed in the posterior segments of two worms (worm A1 and C1). Hooklets were observed in the rostellum of two worms (worm A1 and E1) (table 3, fig. 2).

Two human volunteers orally inoculated with cysticerci shed strobila or segments of *T. asiatica* in their faeces. Gravid proglottid excretion of volunteer A was found from 95 to 122 (number of proglottids excreted in a day ranging from 0 to 3) and that of volunteer B was found from 105 to 125 (ranging from 0 to 35 proglottids) DPI. The prepatent periods of *T. asiatica* infection in the two human volunteers were 95 and 105 days, respectively. The two human volunteers were dewormed using atabrine at 122 (expulsion of three worms) and 125 (expulsion of four worms) DPI, respectively.

Discussion

Mongolian gerbils have been used as a rodent models for the development of human taeniids. Geerts *et al.* (1981/1982) and Wouters *et al.* (1988) reported that the metacestode of *T. saginata* could be recovered from Mongolian gerbils which were subcutaneously inoculated with hatched oncospheres but not from gerbils intraperitoneally inoculated. Although we did not inoculate the hatched oncopheres of *T. asiatica* intraperitoneally into gerbils, the present result concurred with the finding that metacestodes could be recovered from gerbils subcutaneously inoculated with hatched oncospheres. This indicates that the subcutaneous tissue of the Mongolian gerbil may present a suitable site for oncosphere development.

Morphological observations of 45-week-old cysticerci recovered from gerbils showed a similar development to those from SCID mice; the width of scolex of the cysticerci after evagination was similar to those from SCID mice, but the total worm length and the diameter of the suckers were smaller than those from SCID mice (Chang *et al.*, 2005). In addition, calcareous corpuscles were abundant in the neck region and few small granules aggregated in the rostellum of the cysticerci from the gerbils. These morphological features are considered as important criteria for determining the maturity of cysticerci (Chang *et al.*, 2005). Thus, 45–48-week-old cysticerci

Table 2. Morphometrics of *Taenia asiatica* cysticerci recovered from gerbils subcutaneously inoculated with oncospheres.

Age of cysticerci (weeks)	Number of gerbils examined	Total length (μm) (mean ± SD)	Width of scolex (μm) (mean ± SD)	Diameter of sucker (μm) (mean ± SD)	No. of calcareous corpuscles
9	$n = 34$	2,100 ± 410	530 ± 80	170 ± 40	Few
45–48	$n = 44$	4,860 ± 750	660 ± 60	280 ± 40	Abundant

Table 3. Morphometrics of the six adult worms of *Taenia asiatica* recovered from gerbils.

Gerbils	Tapeworms	Length (mm)	No. of proglottids	Genital primordia	Hooklets
A	A1	40	154	+	+
	A2	6	0*	−	−
B	B1	3	0	−	−
C	C1	35	147	+	−
D	D1	4	0	−	−
E	E1	5	0	−	+

* Proglottids were undetectable.

recovered from gerbils in the present study were well developed and infective to humans.

Adult worms of *T. saginata* and *T. solium* had been reportedly recovered from immunosuppressed gerbils after oral inoculation with cysticerci derived from cattle and pigs, respectively (Kamiya *et al.*, 1990; Maravilla *et al.*, 1998). However, the adult stage of *T. solium* could not be recovered from Mongolian gerbils orally inoculated with cysticerci derived from SCID mice (Wang *et al.*, 1999). These reports suggested that the infectivity of cysticerci from alternative intermediate hosts might be much lower than those derived from the natural intermediate hosts. However, the adult stage of *T. asiatica* was recovered from gerbils and gravid proglottids were observed in the faeces of human volunteers after oral inoculation with cysticerci derived from gerbils in the present study. Since Wang *et al.* (1999) did not state the maturity of the cysticerci used, the failure to establish experimental infections in this case might be due to immaturity of the cysticerci. Thus, the present study is the first demonstration of the infectivity of human-infecting *Taenia* cysticerci derived from gerbils to gerbils and humans.

Immature proglottids have been observed in the adult stage of *T. solium* and *T. saginata* recovered from Mongolian gerbils on day 14 and 23 after oral inoculation with cysticerci (Kamiya *et al.*, 1990; Maravilla *et al.*, 1998). In the present study, immature segments with genital primordia were observed in the posterior segments on day 14 after oral inoculation. This indicates that the development of *T. asiatica* in gerbils is as good as those of the other two human taeniid cestodes. Morphological features of *T. asiatica* were described completely by Fan *et al.* (1995). Generally, no hooklets could be observed in the rostellum of *T. asiatica* (Fan, 1988; Eom & Rim, 1993). However, rudimentary hooklets were observed in two specimens of the tapeworms in the present study. Thus, rudimentary hooklets might still be present in the rostellum of *T. asiatica* during the early phase of infection.

In conclusion, the present results show that full mature infective cysticerci can be obtained and maintained for at least 48 weeks in gerbils under laboratory conditions. Moreover, these cysticerci can develop into the adult stage not only in humans but also in rodent alternative definitive hosts, suggesting that this experimental model might be useful for studying *T. asiatica* in the laboratory.

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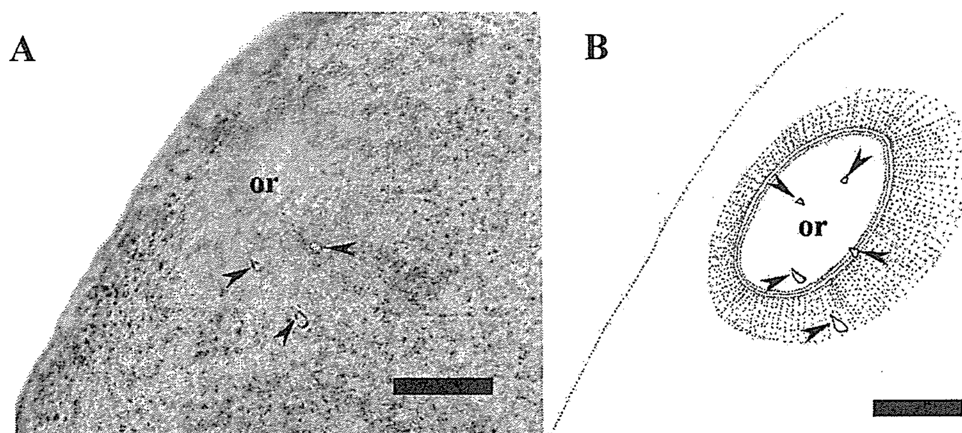

Fig. 2. Rostellar region of *Taenia asiatica* tapeworm from a gerbil. (A) Hooklets (arrowheads). (B) Drawing of the rostellar region. or, ridge of residual rostellar opening.

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Echinococcosis/Hydatidosis

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Summary of general activities related to the disease

1a) Types of test(s) in use/or available, purpose of testing (diagnosis*, surveillance, etc.) and approximate number performed for each purpose

Test	Diagnosis			Surveillance		Total
	Dog	Cat	Others	Fox	Others	
ELISA (coproantigen detection)	1240	234	74	198	24	1770
Post-mortem examination	-	-	-	45	1	46
Faecal examination	931	96	24	244	219	1514

How many confirmed positive results have you reported to the OIE Central Bureau?

From a total of 1240 domestic dogs examined two were positive, of 234 domestic cats one cat have been infected with *Echinococcus multilocularis*.

1b) Agent identification

Agent identification is carried out using:	EmA9-ELISA (coproantigen detection)
Total number of samples tested:	1770

2. Production, testing and distribution of diagnostic reagents

None.

3. Research especially related to development of diagnostic methods and vaccines

Experimental infections of Mongolian gerbils with *E. multilocularis* have been conducted for the study of systemic and cellular immunity to *E. multilocularis*.

Two candidate parasite-specific molecules whose expression levels may represent the viability/activity status of *E. multilocularis* metacestodes were studied under different maintenance conditions.