Table 1　Intestinal parasites diagnosed in canine feces in Saitama Prefecture

Species	Age group				Total (n = 906) Positive	
	Juvenile# (n = 159) Positive		Adult (n = 747) Positive			
	Number (%)	95% CI*	Number (%)	95% CI*	Number (%)	95% CI*
Trichuris vulpis	19 (11.9)	7.8 – 17.9	183 (24.5)	21.6 – 27.7	202 (22.3)	19.7 – 25.1
Toxocara canis	67 (42.1)	34.7 – 49.9	46 (6.2)	4.7 – 8.1	113 (12.5)	10.5 – 14.8
Ancylostoma caninum	19 (11.9)	7.8 – 17.9	75 (10.0)	8.1 – 12.4	94 (10.4)	8.6 – 12.5
Spirometra erinaceieuropaei	1 (0.6)	0.02 – 3.5	8 (1.1)	0.5 – 2.1	9 (1.0)	0.5 – 1.9
Taeniidae †	0 (0.0)	0.0 – 1.9	3 (0.4)	0.1 – 1.2	3 (0.3)	0.1 – 1.0
Dipylidium caninum	0 (0.0)	0.0 – 1.9	2 (0.3)	0.03 – 1.0	2 (0.2)	0.03 – 0.8
Echinostoma sp.	0 (0.0)	0.0 – 1.9	1 (0.1)	0.0 – 0.7	1 (0.1)	0.0 – 0.6
Isospora ohioensis	6 (3.8)	1.7 – 8.0	13 (1.7)	1.0 – 3.0	19 (2.1)	1.4 – 3.3
Cryptosporidium canis ‡	1 (0.6)	0.02 – 3.5	7 (0.9)	0.5 – 1.9	8 (0.9)	0.5 – 1.7
Giardia intestinalis	5 (3.1)	1.4 – 7.2	3 (0.4)	0.1 – 1.2	8 (0.9)	0.5 – 1.7
I. canis	3 (1.9)	0.4 – 5.4	2 (0.3)	0.03 – 1.0	5 (0.6)	0.2 – 1.3
Pentatrichomonas hominis	0 (0.0)	0.0 – 1.9	1 (0.1)	0.0 – 0.7	1 (0.1)	0.0 – 0.6
Total number positive	90 (56.6)	48.8 – 64.1	260 (34.8)	31.5 – 38.3	350 (38.6)	35.5 – 41.8

#Juvenile, under one year old
*CI, confidence interval
† One was identified as *Echinococcus multilocularis* based on the 12S rRNA base sequence
‡ Based on the 18S rRNA base sequence

対象と方法

1．対象

本調査は，1999年5月から2007年12月までの期間に，埼玉県動物指導センターの本所（熊谷市）および3支所（さいたま市，川越市，春日部市）に収容されたイヌとネコを対象とした．収容動物から採取された検体（直腸便）は，採取当日に埼玉県衛生研究所に搬入し，速やかに検査を実施した．検体の採取にあたり，各個体の情報として，性別・推定年齢・収容市町村名を記録した．2006年以降の調査個体に関しては，これらに加え，収容の理由（飼養放棄，捕獲または保護等）も併せて記録した．

2．糞便検査の方法

糞便検査は，直接薄層塗抹法，ホルマリン・エーテル法（MGL法）および比重1.2のショ糖液を用いるショ糖遠心浮遊法を併用した．検出された寄生蠕虫卵と原虫類のシストまたはオーシストは，それぞれの形態学的特徴に基づき，属または種のレベルまで同定を行った．また，テニア科条虫卵が検出された糞便については，CHEKIT-Echinotest（Dr. Bommeli AG）を用いて包条虫属特異的糞便内抗原の測定を行った．分子同定は，糞便内抗原で陽性を示したテニア科条虫卵とクリプトスポリジウムのオーシストについて行った．すなわち，テニア科条虫卵については12Sリボソーム RNA（12S rRNA）領域を，クリプトスポリジウムのオーシストについては18S rRNA 領域をそれぞれターゲットとするプライマー[4][5]を用いてPCR法による増幅を行い，ダイレクトシークエンス法で塩基配列を解読し，種を決定した．

3．統計学的検定および区間推定の方法

寄生虫陽性率の比較にはχ^2検定（両側）を用いた．寄生虫種数の比較にはWilcoxon順位和検定を用いた．陽性率の信頼区間（Confidence Interval, CI）はZar[6]に基づいて信頼率95%で推定した．検定はS-PLUS 6.1 for Windows（Insightful），信頼区間の算出はExcel 2000（Microsoft）をそれぞれ用いた．

結　果

1．イヌにおける寄生虫類の検出状況

調査期間中に採材対象となったイヌは906頭で，そのうち350頭が寄生虫陽性であった（陽性率38.6%）（Table 1）．

蠕虫類ではイヌ鞭虫 *Trichuris vulpis*が最も多く（202頭，22.3%），次に，イヌ回虫 *Toxocara canis*（113頭，12.5%），イヌ鉤虫*Ancylostoma caninum*（94頭，10.4%），マンソン裂頭条虫 *Spirometra erinaceieuropaei*（9頭，1.0%），テニア科条虫 Taeniidae（3頭，0.3%），瓜実条虫 *Dipylidium caninum*（2頭，0.2%）および棘口吸虫類 *Echinostoma* sp.(1頭，0.1%）の虫卵が検出された．テニア科条虫の3頭中2005年度に検出された1頭は，12S rRNA 領域の塩基配列解読により多包条虫 *Echinococcus multilocularis*と同定された．また，1頭では*Taenia*属の片節が見られ，他の1頭では糞便抗原が陰性であったことから，虫卵の分子同定は実施しなかった．

原虫類では *Isospora ohioensis*が最も多く（19頭，2.1%），次に，クリプトスポリジウム属 *Cryptosporidium* sp.（8頭，0.9%），ランブル鞭毛虫 *Giardia intestinalis*（8頭，0.9%），*I. canis*（5頭，0.6%）および腸トリコ

Table 2　Intestinal parasites diagnosed in feline feces in Saitama Prefecture

Species	Age group: Juvenile# (n = 366) Positive		Adult (n = 713) Positive		Total (n = 1,079) Positive	
	Number (%)	95% CI*	Number (%)	95% CI*	Number (%)	95% CI*
Toxocara cati	126 (34.4)	29.7−39.4	109 (15.3)	12.8−18.1	235 (21.8)	19.4−24.3
Ancylostoma tubaeforme	31 (8.5)	6.0−11.8	111 (15.6)	13.1−18.4	142 (13.2)	11.3−15.3
Trichuris sp.	1 (0.3)	0.01−1.5	1 (0.1)	0.0−0.8	2 (0.2)	0.02−0.7
Capillaria sp.	0 (0.0)	0.0−0.8	1 (0.1)	0.0−0.8	1 (0.1)	0.0−0.5
Spirometra erinaceieuropaei	17 (4.6)	2.9−7.3	73 (10.2)	8.2−12.7	90 (8.3)	6.8−10.1
Dipylidium caninum	3 (0.8)	0.2−2.4	12 (1.7)	1.0−2.9	15 (1.4)	0.8−2.3
Taeniidae	0 (0.0)	0.0−0.8	2 (0.3)	0.03−1.0	2 (0.2)	0.02−0.7
Diphyllobothrium nihonkaiense	1 (0.3)	0.01−1.5	0 (0.0)	0.0−0.4	1 (0.1)	0.0−0.5
Pharyngostomum cordatum	3 (0.8)	0.2−2.4	14 (2.0)	1.2−3.3	17 (1.6)	1.0−2.5
Metagonimus yokogawai	0 (0.0)	0.0−0.8	1 (0.1)	0.0−0.8	1 (0.1)	0.0−0.5
Isospora felis	32 (8.7)	6.3−12.1	17 (2.4)	1.5−3.8	49 (4.5)	3.5−6.0
Cryptosporidium sp. †	22 (6.0)	4.0−8.9	8 (1.1)	0.6−2.2	30 (2.8)	2.0−3.9
I. rivolta	12 (3.3)	1.9−5.6	12 (1.7)	1.0−2.9	24 (2.2)	1.5−3.3
Eimeria sp.	0 (0.0)	0.0−0.8	3 (0.4)	0.1−1.2	3 (0.3)	0.06−0.8
Total number positive	188 (51.4)	46.3−56.5	277 (38.8)	35.3−42.5	465 (43.1)	40.2−46.1

#Juvenile, under one year old
*CI, confidence interval
† All but 3 from juveniles were identified as *C. felis* based on the 18S rRNA base sequence

Table 3　Number and percentage of single and multiple infections in 350 parasite-positive dogs and 465 cats in Saitama Prefecture

		Number (%) of parasite species detected: Total number positive	1 species	2 species	3 species	4 species
Dogs	Juvenile#	90 (100)	65 (72.2)	21 (23.4)	3 (3.3)	1 (1.1)
	Adult	260 (100)	189 (72.7)	59 (22.7)	11 (4.2)	1 (0.4)
	Total	350 (100)	254 (72.6)	80 (22.8)	14 (4.0)	2 (0.6)
Cats	Juvenile#	188 (100)	133 (70.7)	48 (25.6)	7 (3.7)	0 (0.0)
	Adult	277 (100)	205 (74.0)	59 (21.3)	11 (4.0)	2 (0.7)
	Total	465 (100)	338 (72.7)	107 (23.0)	18 (3.9)	2 (0.4)

#Juvenile, under one year old

モナス *Pentatrichomonas hominis*（1頭，0.1%）のシストまたはオーシストが検出された．クリプトスポリジウム属は18S rRNA領域の塩基配列解読の結果，すべて *C. canis*と同定された．

2．ネコにおける寄生虫類の検出状況

採材対象となったネコは1,079頭で，そのうち465頭が寄生虫陽性であった（陽性率43.1%）（Table 2）．

蠕虫類ではネコ回虫 *T. cati*が最も多く（235頭，21.8%），次に，ネコ鉤虫 *A. tubaeforme*（142頭，13.2%），鞭虫類 *Trichuris* sp.（2頭，0.2%），毛細線虫類 *Capillaria* sp.（1頭，0.1%），マンソン裂頭条虫 *S. erinaseieuropaei*（90頭，8.3%），瓜実条虫 *D. caninum*（15頭，1.4%），テニア科条虫 Taeniidae（2頭，0.2%），日本海裂頭条虫 *Diphyllobothrium nihonkaiense*（1頭，0.1%），壺形吸虫 *Pharyngostomum cordatum*（17頭，1.6%）および横川吸虫 *Metagonimus yokogawai*（1頭，0.1%）の虫卵が検出された．なお，テニア科条虫卵が検出された2頭の糞便内抗原は陰性であった．

原虫類では *Isospora felis*が最も多く（49頭，4.5%），次に，クリプトスポリジウム属 *Cryptosporidium* sp.（30頭，2.8%），*I. rivolta*（24頭，2.2%）およびアイメリア属 *Eimeria* sp.（3頭，0.3%）のシストまたはオーシストが検出された．クリプトスポリジウム属のオーシストは，検体量の不足により分子同定を実施できなかった3例を除き，すべて *C. felis*であった．

3．動物の種および齢クラスと感染寄生虫種数の検討

イヌおよびネコそれぞれの寄生虫陽性例における，感染寄生虫種数とその内訳をTable 3に示した．イヌ・ネコともに最大4種の感染が確認されたが，動物種間および同一動物種の齢クラス間で比べた場合，検出寄生虫種数の構成割合に有意な差は認められなかっ

平成21年5月20日

た（すべて p>0.05）.

4. 動物の齢クラスと寄生虫陽性率の検討

イヌでは，幼獣（1歳未満）の陽性率は成獣（1歳以上）より有意に高く（p<0.01），また，幼獣はイヌ回虫とランブル鞭毛虫の2種の陽性率においても有意に高い値を示した（いずれも p<0.01）．ただし，イヌ鞭虫の陽性率においては，成獣が幼獣より有意に高い値を示した（p<0.01）．

ネコでも幼獣は成獣より有意に高い寄生虫陽性率を示し（p<0.01），寄生虫種別の比較ではネコ回虫・*C. felis*・*I. felis* 3種で，幼獣が成獣より有意に高い陽性率を示した（いずれも p<0.01）．一方，ネコ鉤虫とマンソン裂頭条虫の2種においては，成獣が有意に高い陽性率を示した（いずれも p<0.05）．

5. 動物の性別，収容地域と寄生虫陽性率の検討

イヌあるいはネコの性別または収容地域と寄生虫陽性率との間には，いずれも有意な関係は認められなかった（いずれも p>0.05）．また，収容理由と寄生虫陽性率との間にも，有意な関係は認められなかった（p>0.05）．

考　察

今回の調査により，最近の埼玉県内のイヌおよびネコにおける寄生虫陽性率が40％前後であることが分かった．さらに県内で実施された過去の類似調査[7)~9)]では，腸管寄生原虫類は一部を除き検索対象外であったので，その流行状況を初めて明らかにすることができた．

過去の調査は検査の方法や対象動物の齢構成が同一でなく，さらに上述のとおり検索対象が蠕虫類に限られているが，まずイヌでは1974年の捕獲犬を対象に，糞便検査と一部剖検を併用した調査が行われ，陽性率は87.9％であった[7)]．ネコでは1983年と1989～1991年に糞便検査と剖検による調査が実施され，陽性率はそれぞれ77.4％と52.4％であった[8)9)]．今回得られた結果をこれらと比較すると，イヌやネコにおける寄生虫陽性率は漸次低減していると考えられるが，本調査の9年間における年度ごとの陽性率では，有意な変化は認められなかった．

寄生虫感染の減少は，同一個体から検出される寄生虫種の数にも見られる．過去の調査では，イヌについては記録がないが，ネコでは2種以上の重複感染は陽性群の43.8％で，最高6種の重複感染が報告されている[9)]．これに対し，今回の調査で2種以上の種が検出されたネコは陽性群の27.3％と低下し，感染機会の減少が示唆された．

しかしながら，結果で示したように，イヌやネコの寄生虫の多くは人獣共通種である．実際，今回検出された寄生虫類の中で，イヌ鞭虫・イヌ回虫・イヌ鉤虫・多包条虫・クリプトスポリジウム・ランブル鞭毛虫および腸トリコモナスの7種，ネコではネコ回虫・ネコ鉤虫・クリプトスポリジウムの3種がそれぞれヒトに経口または経皮的に直接感染する種であった．これらは人体感染時における寄生動態から2種に大別される．すなわち，一つはヒトにおいてもイヌあるいはネコと同様に腸管寄生を行う種であり，もう一つはヒトでは腸管寄生を行わず，それ以外の部位に寄生する種である．

前者には，イヌ鞭虫・クリプトスポリジウム・ランブル鞭毛虫・腸トリコモナスが含まれ，一般に下痢症状を引き起こす．後者では，回虫類や鉤虫類のように虫卵から孵化した幼虫が成虫まで発育せず，体内各部位に侵入して幼虫移行症の原因となり，あるいは多包条虫のように肝臓をはじめとする臓器に幼虫が寄生・発育して多包虫症の原因となるものである．特に，動物由来の回虫類による幼虫移行症は重篤化することが多く，ぶどう膜炎や網膜症を起こす眼トキソカラ症[10)11)]や，肝臓などに好酸球性肉芽腫や膿瘍を起こす内臓トキソカラ症[12)]が知られ，国内でも報告がみられる[13)~15)]．加えて，これらの回虫類の感染経路は，イヌやネコに由来する虫卵を直接経口摂取する場合だけではなく，ニワトリあるいはウシの生肉や生肝の摂食によると考えられる感染例も報告されている[16)17)]．これは，ニワトリやウシがエサと共に回虫類の虫卵を摂取し，人体の場合と同様に幼虫が筋肉や肝臓に体内移行し，それらをヒトが食品として経口的に取り入れた結果生じている．

今回，調査結果には多包条虫やクリプトスポリジウムなど埼玉県内で初めて確認された種が含まれていた．多包条虫は多包性エキノコックス症（4類感染症）の原因種であるが，国内では北海道に限定して分布すると考えられている．しかしながら，既に北海道から移出されるイヌの感染例が報告されており[18)]，北海道以外の都府県で突発的に発生する可能性が指摘されてきた．今回発見された感染例は，登録鑑札やマイクロチップなど個体特定に用い得る情報がなく，詳細な由来は不明であるが，12S rRNA 領域の塩基配列の解読結果が北海道分離株と同一であったことから[19)]，何らかの理由により北海道から運ばれた感染個体が，本県内で遺棄あるいは逃亡したものと推察された．

多包条虫が土着した場合，その根絶は極めて困難である．当該犬は既に虫卵を排出し，中間宿主への感染源となり得た．著者らはこの点を考慮し，本種の中間宿主となる野ネズミ類の捕獲調査を行い，汚染拡散の有無を確認している．現在までのところ野ネズミ類における感染は確認されていないが，北海道と埼玉県におけるヒトやイヌの往来や物流の現状を踏まえ，今後

も積極的な監視を継続していく必要がある．

もう一つの新たな陽性例として，クリプトスポリジウム症（5類感染症）の原因となるクリプトスポリジウム属の2種が確認された．本属には多くの種および遺伝子型が報告され，それぞれ宿主特異性も異なるが，公衆衛生上はヒトを固有の宿主とする *C. hominis*並びにヒトを含む広範な哺乳類を宿主とする *C. parvum*が重要とされる．実際，1996年に本県で水道水に混入し，感染者数9,140名以上と推定される我が国最大のクリプトスポリジウム症の集団感染を引き起こした種は *C. hominis*であった[20)21)]．

大規模な水系感染の原因となる上記2種に対し，今回検出された *C. canis*および *C. felis*の疫学的意義付けは未だ不十分である．しかしながら，著者らは慢性下痢症状を呈したAIDS患者から *C. felis*を検出しており（未発表データ），さらに海外では免疫不全者のみならず健常者が *C. canis*や *C. felis*に感染した事例も報告されている[22)23)]．そのため，イヌあるいはネコ由来のクリプトスポリジウム症について，従来から高リスク者とされてきた免疫不全者は無論のこと，ペット飼育者全てに対し注意を喚起しなければならない．

ペットの飼育が広く普及し，生活圏の共有化が進む一方で，ペットが媒介する寄生虫症に対しては十分な配慮がなされているとは言えない．イヌやネコからの寄生虫感染を予防するためには，第一に飼い主自身がペット由来寄生虫症について，関心と正しい知識を持つことは言うまでもない．その結果，寄生虫検査の積極的な受検と適切な駆虫薬の投与が進み，ペットにおける寄生虫感染の減少が実現される．このことは，飼い主だけでなく地域全体の曝露リスクを低減させることにもつながる．しかしながら，その過程において行政が果たさねばならない役割は大きい．精度の高い監視体制を構築・維持して最新情報の収集に努めるとともに，その結果を適切な対策と併せて速やかに提示していくことが望まれる．

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Prevalence of Intestinal Canine and Feline Parasites in Saitama Prefecture, Japan

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We studied the prevalence of intestinal parasites in animal companions in Saitama Prefecture, Japan, where no detailed data is currently available.

Between May 1999 and December 2007, fecal samples were collected from 906 dogs and 1,079 cats in public animal shelters and examined by microscopy. Overall, prevalence of intestinal parasites in dogs was 38.6% and cats 43.1%. *Trichuris vulpis* was the most prevalent canine parasite species (22.3%), followed by *Toxocara canis* (12.5%), *Ancylostoma caninum* (10.4%), *Isospora ohioensis* (2.1%), *Spirometra erinaceieuropaei* (1.0%), *Crypstosporidium* sp. (0.9%), *Giardia intestinalis* (0.9%), *I. canis* (0.6%), Taeniidae (0.3%), *Dipylidium caninum* (0.2%), *Echinostoma* sp. (0.1%), and *Pentatrichomonas hominis* (0.1%). *T. cati* was the most prevalent feline parasite species (21.8%), followed by *A. tubaeforme* (13.2%), *S. erinaceieuropaei* (8.3%), *I. felis* (4.5%), *Cryptosporidium* sp. (2.8%), *I. rivolta* (2.2%), *Pharyngostomum cordatum* (1.6%), *D. caninum* (1.4%), *Eimeria* sp. (0.3%), Taeniidae (0.2%), *Trichuris* sp. (0.2%), *Capillaria* sp. (0.1%), *Diphyllobothrium nihonkaiense* (0.1%), and *Metagonimus yokogawai* (0.1%). Further molecular analysis to identify canine Taeniidae species and canine and feline *Cryptosporidium* species identified one canine taeniid positive species as *Echinococcus multilocularis*. *Cryptosporidium* species were identified as *C. canis* and *C. felis*. Parasites *E. multilocularis* and *Cryptosporidium* spp. in animal hosts were the first to be recorded in this prefecture.

Compared to previous surveys conducted in the same area, the endemicity of some parasites appeared to have decreased, but some others remain. Given that most of these parasites have zoonotic potential, indicates the importance of having current data on parasite dissemination among animal companions. Government public health agencies should be responsible for educating pet owners about the control and prevention of zoonotic risk from such parasites.

<国内情報>

青森県のと畜場に搬入された豚から検出されたエキノコックス（多包虫）について

1998（平成10）年8月と12月に，青森県十和田食肉衛生検査所（十和田食検）がと畜検査した豚3頭の肝臓から，エキノコックス（多包虫）が青森県で初めて検出された[1)]。これらの感染豚は，青森県内の同一養豚場が出荷したもので，この養豚場での育成中にエキノコックスに感染したとすれば「ある時期あるいは現在も本症の流行があることを強く示唆している」[2)]事例と考えられた。その後，この養豚場周辺の野生動物についての調査が行われたが，感染動物は発見されていない[3)]。一方，十和田食検においても，その後10年間，当該農場からの出荷を含む年間80～90万頭もの豚をと畜検査してきたが，エキノコックス感染豚は確認されなかった。ところが，2008（平成20）年度になって，北海道より直接，青森県のと畜場へ搬入された豚6頭の肝臓からエキノコックス感染が，あらたに十和田食検で確認された。以下にその経緯を報告する。

1999（平成11）年～2004（平成16）年度までは，十和田食検の日常業務が遂行される中でのエキノコックス感染豚の検出報告は無い。2005（平成17）年度に至り，厚生労働省新興・再興感染症研究事業（※）の分担研究として「青森県のエキノコックス調査と監視体制の構築」を実施することとなり，十和田食検の監視体制を強化するため，次の対応がとられた。1）各

表. 十和田食肉衛生検査所における豚のと畜検査頭数およびエキノコックス検査成績

	平成17年度	平成18年度	平成19年度	平成20年度	計
全検査数	888,450	885,430	893,884	910,130	3,577,894
[北海道産]	[0]	[900]	[1,256]	[3,135]	[5,291]
肝の白色結節	27	44	25	13	109
[エキノコックス]	[0]	[0]	[0]	[6]	[6]

検査員に北海道における豚のエキノコックス感染肝の肉眼写真および判定基準を配布して検体採材を行う。2) 採材された肝臓の白色結節病変について，HE 染色およびPAS 染色を施し病理組織学的な検索を実施する。3) 病理学的な診断とともに遺伝子同定も実施する。

このプログラムにもとづいて実施された2005～2008（平成17～20）年度の検査により，肝臓にエキノコックス感染を疑わせる白色結節病変を認めた個体は，年度ごとに27，44，25，13の計109頭であった（表）。これらの病変について病理組織学的な検索を実施した結果，2005～2007（平成17～19）年度ではいずれもエキノコックスは認められず，白色結節はリンパ濾胞，肉芽腫性炎，間質性肝炎，肝嚢胞，寄生虫性肝炎等と診断された。そして，2008（平成20）年度において，採材された肝臓組織13例のうち 6 例にエキノコックスが検出されたのである。この 6 例はすべて北海道産であった。北海道の食肉衛生検査所での豚のエキノコックス検出率は，最近25年間の全道平均で0.1%である[4]。十和田食検が検査した北海道産の豚は，4 年間で5,291頭であり，ここでの検出率もほぼ0.1%となっている。

わが国における人エキノコックス症の大部分は，多包条虫の土着が認められる北海道での発生である（現在までに約500症例）。北海道以外の都府県で発生した多包虫症例は約80で，青森県からの報告がその1/4を占め，しかもそのうち 9 症例は県内での感染の可能性が強い[5]。これは，北海道・青森両地域の人的・物的交流の緊密さに起因すると考えられるが，その具体的な要因については解明されていない。このような状況の中で，1998（平成10）年に十和田食検で青森県産とされる豚からエキノコックスを検出したことから，同県での生活環の定着が強く疑われたのである。しかしながら，これらの感染豚はいずれも青森県内の同一養豚場が出荷したものであるが，その実際の生育地に関しては，当該農場が自家繁殖豚のみならず家畜市場から購入した豚も保有していたために必ずしも確定的ではなかった[1]。今回の調査で，北海道産豚以外からはエキノコックス感染が確認されなかったことからも，青森県内での生活環定着について，現時点ではその可能性は低いと考えられる[6]。我々は，流行地である北海道とは津軽海峡を挟んで隣接する青森県について，今後も継続的に，と畜検査を通じ本州へのエキノコックス伝播の監視と蔓延防止に寄与したいと考えている。

（※）平成17年度「動物由来感染症の流行地拡大防止対策に関する研究（主任研究者：神谷正男酪農学園大教授）」，平成18～20年度「動物由来感染症のコントロール法の確立に関する研究（主任研究者：吉川泰弘東大大学院教授）」

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Laboratory and Epidemiology Communications

Echinococcus multilocularis Detected in Slaughtered Pigs in Aomori, the Northernmost Prefecture of Mainland Japan

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Echinococcus multilocularis is a causative agent of human alveolar echinococcosis. The distribution of the parasite in Japan was thought to be limited to the northernmost insular prefecture of Hokkaido, where the Tsugaru Strait acts as a natural physical barrier against migration to Honshu, the mainland of Japan; however, in Aomori Prefecture, situated in the northernmost part of Honshu, *E. multilocularis* infection in pigs was first reported in August and December 1998, when Aomori Prefectural Towada Meat Inspection Center (Towada MIC) detected the parasite during postmortem inspections of livers from three pigs. The infected pigs had all been transported from the same piggery in Aomori, so the implication of this case was that if the pigs had been infected while being reared on the farm, then either there had been an epidemic of *E. multilocularis* in Aomori some time previously or else the infection was epidemic at that time (1). An intensive epizootiological survey of the potential definitive and intermediate hosts in the area surrounding the piggery was undertaken, revealing no infected animals (2,3). Over the subsequent decade, Towada MIC has performed postmortem inspections of 800,000–900,000 pigs annually, including animals from the same piggery, and no pigs were found to be infected with *E. multilocularis*. However, *E. multilocularis* infection was again confirmed in the fiscal year (FY) 2008 by Towada MIC in the livers of six pigs that were transported to a slaughterhouse in Aomori directly from Hokkaido. The details of the case are given below.

From 1999 until FY2004, no pigs infected with *E. multilocularis* were reported during the routine work of Towada MIC. In FY2005, a system for surveying and monitoring *E. multilocularis* in Aomori Prefecture was put in place as part of a domestic zoonosis survey program, and the following measures were taken to bolster the monitoring system at Towada MIC. First, macroscopic photos of the livers of pigs from Hokkaido infected with *E. multilocularis* and diagnostic criteria were to be distributed to all inspectors, and samples were to be collected from livers showing signs of infection. Second, white nodular lesions in liver samples were to be stained with hemotoxylin-eosin and/or periodic acid Shiff (PAS) stain for histological examination. Third, molecular identification was to be performed together with a pathological diagnosis.

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Among inspections carried out from FY2005 to FY2008 under this program, the number of pigs with liver samples displaying white nodular lesions suggestive of *E. multilocularis* infection for each year was 27, 44, 25, and 13, representing a total of 109 (Table 1). Histopathological examination of the lesions did not confirm a single case of *E. multilocularis* infection, and the whitish nodules were diagnosed as lymph follicle formation, granulomatous inflammation, interstitial hepatitis, hepatic cysts, parasitic hepatitis (probably caused by the passage of ascarid larvae), etc. In FY2008, liver tissue was analyzed in 13 cases, and *E. multilocularis* cysts with PAS-positive laminated layers were detected in six of these. All of the six cases had been transported directly from Hokkaido. The cysts had obviously disrupted in the liver tissues, and neither brood capsules nor protoscoleces were observed in any of the investigated lesions. These pathological findings (Fig. 1) were consistent with those previously described in spontaneously infected pigs in Hokkaido (4). For the positive specimens, molecular confirmation of the causative agents was performed based on the method of Yamasaki et al. (5). Briefly, genomic DNAs from ethanol-fixed samples were prepared using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), and DEXPAT (TaKaRa Bio, Shige, Japan) was used for formalin-fixed and paraffin-embedded sections. Mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) was amplified by PCR. Samples for direct sequencing were prepared using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif., USA), and sequencing was performed on an ABI PRISM 3100-*Advant* Genetic Analyzer (Applied Biosystems). Sequence data were analyzed using EditSeq and MegAlign software (DNASTAR, Madison, Wis., USA). We amplified ~1.7 kb *cox1* in ethanol-fixed specimens,

Table 1. Number of pig postmortem inspections and results of *E. multilocularis* tests at Aomori Prefectural Towada Meat Inspection Center

	FY2005	FY2006	FY2007	FY2008	Total
Total no. of inspections	888,450	885,430	893,884	910,130	3,577,894
No. of pigs from Hokkaido	0	900	1,256	3,135	5,291
Whitish nodules in the liver	27	44	25	13	109
Positive for *E. multilocularis*	0	0	0	6	6

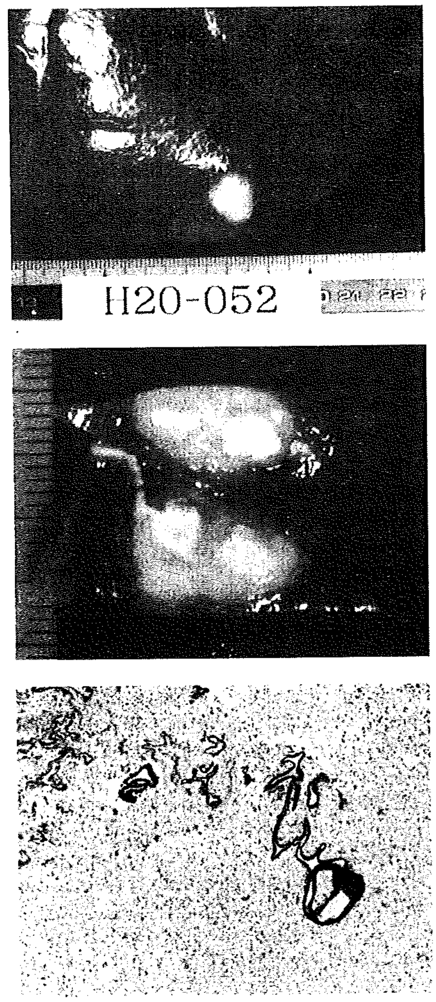
(A)
(B)
(C)


Fig. 1. Pathological findings of a hepatic echinococcal nodule. (A) Macroscopic appearance of a whitish nodule caused by *E. multilocularis*. (B) Cut surface of a nodule. (C) Magnification of a nodule showing regressive cyst of *E. multilocularis*. The cuticular layer of the cyst was strongly PAS positive. PAS stained. ×40.

and shorter sizes of *cox1* fragments (108–110 bp) were successfully amplified in formalin-fixed specimens (data not shown). DNA sequencing of the amplicons confirmed that the causative agents was *E. multilocularis* in all cases, with identical nucleotide sequences to that of *E. multilocularis* isolates from Hokkaido (GenBank accession no. AB018440).

The majority of cases of human alveolar echinococcosis in Japan have occurred in Hokkaido (approximately 500 cases to date), where *E. multilocularis* is recognized as indigenous. Approximately 80 cases of alveolar echinococcosis have been encountered in other prefectures, of which a quarter have been reported from Aomori Prefecture. Moreover, in nine of these cases there is a strong possibility that infection occurred within the prefecture (6). This is believed to be a result of the closeness with which people and goods circulate between Hokkaido and Aomori, although the specific factors responsible for infection are not known. Given this situation, the fact that *E. multilocularis* was detected in 1998 in pigs that were believed to have come from Aomori gave rise to the strong suspicion that the parasite had established its life cycle within the area. However, this could not be determined for certain; although the infected pigs were all transported from the same piggery in Aomori, the piggery in question not only bred pigs but also possessed pigs that had been purchased at livestock markets (1). The fact that in the present survey *E. multilocularis* infection was not found in pigs from outside Hokkaido suggests that there is at present a very low probability that *E. multilocularis* has established its life cycle in Aomori Prefecture. Data obtained from an inspection of 26,380,171 pigs in Hokkaido between 1983 and 2007 by the prefectural government revealed that 29,344 (0.1%) were infected with *E. multilocularis*. As shown in this report, Towada MIC has examined 5,291 pigs from Hokkaido over the last 4 years, and the rate of *E. multilocularis* detection is also approximately 0.1%. Pigs do not appear to play any role in transmission of the parasite, as the metacestode develops no brood capsules or protoscoleces in the host. However, detection of swine echinococcosis can be used as an indicator for the environmental egg contamination. Aomori is just across the Tsugaru Strait from Hokkaido, where *E. multilocularis* is endemic, and we intend to make a continual contribution to monitoring and preventing the spread of *E. multilocularis* to Honshu via postmortem inspections.

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Laboratory and Epidemiology Communications

Detection of *Paragonimus* Metacercariae in the Japanese Freshwater Crab, *Geothelphusa dehaani*, Bought at Retail Fish Markets in Japan

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Metacercariae, the encysted larval stage of flukes capable of infecting the final and/or paratenic hosts, of *Paragonimus miyazakii* and of both diploid and triploid forms of *P. westermani* are found in the Japanese freshwater crab, *Geothelphusa dehaani*, which acts as the second intermediate host in Japan. This crab is known as Sawagani in Japanese and is widely distributed in Japan, from Hokkaido to Kyushu islands, including Yakushima Island. Both *Paragonimus* spp. are known to be medically important causes of human infection, although the respiratory symptoms that develop in patients vary according to the form and species of the causative lung fluke. Chronic cough with rusty-colored sputum is the most common symptom of patients infected with the triploid form of *P. westermani*, while infection with *P. miyazakii* and the diploid form of *P. westermani* usually causes pleural effusion without remarkable lesions in the lung parenchyma (1).

In Japan, the incidence of *Paragonimus* infection has increased among long-term foreign residents (2,3). It is postulated that long-term residents from Asian countries such as China, Korea and Thailand maintain their dietary habits in Japan and, thus, ingest uncooked Sawagani in their ethnic dishes. Infection of people outside of these groups who eat these dishes has also been reported. There is a need for caution regarding paragonimiasis associated with these eating habits. In some cases, the causative foodstuff included in these dishes was identified as Sawagani sold at local retail fish markets.

In the present study, we purchased Sawagani originating from three prefectures (Shizuoka Prefecture in the Tokai district, and Miyazaki and Nagasaki prefectures in the Kyushu district) at retail fish markets in the Tokyo metropolitan area between April 2004 and February 2008 and examined these crabs for the prevalence of *Paragonimus* metacercariae (Table 1). Lung fluke metacercariae were detected in 44 (17%) of 266 examined crabs. The positive crabs harbored a total of 169 metacercariae, with the average numbers of metacercariae being 3.8 and 0.64 per positive crab and per crab of the total number of crabs examined, respectively. The maximum number of metacercariae in a single crab was 23 in a crab originating in Miyazaki Prefecture that was purchased in February 2008.

Individual metacercariae isolated from the crabs were identified to the species (*P. westermani* or *P. miyazakii*) and, further, to the form (diploid or triploid) for *P. westermani*. The metacercariae of *P. miyazakii* could be morphologically discriminated from those of *P. westermani* by the presence of a membranous substance, as well as by the absence of a stylet (1). Of a total of 169 isolated metacercariae, both of these characteristics were confirmed in only 20 metacercariae, which were identified as *P. miyazakii*. The remaining metacercariae were subjected to molecular identification by PCR-restriction fragment length polymorphism (RFLP) analysis and sequencing. First, the total genomic DNA was prepared from individual metacercariae following our previously described method (4). The ITS2 region of the nuclear ribosomal DNA (rDNA) and a portion of the 16S mitochondrial rDNA were amplified by PCR using primer pairs 3S (forward: 5′-GGTACCGGTGGATCACTCGGCTCGTG-3′) with A28 (reverse: 5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′) (5) and T7-1 (forward: 5′-ATTTACATCAGTGGGCCGTC-3′) with SP6-1 (reverse: 5′-GATCCAAAAGCATGTGAAAC-3′) (6), respectively. The amplified products were treated with restriction enzymes and separated by electrophoresis on agarose gel (RFLP analysis). For the RFLP analyses, we selected restriction enzymes *Sna*BI and *Bss*SI to digest the ITS2 PCR products from *P. westermani* and *P. miyazakii* (4). We selected enzymes *Sna*BI and *Bsr*DI based on the theoretical restriction maps generated from the 16S mitochondrial rDNA sequences of diploid and triploid forms of *P. westermani* (6,7). Undigested amplicons were sequenced using the corresponding primers to verify the identification made by RFLP analy-

Table 1. Prevalence, number and species of *Paragonimus* metacercariae in Japanese freshwater crabs, *Geothelphusa dehaani*, sold at retail fish markets in the Tokyo metropolitan area, Japan

Month of purchase	Origin (Prefecture)	No. of crabs		No. of Mc[1)] detected	Species[2)] of Mc
		examined	infected		
Apr. 2004	Shizuoka	48	0	0	
Apr. 2007	Miyazaki	46	0	0	
Apr. 2007	Miyazaki	16	7	29	Pm
Apr. 2007	Nagasaki	21	5	9	Pm
June 2007	Shizuoka	35	0	0	
June 2007	Miyazaki	44	5	9	Pw (3n)
Jan. 2008	Miyazaki	30	4	6	Pm, Pw (2n)
Feb. 2008	Miyazaki	26	23	116	Pm
Total		266	44	169	

1): Metacercariae.
2): Pm, *P. miyazakii*; Pw (2n), the diploid form of *P. westermani*; Pw (3n), the triploid form of *P. westermani*.

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Fig. 1. RFLP patterns of PCR products amplified from the DNA of *P. westermani* metacercariae (lanes 1 - 3 for both the diploid and triploid forms; lanes 7 - 9 for the diploid form; lanes 10 - 12 for the triploid form) or *P. miyazakii* metacercariae (lanes 4 - 6). The ITS2 PCR products were untreated (lanes 1 and 4) or treated with endonucleases *Sna*BI (lanes 2 and 5) or *Bss*SI (lanes 3 and 6). The 16S rDNA PCR products were also untreated (lanes 7 and 10) or treated with endonucleases *Sna*BI (lanes 8 and 11) or *Bsr*DI (lanes 9 and 12). A 100-bp DNA ladder marker was used to estimate the size of the fragments.

sis.

PCR amplification with the primer pair 3S and A28 generated single 520-bp products from the metacercarial DNA samples. Electrophoresis of the restriction enzyme-digested products resulted in two species-specific RFLP patterns, as previously described (4). Species identification of the metacercariae was made based on the digestion patterns of amplification products. Products that were digested with *Sna*BI to produce 2 fragments (about 420 bp and 100 bp) but remained undigested with *Bss*SI were identified as those of *P. westermani*. Products that were undigested with *Sna*BI but were digested with *Bss*SI to produce 2 fragments (about 300 bp and 220 bp; Fig. 1) were identified as those of *P. miyazakii*.

DNA samples prepared from *P. westermani* metacercariae were further analyzed to determine the form, i.e., diploid or triploid. PCR amplification of mitochondrial DNA with the primer pair SP6-1 and T7-1 produced a single 840-bp product. Restriction digestion of PCR products was used to identify the diploid and triploid forms. Products that were digested with *Sna*BI to produce 2 fragments (about 550 bp and 290 bp) but remained undigested with *Bsr*DI were identified as those of the diploid form. Products that remained undigested with *Sna*BI but were digested with *Bsr*DI to produce 2 fragments (about 560 bp and 280 bp; Fig. 1) were identified as those of the triploid form. The species and forms identified by the RFLP analyses were verified by sequencing of the respective PCR products.

Consequently, as shown in Table 1, most of the metacercariae were identified as *P. miyazakii* (157 metacercariae from 36 positive crabs), while the others were *P. westermani* (3 metacercariae from 3 positive crabs and 9 metacercariae from 5 positive crabs were of the diploid and triploid forms, respectively). However, there were no mixed infections either with *P. miyazakii* and *P. westermani* (diploid and/or triploid forms) or with both forms of *P. westermani* in any crab examined in the present study.

Sawagani from Miyazaki Prefecture were also purchased at a retail fish market in Fukuoka City in April 2008 and were examined for *Paragonimus* metacercariae. *P. miyazakii* metacercariae (35 in total) were detected in 15 of 30 examined crabs. This finding implies that Sawagani with *Paragonimus* metacercariae that are responsible for human infections are likely also sold in retail fish markets in areas other than Tokyo.

The heat resistance of *P. westermani* metacercariae within the crab hosts was investigated almost a century ago (8). The Japanese mitten crab, *Eriocheir japonicus*, which played a major role as the second intermediate host in spreading the human infection of *P. westermani* at that time in Japan was investigated (*P. miyazakii* metacercariae have never been isolated from this crab species). It was shown that boiling infected crabs at 55°C for 5 min killed all the metacercariae (8). However, to the best of our knowledge, the conditions required to kill metacercariae of *P. westermani* and *P. miyazakii* in Sawagani have not yet been well examined, although we are currently investigating these conditions. Therefore, the implementation of a health education campaign is recommended throughout Japan to emphasize that Sawagani, even those sold at retail fish markets, are potential sources of lung fluke infection in humans. Special attention should be paid to ethnic dishes that are prepared with uncooked Sawagani.

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Ectopic (Subcutaneous) *Paragonimus miyazakii* Infection in a Dog

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Abstract. Ectopic infection with *Paragonimus miyazakii* was determined to be the cause of a subcutaneous inguinal mass in a 15-month-old, male, boar-hunting dog. On histologic examination, the mass comprised granulomatous panniculitis, intralesional adult trematodes and eggs, and lymphadenitis. Extrapulmonary paragonimosis in animals is rare. This appears to be the first report in a dog of ectopic *P. miyazakii* infection with mature trematodes and eggs that involved the inguinofemoral lymphocenter and surrounding subcutis.

Key words: Dogs; ectopic parasitism; histopathology; lymphadenitis; *Paragonimus miyazakii*; subcutis.

Paragonimosis is a parasitic disease caused by trematodes of the genus *Paragonimus* and is an important food-borne endemic zoonosis worldwide.[6,8] Adult trematodes reside in the lungs of definitive hosts (humans and various wild and domestic animals, including dogs).[6,8] Ectopic paragonimosis with larvae and/or adults is well recognized in humans;[3,6,8] however, only a few cases of natural or experimentally induced ectopic paragonimosis were reported in dogs.[1,2,5,7,14] To the best of our knowledge, this represents the first report of extrapulmonary *P. miyazakii* infection in a dog with mature trematodes and eggs in an inguinal mass that involved lymph nodes and surrounding subcutis.

A 15-month-old, setter-type, male dog that had been used for boar hunting was presented to a private animal hospital for examination of a palpable inguinal mass. Reportedly, the dog occasionally ate raw wild boar meat. Lymphoma was suspected clinically, so a core biopsy of the inguinal mass was performed. After unidentifiable fragments of a parasite were detected on histologic examination, excisional biopsy was performed for diagnosis and therapy.

On gross examination, the formalin-fixed subcutaneous mass was gray and lobulated, approximately 16 × 10 × 10 cm. The cut surface of the mass was mottled yellow-brown to dark-red and contained trematodes and enlarged lymph nodes. By stereomicroscopy, the trematodes, encapsulated in cysts, were whitish, had a thick ellipsoidal body and a small, reddish, crater-shaped acetabulum. Eggs were operculate, irregularly barrel shaped, and averaged 77.7 μm × 48.0 in length and width.

On histologic examination, the subcutaneous mass comprised granulomatous panniculitis with cysts that contained adult trematodes and eggs, and lymphadenitis associated with the eggs. Dense, broad bands of fibrous tissue encapsulated the mass and dissected between the cysts and nodules (Figs. 1, 2). Cysts contained single or paired adults, scattered mature eggs, mixed inflammatory cells, red blood cells, and necrotic debris. Nodules consisted of numerous mature and degenerated eggs in granulomatous inflammation (Fig. 2). Some nodules contained a central mass of eggs and a thin peripheral rim of fibrosis; in others, solid nodules of eggs were separated by fine fibrous septa. Infiltrating leukocytes included a variable number and mixture of plasma cells, lymphocytes, neutrophils, eosinophils, and macrophages, with a few multinucleated giant cells. The affected lymph nodes had both small and massive clusters of eggs scattered throughout the nodal sinuses (Fig. 3). Some egg clusters elicited granulomatous response. Follicles were hyperplastic with prominent germinal centers; nodal fibrosis was severe and diffuse.

On histologic examination, the adult trematodes had tegument, with a single spine, well-developed oral and ventral suckers, uterus, ovary, and intestine. Numerous mature eggs were in the uterus; lobes of the ovary were moderately branched. Testes and vitelline ducts were also fully developed and contained mature sperm and vitelline cells, respectively.

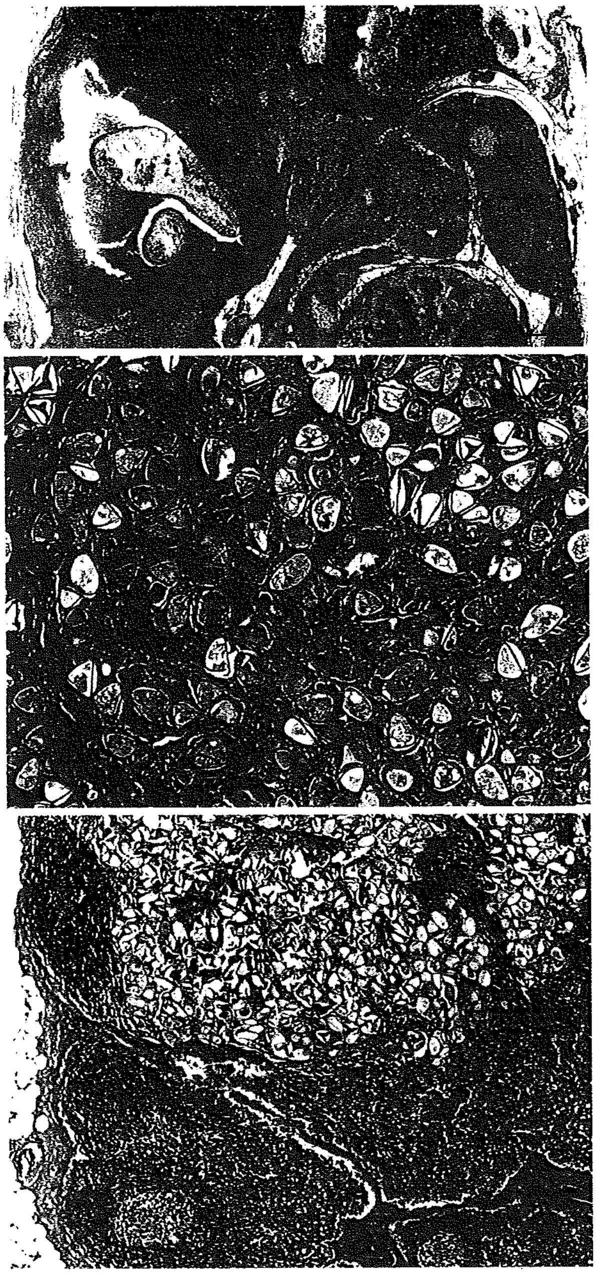

Fig. 1. Inguinal subcutaneous mass; dog. Granulomatous panniculitis and lymphadenitis associated with adult trematodes and eggs. HE. Bar = 1 mm.

Fig. 2. Inguinal subcutaneous mass; dog. Clusters of trematode eggs in granulomatous inflammation. HE. Bar = 100 μm.

Fig. 3. Lymph node of the inguinofemoral lymphocenter; dog. Granulomatous lymphadenitis with trematode eggs. Lymphoid follicles have prominent germinal centers. HE. Bar = 400 μm.

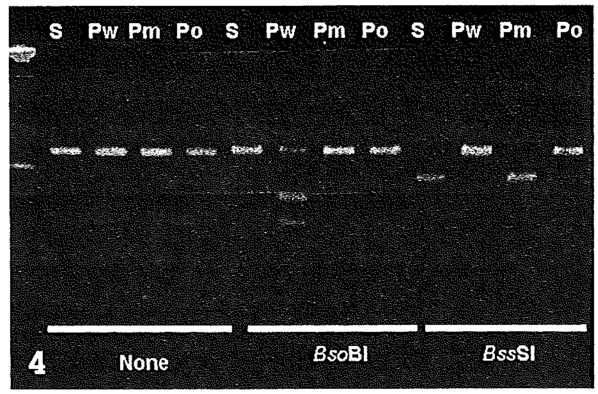

Fig. 4. RFLP analysis of ITS2 PCR products. The DNA from the sample (S), *P. westermani* (diploid type) (Pw), *P. miyazakii* (Pm), and *Paragonimus ohirai* (Po) was amplified with ITS2-specific PCR primers and digested with endonucleases, *Bso*BI, and *Bss*SI. The restricted fragments of the sample are identical to those for *P. miyazakii*.

Microscopic features of the adult parasites and stereomicroscopic features of the eggs were characteristic of *Paragonimus* spp., so ectopic paragonimosis was diagnosed. After approximately 2 weeks, the excision site swelled again to approximately two thirds of its size before surgery. Oral praziquantel treatment (10 mg/kg, once a day, for 10 days) was administered. The swelling subsided after the treatment, and the dog has remained free of clinical disease, without further treatment, for more than 2 years. Additional clinical examinations, including chest radiography or fecal examination for *Paragonimus* eggs, were not performed.

The *Paragonimus* sp. was identified from the formalin-fixed specimen by polymerase chain reaction (PCR) linked restriction fragment length polymorphisms (RFLPs) and DNA sequencing.[12] Restricted fragment length polymorphisms of the second internal transcribed spacer (ITS2) from nuclear ribosomal DNA were identical to those of *P. miyazakii* (Fig. 4), and the sequences of ITS2 were identical to those deposited in the GenBank/EMBL/DDBJ nucleotide database (accession number U96912) for *P. miyazakii*. The species was thus identified as *P. miyazakii*.

Paragonimosis is an important food-borne parasitic zoonosis caused by trematodes (genus *Paragonimus*), which infect the lungs of humans and various other animals.[6,8] At least 28 species of *Paragonimus* have been identified,[8] and 10 species are recognized as causing human disease.[6] *Paragonimus* spp. are mainly parasites of cats, dogs, and various mammals that eat freshwater crabs and crayfish (the second intermediate host), as well as the raw meat of wild boars (the paratenic host).[9] Occasionally, humans become accidental hosts.[8] With approximately 200 million people at risk and 22 million people infected worldwide, paragonimosis still is an important public health threat.[6] In the Far East, including Japan, the prevalence of human parasitic diseases has been greatly reduced as living standards improved, but pulmonary paragonimosis remains an important endemic parasitic disease.[11] Dogs and cats, as more likely definitive hosts, generally play a greater role than humans in the *Paragonimus* life cycle.[6]

In veterinary medicine, 2 *Paragonimus* spp., *Paragonimus westermani* and *Paragonimus kellicotti*, are of particular interest.[4] *P. westermani* is the best-known species in Asia; *P. kellicotti* occurs in North America. In addition, in Japan, *P. miyazakii* is another important species responsible for human and animal paragonimosis.[3,6,10]

Paragonimus trematodes usually infect the lungs of the mammalian host.[3] However, aberrant migration may be more likely in accidental hosts,[8] and, because humans are less suitable than other mammals as a definitive host for *Paragonimus*, they may be more commonly affected by ectopic paragonimosis. In human extrapulmonary paragonimosis, various sites and tissues, including the brain, spinal cord, abdominal cavity, or subcutis, may be involved;[3,6,8] pathologic effects are principally caused by the presence of adult trematodes and eggs, the movement of trematodes through tissues, and the metabolites produced by trematodes.[3] Some species differences exist in the frequency and effect of ectopic paragonimosis. Subcutaneous masses associated with parasitic migration reportedly occur in 20–60% of *Paragonimus skrjahbini* infections, compared with approximately 10% with *P. westermani* and 2.4% with *P. miyazakii* (2/82).[6,10] For those *Paragonimus* spp., such as *P. miyazakii* and *P. skrjahbini*, for which humans are an unsuitable host, the trematodes are unlikely to mature, so eggs are rarely found in extrapulmonary sites.[3]

In dogs, ectopic paragonimosis is rare.[1,2,5,7,14] In reported cases, extrapulmonary lesions were mostly produced by migration of immature trematodes or by eggs;[5,14] exceptional cases had *P. westermani* cysts and eggs in trachobronchial lymph nodes[7] or spleen.[2,5] In contrast, the present case developed an inguinal subcutaneous mass that contained encysted solitary or pairs of adult trematodes. The trematodes had matured in this ectopic location, forming cysts and producing eggs. Granulomatous panniculitis and lymphadenitis were mainly attributed to the presence of eggs. Because dogs are definitive hosts for *P. miyazakii*, the incidence of canine extrapulmonary paragonimosis should be low. However, once *P. miyazakii* settles in an extrapulmonary site, maturation and egg laying may occur, as in the present case.

In this dog, the trematodes may have migrated to an inguinal lymph node, mated, deposited eggs, and provoked lymphadenitis and regional panniculitis. In human medicine, lymphadenitis because of the presence of metazoan parasites, including *Paragonimus* or their eggs, is rare but has been described.[13] In dogs, such lymphadenitis is also rare but has been described with *P. westermani*[7] and *P. kellicotti* infection.[1] However, lesions in those cases were much less severe than in the present case.

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Original Article

PARAGONIMUS HETEROTREMUS INFECTION IN NAGALAND: A NEW FOCUS OF PARAGONIMIASIS IN INDIA

***TS Singh, H Sugiyama, A Umehara, S Hiese, K Khalo**

Abstract

Purpose: To determine the prevalence of paragonimiasis among the patients who were attending the tuberculosis (TB) clinics at the Community Health Centre, Pfutsero, Phek District, Nagaland. To determine the species of *Paragonimus* that cause infection in humans and the crustacean host that acts as the infectious source for humans. **Materials and Methods:** Sputum specimens were examined microscopically for Paragonimus eggs and acid fast bacilli. Blood samples were tested by microenzyme-linked immunosorbant assay for Paragonimus-specific immunoglobulin G antibodies. Crab extracts prepared by digestion with artificial gastric juice were examined for *Paragonimus metacercariae* under a stereoscopic microscope. The species identification of the parasite was based on morphological and molecular characterizations of eggs and metacercariae employing polymerase chain reaction and DNA sequencing. **Results:** Seven out of the 14 patients tested seropositive for paragonimiasis and Paragonimus eggs were detected in sputum of two out of the seven seropositive patients, indicating a prevalence of 50% and an egg detection rate of 14%, respectively. The prevalence was highest in the 10-30 year age group. More males got the infection than females, the ratio being 5:2. *P. heterotremus* was identified as the causative agent of human paragonimiasis and *Potamiscus manipurensis* as the crab host. **Conclusions:** The study revealed that paragonimiasis has been endemic in Pfutsero, Nagaland, and half of the patients attending the TB clinic were actually suffering from pulmonary paragonimiasis. This is the first confirmed report of an endemic focus of paragonimasis and description of *P. heterotremus* as the causative agent in Nagaland, India.

Key words: *India, lung fluke, Nagaland, paragonimiasis, P. heterotremus, tuberculosis*

Introduction

Lung flukes have been described in the world, mainly from East and Southeast Asia and also from Africa and Americas. *Paragonimus westermani*, the most widely distributed species in Asia, was first described by Kerbert from the lungs of a Bengal tiger, which was captured in India and died at a zoo in Amsterdam more than a century ago. However, very little attention has been paid to this parasite because paragonimiasis was never considered to be a public health problem in India and had remained a neglected disease until the first case was reported from Manipur in 1982.[1] After that, many cases were reported from several parts of Manipur.[2-4] Subsequently, endemic foci of paragonimiasis were also discovered in Arunachal Pradesh.[5] Most interestingly, *P. heterotremus* has been identified as the causative agent of human paragonimiasis in this part of India against the widely believed *P. westermani*, which was reported from many mammals in India.

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In the past, some occasional cases from Nagaland, initially diagnosed as pulmonary TB by clinical symptoms and chest X-rays, were referred to the Regional Institute of Medical Sciences, Imphal, Manipur. The cases were parasitologically confirmed as pulmonary paragonimiasis. However, detailed information on the prevalence of paragonimiasis in the northeast states of India other than Manipur and Arunachal Pradesh were limited. The present study was, therefore, performed to ascertain the prevalence of paragonimiasis among the patients who were attending the TB clinic at the Community Health Centre, Pfutsero, Nagaland, and to determine the causative species and some of the epidemiological factors responsible for the infection.

Materials and Methods

Patients and clinical examination

The senior author visited the Community Health Centre at Pfutsero town, Phek district, Nagaland, during March 27-28, 2008 to investigate paragonimiasis among the patients attending the TB clinic at the health centre. Pfutsero town is located in southeast Nagaland, bordering Manipur in the south and Myanmar in the east.

Detailed clinical history taking and physical examination of all the patients were performed by the medical officers. The findings were recorded in a pre-designed profoma printed in English. Informed consent about the examination and procedures was obtained from each patient after proper explanation in their own dialect. Postero-anterior chest roentgenograms were taken for all the patients to evaluate any abnormal lesion in the chest.

Sputum examination

The sputum samples of the patients were collected in sterile plastic screw-capped containers. The specimens were examined microscopically for Paragonimus eggs and also for acid fast bacilli (AFB) using the wet cover slip smears and Ziehl-Neelsen-stained smears, respectively. The Paragonimus eggs were then preserved in two parts, one portion in equal volume of 10% phosphate-buffered formalin for morphological study and another in equal volume of 70% ethanol for molecular characterization.

Microenzyme-linked immunosorbant assay (ELISA) test

The blood samples of all the patients were tested for Paragonimus immunoglobulin G antibodies by micro-ELISA using antigens prepared from adult *P. heterotremus* worms.[6] Optical density (OD) values higher than 0.300 were taken as positive.

Examination of crabs

A total of 20 fresh water crabs were collected from a "Zachughie" mountain stream near the Pfutsero town. After morphological examination, the crabs were extracted and then digested with artificial gastric juice, followed by differential filtration.[7] The filtrates were examined under a stereoscopic microscope for *P. metacercariae*. The isolated metacercariae were preserved in two separate vials containing 10% formol-saline and 70% ethanol for morphological and molecular characterization, respectively.

Morphological and molecular characterization

Morphological features of eggs from the patients and metacercariae from the crabs were examined microscopically. Molecular characterization of eggs and metacercariae was performed by DNA isolation, amplification of the ITS2 regions of the ribosomal DNA by polymerase chain reaction (PCR)-linked restriction length polymorphism method and sequencing.[8,7] To be more precise, the primers used were 3S (forward, 5'-GGTACCGGTGGATCACTCGGCTCGTG-3')[9] and A28 (reverse, 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3').[10] The PCR amplification was performed using 0.25 μm of each primer and 2.5 U of Taq polymerase (Invitrogen Corp., Carlsbad, CA, USA). The amplified products were extracted from agarose gels (Lonza, Rockland, ME, USA) and sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI 310 Genetic Analyzer; Applied Biosystems). The amplified products (10 μm) were also treated with 5 U of the restriction enzyme *Apa*LI (New England Biolabs, Beverly, MA, USA) at 37°C for 1 h. The amplicons with or without the enzymatic treatment were then separated by electrophoresis on 2% (w/v) agarose gels.

Results

A total of 14 patients who attended the TB centre with some respiratory symptoms were investigated. The major clinical manifestations presented by them are shown in Table 1. Chronic productive cough was the most common of all the complaints followed by difficulty in breathing and recurrent haemoptysis. In three patients, the sputum smears showed AFB while Paragonimus eggs were all negative. In 11 patients who were negative for AFB, two patients discharged Paragonimus eggs in the sputum. These two patients and four other patients who were negative for Paragonimus egg and AFB were positive for antibodies against the Paragonimus antigen. One smear-positive TB patient was also seropositive against the Paragonimus antigen. In summary, seven out of the 14 patients were positive for antibodies against the Paragonimus antigen. The OD values of the seropositive cases varied from 0.34 to 1.53, with 0.82 on average. Two patients who were egg positive showed much higher OD values (1.53 and 1.36) than the egg negative but seropositive patients.

Of the seven paragonimiasis cases, there were five male and two female, making a male-to-female ratio of 5:2. In addition, a higher prevalence of paragonimiasis was detected among children and young adults in the age group of between 7 and 32 years and rare after 40 years of age. The chest roentgenograms showed abnormal areas in three of the seven seropositive patients (paragonimiasis). Left-sided pleural effusions were seen in two patients whose sputa were Paragonimus egg positive and right lung pneumonia in another seropositive patient. Out of the three TB patients, no abnormal lesions were detected in two while nodular shadows were seen in the right upper lung in one. This patient was infected with both Paragonimus (seropositive) and TB. Fever, weakness, weight loss and loss of appetite were found as other associated symptoms in this patient.

Table 1: Major clinical manifestations and laboratory examination findings observed in 14 patients

Clinical manifestations and laboratory findings	No. of patients (%)
Cough	14 (100)
Difficult breathing	9 (64)
Recurrent haemoptysis	6 (43)
Fever	6 (43)
Pain in the chest	3 (21)
Acid fast bacilli (AFB)	3 (21)
Anti-Paragonimus antibodies (Ab)	7 (50)
Paragonimus eggs	2 (14)
Both AFB and Ab	1 (7)

The morphological features of the eggs from the two patients [Figure 1a] were found to be characteristic of *P. heterotremus* with some variations in the shape and size. They were oval and elongated in shape, golden-yellow in colour and operculated and measured 82-95 µm (average = 82 µm) in length and 45-58 µm (average = 49 µm) in width. The eggshell thickness was almost uniform and indiscernible at the non-operculated end.

The freshwater crabs captured in the stream near Pfutsero town were morphologically identified as *Potamiscus manipurensis* [Figure 1b]. Of the 20 freshwater crabs examined, 48 *P. metacercariae* were isolated. Five smaller crabs (carapace size: 20.5 mm × 24.5 mm on average) and 15 larger crabs (carapace size: 29 mm × 37 mm on average) yielded 30 and 18 metacercariae, respectively. The number of metacercariae per crab was higher in smaller crabs (average = 6) than in bigger crabs (average = 1.2). The fresh metacercariae [Figure 1c] were oval to suboval in shape, with a thin outer cyst wall and a thicker inner cyst wall, which was typically thickened at both poles, better defined in Figure 1d. The inner cyst measured on average 197 µm in the long axis and 163 µm in the transverse axis. The thickness of the inner wall was on average 6.4 µm on the side and gradually thickened at the pole to 18.5 µm on average. The oral sucker was smaller than the ventral sucker and was provided with a stylet. The morphological features of the metacercariae were characteristic of *P. heterotremus*.

By PCR amplification, the ITS2 PCR products of about 520 bp were generated from the DNA samples prepared from the eggs from patients [Figure 2, lane 1] and metacercariae. Two fragments (about 350 bp and 170 bp, Figure 2. lane 2) were generated from the PCR products (520 bp) after digestion with a restriction enzyme *Apa*LI, which recognizes the sequences from *P. heterotremus*.[11] The PCR products were excised from agarose gels after electrophoresis and were used for sequence analysis. The analysis revealed that the aligned ITS2 regions were 461 bp (without primer sequences) for both eggs and metacercariae. The obtained sequence data were deposited in the database GenBank/EMBL/DDBJ under accession numbers AB456558 and AB456559 for the metacercariae and eggs, respectively. They were the identical sequences. Similarity searches of

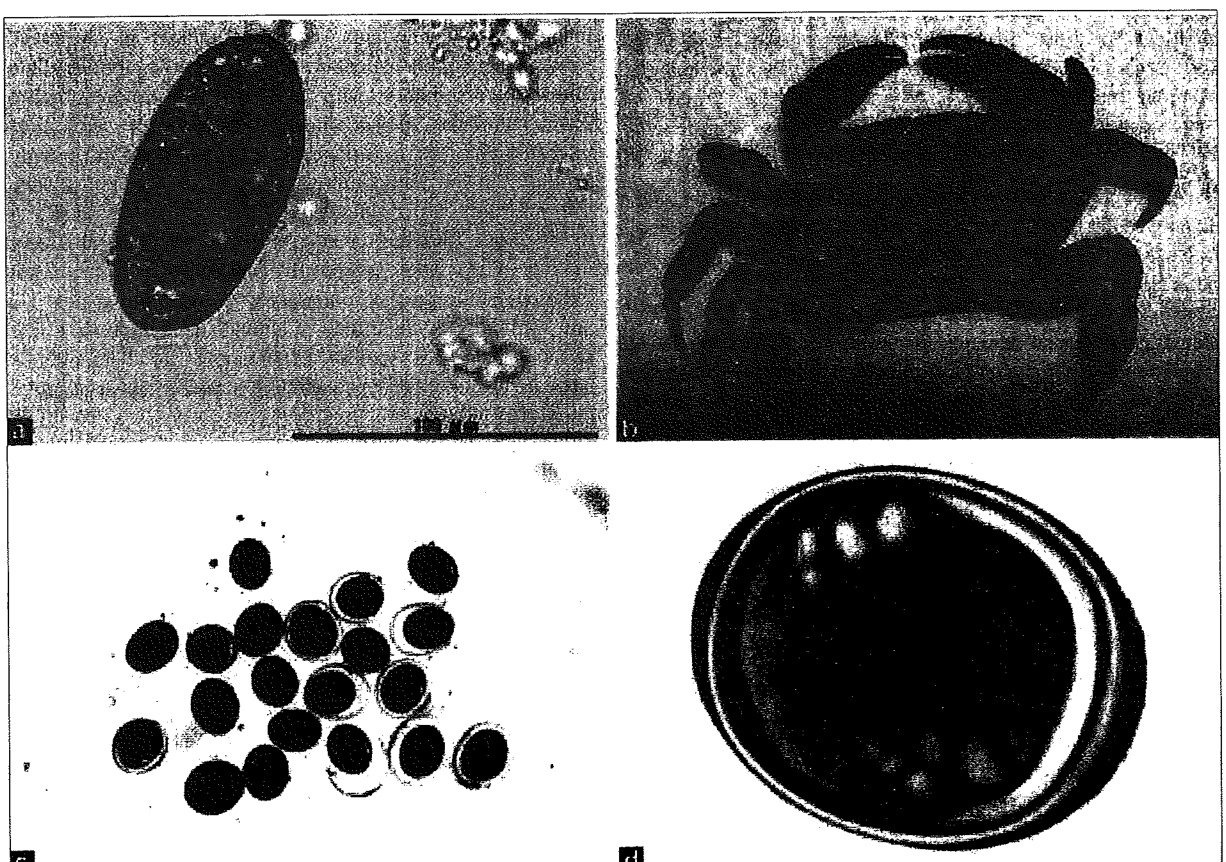

Figure 1: (a) Photomicrographs of formalin (10%)-preserved Paragonimus eggs (×40) found in the sputum examination. (b) Photograph of *Potamiscus manipurensis*, the crab host of *Paragonimus heterotremus*, collected from a mountain stream in Pfutsero town, Nagaland. (c) *P. heterotremus* metcarcariae (×10) isolated from *Potamiscus manipurensis*. (d) Single metacercariae (×40). Note the oval-shaped and thickened cyst wall at either pole

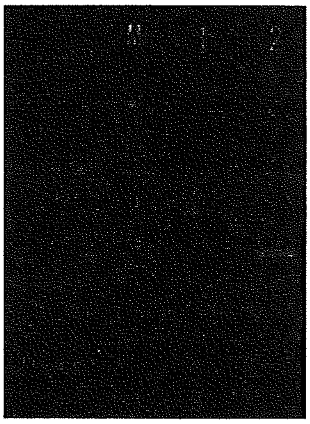

Figure 2: Results of polymerase chain reaction (PCR) (lane 1, a band of about 520 bp) and PCR-restriction fragment length polymorphism with *Apa*LI (lane 2, two fragments of about 350 bp and 170 bp) using the DNA sample isolated from the eggs from one patient. The same results were obtained when we used DNA samples isolated from the eggs of another patient or those from the metacercariae. Hundred basepair DNA ladders (Invitogen) were used to estimate the sizes of the bands (lane M)

the database revealed that the obtained sequences were identical to those from the metacercariae (AB308377) and eggs (AB308378) of *P. heterotremus* occurring in Manipur, India.[7]

Discussion

Although some occasional cases of paragonimiasis, which diagnosed initially as pulmonary TB, were already discovered in Nagaland, the detailed information about the disease was not available. The senior author, therefore, visited the health centre at Pfutsero in Nagaland to investigate further for paragonimiasis and Paragonimus during March 28-29, 2008. We determined the prevalence of paragonimiasis, and the egg detection rate of 14 patients who attended the health centre was 50% and 14%, respectively. The results of morphological and molecular characterization of Paragonimus eggs from sputum samples have established that *P. heterotremus* was the causative agent of paragonimiasis in Nagaland. This species has also been identified as a significant cause of human pulmonary paragonimiasis in Manipur and Arunachal Pradesh, India,[1-5] as well as in Southeast Asian countries like Thailand, Lao PDR and Vietnam.[12]

We also determined the epidemiological factors responsible for infections with *P. heterotremus*. A high prevalence rate of 64% was observed in children and young adults (age ≤ 30). This finding was in agreement with that in Manipur in which two-thirds of the patients were in the age group of 11-30 years[2] and in Arunachal Pradesh in which the infection was higher (52%) in children (age ≤ 15).[13] Crabs are abundant in most of the mountain streams in the endemic areas in Nagaland. The villagers believed that raw crabs or its extract and soup provided them strength and nutrition. Some believed that ingestion of raw crab extract can cure fever and allergy. These activities are important modes of infection for local people, especially for the young adults. Therefore, it is imperative to undertake health educational programs for the prevention of paragonimiasis in this endemic area.

General physical conditions of paragonimiasis were relatively good. The patients were quite ambulatory and apparently healthy looking. The symptoms were exacerbated just by hard physical activities, which often initiated bouts of haemoptysis. Generally, clinical symptoms and radiological appearances of paragonimiasis were overlapping with pulmonary TB thus resulting in an overdiagnosis of the non-tubercular cases as smear-negative pulmonary TB. Therefore, a detailed clinical history of illness, including dietary habit of consumption of crabs and laboratory investigation such as sputum examinations for Paragonimus eggs and serodiagnoisis, are essentially important in all cases with respiratory symptoms to avoid misdiagnosis. Once diagnosed as paragonimiasis, the disease can be effectively treated with praziquantel.

Conclusion

The result of this investigation revealed the first recognized endemic area of paragonimiasis in Nagaland. Fifty per cent of the patients who were attending the TB clinic with some respiratory symptoms were found to be suffering from pulmonary paragonimiasis based on a serological micro-ELISA test. Two patients who presented with bloody sputum showed Paragonimus eggs in the sputum smears. The infection was common in children and young adults up to 30 years. The chest roentgenograms were normal except in four of the seven seropositive patients. The clinical and radiological features of pulmonary paragonimiasis and TB are similar and, therefore, it should be emphasized that serodiagnosis and sputum examination for Paragonimus eggs are essential before concluding a case as smear-negative pulmonary TB.

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I, hereby declare that the particulars given above are true to the best of my knowledge and belief.

Date: 30th March 2009 **Dr. Reba Kanungo**