99%で *Avibacterium paragallinarum* と同定された。しかしながらこの計2株も，*C. canimorsus* と同定される，あるいは *C. canimorsus* が鑑別候補菌種となるコード番号とは1テスト項目（H-7: キシロース，D-4: ホスファターゼ）の陽性/陰性の違いであった。D-1株はチョコ培地増菌，rb加HI培地増菌ともに同じコード番号となり，*C. cynodegmi* と同定された。上記の結果をまとめると，rb加HI培地増菌で *C. canimorsus* と同定されるか，*C. canimorsus* が鑑別候補菌種の一つとなったのは24株中23株であった。この中でチョコ培地増菌ではrb加HI培地増菌とは異なるコード番号となった12株のうち，2株はチョコ培地増菌では該当菌種なし(H-7)，あるいは他の菌種(D-4)であると判定された。β-ラクタマーゼ試験では，イヌ由来株の2株(D-2, D-5)およびネコ由来株の2株(C-2, C-4)の計4株はβ-ラクタマーゼ陽性を示し，ヒト分離株12株を含めた他の20株は陰性であった。HNニッスイ-20ラピッドでβ-ラクタマーゼ陽性となった4株は，セフィナーゼディスクによる検査でも陽性を示し，また他の20株はいずれもセフィナーゼディスクによる検査でも陰性であり，両試験の判定結果は完全に一致した。

C. cynodegmi

7株いずれもrb加HI培地による増菌とチョコ培地による増菌で判定結果のコード番号が一致した（表5）。7株中5株は *C. cynodegmi* と同定され，2株(C-5, C-6)は *C. cynodegmi* あるいは *C. canimorsus* が鑑別候補菌種であると同定された。β-ラクタマーゼ試験で陽性を示した菌株はなかった。

C. canimorsus および *C. cynodegmi* の各菌株について，rb加HI培地とチョコ培地の2種類の培地を用いて増菌培養を行い，HNニッスイ-20ラピッドを用いた生化学的性状検査による同定結果を検討した。どちらの培地を用いた試験でもおおむね良好な結果を得たが，生育が比較的遅い菌株の場合，チョコ培地での48時間培養では増殖がやや不十分であるためか，一部の菌株では正確な同定結果が得られなかった。*C. canimorsus* には元来の性質として糖の分解能の乏しい菌株も存在するため，糖の分解能のテスト項目が陰性化しても同定結果自体には直接影響しない場合が多い。しかしながら，rb加HI培地増菌では *C. canimorsus* と同定されたにもかかわらず，チョコ培地増菌では他属の菌や該当なしと同定された2株は，フォスファターゼが陰性化したケースと，糖の分解能の陽性パターンがイレギュラーに変化したケースであった。また，H-7株のように，同じ *Capnocytophaga* 属菌のうち，ヒトに常在する菌種とイヌ・ネコに常在する菌種の双方が候補菌種となることがあったが，ヒト保有菌種ではカタラーゼ・オキシダーゼともに陰性，イヌ・ネコ保有菌種ではどちらも陽性であるため，これらの追加検査を行うことによって，鑑別が可能である。臨床分離株はイヌ・ネコ分離株と比較して，糖の分解能に乏しい株が認められる傾向があったが，これは本来の宿主および常在部位でないヒトの末梢血中から分離されたことによる影響なのか，ヒトに病原性を有するタイプの菌株がもともともっている性質であるのかは不明である。*C. cynodegmi* の各菌株では2種の増菌培地を用いた試験結果の間に違いが認められなかったが，このことは *C. cynodegmi* が *C. canimorsus* よりも一般的に生育が良好で，チョコ培地上でも各株とも十分に生育したためと思われる。イヌ・ネコの保有する2菌種である *C. canimorsus* と *C. cynodegmi* は遺伝学的にも極めて近縁で，生化学的性状も類似している。しかし，適切な増菌培養を行ったのちにHNニッスイ-20ラピッドによる検査を行えば，両種をかなり高率に同定することが可能であることが示され

表5. *C. cynodegmi* 検査成績

菌株*	5%ウサギ血液加ハートインフュージョン寒天培地					チョコレート寒天培地EX				
	コード	同定菌名	確率	同定菌名	確率	コード	同定菌名	確率	同定菌名	確率
D-8	3017131	*C. cynodegmi*	>99%	—		3017131	*C. cynodegmi*	>99%	—	
D-9	3017131	*C. cynodegmi*	>99%	—		3017131	*C. cynodegmi*	>99%	—	
D-10	3017131	*C. cynodegmi*	>99%	—		3017131	*C. cynodegmi*	>99%	—	
C-5	3017121	*C. canimorsus*	67%	*C. cynodegmi*	33%	3017121	*C. canimorsus*	67%	*C. cynodegmi*	33%
C-6	3017121	*C. canimorsus*	67%	*C. cynodegmi*	33%	3017121	*C. canimorsus*	67%	*C. cynodegmi*	33%
C-7	3017131	*C. cynodegmi*	>99%	—		3017131	*C. cynodegmi*	>99%	—	
A-2	3017131	*C. cynodegmi*	>99%	—		3017131	*C. cynodegmi*	>99%	—	

*: D-8–10: イヌ由来，C-5–7: ネコ由来，A-2: ATCC49044株(Type strain)

た。細菌検査室においては一般的にヒツジ血液寒天培地が用いられることが多いと思われるが，われわれが国内で市販されている数種類のヒツジ血液寒天培地を*Capnocytophaga*属菌の増菌培養に用いたところ，成分として，ハートインフュージョンベースで作られたものでは比較的良好に生育した。しかしながら，その他トリプチケースソイベースのものなどでは，今回用いたチョコ培地と同じかやや良好な生育しか認められず，特に一部の菌株では十分な増菌が得られないものもあった。すなわち，一口にヒツジ血液寒天培地といっても，その成分の違いにより，生育に大きな違いが見られ，使用には注意を必要とすることがわかった。HNニッスイ-20ラピッドを用いた同定では，正確な結果を得るには48時間以内の培養時間でマクファランド3相当の菌液を調整できる菌量を得る必要がある。生育の遅い菌株の場合には今回のチョコ培地と同様，正確な同定結果が得られない場合もあることから，各検査室で使用している培地の特性に留意する必要がある。さらに，HNニッスイ-20ラピッドには，菌種同定用のコード番号として表される19種類のテスト項目のほかに，β-ラクタマーゼ保有の有無についての項目がある。イヌ・ネコ口腔内分離株の計11株のうち，イヌ・ネコ口腔由来の4株はβ-ラクタマーゼ陽性を示したが，ヒト患者由来の12株はいずれもβ-ラクタマーゼ陰性であった。また，別途実施したセンシディスク（日本ベクトン・ディッキンソン）を用いた薬剤感受性試験では，これら4株はペニシリン系の抗菌薬であるペニシリン，アンピシリンおよびアモキシシリンに耐性であった（データ未公表）が，β-ラクタマーゼ阻害剤との合剤であるオーグメンチンには感性であった。このことから，本キットはペニシリン系抗菌薬に対する薬剤感受性の簡易的なチェックにも有用であると考えられる。

イヌ・ネコが高率に保有する*C. canimorsus*がヒトに重篤な感染を起こしうる病原性を，一般的，基本的性質として有するのか，何らかの病原因子などを有する一部の菌株に限ってヒトに重篤な症状を引き起こすのかは現在のところ不明である。現在，生化学的性状を含めた各種性状と病原性との関連性についても解析を進めている。

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ヒストプラズマ症（皮膚，呼吸器，全身）

Histoplasmosis

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病　態

原因菌は高度病原性真菌症原因菌のひとつ*Histoplasma capsulatum*である。かつてはわが国に存在せず，輸入真菌症と考えられていたが，ヒト，馬，牛をはじめ犬，猫ともに国内感染例が報告されていることから，近年，わが国に存在する唯一の高度病原性真菌症であると認識されるようになった（表）。

教科書に書かれている呼吸器症状，各種リンパ節，肝臓，脾臓の腫脹ならびに全身症状は米国において主に報告されている症状で，わが国で発生している主症状とは異なる。日本で確認された犬のヒストプラズマ症は，皮膚潰瘍と粘膜の病巣を特徴とし（**図1**），多発性皮膚結節を生じることもある。一方，悪性腫瘍などの免疫不全が基礎疾患にある場合は重篤となり呼吸器症状，全身症状

表　国内で感染したと推測されるヒストプラスマ症

症例	年齢（歳）	性別	発病地	症状	診断方法	予後	文献
ヒト							
1	17	F	岡山	全身播種	H	死亡	Acta. Med. Okayama, 11: 347-364, 1957.
2	24	M	熊本	呼吸器	H	不明	結核 36: 194, 1961.
3	67[a]	M	新潟	全身播種	H	死亡	結核 59: 256-257, 1984.
4	72	M	群馬	皮膚	C, CL, H	治癒	J. Dermatol. 21: 586-589, 1994.
5	84	M	大阪	全身播種	H, MB	死亡	日本病理学会会誌 93: 387, 2004.
6	78	M	京都	全身播種	C, CL, H, MB	死亡	Tropical Medicine and Health 33: 40, 2005.
7	47	M	岡山	肺	H, MB	経過観察中	日本呼吸器外科学会雑誌 22: 92-96, 2008.
8	59	M	奈良	全身播種	H, MB	経過観察中	感染症学会雑誌 82: 588, 2008.
馬[b]							
1	4	F	栃木	全身播種	H, IA	死亡	Jpn. Vet. Med. Sci. 63:1229-31, 2001.
牛[c]							
4	3カ月	F	岩手	全身播種	H	死亡	Jpn. J. Vet. Sci. 34: 333-339, 1972.
犬							
1	8：雑種	F	東京	粘膜・皮膚	H, IH	治癒	J. Vet. Med. Sci. 60: 863-5, 1998.
2	2.6：MD	M	東京	皮膚	H, MB	治癒	真菌誌 42: 229-35, 2001.
3	2.3：SZ	F	熊本	皮膚	H, MB	緩解・増悪	真菌誌 42: 229-35, 2001.
4	5：柴	F	東京	皮膚	H, MB	起立不能により安楽死	Vet. Microbiol., 94: 219-24, 2003.
5	4：SH	M	東京	皮膚	C, H, MB	緩解その後追跡不可能	J. Vet. Med. A. 52: 472-480, 2005.
6	12：SZ[d]	M	東京	皮膚	C, MB	緩解・増悪	Medical Mycology 43: 233-245, 2007.
7	8：BT	F	千葉	皮膚・全身播種	H, MB	死亡	Medical Mycology 43: 233-245, 2007.
8	13：LR	F	千葉	肺	H, MB	死亡	日本獣医歴史学雑誌. 48: 1-21, 2011.
ラッコ[e]							
1	4.75	F	新潟	全身播種	H, IH	死亡	J. Comp. Pathol. 125: 219-23, 2001.
猫							
1	10：日本	F	不明	消化器	H, IH	死亡	J. Vet. Med. Sci. 71: 1669–1672, 2009.

a：1984年までに岡山，鹿児島，熊本，長崎，久留米，福岡，大宮，新潟，山形など国内発症例 13例（9例は病理組織，4例は臨床診断）が確認されていたと報告されている。b：この他に仮性皮疽として国内および外地の症例として20,000頭以上が記録されていた。c：明治時代に5例が記載されていた（時重獣医学博士論文集：214-216,1918）。d：2005年2月老衰のため死亡。e：症例個体は国内で出生，親は輸入個体で，ヒストプラスマ症により死亡と推定されている。MD：ミニチュア・ダックスフンド，SZ：シー・ズー，SH：シベリアン・ハスキー，BT：ボストン・テリア，LR：ラブラドール・レトリーバー，C：細胞学的診断，CL：培養陽性，H：病理組織学的診断，MB：分子生物学的診断，IH：免疫組学的診断。

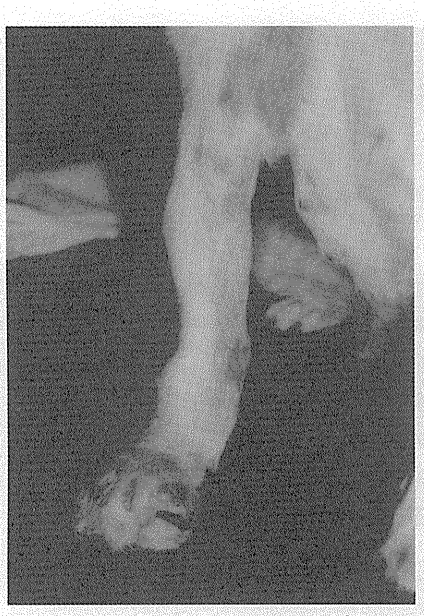

図1　乳癌の転移にともなったヒストプラズマ症の多発性肉芽腫性皮膚潰瘍の1例（ボストンテリア，8歳）

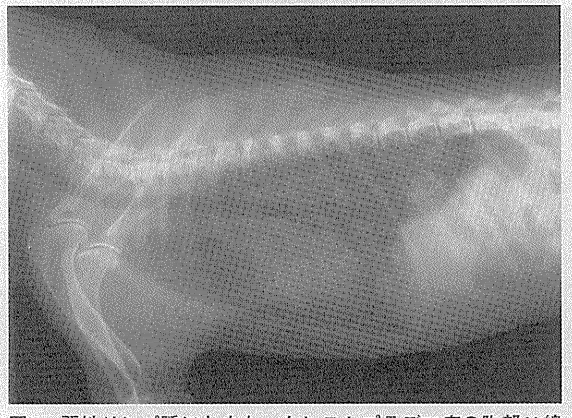

図2　悪性リンパ腫にともなったヒストプラズマ症の胸部X線像（ラブラドール・レトリーバー，13歳）

に至る。猫では急性致死症例が報告されている。

診　断

1. 臨床症状

難治性潰瘍性結節性の皮膚病変で抗菌薬に反応しない症例は本症を疑い，真菌要素の検出を目的とした細胞診，病理組織学的検査に進むべきである。

また本症が疑われる場合，呼吸器症状が出ていなくとも，症状が進行した時の比較に備えて必ず胸部X線撮影を行うべきである（図2）。眼底検査も点状の陰影を認めることがあり，全身播種を判定するために推奨したい。

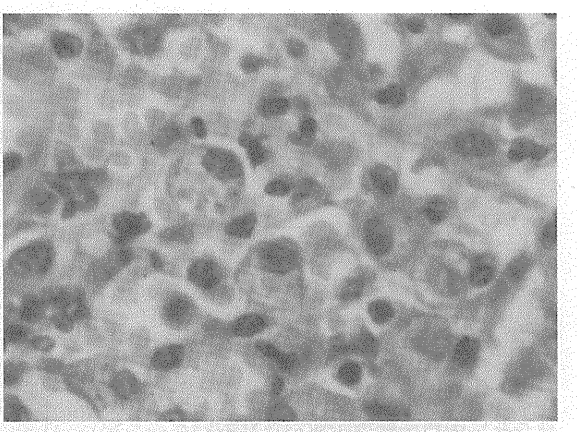

図3　ヘマトキシリン・エオジン染色での細胞内寄生性酵母様細胞の確認

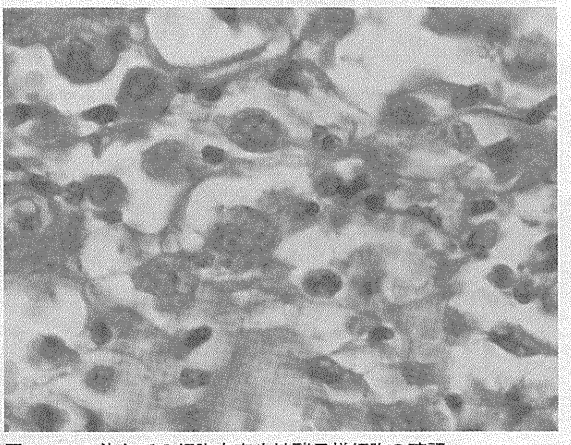

図4　PAS染色での細胞内寄生性酵母様細胞の確認

2. 病理学的検査

病理組織では著しい肉芽腫性炎症反応が特徴的である。これら肉芽腫を形成している組織球は細胞性免疫不全の基礎疾患をともなう場合，取り込んだ酵母細胞を殺すことができず，菌は組織球内で増え続ける。生検組織（骨髄も含む），血液塗抹，肺洗浄液，皮膚病巣浸出液などがその材料となる。

まず，ヘマトキシリン・エオジン染色ではマクロファージに取り込まれた菌体の周囲にハローを形成することが特徴であるが，判定には熟練を要する（図3）。真菌を特異的に染色するPAS染色は菌体の確認には有用である（図4）が，染色性が弱いものもあり，グロコット染色で菌体を確認することを推奨したい（図5）。また，犬の場合，潰瘍部の浸出液の塗抹をギムザ染色により観察すると，マクロファージ内に酵母細胞を確認できることもある（図6）。

また，抗ヒストプラズマ血清を用いた免疫染色も有用

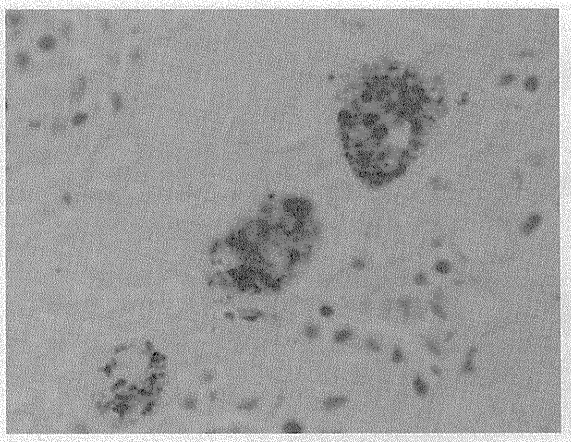

図5　グロコット染色での細胞内寄生性酵母様細胞の確認

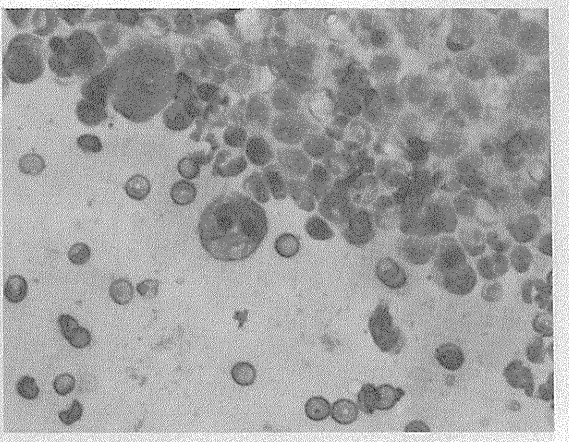

図6　塗抹標本でのマクロファージ内の酵母様菌体

である。

3. 遺伝子診断

パラフィン包埋組織，生検組織，浸出液などからの遺伝子検出による診断が可能である。

方法は菌体や臨床検体から抽出したDNAをnested-PCRにかけ，塩基配列を決定し，BLASTサーチ（http://blast.ncbi.nlm.nih.gov/Blast.cgi）を行い，そこから得られた相同性と簡易系統樹を用いる方法である。相同性は原因菌*Histoplasam capsulatum*の有性型*Ajellomyces capsulatus*（この菌名でデータベースは登録されている）と95％以上で本症と推定できる。PCRは，はじめITS-5（5'-GGA AGT AAA AGT CGT AAC AAG G-3'）とITS-4（5'-TCC TCC GCT TAT TGA TAT GC-3'）によりファーストPCRを行い，このPCR産物を10～100倍に希釈して，HcAF（5'-CAC GCC GTG GGG GGC TGG GAG CCT-3'）とHcCR（5'-ATG GTG GGC RGG AGC CGG CC-3'）のプライマーで増幅させ，バンドを検出する。PCR条件はいずれも95℃：4分ののち，94℃：1分―50℃：1分半―72℃：2分を40回，最後に72℃：10分である。遺伝子配列決定はこのPCR産物を外注することで解決できる。ただし，バンド検出だけでは確定診断とならない。必ず300塩基以上の配列を決定することである。

4. 培養検査

本菌種は高度病原性真菌であるから，実験室内感染には十分注意しなければならないため，必要な場合は専門機関に相談し，現在，培養は行わない。なお現在までに小動物臨床領域では，培養を試みて陽性となった症例はない。

5. 血清学的診断

血清中や尿から抗原を検出する方法と，抗体を検出する方法がある。ラテックス凝集法，免疫拡散法，補体結合法などのキットが海外で販売されているが，日本での入手は数週間以上を要する。

さらにほとんどの犬の症例は血清学的診断法で陰性を示すことが知られているばかりでなく，海外でも犬の症例での血清学的診断法は陰性を示すことがあると報告されているので，血清学的診断は小動物臨床領域では有用とはいい難い。

治　療

抗真菌薬の長期投与が必要である。犬ではイトラコナゾールを5 mg/kg，1日1回で2週間投与して症状が軽減した症例もある。通常3カ月以上，可能ならば6カ月以上の連続投与が望ましいと考えている。

しかしながら，副作用として皮膚潰瘍の悪化が認められることもあり，ヒストプラズマ症そのものの悪化か副作用かを見極めるのが難しい。なお，現在多くの真菌症治療で使われているイトラコナゾールを1週間連続投与し，その後は週末だけ投与する方法による治療例はいまのところ報告されていない。また，アムホテリシンBを0.5 mg/kgで2週間，静脈内投与が試された例もあるが，乳癌の基礎疾患があったため，効果は得られなかった。外科的切除も有効である場合もあるが，基礎疾患がある場合には無効であり，完治した例も再手術を施している。

一方，局所療法として注射用のケトコナゾール（20 mg/g）とアムホテリシンB（1 mg/g）をワセリンに練り込み，患部に塗布する方法が試された。この方法は高齢により生涯を閉じるまで続けられたが，患部の増悪

により全身症状に至ることはなかった。

ヒストプラズマ症の治療

処　方　例

―①を通常3カ月，可能ならば6カ月以上，投与する。②は病巣が繰り返し発現するようであれば，適宜使用する。

①イトラコナゾール（イトリゾール）
5 mg/kg，経口，1日1回［犬／猫］

②ケトコナゾール（ニゾラール）とアムホテリシンB（ファンギゾン注）をワセリンなどの基剤に練り込み，患部に適宜塗布する。なお，ケトコナゾールを含有するニゾラールクリームにアムホテリシンBを練り込んでも使用できると考えている。
ケトコナゾール（ニゾラール）20 mg/g
アムホテリシンB（ファンギゾン注射用）1 mg/g

予　防

原因菌は土壌に生息すると考えられ，生活環境そのものが感染源であるため，疾患そのものの予防方法はない。とくに戦前に馬を飼育していた土地では散歩，アジリティー競技，屋外飼育などに注意を要する。

またヒストプラズマ症は接触感染が否定できないため，発症した動物からの飼育関係者，他の動物への感染防止が重要である。

現在までに愛玩動物からヒストプラズマ症に感染した症例は報告されていないが，とくに，家族に免疫低下をともなう疾患の方が同居している場合は，感染動物の入院施設を備えた動物病院に症例の管理をゆだねることを推奨する。

また，経口感染も海外では確認されていることから，多頭飼育の場合，隔離飼育が望ましい。

したがって，動物が使用していたタオル，寝具などの滅菌（オートクレーブをかける，消毒液に漬け込むなど），生活環境の消毒などは飼育家族に任せるのではなく，感染症予防の専門家（医師・獣医師）の指導のもとに行われることが望ましい。

予　後

現在までに完治した犬の症例は9例中2例であることから，必ずしも治癒できる保証はない。また長期入院はもちろん，安全な飼育管理が可能な場合でも愛玩動物では治療費の問題が深刻となることが想定される。通院治療し，各種検査と薬剤投与だけを受けた場合，体重5 kg程度の犬で数十万円以上と推定される。飼育者を経済的に圧迫することになるため，海外では安楽死も選択肢のひとつである。わが国でも管理と経済の両面からこの決断に至った症例が報告されている。

エキノコックス症

Echinococcosis

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環境動物フォーラム／OIE（国際獣疫事務局）エキノコックス症リファレンスラボラトリー

エキノコックスは寄生虫（条虫：サナダムシ）の仲間でヒトへ感染すると重篤な疾病を引き起こす。エキノコックスには単包条虫と多包条虫があり，日本でとくに問題となっているのは多包条虫である。ヒトのエキノコックス症は1999年4月施行の「感染症の予防及び感染症の患者に対する医療に関する法律」で四類感染症に分類され，医師が診断した場合，厚労省令で定める事項（患者の年齢，性別など）を最寄りの保健所長を経由して都道府県知事に届け出なければならない疾病となった。さらに2004年10月には「感染犬の届出」が獣医師に義務づけられ，翌年1月「第1号の届出」があった。このようにわが国では，世界に先駆けて法律の裏付けのもと「エキノコックス症感染源対策」が整備されつつある。一方，流行地において主要な感染源となっている。キツネ対策（駆虫薬による）についても地域の住民，獣医師の参加によって汚染環境の修復が進んでいる（参照：本誌36号（2005年4月）特集「ズーノシス」）。

病　態

多包条虫は本来キツネと野ネズミの間で伝播する。**図1**に成虫から次の世代の成虫になるまでの過程（生活環）を示す。生活環は，1）虫卵（終宿主の糞便とともに

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Coprological survey of alimentary tract parasites in dogs from Zambia and evaluation of a coproantigen assay for canine echinococcosis

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Faecal samples were collected from the rectum of 540 domestic dogs from four districts (Lusaka, Katete, Petauke and Luangwa) in Zambia between 2005 and 2006 and prevalences of canine alimentary tract parasites were determined by coprological examination. Thirteen different ova and parasites including strongyle (43.3%), *Spirocerca lupi* (18.7%), taeniid (13.1%), *Toxocara canis* (7.6%), *Sarcocystis* sp.* (7.5%), *Isospora* sp.* (5.7%), *Physaloptera* sp.* (4.6%), *Capillaria* sp.* (2.8%), *Dipylidium caninum* (2.2%), *Mesocestoides* sp.* (2.0%), *Ascaris* sp.* (1.7%), *Trichuris vulpis** (0.4%) and *Schistosoma mansoni** (0.4%) were detected, *Ascaris* and *Schistosoma* probably originating from coprophagy. The species with asterisks and later-described *Taenia multiceps* are for the first time reported from dogs in Zambia. A coproantigen enzyme-linked immunosorbent assay (CoproAg-ELISA) developed for *Echinococcus* spp. revealed 43 positive dogs and 37 of these harboured taeniid eggs. From 63 of the 71 taeniid egg-positive samples, eggs and DNA thereof were isolated and subjected to a multiplex polymerase chain reaction for differentiating *E. granulosus sensu lato*, *E. multilocularis* and *Taenia* spp. Amplicons indicative for *Taenia* spp. were obtained from 60 samples. Sequencing of amplicons spanning part of the mitochondrial cytochrome *c* oxidase subunit 1 gene, which was possible with 38 samples, revealed 35 infections with *T. hydatigena* and 3 with *T. multiceps*. Therefore, the CoproAg-ELISA showed some positives, but concrete evidence for the existence of canine *E. granulosus* infection could not be established. Comparison of the results of the CoproAg-ELISA and *Taenia* species identification indicated that the CoproAg-ELISA cross-reacts with patent infections of *T. hydatigena* (57%) and *T. multiceps* (33%).

INTRODUCTION

It is very common in most African countries to see free-ranging dogs under poor hygiene condition and in close contact with people, especially in rural settings. Although the potential role of dogs as source of zoonotic parasites to humans has been recognized as a significant public health problem, investigation on the prevalence of such parasite infections has hardly been conducted in most African countries. The conditions combined with poor veterinary services and a lack of awareness of zoonotic diseases exacerbate the risks of disease transmission from dogs to humans.

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Echinococcus spp. belonging to such canine zoonotic parasites can cause serious diseases in humans. Therefore, the precise diagnosis of definitive hosts that are shedding infective eggs is of primary importance for evaluating the endemicity and the risk posed to humans in a region, and for assessing control programs. For detecting *Echinococcus* adult infections, we developed a monoclonal antibody-based coproantigen detection assay (Morishima *et al.*, 1999), which in turn has been used for epidemiological studies in foxes, dogs and cats mainly in Hokkaido, Japan (Tsukada *et al.*, 2000; Nonaka *et al.*, 2009a). The assay showed high specificity against antigens of common canine parasites including *Toxocara canis*, *Dipylidium caninum*, *Spirometra erinaceieuropaei* and some *Taenia* species (Sakashita *et al.*, 1995); however, cross-reactivity of the assay was recognized against patent *T. hydatigena* infections (Malgor *et al.*, 1997). Nevertheless, there have been few practical problem in using this assay in Hokkaido because the prevalences of most *Taenia* species are very low there. Only *T. taeniaeformis* is commonly found in cats, but the assay showed no cross-reactivity with this parasite.

When considering using the assay in other regions of the world, its specificity should be further evaluated with samples from regions where common canine parasites, including *Echinococcus* and *Taenia* species, are prevalent. In Zambia, it has been estimated that *E. granulosus* G1 strain (sheep strain), which has a broad range of intermediate hosts including sheep, cattle, goats and pigs, is prevalent (Macpherson and Wachira, 1997); thus, the parasite might be present in most of the typical villages in Zambia where animal husbandry of cattle, goats, and pigs is common. Indeed, it has been reported that metacestodes of *E. granulosus* were routinely found in lungs and livers of cattle at abattoirs in Lusaka (Pandey, 1987; Pandey and Sharma, 1987). Unfortunately, no information is available on the prevalence of canine parasites from that region except for one report in which a parasitic helminth necropsy survey was conducted with 85 dogs in Lusaka, revealing the existence of various parasites such as *D. caninum* (25% in prevalence), *T. hydatigena* (18%), *Toxocara canis* (14%), *Ancylostoma caninum* (8%), *Toxascaris leonina* (7%), *Ancylostoma braziliensis* (2%), *Spirocerca lupi* (2%) and *E. granulosus* (1%) (Islam and Chizyuka, 1983).

In this survey, at first, in order to clarify the prevalence of alimentary tract parasites in dogs in Zambia, fresh faecal samples from dogs raised in rural villages and in Lusaka were examined for faecal helminth eggs and coccidian oocysts/sporocysts. Samples containing taeniid eggs were further characterized for identifying the species of taeniid cestodes by multiplex polymerase chain reaction (PCR) and nucleotide sequence analysis of part of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene. To evaluate its specificity, the developed coproantigen detection assay for canine echinococcosis was performed on all faecal samples.

SUBJECTS AND METHODS

Collection of Faecal Samples and Questionnaire

Since we obtained local information (unpublished) that *E. granulosus* cysts were found in cattle from the Tete Province in Mozambique, we selected for this study two provinces of Zambia that are located next to this province. Fresh faecal samples were collected per rectum from 540 dogs raised in rural villages in Katete and Petauke (eastern province) and Luangwa and Lusaka (Lusaka province) between 2005 and 2006 (Fig.). In order to facilitate the capture and holding of these free-ranging dogs, sample collections were announced to village residents 1 day before. The purpose of the survey was explained and a simple questionnaire was administered to dog owners at the day of sample collection. The questionnaire included the dog's age and sex.

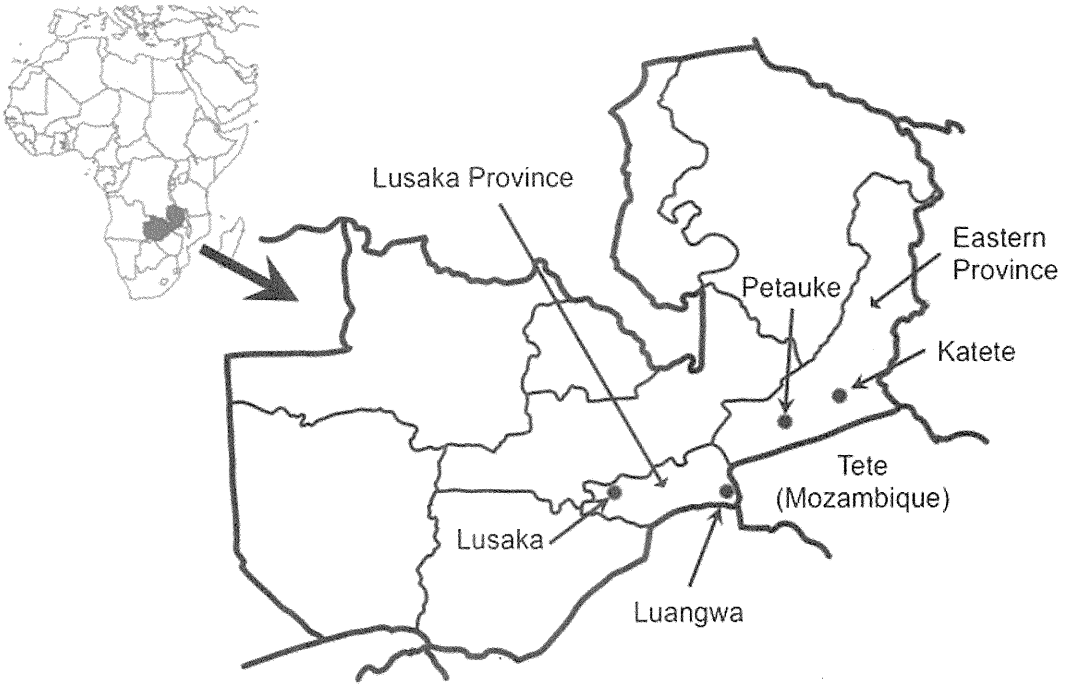

FIG. Study area in Zambia.

Examination for Faecal Helminth Eggs and Coccidian Oocysts/Sporocysts, Coproantigen ELISA

Coproscopic examination (ova-examination) and a coproantigen sandwich enzyme-linked immunosorbent assay (CoproAg-ELISA) were performed as described by Morishima *et al.* (1999). In order to kill *Echinococcus* eggs, all faecal samples were stored at −80°C for more than 7 days before examination. Briefly, 0.5 g of faecal samples were weighed and put into plastic tubes. Then 1% formalin containing 0.3% Tween 20 were added to make a total volume of 15 ml. After a centrifugation step (1000*g* for 10 minutes), the centrifugal sucrose (specific gravity =1.27) flotation method (Ito, 1980) was applied to the resultant sediments for ova-examination. The supernatants were used for CoproAg-ELISA developed for detecting *Echinococcus* coproantigen using the monoclonal antibody EmA9. Two cutoff values were used in CoproAg-ELISA to discriminate between negative and suspicious samples [mean (μ)+3 standard deviations (SD) of negative controls; OD=0.206] and between suspicious and positive samples (μ+5SD; OD=0.289). The negative controls used were faecal samples from 605 companion dogs raised and kept only on the main island of Japan that is free of *Echinococcus* infections.

Preparation of Egg DNA

From all samples in which taeniid eggs were detected, 1–10 eggs per sample were collected manually from the cover glass used for ova-examination under a stereomicroscope. Egg DNA was then extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction.

Multiplex PCR for Discriminating *E. multilocularis*, *E. granulosus sensu lato* and *Taenia* species

The multiplex PCR for discriminating taeniid eggs was done as described by Trachsel *et al.* (2007) using egg DNA as template.

This method can distinguish *E. multilocularis, E. granulosus* s.l. and *Taenia* species. The amplified products [395 base pairs (bp) for *E. multilocularis*, 117 bp for *E. granulosus s.l.* and 267 bp for *Taenia* spp.] were examined by agarose gel electrophoresis. Positive controls of DNA extracted from metacestodes of *E. granulosus* genotype G1, *E. multilocularis* and *T. hydatigena* using QIAamp DNA Mini Kit (Qiagen) and a negative control without DNA were included in all the tests.

Nucleotide Sequencing

Egg DNA identified as *Taenia* sp. by the multiplex PCR was subjected to another PCR and sequence analysis. Part (491 bp) of the mitochondrial cytochrome CO1 gene was amplified using the primers PR-A (5′-TGG TTT TTT GTG CAT CCT GAG GTT TA-3′) and PR-B (5′-AGA AAG AAC GTA ATG AAA ATG AGC AAC-3′) according to Okamoto *et al.* (1995). Nucleotide sequences of the products were determined by a Beckman CEQ 8000 DNA analyser using a GenomeLab DTCS Quick Start kit (Beckman Coulter, Fullerton, CA, USA) following the manufacturer's instruction. The sequences obtained were subjected to BLAST sequence similarity search (National Center for Biotechnology Information, Bethesda, MD, USA) to identify the species of *Taenia*.

Statistical Analysis

Ninety-five per cent confidence intervals of prevalence were calculated on the basis of binomial distributions using the software program R (version 2.8.1, R Development Core Team, 2008). For the ova and parasites showing more than 5% in overall prevalence, logistic regression analysis was performed to evaluate the effect of site (district), sex and age of dogs on the prevalence using the software program JMP 8 (SAS Institute, Tokyo, Japan). Since sex was not recorded from the dogs from Lusaka, the data of these dogs were excluded in the above analysis. Dogs were grouped as younger or older than 6 months ($\leqslant$6 months old versus $>$6 months old) for comparing *T. canis* prevalences, and younger or older than one year ($\leqslant$1 year old versus $>$1 year old) for the other parasites. If the effects of interaction between sites and other factors were significant ($P<0.05$), the same analysis was further performed on the effect of sexes and ages at each site separately. For dogs from Lusaka, the difference in prevalence between age groups was analysed by Fisher's exact test using the software program R.

RESULTS

Faecal Examination for Helminth Eggs and Coccidian Oocysts/Sporocysts

From 540 canine faecal samples examined, 13 kinds of helminth eggs and coccidian oocysts/sporocysts were detected (Table 1). Among all the ova and parasites found, strongyle eggs were most abundant and were found in 43.3% of the dogs. The prevalence was, however, significantly lower in Luangwa district (17.0%) as compared to the other districts. Taeniid eggs were discovered in 71 (13.1%) dogs in total, and a significantly higher prevalence (33.9%) was observed in Luangwa district where goat husbandry is more prominent than in other districts. The number of taeniid eggs detected ranged from 1 to more than 1000 with a median of 20. Less than 10 eggs were found in 24 dogs. Eggs of the other potential zoonotic parasites such as *T. canis*, *D. caninum* and *Schistosoma mansoni* were also found in less than 10% of the dogs. The prevalence of *T. canis* was significantly higher in Lusaka (25.8%) than in the other districts. Interestingly, *Ascaris* sp. eggs were found in nine dogs (four in Katete and five in Lusaka), thus in 1.7% of the samples.

In the logistic regression analysis (n=339), significant differences in prevalences among districts were obtained for taeniids, *Spirocerca lupi* and strongyle eggs.

TABLE 1. *Prevalences based on detecting helminth eggs and coccidian oocysts/sporocysts in rectal faeces of free-ranging dogs from four districts in Zambia*

Parasite	Prevalence (%) (95% confidence interval)				
	Katete (n=224)	Petauke (n=89)	Luangwa (n=165)	Lusaka (n=62)	Total (n=540)
Strongyle*	60.7 (54.2–66.9)	48.3 (38.2–58.5)	17.0 (12.0–23.4)	43.5 (31.9–55.9)	43.3 (39.2–47.5)
Spirocerca lupi	7.1 (4.4–11.3)	41.6 (31.9–52.0)	26.1 (20.0–33.2)	8.1 (3.5–17.5)	18.7 (15.6–22.2)
Taeniid†	4.5 (2.2–8.1)	4.5 (1.2–11.1)	33.9 (27.2–41.5)	1.6 (0.0–8.7)	13.1 (10.6–16.3)
Toxocara canis	9.8 (6.6–14.4)	2.2 (0.6–7.8)	0.6 (0.0–3.3)	25.8 (16.6–37.9)	7.6 (5.6–10.1)
Sarcocystis sp.‡	ND‡	9.0 (4.6–16.7)	6.7 (3.8–11.5)	ND‡	7.5 (4.8–11.4)
Isospora sp.	6.3 (3.8–10.2)	10.1 (5.4–18.1)	4.2 (2.1–8.5)	1.6 (0.0–8.7)	5.7 (4.1–8.0)
Physaloptera sp.	4.0 (2.1–7.5)	4.5 (1.2–11.0)	7.3 (4.2–12.3)	0 (0.0–4.7)	4.6 (3.2–6.7)
Capillaria sp.	2.2 (1.0–5.1)	11.2 (6.2–19.5)	0 (0.0–1.8)	0 (0.0–4.7)	2.8 (1.7–4.5)
Dipylidium caninum	2.2 (1.0–5.1)	4.5 (1.2–11.0)	1.2 (0.1–4.3)	1.6 (0.0–8.7)	2.2 (1.3–3.8)
Mesocestoides sp.	2.7 (1.2–5.7)	4.5 (1.2–11.0)	0.6 (0.0–3.3)	0 (0.0–4.7)	2.0 (1.1–3.6)
Ascaris sp.	1.8 (0.5–4.5)	0 (0.0–3.3)	0 (0.0–1.8)	8.1 (3.5–17.5)	1.7 (0.9–3.1)
Trichuris vulpis	0 (0.0–1.3)	1.1 (0.0–6.1)	0 (0.0–1.8)	1.6 (0.0–8.7)	0.4 (0.0–1.3)
Schistosoma mansoni	0.4 (0.0–2.5)	0 (0.0–3.3)	0.6 (0.0–1.8)	0 (0.0–4.7)	0.4 (0.0–1.3)

*Strongyle possibly includes the genera *Ancylostoma*, *Uncinaria* and others whose eggs cannot be distinguished morphologically.
†Taeniid possibly includes the genera *Taenia* and *Echinococcus* whose eggs cannot be distinguished morphologically.
‡The samples of Katete and Lusaka were not carefully examined for the small sporocysts of *Sarcocystis* sp.

Differences in prevalences between age groups were significant for *S. lupi* in Luangwa, and *Sarcocystis* sp. in Petauke. However, in Lusaka (n=51), no parasites showed significant differences between age groups. Moreover, differences in prevalences between sex groups were not significant for any parasite.

Among the 540 samples, 160 (29.6%) samples showed neither parasite, a single parasite kind was diagnosed in 244 (45.2%) samples and multiple different ova and parasites were found in 136 (25.2%) samples (two kinds in 100, three kinds in 25, four kinds in 10 and five kinds in 1).

Multiplex PCR and Nucleotide Sequencing

From 63 of the 71 samples containing taeniid eggs, DNA was isolated from the eggs. PCR products were obtained from 60 samples in the multiplex PCR, and all were of the size specific for *Taenia* spp. There were 18 samples with less than 10 eggs detected, and three of them showed no products in the multiplex PCR.

PCR targeting part of the mitochondrial CO1 gene was successful with 38 samples out of the available 63 DNA samples. Sequencing and BLAST sequence similarity searches showed that 35 sequences had 98.4–100% identity with those of *T. hydatigena* registered in GenBank (accession no. DQ995656, AM503318 or EU544552). The sequences of three samples revealed *T. multiceps* with identities of 94.4–100% (GenBank accession no. EF393620). For the remaining 25 samples, no amplicons (n=11) were obtained in the PCR with primers PR-A and PR-B or no clear sequences (n=14) were obtained by direct sequencing. *T. hydatigena* was found in three districts (Katete, Petauke and Luangwa), whereas *T. multiceps* was identified only in the Luangwa district.

CoproAg-ELISA

The CoproAg-ELISA for *Echinococcus* spp. was positive in 43 dogs and suspicious in 6 dogs, while 37 and 5 of those had taeniid eggs, respectively, after coproscopic examination

(Table 2). The multiplex PCR performed on taeniid egg DNA of the 32 CoproAg-ELISA-positive and the 4 CoproAg-ELISA-suspicious samples, however, showed that the eggs of all samples were those of *Taenia* spp.

Among the 38 samples identified as harbouring taeniid species by nucleotide sequence analysis, 20 of 35 samples (57%) identified as *T. hydatigena* and 1 of 3 samples (33%) as *T. multiceps* showed positive reactions in the CoproAg-ELISA.

DISCUSSION

The coproscopic examination revealed that the dogs in the study area of Zambia were infected with a variety of zoonotic parasites. In the previous study conducted in Lusaka, Islam and Chizyuka (1983) found nine helminth species, of which all but two (*T. leonina* and *E. granulosus*) also were identified in the present study. In addition, *Sarcocystis* sp., *Isospora* sp., *Physaloptera* sp., *Capillaria* sp., *Mesocestoides* sp., *T. vulpis*, *S. mansoni*, *Ascaris* sp. and *T. multiceps* were observed, which are thus the first reports from dogs of Zambia.

The highest prevalence was observed for strongyles (43.3%). From the study of Islam and Chizyuka (1983), it can be speculated that *A. caninum* and *A. braziliense* are the dominant species among strongyles. Since *A. braziliense* is more frequently involved in cutaneous larva migrans than *A. caninum* (Bowman, 2009), species identification of *Ancylostoma* by molecular methods should be considered in future studies for public health risk assessment. In this study, we detected lower prevalence of strongyles in the Luangwa district than in other districts. Luangwa is a valley with a very hot and humid climate throughout the year. Therefore, taking into account that climatic conditions of Luangwa are suitable for parasite transmission, results obtained in this study need further clarification.

TABLE 2. *Comparison of the results of coproantigen ELISA and faecal examination for taeniid eggs*

Results of coproantigen examination	Results of faecal examination for taeniid eggs		
	Positive	Negative	Total
Positive	37	6	43
Suspicious	5	1	6
Negative	29	462	491
Total	71	469	540

For infections with *T. canis*, an age resistance limit of 6 months has been indicated (Webster, 1956) and therefore, prevalences for this parasite of age groups younger and older than 6 months were compared. However, no significant difference was observed in this study which is in agreement with recent findings (Fahrion *et al.*, 2008) demonstrating that a low infective dose of 100 embryonated eggs consistently induced patency in adult dogs with and without previous exposure to the parasite. Since an overall prevalence of 5.1% (19/371) was observed in dogs older than 6 months, infection with *T. canis* in older dogs may constantly be occurring in Zambia. It is noteworthy that a higher prevalence of *T. canis* was observed in Lusaka, but the reason for this observation was not further elucidated in this study. Nevertheless, *T. canis* is one of the most important zoonotic parasites, and thus, the high prevalence in Lusaka would be of significance in public health because this district is the most populated city in Zambia and holds many high-density residential areas.

Eggs of *S. mansoni* were found in two dogs, but without intact miracidium inside the eggs. In general, trematode eggs are not isolated by the flotation techniques. It is known that dogs are not an appropriate host for *S. mansoni*, and combined with the absence of an intact miracidium in our results, it is most probable that the observation could be a result of coprophagy of human faeces by the dogs. Coprophagy of either human or pig faeces by dogs is also suspected by the finding of *Ascaris* sp. eggs

in this study. A similar observation was reported from India (Traub *et al.*, 2005). Recently, infection of *A. lumbricoides* in dogs was reported in an endemic region for this human parasite (Shalaby *et al.*, 2010), suggesting that active infection with *A. lumbricoides* may occur in dogs in the study area. The finding of *S. mansoni* and *Ascaris* sp. eggs in dog faeces could also indicate their endemicity in the region.

It should be also noted that we used frozen samples for the faecal egg examination because of the biohazard concern of *Echinococcus* eggs. As it was observed that freezing of faeces reduced significantly the egg count of ovine gastro-intestinal strongyles in flotation techniques (van Wyk and van Wyk, 2002; Rinaldi *et al.*, 2011), the result obtained in this study may be an underestimation.

Taeniid eggs were found in 71 samples. Canine taeniid species include both zoonotic and non-zoonotic species. Since taeniid eggs cannot be distinguished by their morphology, we used immunological and molecular techniques that were recently developed for distinguishing the genus and species of taeniid cestodes, and the result was used for evaluating potential cross-reactivity of the CoproAg-ELISA.

Various coproantigen detection methods for adult *Echinococcus* spp. infection have been developed (Deplazes and Eckert, 1996; Benito and Carmena, 2005; Benito *et al.*, 2006; Huang *et al.*, 2007). The CoproAg-ELISA used was developed to detect adult infections of *E. multilocularis* (Sakashita *et al.*, 1995) and *E. granulosus* (Malgor *et al.*, 1997). The sensitivity and specificity of the test had been evaluated for *E. multilocularis* infection using fox samples in Hokkaido, measuring 92.2% in sensitivity and 96.6% in specificity (Morishima *et al.*, 1999; Yimam *et al.*, 2002). Cross-reactivity with patent *T. hydatigena* (Malgor *et al.*, 1997) and *T. pisiformis* infections (unpublished) has been observed, but not with *T. taeniaeformis* or *T. crassiceps* (Sakashita *et al.*, 1995). In this study, positive reactions in the CoproAg-ELISA were observed in 43 samples, of which taeniid eggs were detected in 37 samples. The multiplex PCR revealed that all of the egg DNAs extracted were those of *Taenia* spp. These results indicate that the prevalence of *E. granulosus* in the study area was low or negligible. On the other hand, among 35 samples containing *T. hydatigena* eggs, 20 (57%) showed positive reactions in the CoproAg-ELISA. In addition, one of three samples containing *T. multiceps* eggs was positive. These results suggest a cross-reactivity of the test in patent infections with *T. hydatigena* and *T. multiceps*. Higher specificities for the detection of *E. granulosus* coproantigen were reported for a test system using polyclonal antibodies (Deplazes *et al.*, 1992; Deplazes *et al.*, 1994).

The multiplex PCR on egg DNA yielded products in 60 out of 63 samples. It is known that PCR on copro-DNA is often unsuccessful because of the presence of a variety of PCR inhibitors in faeces (Monteiro *et al.*, 1997). In this study, individual eggs were picked up under a stereomicroscope, and this method for isolating taeniid eggs effectively excludes PCR inhibitors.

Sequence analysis of the partial CO1 gene revealed the occurrence of *T. hydatigena* and *T. multiceps*. Especially, in Luangwa where a significantly higher prevalence of taeniid eggs was observed than in other districts, both *Taenia* species were found with *T. hydatigena* being more prevalent. The observed higher prevalence of *Taenia* spp. in Luangwa could be related to the difference in the local animal husbandry system. In typical local villages in Zambia, it is common to raise cattle, goats and pigs. However, in Luangwa, goats, which can serve as an intermediate host of both *T. hydatigena* and *T. multiceps*, are the main animal in the local husbandry system, and self-consuming of goats is more popular. In contrast, pigs are the main animal in Katete and Petauke, whereas goat husbandry is less popular there. Although pigs can also serve

as an intermediate host of *T. hydatigena*, self-consuming of pigs, or even goats and cattle are limited in those districts. In Lusaka, the capital urban city, animal husbandry is not popular.

We tried to detect *E. granulosus* infection in dogs in the study area in Zambia using recently developed diagnostic techniques for *Echinococcus* spp.; however, concrete evidence of the infection could not be obtained. Nevertheless, the study revealed the potential cross-reactivity of the CoproAg-ELISA with patent *T. hydatigena* and *T. multiceps* infections. The previous experimental infection study showed that the cross-reactivity of the CoproAg-ELISA with *T. hydatigena* was only observed in its patency period (Malgor *et al.*, 1997). Therefore, the relationship of the cross-reactivity with *T. multiceps* and the patency should also be clarified. Moreover, the evaluation of the antigens playing a role in this cross-reactivity is also of future interest.

Since the CoproAg-ELISA showed cross-reactivity with patent infection with *Taenia* spp., similar surveys in future should be conducted in combination with molecular techniques that enable genus and species identification of taeniid eggs. A technique using PCR-based restriction fragment length polymorphism has been developed for this purpose (Trachsel *et al.*, 2007). With the PCR-based restriction fragment length polymorphism, mixed infection with multiple species of *Taenia* can be identified simultaneously.

For conducting a survey for gastrointestinal parasite infection, faeces would provide valuable information about the animal itself and the aetiological agents that it harbours (Nonaka *et al.*, 2009b). Moreover, a survey upon faeces is a non-invasive method, thus causing minimal disturbance to the animal condition, ecology and life. Classical approaches such as conducting faecal egg examination alone did not provide adequate information for parasite identification especially for the parasites producing morphologically similar eggs. However, as shown in this and in earlier studies (Bruzinskaite *et al.*, 2009; Davidson *et al.*, 2009; Ziadinov *et al.*, 2008), faecal egg examination in combination with the recently developed molecular techniques would provide results with more accuracy, and being a useful tool in surveys of zoonotic parasite infections in dogs.

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Identification of genetic loci affecting the establishment and development of *Echinococcus multilocularis* larvae in mice

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ABSTRACT

Alveolar echinococcosis (AE) is a severe hepatic disorder caused by larval infection by the fox tapeworm *Echinococcus multilocularis*. The course of parasitic development and host reactions are known to vary significantly among host species, and even among different inbred strains of mice. As reported previously, after oral administration of parasite eggs, DBA/2 (D2) mice showed a higher rate of cyst establishment and more advanced protoscolex development in the liver than C57BL/6 (B6) mice. These findings strongly suggest that the outcome of AE is affected by host genetic factor(s). In the present study, the genetic basis of such strain-specific differences in susceptibility/resistance to AE in murine models was studied by whole-genome scanning for quantitative trait loci (QTLs) using a backcross of $(B6 \times D2)F_1$ and D2 mice with varying susceptibility to *E. multilocularis* infection. For cyst establishment, genome linkage analysis identified one suggestive and one significant QTL on chromosomes (Chrs.) 9 and 6, respectively, whereas for protoscolex development, two suggestive and one highly significant QTLs were detected on Chrs. 6, 17 and 1, respectively. Our QTL analyses using murine AE models revealed that multiple genetic factors regulated host susceptibility/resistance to *E. multilocularis* infection. Moreover, our findings show that establishment of the parasite cysts in the liver is affected by QTLs that are distinct from those associated with the subsequent protoscolex development of the parasite, indicating that different host factors are involved in the host–parasite interplay at each developmental stage of the larval parasite. Further identification of responsible genes located on the identified QTLs could lead to the development of effective disease prevention and control strategies, including an intensive screening and clinical follow-up of genetically high-risk groups for AE infection.

1. Introduction

Alveolar echinococcosis (AE) is a severe hepatic disorder caused by the metacestode stage of the tapeworm *Echinococcus multilocularis*. This zoonotic disease is endemic in many regions of the northern hemisphere and is one of the most significant life-threatening helminth diseases (Craig et al., 1996). The intermediate hosts, mainly rodents and occasionally humans, become infected by oral uptake of the eggs excreted in the feces of final host carnivores. The larval oncospheres hatch from eggs in the intestine, most probably penetrate the intestinal wall and disseminate primarily into the liver. The larvae establish in the liver and develop to harbor mature protoscoleces.

Many researchers have used inbred mice as experimental models of AE to study the host–parasite interplay. However, the course of parasitic development and host reactions are known to vary significantly among mouse strains (Yamashita et al., 1958; Webster and Cameron, 1961; Gottstein and Felleisen, 1995; Bauder et al., 1999; Hildreth and Granholm, 2003; Matsumoto et al., 2010). Previous study showed that the two inbred strains, DBA/2 (D2) and C57BL/6 (B6), differ markedly in their susceptibility to *E. multilocularis* infection (Matsumoto et al., 2010). When infective eggs were administered orally, D2 mice had a higher number of cysts established in the liver than did B6 mice, and moreover, mature protoscoleces were observed only in D2 mice at 16 weeks p.i. Although this observation supported the belief that the susceptibility to *E.*

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multilocularis infection is genetically controlled, as has been previously suggested for animals and humans (Nakaya et al., 1997; Hildreth and Granholm, 2003; Vuitton, 2003), genetic factors responsible for the differences in susceptibility remain unknown.

Quantitative trait locus (QTL) mapping is a promising tool for the detection of genetic loci that contribute to differences in phenotypic variation. In this study, the traits and genotypes of offspring derived from two different strains of animals were investigated on a genome-wide scale to determine statistical linkages and to identify loci which contained genes affecting the traits. To date, the genetic background of susceptibility to nematode infection has been investigated using this approach in animals (Iraqi et al., 2003; Suzuki et al., 2006; Beraldi et al., 2007) as well as in humans (Williams-Blangero et al., 2008).

In this study, QTL analysis was conducted on offspring derived from D2 and B6 mice with varying susceptibility to *E. multilocularis* infection. Genetic linkage analysis identified one significant and one highly significant QTL for the establishment and development of *E. multilocularis* larvae in mice. Furthermore, our data show that establishment and development of parasites are controlled by distinct and multiple genetic factors.

2. Materials and methods

2.1. Mice

D2, B6 and (B6 × D2)F_1 (F_1) mice were purchased from Japan SLC (Shizuoka, Japan). F_1 × D2 backcross (N_2) mice were generated to conduct the genetic and phenotypic assessment experiments. All animal experiments were performed in accordance with the guidelines of Hokkaido Institute of Public Health, Japan and were approved by the ethics committee of the Institute.

2.2. Experimental infection

Echinococcus multilocularis eggs were obtained from the feces of two beagle dogs that had been orally inoculated with mature metacestode tissue containing protoscoleces (Hokkaido isolate). The eggs were then washed several times with physiological saline and kept at 4 °C until use for experimental infections in mice. The experimental infections were performed in three independent and identical trials using these infective eggs, prepared 1–3 months prior to each experimental infection. The anesthetized mice, aged 4–5 weeks, were administered with either 200 or 2,000 eggs suspended in 0.5 ml of physiological saline orally. The animals were handled within a safety facility (Biosafety Level 3) at the Hokkaido Institute of Public Health, Japan.

2.3. Examination of cyst establishment and protoscolex development

A total of 148 mice (D2 ($n = 10$); B6 ($n = 10$); F_1 ($n = 10$); N_2 ($n = 118$)) infected with 200 eggs were sacrificed and necropsies were performed at 4 weeks p.i. for the assessment of cyst establishment. The livers were cut into approximately 0.5 mm-thick slices and the total numbers of cysts were counted. A total of 283 mice (D2 ($n = 11$); B6 ($n = 10$); F_1 ($n = 12$); N_2 ($n = 250$)) infected with 2,000 eggs were sacrificed and necropsies were performed at 16 weeks p.i. for the assessment of protoscolex development. The livers were collected and the total weight of the organs and that of host liver tissue and parasitic cysts were measured. Several parts of the cysts (total weight 1–2 g) were minced, passed through a 125 μm sieve, and then washed repeatedly with physiological saline. The numbers of mature protoscoleces were counted under a stereoscopic microscope as an index of the parasite development. The number of eggs used for the first experiment ($n = 200$) was expected to produce a macroscopically countable number of cystic lesions in the host liver, while the number used in the second experiment ($n = 2,000$) was expected to reduce the effect of the difference in the numbers of cysts established in individual mice.

2.4. Genotype analysis

To identify and map QTLs, a total of 121 informative microsatellite markers – 113 for cyst establishment and 117 for protoscolex development – were chosen from a database maintained by Mouse Genome Informatics (MGI), The Jackson Laboratory, USA (http://www.informatics.jax.org/), as listed in Table 1. After the initial QTL scan, extra markers were included in the vicinity of the provisional QTLs to refine their position. A total of 232 N_2 mice, 118 for cyst establishment and 114 for protoscolex development, were genotyped using each microsatellite marker. After genomic DNA extraction and PCR amplification, amplified PCR products were electrophoresed in 10% polyacrylamide or 3% agarose gels, stained with ethidium bromide and visualized under UV light. The genotype at each marker locus was determined by the size of the PCR product.

2.5. Data analysis

Two phenotypes – cyst establishment and protoscolex development in mice – were analyzed separately and the following phenotypic parameters were used for the analyses. For the analysis of cyst establishment, the number of cysts in the liver was converted to a percent value against a mean number of cysts in susceptible D2 mice to avoid the bias resulting from egg infectivity between two independent experimental infections. For the analysis of protoscolex development, the number of mature protoscoleces in the liver was indicated in terms of number/gram of cystic lesion, which was subsequently log-transformed for QTL analysis. Zero values were assigned a value of 1 to allow log transformation. Linkage analyses of two phenotypes to chromosomal loci were performed using the MapManager QTXb20 software (Manly et al., 2001). Recombination frequencies (%) were converted into genetic distances (centiMorgan; cM) using the Kosambi map function, in which linkage data are provided as likelihood ratio statistic (LRS) scores. Genome-wide significance thresholds were calculated in terms of LRS by carrying out permutation tests for 1,000 permutations based on the established guidelines (Lander and Kruglyak, 1995). The thresholds in the backcrossed progenies were determined by the QTL software program for finding suggestive, significant and highly significant linkages. Two-way interactions (epistasis) were estimated with a QTL scan and statistical significance for these gene to gene interaction tests were based on $P < 10^{-5}$ using 1,000 permutations of the observed data. Statistical analysis was performed using the Stat-View program (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered to be significant. A Scheffé's F test was conducted for multiple comparisons between the mean values of genotypes at the detected QTLs.

3. Results

3.1. Establishment of E. multilocularis cysts

At 4 weeks after oral administration of 200 eggs, the total numbers of cysts in the liver were counted. In two independent experiments, the average numbers of cysts in D2 mice were different (average ± S.D.): 79.0 ± 30.4 and 60.6 ± 13.5 for the first and second experiments, respectively. Therefore, the numbers of cysts

Table 1
List of microsatellite markers used for whole genome scanning in this study.

Markers	cM	CYS	PSC	Markers	cM	CYS	PSC	Markers	cM	CYS	PSC
D1Mit118	1.65	+	+	*D6Mit159*	12.36	+	+	*D13Mit17*	7.73	+	+
				D6Mit74	23.70	+	+	*D13Mit60*	14.44	+	+
D1Mit324	29.13	+	+	*D6Mit188*	32.53	+	+	*D13Mit63*	21.00	+	+
D1Mit415	43.94	+	+	*D6Mit104*	51.53	+		*D13Mit9*	42.19	+	+
D1Mit191	52.66	+	+	*D6Mit150*	53.75	+	+	*D13Mit148*	59.69	+	+
D1Mit30	57.91	+	+	*D6Mit254*	59.32	+		*D13Mit262*	63.93	+	+
D1Mit445	58.27		+	*D6Mit374*	64.60	+					
D1Mit14	67.71	+	+	*D6Mit59*	69.36	+		*D14Mit10*	6.41	+	+
D1Mit145	74.68		+	*D6Mit15*	77.70	+	+	*D14Mit120*	20.88	+	+
D1Mit355	80.36		+					*D14Mit102*	34.36	+	+
D1Mit150	81.08		+	*D7Mit114*	15.42	+	+	*D14Mit225*	39.46	+	+
D1Mit291	88.97	+	+	*D7Mit82*	32.76	+	+	*D14Mit165*	56.16	+	+
D1Mit511	93.70		+	*D7Mit318*	42.27	+	+	*D14Mit266*	64.86	+	+
				D7Mit66	64.30	+	+				
D2Mit293	17.24	+	+	*D7Mit333*	82.25	+	+	*D15Mit12*	1.80	+	+
D2Mit296	21.81	+	+					*D15Mit5*	16.74	+	+
D2Mit91	39.24	+	+	*D8Mit4*	18.89	+	+	*D15Mit156*	32.19	+	+
D2Mit185	55.23	+	+	*D8Mit100*	29.70	+	+	*D15Mit159*	41.96	+	+
D2Mit62	59.34	+	+	*D8Mit234*	39.33	+	+	*D15Mit161*	52.78	+	+
D2Mit286	76.74	+	+	*D8Mit242*	50.07	+	+				
D2Mit229	88.99	+	+	*D8Mit200*	61.37	+	+	*D16Mit182*	2.57	+	+
D2Mit200	102.29	+	+					*D16Mit59*	26.86	+	+
				D9Mit90	17.80	+	+	*D16Mit140*	40.30	+	+
D3Mit164	2.01	+	+	*D9Mit91*	20.74	+	+	*D16Mit152*	48.23	+	+
D3Mit182	21.73	+	+	*D9Mit302*	36.36	+	+	*D16Mit106*	57.68	+	+
D3Mit28	39.27	+	+	*D9Mit133*	45.80	+	+				
D3Mit14	61.32	+	+	*D9Mit355*	51.41	+	+	*D17Mit113*	8.14	+	+
D3Mit129	80.49	+	+	*D9Mit18*	71.49	+	+	*D17Mit198*	14.59		+
								D17Mit139	27.40		+
D4Mit235	3.57	+	+	*D10Mit248*	5.21	+	+	*D17Mit89*	32.82	+	+
D4Mit237a	22.38	+	+	*D10Mit61*	34.80	+	+	*D17Mit218*	43.76		+
D4Mit139	29.65	+	+	*D10Mit186*	38.56	+	+	*D17Mit187*	50.17	+	+
D4Mit152	39.46	+	+	*D10Mit14*	66.75	+	+	*D17Mit221*	59.77	+	+
D4Mit303	45.55	+	+	*D10Mit297*	72.31	+	+				
D4Mit308	57.66	+	+					*D18Mit132*	11.92	+	+
D4Mit54	70.02	+	+	*D11Mit226*	5.64	+	+	*D18Mit17*	21.09	+	+
D4Mit42	82.64	+	+	*D11Mit21*	25.94	+	+	*D18Mit124*	32.15	+	+
				D11Mit140	32.13	+	+	*D18Mit184*	39.70	+	+
D5Mit180	11.93	+	+	*D11Mit4*	41.87	+	+	*D18Mit7*	51.92	+	+
D5Mit108	23.91	+	+	*D11Mit212*	54.34	+	+				
D5Mit258	33.95	+	+	*D11Mit199*	65.48	+	+	*D19Mit69*	8.93	+	+
D5Mit208	48.51	+	+	*D11Mit48*	82.96	+	+	*D19Mit80*	18.24	+	+
D5Mit188	57.51	+	+					*D19Mit33*	51.76	+	+
D5Mit370	65.23	+	+	*D12Mit219*	9.69	+	+				
D5Mit222	81.53	+	+	*D12Mit172*	21.09	+	+	*DXMit166*	28.26	+	+
				D12Mit5	37.16	+	+	*DXMit130*	55.45	+	+
				D12Mit101	51.55	+	+	*DXMit186*	76.75	+	+

cM, centiMorgan; CYS, markers used for Quantitative trait loci (QTLs) analysis of cyst establishment; PSC, markers used for QTL analysis of protoscolex development.

observed in B6, F_1 and N_2 mice were converted to percent values against a mean number of cysts in susceptible D2 mice in each experiment. The distribution of percent values are shown in Fig. 1A. All animals had cestodes in the livers. The mean values for B6 mice (40.2%) were significantly lower than those of D2 mice ($P < 0.01$, Scheffé's F test), while those of N_2 progenies varied from 1.7% to 182.5% with an average of 72.0%.

3.2. Identification of QTLs and their effects on cyst establishment

The genome-wide linkage analysis of cyst establishment was performed using 118 N_2 mice, where one suggestive and one significant QTL were detected on chromosomes (Chrs.) 9 and 6 (*Emcys1*), respectively (Figs. 2A and 3A). The significant QTL had a peak LRS score of 15.0 and accounted for 12% of the total variance (Table 2). No significant epistatic interactions were observed between all of the tested markers (data not shown). Segregation of N_2 mice according to their genotypes at *Emcys1* (*D6Mit150*) showed that individuals homozygous for the D2 allele (DD) had a statistically higher number of cysts than did the heterozygous mice (DB) ($P < 0.01$, Scheffé's F test), and that there were no significant differences between D2 and DD, and between B6, F_1 and DB (Fig. 4A). A list of *Emcys1* candidates on Chr. 6 is shown in Table 3.

3.3. Development of E. multilocularis protoscoleces

At 16 weeks after oral administration of 2,000 eggs, the total numbers of mature protoscoleces in the liver were calculated. The distributions of numbers of mature protoscoleces per gram of cystic tissue are shown in Fig. 1B. Although all animals had *Echinococcus* cysts in their enlarged livers, the number of mature protoscoleces differed greatly among individual mice even within the same mouse group. In D2 mice, 1.5×10^4–8.5×10^5 protoscoleces were recovered per gram of cystic lesion, whereas in all B6 mice and some N_2 progenies, protoscolex formation was completely absent (Fig. 1B).

3.4. Identification of QTLs and their effects on protoscolex development

The genome-wide linkage analysis of protoscolex development using 114 N_2 mice identified two suggestive and one highly

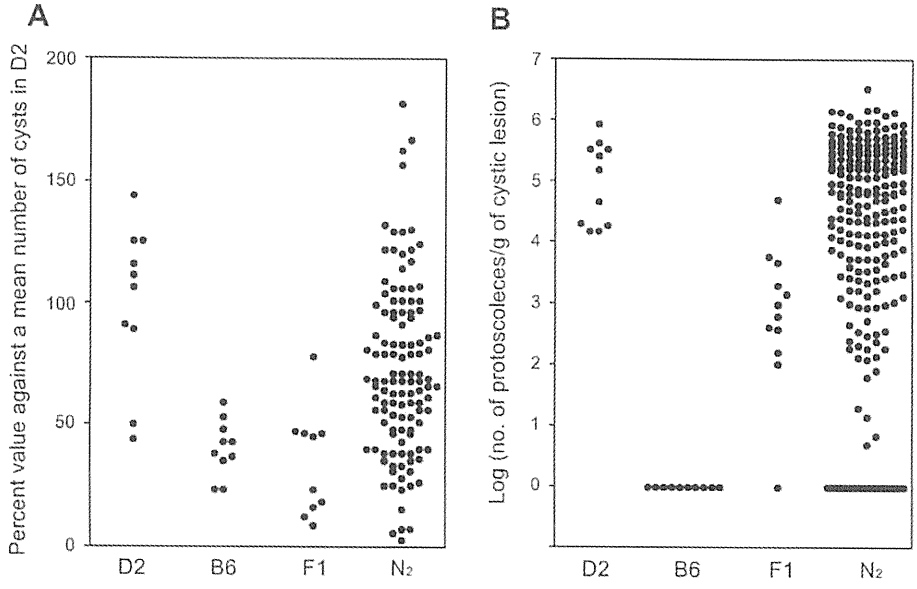

Fig. 1. Distribution of cyst establishment (A) and protoscolex development (B) of *Echinococcus multilocularis* in the livers of D2, B6, F_1 and N_2 mice. (A) The number of cysts in the liver was counted 4 weeks p.i. with 200 eggs for the assessment of cyst establishment. The numbers were converted to percent values against a mean number of cysts in susceptible D2 mice. (B) The number of protoscoleces was counted 16 weeks p.i. with 2,000 eggs for the assessment of protoscolex development. The numbers were indicated as number/gram of cystic lesion and subsequently log-transformed. Zero values were assigned a value of 1 to allow log transformation.

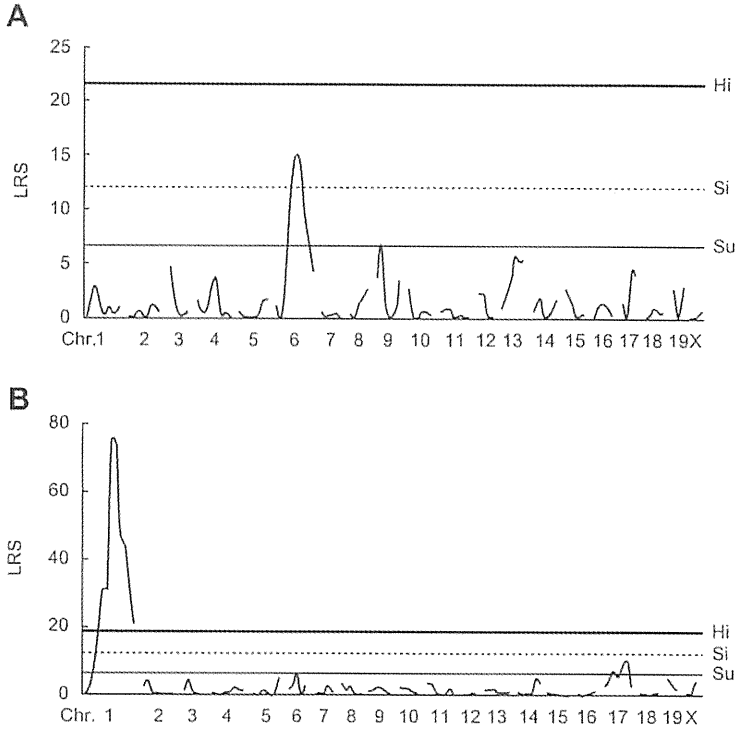

Fig. 2. Whole genome scans for chromosome sites associated with cyst establishment (A) and protoscolex development (B) of *Echinococcus multilocularis* in the liver of N_2 mice. Analyses of linkage of cyst establishment (A) and protoscolex development (B) in the liver of N_2 mice to chromosomal loci were performed using the MapManager QTXb20 software. Recombination frequencies (%) were converted into genetic distance (centiMorgan; cM) using the Kosambi map function, in which linkage data are provided as likelihood ratio statistic (LRS) scores. Genome-wide significance thresholds were calculated in terms of LRS by carrying out permutation tests for 1,000 permutations. The thresholds for suggestive (Su), significant (Si) and highly significant (Hi) linkages are indicated in thin, dotted and thick lines, respectively.

significant QTLs on Chrs. 6, 17 and 1 (*Empsc1*), respectively (Figs. 2B and 3B). The highly significant QTL had a peak LRS score of 75.4 and accounted for 48% of total variants (Table 2). The two suggestive QTLs with LRS scores of 7.1 and 10.3 explained only 6% and 9% of the variants, respectively. Although weak epistatic interaction was observed between *Empsc1* and suggestive QTLs, the scores did not reach the significant level (data not shown). N_2 carrying D2 mice homozygous at *Empsc1* (*D1Mit14*) had statistically higher numbers of mature protoscoleces than did DB heterozygous mice ($P < 0.001$, Scheffé's F test), and there were no significant differences between