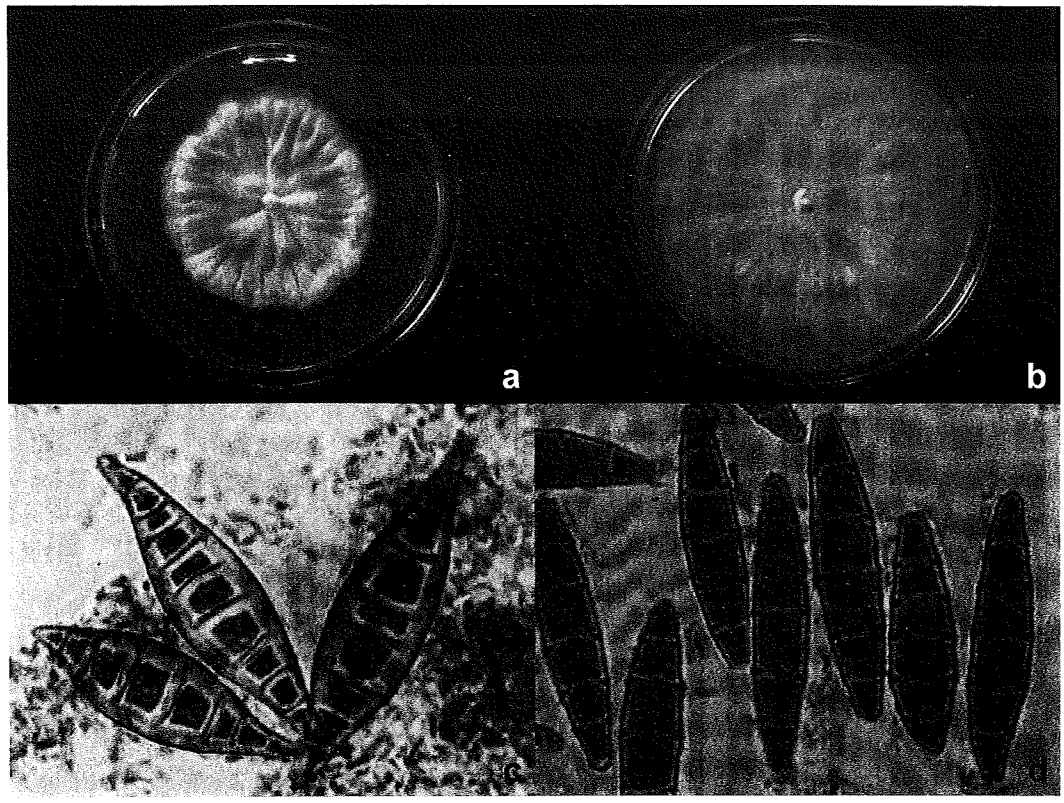

写真2　*Microsporum canis* の集落
サブロー培地（a），ポテト・デキストロース培地（b）での集落，25℃，14日．*Microsporum canis* の分生子（c）と *M. gypseum* の大分生子．*M. canis* の大分生子（d）は紡錘形，中心から先端部にかけて非対称性にやや腫大し，細胞外壁は厚く，隔壁はやや薄い．一方 *M. gypseum* の大分生子は細胞壁が薄く，左右はほぼ対称で先端が鈍い．

国からエキゾチックペットといわれるハムスター，ウサギ，ラット，チンチラ，ハリネズミなどの動物が次々と輸入されることに伴って，海外で流行している *A. benhamiae* による感染の報告も相次ぐようになった[3]。**写真3**は *A. benhamiae* の初代分離状況を示す。

Trichophyton mentagrophytes による皮膚糸状菌症の場合，最近の動向として，交配試験とあわせてリボゾーム RNA 遺伝子の internal transcribes spacer 領域の配列を決定し，web 上で BLAST サーチをかけて，有性型の菌種名を推定し，原因菌を記載することが求められている。

本来，有性型は交配試験により決定するものであるが，結果を得るまでに数カ月以上要することから，敬遠されていることに加え，分子生物学的同定が進んだ今日，有性型の推定まで1～2日で到達するので，学術的な発表データ以外，交配試験はあまり行われなくなっている。

交配試験の概略は，皮膚糸状菌には交配型（性別：“＋”と“－”で表す）があらかじめわかっている菌株と高塩培地を用いて，対峙培養を行う。交配に使われる菌は *Arthroderma vanbreuseghemii*，*A. benhamiae*，*A. simii* などで，シャーレを二分し，片側に被験菌を，反対側に交配株を植え，交配成立時に形成される裸子嚢核内部に形成される子嚢胞子の有無により，交配型を決定する（**写真3**）[1]。

たとえば *A. benhamiae* の交配型“＋”と対峙培養したときに裸子嚢殻を形成し，その

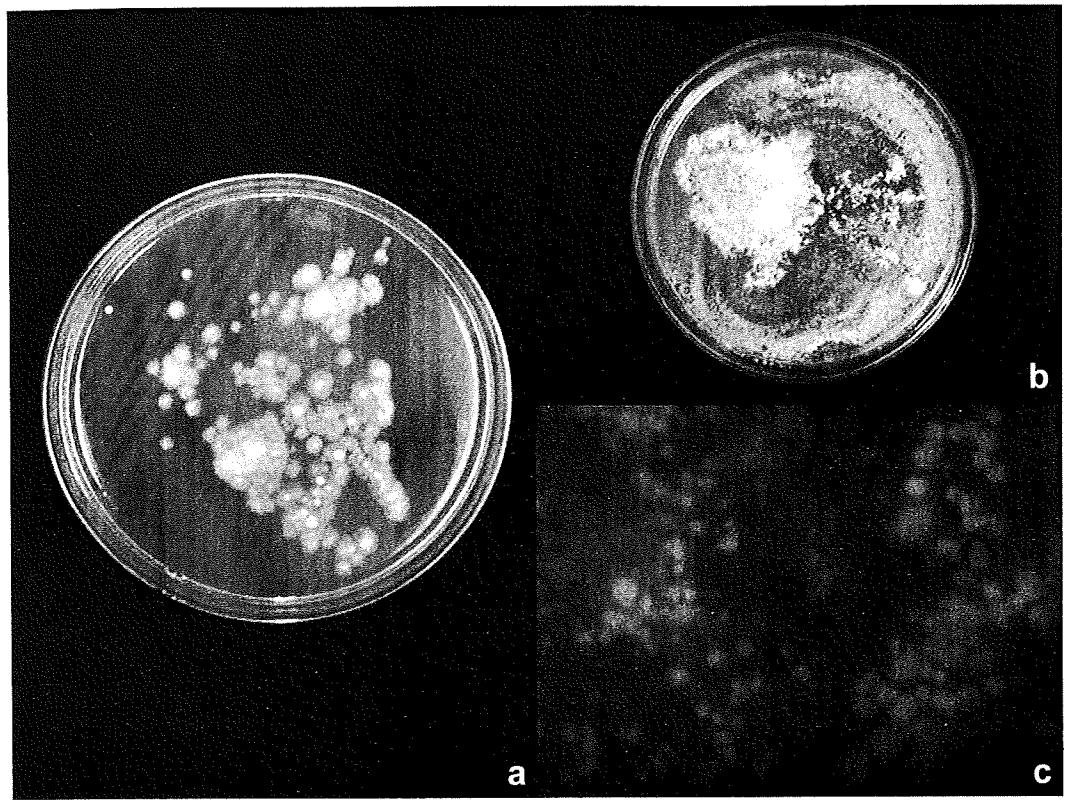

写真3　*Trichophyton mentagrophytes* 初代分離状況
シクロヘキシミドとクロラムフェニコールを添加したポテト・デキストロース寒天培地，35℃，10日．動物の被毛から分離する場合，室温で生育する環境菌が多数付着しているため，35℃を用いたほうが，分離しやすい（a）．対峙培養により交配させテスター株との間に形成された裸子嚢核（b）およびその拡大（c）．

内部に子嚢胞子が形成された場合，被験株の交配型は“－”で，完全型は *A. benhamiae* と判定する。また，同じ被験菌を *A. vanbreuseghemii* や *A. simii* の交配型“＋”と同様に対峙培養した場合，裸子嚢殻は形成するが，内部に子嚢胞子を形成しないこともある。この場合，交配型を“－”と判定することはできるが，有性型を同定することはできない。このように複雑なうえ，使用する交配株の状態によっても左右されることが多いので，分子生物学的同定法が好まれている。

T. mentagrophytes の生育は中程度，扁平，粉状あるいは綿毛状で白色～淡黄色を呈する。粉状の集落の場合，同心円状の輪が形成されることもある。裏面は黄色，黄赤褐色，褐色と様々である。毛髪穿孔テストは陰性で，生育にニコチン酸を要求しない。

顕微鏡的には大分生子は比較的少ない。3～8細胞性，棍棒形～葉巻形 8～50×4～12μmである。壁は薄く，表面は平滑である。小分生子は豊富に産生され，1細胞性，球形，亜球形，3～4×2～3 μm，ブドウの房状に産生されているものと，菌糸側壁に直角に単生しているものがある。コイル状の菌糸（らせん体）がしばしば認められる（**写真4**）。

4．*Trichophyton verrucosum* による皮膚糸状菌症

Trichophyton verrucosum は世界的に分布し，牛や馬などとの接触により感染する。酪農家に多く，就業間もない方に発症することが多い。ヒトに感染した場合，著しい炎症反応が起こる。治療しても円形の色素斑は数十年にわたって消えない。我が国でもウシから

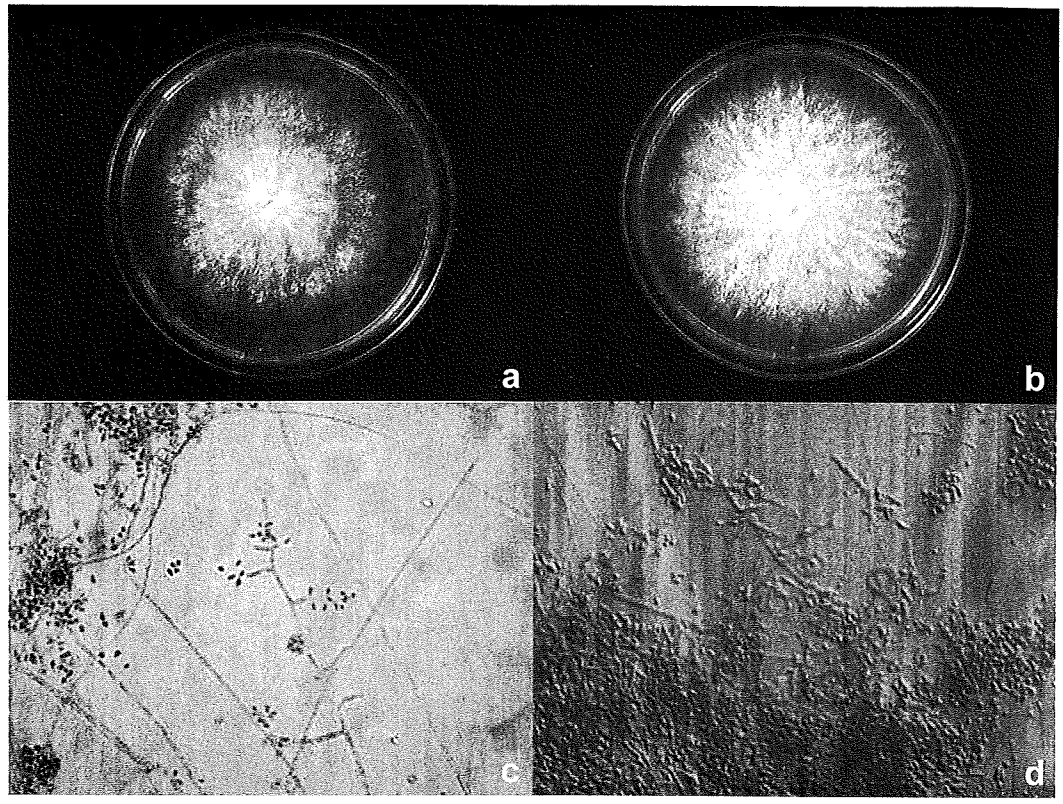

写真4　*Trichophyton mentagrophytes* の集落
サブロー培地（a），ポテト・デキストロース培地（b）での集落，菌糸に直角に単生した小分生子（c）とらせん体（d），25℃，21日．

高頻度に分離される（**写真5**）[1)5)]。

T. verrucosum は生育が遅く，初め無毛性，次第に隆起すると同時に皺ができ塊状になる。表面の色調は白色であるが，時にサーモン色～黄色となる。裏面は無色またはサーモン色を呈する。37℃での生育は良好で，栄養素としてチアミンを要求する。なお，株によりイノシトールを必要とするものもある。よって *T. verrucosum* は一般に使用されている抗生物質とアクチジオンが添加されたサブロー培地を用いて分離することは難しい場合がある。菌分離が必要な場合には，健常部に近いところで落屑を採集し，専門機関に依頼するほうが失敗は少ない。毛髪穿孔テストは陰性である。

顕微鏡的には大分生子は極めて稀で，富栄養培地で時に産生され，紡錘形で先端部は先細りとなる。4～7細胞よりなり表面は平滑で壁は薄い。小分生子の着生も悪く，1細胞性で，卵円形～洋梨形である。厚膜胞子（chlamydospores）が多数産生されるのが特徴である。

II．皮膚糸状菌症原因菌以外の菌種による皮膚病

1．*Histoplasma capsulatum*

H. capsulatutum を原因菌とするヒストプラズマ症は高度病原性真菌症の1種で，かつては輸入真菌症として取り扱われてきたが，我が国でもヒト，動物ともに土着症例が報告されている。なかでもイヌのヒストプラズマ症は皮膚潰瘍を主徴とし（**写真6**），原因菌の遺伝子型がウマの仮性皮疽と近縁であることがわかっているため，接触感染の危険性が示唆されている[6)]。

原因菌の *H. capsulatutum* は温度依存性の

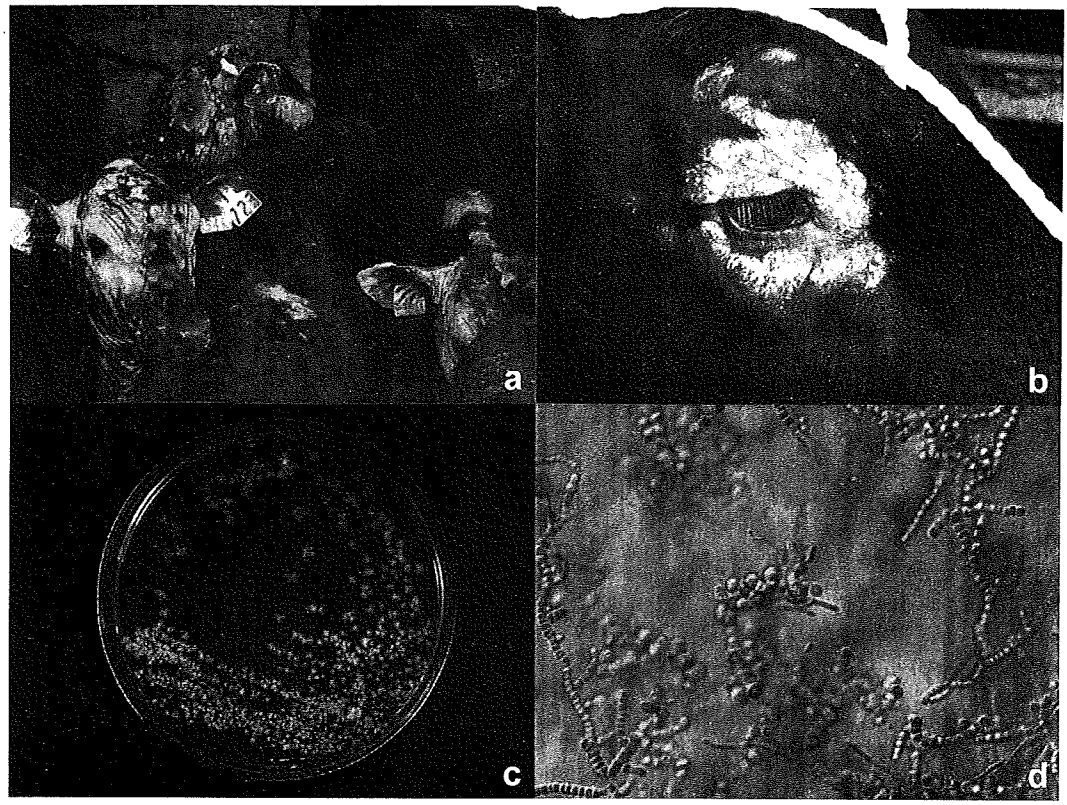

写真5 *Trichophyton verrucosum* に感染したウシ症例（a，b）
初代分離集落（c）（ブレイン・ハート・インフュージョン寒天培地，35℃，28日）とかき取り標本のでの厚膜胞子（d）.

二形性真菌で，室温では菌糸形，宿主内や特種な培地で35℃ないし37℃で培養すると酵母様集落となる。室温での発育は遅く，集落は粉状から綿毛状で次第に黄褐色を帯びてくる。顕微鏡的に大，小の分生子が形成され，大分生子の細胞壁は厚く表面には多くの指状の突起を持つのが特徴である。富栄養の培地を用い35～37℃で培養すると白色～淡黄色の酵母様集落を形成する株もある。酵母細胞は球形または卵円形，直径は数μmである（**写真6**）。しかし，高度病原性真菌であるため，一般の検査室での培養は危険である。すでに培養して生育している集落がある場合や本菌種による感染が疑われた場合は，専門機関に相談することが重要である。

病理組織学的に細胞内寄生性で，マクロファージ内に酵母細胞を認める。

また，飼育動物が日常使用しているタオルや寝具は滅菌処理しなければならない。小児，高齢者，免疫疾患をもつ家族が同居している場合，罹患した動物の治療は，感染動物の入院施設をもつ病院で行い，自宅での飼育管理は避けることを推奨する。

イヌの治癒率は約1/3で，ペットロスによる別の心理的ケアも必要となる場合が想定されるばかりでなく，生涯にわたって緩解と増悪を繰り返す症例も知られている。さらに治療費も体重5キロ程度のイヌでおおよそ年間50～60万円以上かかり，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1つに入っている。

現在，飼育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，小児科領域でのアドバイスとして，安全性を重要視

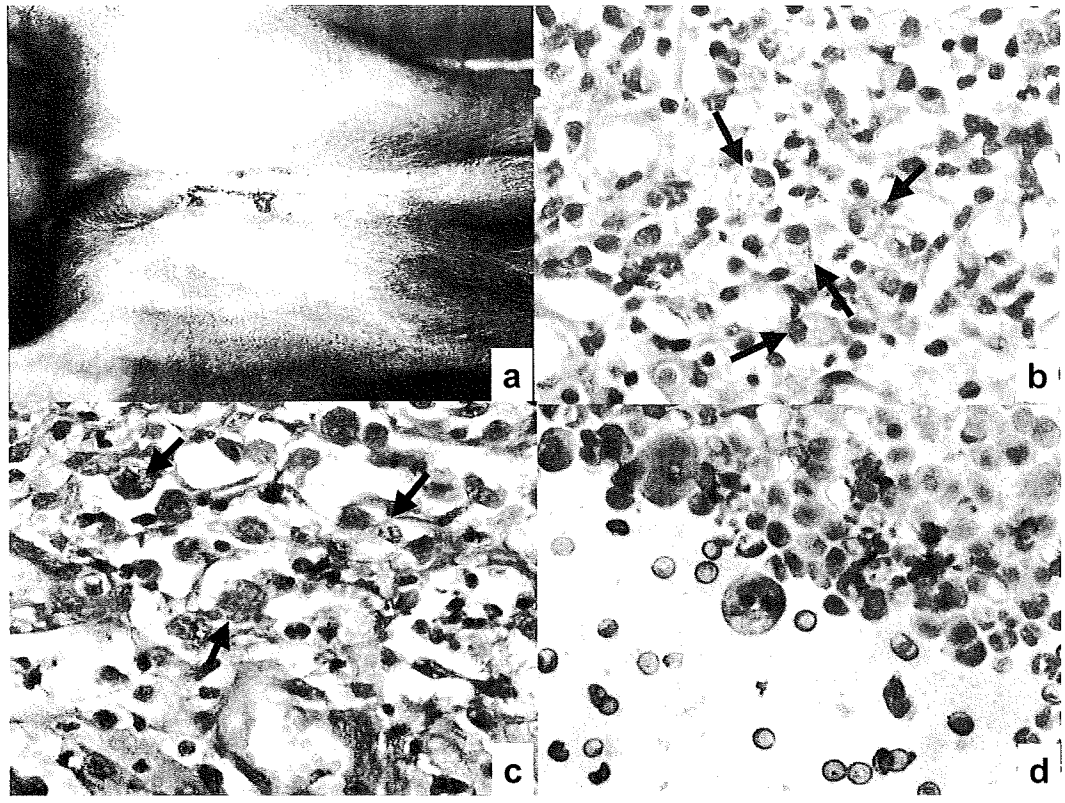

写真6　ヒストプラズマ症のイヌ症例（a）
病理組織でのマクロファージ内の酵母細胞（HE（b），PAS（c）染色，矢印），膿の塗抹でのマクロファージ内の酵母細胞（ライトギムザ染色（d））．

し，ヒストプラズマ症だけは，飼育動物の安楽死を第一の選択肢として考慮されたい。

2．***Chaetomium globosum***

C. globosum はセルロース分解菌として産業用有用微生物であり，香料成分や抗生物質を産生することも知られている。一方，この菌種および近縁菌により，ヒトの皮膚炎，爪感染，副鼻腔炎，肺炎，免疫状態の低下した場合の全身感染などの原因菌として知られているほか，最近ではイヌの落屑・脱毛・紅斑が報告されている（**写真7**）。また，感染症例はウッド灯にわずかに反応するため，臨床的にも鑑別がむずかしい[7]。

原因菌の *C. globosum* は，皮膚科領域で分離・培養用に使用されているアクチジオンと抗生物質を添加したサブロー培地では，*M. canis* と類似した白色集落が生育してくる。本菌種に特徴的な構造物（子嚢果と子嚢胞子など）を観察するにはポテト・デキストロース寒天培地を推奨する（**写真7**）。

3．***Hortaea werneckii***

H. werneckii はヒトの黒癬の原因菌で，手掌，足底の角層内に褐色の菌糸と胞子で寄生する。世界的な本菌の分布は熱帯および亜熱帯地域とされ，とくに海岸地方から症例が報告されている。我が国でも1980年代に沖縄で症例が報告されて以来，九州，四国，関東から次々に症例が報告されている。「はいはい」をしている乳児での報告もある。また環境中では，ハウスダストやプールからも分離されている。家庭内飼育動物では手掌と背部に角化した黒色の病変をともなったモルモットの症例が報告されている（**写真8**）が[8]，動物との接触による感染は，今のところ報告

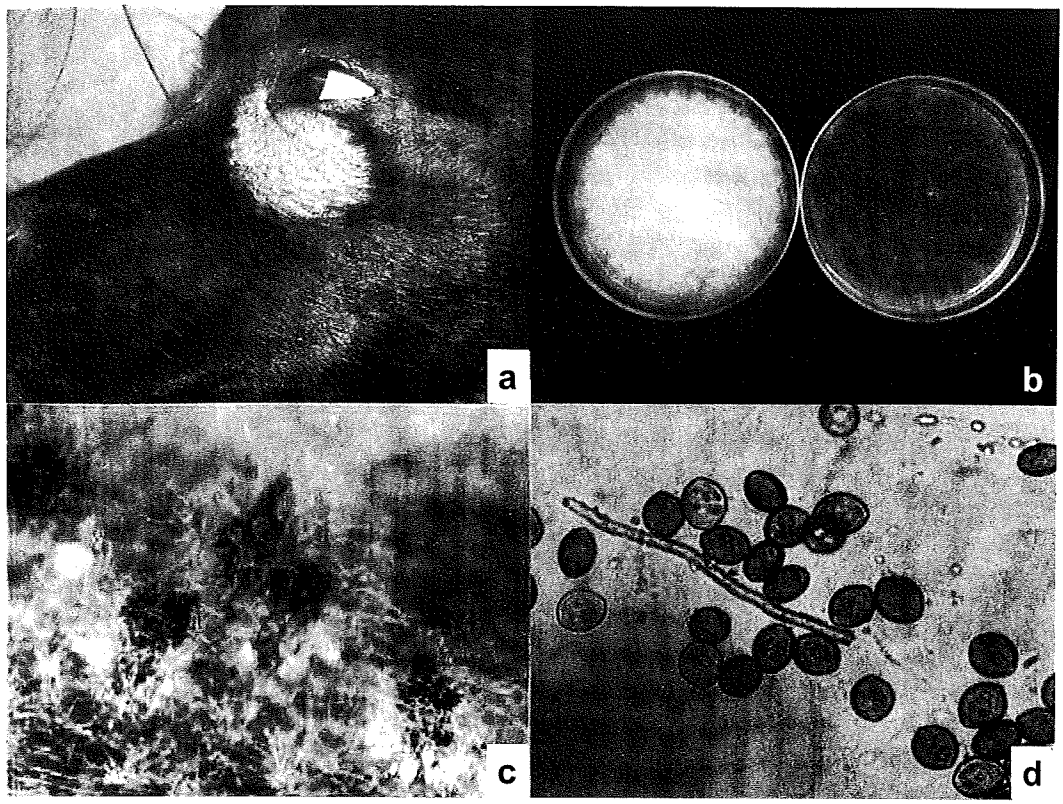

写真7　*Chaetomium globosum* イヌ感染症例（a）
サブロー培地（b左），ポテト・デキストロース培地　（b右）での集落　25℃，4週間，ポテト・デキストロース培地上集落の実体顕微鏡下での閉子嚢核（c），かき取り標本による子嚢胞子（ラクトフェノール固定（d））．

されていない。

また，熱帯の水族館では飼育海水中に本菌種が棲息していることが知られているので，タッチングプールで水棲生物を触った後の手洗いの励行が徹底されている。しかし，一般家庭での熱帯水棲動物の飼育管理状況は不明であるため，黒癬が疑われた場合，熱帯水棲動物飼育状況も感染源特定の鍵となるかもしれない。

原因菌の *H. werneckii* の集落は初め黄緑色，黒緑色，やがて金属性光沢のある漆黒糊状（黒色酵母）集落を形成する。しばらくすると菌糸が成育し，気菌糸を形成する株も見られる。顕微鏡的には菌糸は太く隔壁がある。分生子は初め1細胞性で淡褐色，楕円形，成熟すると2細胞性で，褐色，暗褐色，ピーナッツ形，紡錘形になる（**写真8**）。

4．その他

S. schenkii によるスポロトリコーシスは，擦過傷などを介して真皮内に感染・増殖して発症する。リンパ管の走行に沿って皮疹が生じるリンパ管型と，単発の皮疹が増大していく限局型がある。症状は，紅色の浸潤を伴う丘疹とで，しだいに増大して結節となり，やがて自壊しやすく浅い潰瘍を形成し，軽度の浸出液を伴う。

我が国では植物の刺からの感染は報告されているが，動物との接触による感染は報告されていない。一方，ネコ症例は報告されている[9]。しかしながら，海外ではネコの引っ掻き事故による本菌種の感染が多発しているので[10]，今後，動物との接触により発生が懸念される。

原因菌の *S. schenkii* は温度依存性の二形

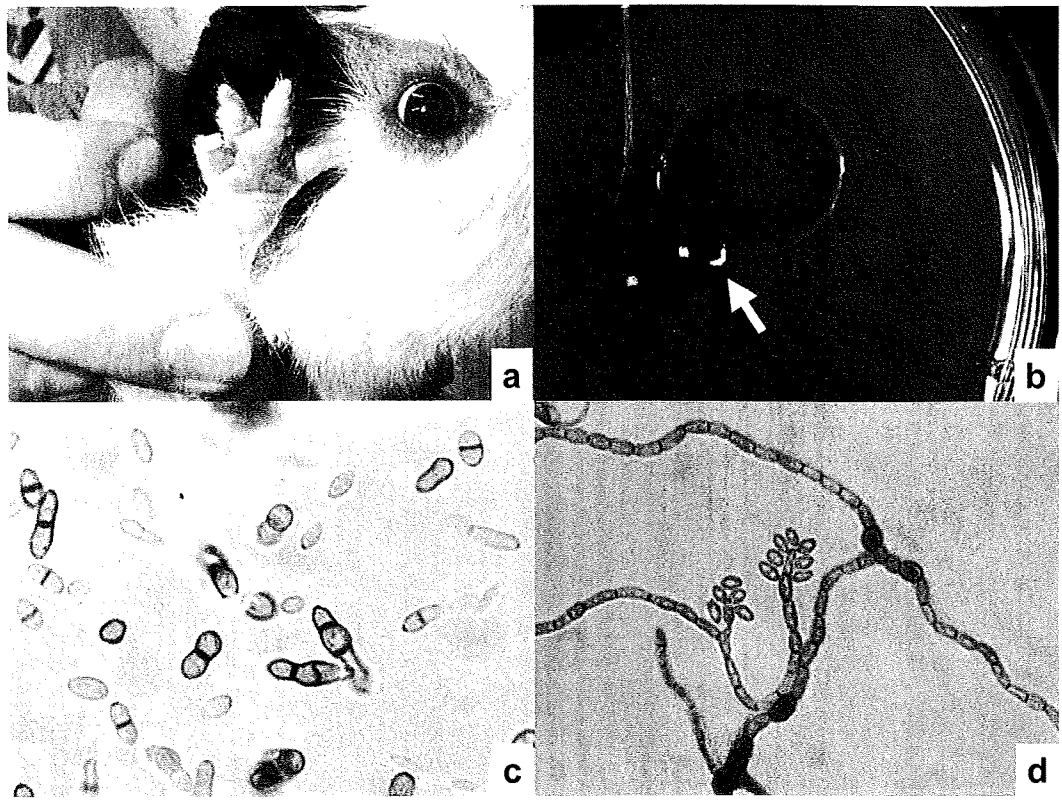

写真8 *Hortaea werneckii* モルモット感染症例（a）とクロラムフェニコール添加ポテト・デキストロース寒天培地上での初代分離状況
黒く光っている集落が本菌種（b：矢印），集落かき取りによる顕微鏡所見（c）とスライドカルチャーによる菌糸と分生子（d）．

性真菌で，室温では菌糸形，宿主内は特種な培地で35～37℃で培養すると酵母形となる。病理組織学的にはアステロイド体の出現が特徴である。

治療は抗真菌薬の投与のほか，ヨード剤の内服，使い捨てカイロによる温熱療法が用いられている。

このほかに，黒色真菌症原因菌の *Exophiala dermatitidis*[11]，*Scopulariopsis brevicaulis*[12]，*Candida* 属菌種[13]などの皮膚症例が愛玩動物や産業動物で報告されている。

III．診療上のポイント

1．感染源の推定

皮膚病を示した症例では，交友関係のある方の家庭も含めて，ペットの飼育の有無を問診することが重要な鍵である。

家庭内飼育動物から感染した皮膚糸状菌症で，イヌを原因とするものは極めて少ないが，ネコを原因とする例は毎年，発生している。背景に，家庭内で飼育されているイヌの多くは1カ月に1～2回のシャンプーが行われている一方，ネコは全くしない，もしくはしても年1～数回程度である（http://www.nekoneko-felis.com/nekoroom/vote/003.html）ことに関係すると推測している。しかし，仔犬が皮膚糸状菌症を発症している例もあるので，イヌはネコより清潔だからと感染原因から除外するのは早計である。

さらに，シャンプーを過信するのも危険である。表面の被毛に付着している真菌は除去できても毛胞深部まで入り込んだ菌糸の除去はシャンプーだけでは困難で，動物の治療に

は抗真菌薬の内服と外用が必要である。動物由来の真菌症と診断されたら，飼育動物は獣医師の診療を受けるように指導し，動物の治療を徹底することが大切である。

しかし，最近ではペットの飼育や動物との接触が全くなくても，学童間で動物由来性の皮膚糸状菌（*Arthroderma benhamiae*）が感染することも知られていることを付け加えておく(http://www.jstage.jst.go.jp/article/jsmm/49/0/49_151/_article/-char/ja/)。

2．感染菌種の同定

かつては，形態学的特徴により同定されてきたが，迅速に同定するには，初代培養物上に糸状菌の発育を認めた時点で，その一部からDNAを抽出し，分子生物学的同定を行うことを推奨したい。特に症例報告のレポートでは場合，リボゾームRNA遺伝子配列よる同定が求められることが多い。

しかし，形態学的特徴や生理生化学的性状による同定は遺伝子データの裏付けとして重要である。最終的な同定は両者の整合性によって決めることが必須である。

なお，ヒストプラズマ症が疑われた場合，培養はせず，専門機関に問い合わせることが重要である。

3．治療法

真菌症と診断されたら，抗真菌薬の外用および内服，菌種によっては温熱療法も有効である。

4．予防法

外出先から帰ったら，手洗い，うがいなど日常的な衛生管理はもちろんのこと，場合によってはシャワーを浴び，衣服の交換も必要である。

また，ヒストプラズマ症と診断された個体を飼育していた場合，家族への感染をふせぐことが第一である。動物が使用していたタオル，寝具などは滅菌すること（オートクレーブをかける，消毒液に漬け込むなど），あわせて生活環境の消毒も必要である。ただし，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）の指導のもと行われることがのぞましい。

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特集

人と動物の共通感染症最前線 6

小動物臨床獣医師を対象としたヒストプラズマ症に関する認識調査

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要　約

ヒストプラズマ症は我が国の小動物臨床領域で遭遇しうる最も危険度レベルの高い真菌症である。本症に関するアンケート調査を小動物臨床獣医師に行ったところ，257名の回答を得た。その結果，病名は広く認識されているものの，①真菌による感染症であること，②馬の仮性皮疽がその病型の1つであること，③国内にも存在し人と動物で発症していること，④仮性皮疽は届出伝染病であることを総合して理解している割合は極めて低く5.8%であった。

はじめに

ヒストプラズマ症は炭疽菌と同等のバイオセーフティレベル3（健常個体でも感染し，死に至ることもある）の高度病原性真菌 *Histoplasma capsulatum* を原因とする真菌症である。従来は輸入真菌症として取り扱われてきたが，人も動物も国内で感染した症例（動物に限ると馬1例，犬8例，牛4例以上）が確認されていることから，ヒストプラズマ症は，我が国に存在する最も危険な真菌症であると認識されるようになってきた[1-4]。

ヒストプラズマ症とは

ヒストプラズマ症の原因菌 *H. capsulatum* は温度依存性の二形性真菌で，宿主内では酵母形，室温では菌糸形となる。宿主は人および各種動物で，全世界的に主に温帯から熱帯に分布し，大河の流域に多い。宿主，流行地域により var. *capsulatum*（全世界的分布，各種動物が罹患），var. *duboisii*（アフリカに分布し人とヒヒが罹患），var. *farciminosum*（全世界的分布，馬科動物が罹患）に分けられている。感染経路は，経気道感染，創傷感染，接触感染で，ハエやアブによる昆虫による媒介，犬では経口感染も示唆されている。

国内感染例の人および犬から検出した遺伝子は馬の仮性皮疽の原因の *H. capsulatum* var. *farciminosum* と近縁なため，特に接触感染の危険が示唆されている[1)]。また，戦前は全国的に馬の仮性皮疽の流行地であったことから，土壌から原因菌が分離されたとの報告はないものの，馬の飼育が盛んだった地域，往来が多かった場所などの土壌は汚染地域と考えられ，そこで人や牛および犬が異種感染していると考えられている[1,2)]。

ヒストプラズマ症の症状は風邪に似た呼吸器症状，肺炎，播種性全身感染である。一方，馬はリンパ管行性に潰瘍，肉芽腫性病変となり，重篤な場合は全身感染に至る。興味あることに我が国の犬症例は粘膜・皮膚の膿瘍性・肉芽腫性潰瘍が主な症状で（図1），肺病巣を欠くことが多い。また，腫瘍など基礎疾患のある場合は全身播種性となることもある。治療法は抗真菌薬の長期投与が有効であるが，再燃を繰り返す症例も多い。基礎疾患がある場合は予後不良となる。

調査目的

2002年の日本医真菌学会教育委員会の報告によれば[5)]，獣医微生物学の講義中に約6割の大学でヒストプラズマ症の病名が紹介されている。一方，小動物臨床現場の獣医師の認識度は不明である。

今回，ヒストプラズマ症を我が国の小動物臨床領域で遭

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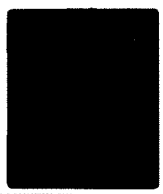

ヒストプラズマ症に取り組んで足掛け10年。はじめ「こんな病気日本に無い！」「死亡繊炎の誤診じゃないの！」と，なかなか信じてもらえませんでしたが，少しずつこの感染症の存在を理解していただける先生が増えてうれしい限りです。モット多くの方に正しく理解していただけるとモット^{2}うれしいです。

皮膚に多発した難治性，膿性，肉芽腫性潰瘍。(a) 前肢，(b) 後肢，(c) 頸部。この症例は乳癌の転移にともなった日和見感染で，全身播種となり，死の転帰となった。

図1　ヒストプラズマ症の1例

遇しうる最も危険度レベルの高い真菌症と考え，小動物臨床獣医師における本症への認識度をアンケート調査することにより，①本症への関心を高め，②調査結果に基づいた安全対策等を考察し，③実態に則したコントロール法および予防法の提言ための基礎データを得ることを目的としてメールまたはファックスによる任意転送によるアンケート調査を行った。

結　果

257通（平成18年小動物臨床従事者約13,200名の1.9%に相当）の回答を得た。

回答者の性別は男性85.6%，女性14.4%，年齢は40歳代（34.2%）を中心に20～70歳代，出身大学は全16大学，卒後年数は21～25年（17.8%），臨床経験は21～25年（18.3%）を中心に各年数を網羅し，病院所在地は関東地方（58.3%）を中心に四国，九州の一部を除いた全国であった。

各項目別認識度は，ヒストプラズマ症の病名を知っていると答えた方は78.6%，原因菌が真菌であると知っている— 74.3%，仮性皮疽の病名を知っている— 48.6%，仮性皮疽の原因菌が菌であると知っている— 53.3%，仮性皮疽は家畜伝染病法により届出伝染病とされていることを知っている— 29.6%，仮性皮疽とヒストプラズマ症は広義に同じ疾病であることを知っている— 24.1%であった。

ヒストプラズマ症も感染症法，家畜伝染病法などで管理する必要がある疾患かという問いに「必要ある」と答えた方は34.6%，日本国内で感染してヒストプラズマ症が発生していること裏付ける学説を知っている— 15.2%，我が国のヒストプラズマ症は人の他，犬，馬，ラッコで発症していることを知っている— 21.4%，現在までにヒストプラズマ症の診断経験（近医での症例の見学，疑わしい症例の経験を含む）がある— 2.7%であった。なお，今回の調査で疑症例3例（いずれも犬）が示唆された。

一方，回答の個別解析から，ヒストプラズマ症と仮性皮疽の病名と原因菌，両者が広義に同じ疾病であることを正しく認識している方は10.5%，これらに国内感染例の発生に関する情報と仮性皮疽が届出伝染病であることを加えて総合的に理解している方は5.8%であった。

考　察

ヒストプラズマ症の病名についての認識は78.6%であった。この数字は大学教育より高いことから病名は広く浸透していると思われた。しかし，ヒストプラズマ症と仮性皮疽の病名と原因菌，両者の関係，国内感染症例の存在を総合的に理解している割合は極めて低く，小動物臨床現場ではヒストプラズマ症は正しく理解されていない。

まず，より多くの小動物臨床現場に関係される方々にヒストプラズマ症の存在を認識してもらうことが大切である。さらに安全性の面から臨床従事者，飼育家族，他の患者などへの二次感染防止を考慮した安全対策が必要である。現在，不安を煽動しない方策を慎重に考えている。

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Evaluation of *Echinococcus multilocularis* tetraspanins as vaccine candidates against primary alveolar echinococcosis

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ABSTRACT

Echinococcus multilocularis causes an important zoonotic cestode disease. The metacestode stage proliferates in the liver of intermediate hosts including human and rodents and forms multiple cysts. Recently, members of a transmembrane protein tetraspanin (TSP) family have been used as vaccines against schistosomosis, or as diagnostic antigens for cysticercosis. In this study, seven tetraspanins of *E. multilocularis*, designated as TSP1 to TSP7, were evaluated for their protective potential against primary alveolar echinococcosis. The large extracellular loop (LEL) region of these tetraspanins was cloned from a full-length enriched cDNA library of *E. multilocularis* metacestodes and expressed in *Escherichia coli* as a fusion protein with thioredoxin. Recombinant TSPs were applied as vaccines against an *E. multilocularis* primary experimental infection in BALB/c mice. Cyst lesions in the livers of vaccinated and non-vaccinated mice were counted. The cyst lesion reduction rates induced by the seven tetraspanins in vaccinated vis-à-vis non-vaccinated mice were: 87.9%, 65.8%, 85.1%, 66.9%, 73.7%, 72.9% and 37.6%. Vaccination conferred protective rates to mice ranging from 0% (TSP5, 6, 7) to maximally 33% (TSP1, 3). The results indicated that recombinant tetraspanins have varying protective effects against primary alveolar echinococcosis and could be used in vaccine development.

1. Introduction

Echinococcus multilocularis is a cestode parasite, whose larval stage causes hepatic alveolar echinococcosis in humans and rodents, and thus it represents an ecological and public health problem in many countries of the Northern Hemisphere [1]. Although prevention of this tumor-like disease is very important, most studies have concentrated on the parasite's morphology and immunology [2,3]. The application of protective proteins as vaccines against other cestode parasites, especially *Taenia spp.* [4–10] and *E. granulosus* [11–14] suggests that vaccine development for *E. multilocularis* is also possible. Great success has been achieved in vaccinating against *E. granulosus* infection using the recombinant protein EG95 [12,14]. In similar studies, it has been reported that recombinant proteins EMY162 [15], EM95 [15,16] and 14-3-3 [17] protect against primary (egg) *E. multilocularis* infection.

Recently, many studies have focused on the tetraspanins, a transmembrane protein family, for their multiple functions involved in the coordination of intracellular and intercellular processes, including signal transduction, cell proliferation, adhesion, migration, cell fusion, and host–parasite interactions [18]. The host–parasite interactions are thought to be associated with immune evasion [19], which has resulted in the use of tetraspanins as vaccines interfering with the schistosome survival strategy [19–22]. Several tetraspanins, TSP1, TSP2 [19] and Sm23 [20] of *Schistosoma mansoni*, and SJ23 of *S. japonicum* [21,22] have been reported as potential vaccine candidates against schistosomosis. It is notable that these tetraspanins caused varying reductions in dif-

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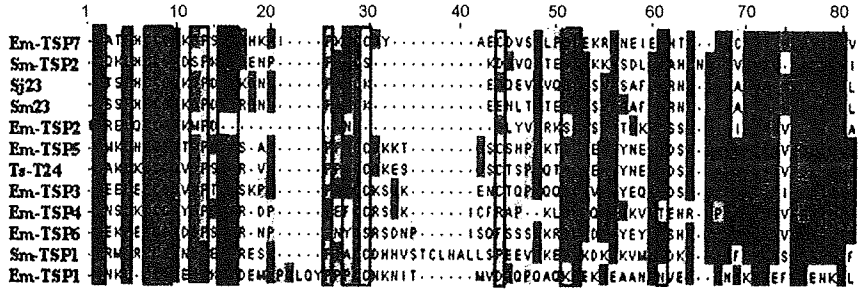

Fig. 1. Comparison of the LEL domain amino acid sequences for the seven cloned transmembrane proteins (Em-TSP1 to TSP7) and those of Sm-TSP1, Sm-TSP2, Sm23, Sj23 and Ts-T24. Alignment of these amino acid sequences was performed with the Clustal W2 on-line service. Identical amino acid residues are marked with colored shadow. Key residues are marked with a red frame. Extra conserved residues in *Schistosoma*, *Echinococcus* and *Taenia* are marked with a black frame. *Echinococcus* and *Taenia* specifically conserved amino acids are in shorter black frames. Em, *Echinococcus multilocularis*; Sm, *Schistosoma mansoni*; Sj, *Schistosoma japonicum*; Ts, *Taenia solium*. The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for *E. multilocularis* tetraspanin 1–7 and Sm-TSP1, Sm-TSP2, Sm23, Sj23 and Ts-T24 are FJ384717 , FJ384718 , FJ384719 , FJ384720 , FJ384721 , FJ384722 , FJ384716 , AF521093 , AF521091 , M34453 , M63706 and AY211879 respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ferent parasitic stages: adult worms (TSP1, TSP2, Sm23 and SJ23), liver eggs/granulomas (TSP1, TSP2 and SJ23) and intestinal/fecal eggs (TSP2 and SJ23).

Although such successes have been achieved using tetraspanins to protect mice against different stages of schistosome infections, no studies have been made on tetraspanins in *Echinococcus*, especially with a view to exploiting their vaccine potential. As a preliminary study, we evaluated the immunogenicity and protective ability of immunization with seven tetraspanins against *E. multilocularis* infection in a murine model.

2. Materials and methods

2.1. Molecular analysis of TSP amino acid sequences by biological prediction servers

A full-length enriched cDNA library of *E. multilocularis* metacestodes (Hokkaido isolate) was constructed using a vector-capping method (Hitachi Biotechnologies, Tokyo, Japan) and their 5′ ends were sequenced. We identified members of the tetraspanin family by BLAST searching on-line using partial sequences, picked seven representative clones from the library and sequenced them in full. The conserved, crucial parts of these proteins-LEL domain (aa 1 to aa 81 in Fig. 1) were compared with other known tetraspanins from helminthes using the Clustal W2 on-line service. Then we used the MegAlign component of DNAStar programme (Version 4.01 DNASTAR, Madison, WI) to construct a phylogenetic tree of transmembrane proteins, including the TSP1 to TSP7 tetraspanins of *E. multilocularis*; the Sm23 [20], Sm25 [23], Sm-TSP1 and Sm-TSP2 proteins of *S. mansoni* [19]; the Sj23 [21,22] and Sj25 proteins of *S. japonicum* [24], the Sh23 protein of *S. haematobium* [25] and T24 protein of *Taenia solium* [26]. We also used the DNAStar program to determine the percentage identity of the several TSPs of *E. multilocularis*.

2.2. Cloning, expression and purification of recombinant proteins

For facilitating solubilization and purification of expressed proteins, the regions encoding the extracellular loop (LEL) were amplified by RT-PCR with Taq polymerase to fuse them in-frame with the N-terminal *E. coli* thioredoxin (TRX) and the C-terminal V5 and 6-His epitopes in the pBAD/Thio-TOPO plasmid (Invitrogen, USA). The primers used are listed in Table 1. The cDNAs were ligated into the plasmid and were transformed into *E. coli* TOP10 cells (Invitrogen, USA) with recombinant plasmids according to the manufacturer's instructions (pBAD/TOPO® ThioFusion™ Expression Kit, Invitrogen, USA). The recombinant bacteria were cultured in Lennox broth at 37 °C for 16 h, complemented with 0.05% arabinose as an inducer. Recombinant fusion proteins from *E. coli* lysates were purified with a HisTrap affinity column under nondenaturing conditions (HisTrap FF crude 1 ml, GE Healthcare, USA). The purified proteins were dialyzed in PBS with Snake Skin Pleated Dialysis Tubing (10,000 MWCO, PIERCE, USA) and stored at −80 °C.

Table 1
Oligonucleotide primers for amplification of the LEL domain and TSP ORFs.

Primer[a]	Sequence[b]			
	LEL domain	Product (bp)	TSP ORF	Product (bp)
TSP1-s	CCTGATAACCTAAACAAAGC	330	AGCTATCTTCTCGGATGTGCTAGAC	792
TSP1-a	GAGGGTTTTGTTCTCTGCCAA		TTCCGCTTTCCACTGCTTGTACAGC	
TSP2-s	TACAAGGATGAGTTGATGGAC	195	GGTGTTCTTCAAGGTGGAATGAAGTG	621
TSP2-a	CATGGAGGACTCAACTTTGCC		CGCATAACTTCG AATTTTGGACTGC	
TSP3-s	CATGAGTTTGTCGGTCTTGTG	264	CTGAAGACGTTCGCGGTCATTGT	447
TSP3-a	CAGAGAGGAATCCTTAATTTG		CTTCTCGTATTCGTTGACCTTCTTGGCC	
TSP4-s	TTCGCGCTGAAAAATCAGTCC	267	GCAATGCTGCGCTTTAGCGGTTGCT	687
TSP4-a	AGGTAGTCTATTTTCCGTCAA		GGTTACAGATGCTGTCCTAGGGCCAA	
TSP5-a	CACGACTTCGTTCGTCTTGTT	261	GCTCTCTCTTGCGGTGGTAACTT	678
TSP5-s	CAGGGCAGAATCCTTTATCTC		GACCTTCTCATATTCGCCGACCTTCTTG	
TSP6-s	GACAAGCAGGGTAAAGACTTT	252	GTTCTGACATGCGGAGAGCAGT	669
TSP6-a	GCTTCGAAGGTATTCGTAGAT		GCTCCGCTTGCCGGCCAAACAGCA	
TSP7-s	GTTTTCAAAGATGAGGTGCGA	249	TCGAAAACGTGTGCTGGTTGTATCAG	690
TSP7-a	CAACGTATGTTTCTCAATCTC		CACAGGTTCATAAGATTCAATGTCGG	

[a] s, sense; a, antisense.
[b] Sequences are 5′–3′.

2.3. Animal experiments

All animal experiments were performed in accordance with the guidelines of Hokkaido University and Hokkaido Prefecture.

2.4. Antigen preparation from infected cotton rats

Cotton rats were infected with the eggs of *E. multilocularis* (Hokkaido isolate). Nine weeks post-infection, the brood capsules were formed and the protoscolex appeared. Cotton rats were then dissected and cyst blocks were collected from the abdominal cavity, removing as much host tissue as possible. After washing several times with 0.15 M PBS (pH7.4), the cyst blocks were cut with scissors and treated to several cycles of freeze/thawing in liquid nitrogen. We then added 9 volumes of PBS (pH7.4), containing protease inhibitors (5 mM ethylenediaminetetraacetic acid, 5 mM iodoacetamide, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin) and 1% Triton X-100, and homogenized the cyst blocks with a glass-homogenizer and subsequently with a teflon-homogenizer. The tissues were further homogenized using the ultrasonic disruptor (UR-200P, Tomy Seiko, Tokyo, Japan) at 40 W for 3 min. The lysate was centrifuged at 10,000 × *g* for 30 min at 4 °C and the supernatant was dialyzed for 48 h in PBS (pH 7.4). Dialyzed proteins were finally filtered through a nitrocotton filter (1 μm pore size). The cyst-extracted antigen was stored at −80 °C.

2.5. Rabbit immunization and polyclonal antisera generation

For the studies on specific antibody response and cross-reactivity of the seven tetraspanin proteins, polyclonal antisera directed against the seven TSPs were generated in seven 15 weeks old male rabbits. One rabbit for each recombinant TSP was immunized by three successive intramuscular injections of purified recombinant fusion TSP proteins at days 0, 14 and 28 separately at a dosage of 150 μg/rabbit. TSP proteins for primary immunization were emulsified in Freund's complete adjuvant, while those for the second and third boosters were emulsified in Freund's incomplete adjuvant. Immunization was completed within six weeks. Pre-immune serum was collected as a negative control and all sera were aliquoted and stored at −80 °C. For immunoblotting analysis and ELISA tests, the antisera to TSP proteins were absorbed by affinity chromatography with HiTrap affinity columns (HiTrap NHS-activated HP 1 ml, GE Healthcare, USA), coupled with purified TRX. This absorption was performed in order to deplete the antisera of antibodies directed to the fusion partner protein TRX.

2.6. SDS-polyacrylamide gel electrophoresis and immunoblotting

Recombinant and cyst-extracted proteins were treated with Laemmli sample buffer (BIO-RAD, USA), heated at 98 °C for 5 min, and then separated on a 12% SDS–polyacrylamide gel. For immunoblotting analysis, proteins were transferred from the gel to a PVDF membrane (BIO-RAD, USA) using the Trans-Blot™ SD Semi-Dry Transfer Cell (Bio-Rad, USA). The membrane was blocked with 5% skimmed milk in TBS-T for 1 h and was subsequently incubated with the anti-TSP antibody at a dilution of 1:5000 in 3% bovine serum albumin/PBS for 1 h. This was followed by incubation with horseradish peroxidase (HRP)-conjugated goat-anti-rabbit antibody at a dilution of 1:10,000 in 3% BSA/PBS for 1 h. Finally, an HRP substrate Luminol Reagent (Millipore Corporation, USA) was added and the reaction was detected by chemiluminescence using the LAS1000 Mini Reader (FUJIFILM, Japan). Three washes with TBS-T were performed after every step. Immunoblotting was performed at room temperature.

2.7. Vaccination procedure

Seventy female BALB/c mice were divided into 10 groups at seven weeks old, each having seven mice. Groups one, two and three were non-immunized or immunized with PBS plus adjuvant, and TRX plus adjuvant, respectively as controls, while groups four to ten were immunized with respective TSPs (TSP1 to TSP7) plus adjuvant. For groups four to ten, each mouse received a subcutaneous injection of 20 μg of protein dissolved in 100 μl PBS, and emulsified in an equal volume of Freund's complete adjuvant (primary immunization) and Freund's incomplete adjuvant (booster). PBS and TRX controls were similarly treated by replacing TSP proteins with PBS or TRX. The primary immunization was done on day 0. Boosters were given twice on days 21 and 42. Sera were collected and tested for the antibody by ELISA on day 49 post-immunization. One mouse from group one, four, five, six and nine died during the etherizing pre-challenge.

2.8. Evaluation of vaccine efficacy

Experimental infection was done using *E. multilocularis* eggs collected from the feces of an experimentally infected dog. The etherized mice were orally administrated with 0.5 ml of eggs suspension at a concentration of 400 eggs per ml in physiological saline 56 days post-immunization. One month post-infection, all mice were sacrificed and necropsies were performed. Livers were collected in plastic dishes and cut into approximately 0.5 mm thick slices, from which the numbers of all detectable lesions were counted. Based on these data, the cyst reduction rate of each tetraspanin was calculated as a percentage reduction in cyst lesion counts in vaccinated groups as compared to non-vaccinated ones.

2.9. Cross-reaction among TSPs determined by ELISA

We used ELISA to analyze sera from immunized rabbits for their cross-reactivity to heterologous TSPs. Prior to ELISA, the sera were absorbed with TRX as described above. Ninety-six well Costar® plates (Corning Inc., USA) were coated with recombinant TSPs at a concentration of 0.1 μg/ml in carbonate buffer (pH 9.6) for 2 h at room temperature. This was followed by blocking with 5% skimmed milk overnight at 4 °C and washing four times with PBS-Tween. Then serum samples, diluted 1:5000 in 3% BSA/PBS, were added and the plates were incubated for 1 h at 37 °C before washing four times. Goat-anti-rabbit IgG conjugated with horseradish peroxidase (Invitrogen, USA), diluted 1:10,000 in 3% BSA/PBS, was then added and incubated at 37 °C for 1 h. This was followed by washing four times and the addition of 3,3′,5,5′-tetramethylbenzidine substrate before incubation for 20 min at room temperature. The reaction was stopped using 1N HCL and color development was measured at 450 nm with the Biotrack II plate reader (Amersham Biosciences, USA). Antisera to TSP proteins were absorbed with TRX-coupled HiTrap affinity columns (HiTrap NHS-activated HP 1 ml, GE Healthcare, USA) to deplete the antibodies directed to the fusion partner protein TRX. Pre-immune serum was used as a negative control. The mean OD value of pre-immune sera was calculated and used as a cut-off point (threshold value) to discriminate between "positive" and "negative".

2.10. Detection of TSPs transcripts in oncosphere by RT-PCR

Eggs were treated with artificial gastric fluid for 1 h at 37 °C and centrifuged at 1000 × *g* for 5 min. The pellet was used to extract total RNA using a TRIzol plus RNA Purification Kit (Invitrogen, USA), and cDNA synthesis was carried out using the SuperScript III First-strand Synthesis System Kit (Invitrogen, USA) according to the manufacturer's instructions. Sense and anti-sense primers

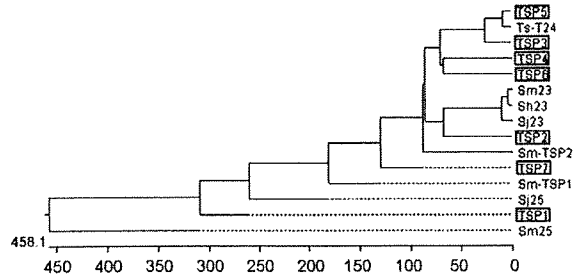

Fig. 2. Phylogenetic tree of transmembrane proteins constructed by the MegAlign component of the DNAStar program (Version 4.01 DNASTAR, Madison, WI). TSP1 to TSP7: tetraspanins of *E. multilocularis* (in marked frame); Sm: *Schistosoma mansoni*; Sj: *Schistosoma japonicum*; Ts: *Taenia solium*; Sh: *Schistosoma haematobium*. GenBank accession numbers for Sm25, Sh23 and Sj25 are AF028730 , U23771 and U77941 respectively.

Table 2
Percentage identity analysis of the entire ORF of TSP by DNASTAR (Version 4.0).

1	2	3	4	5	6	7		
100	13.0	17.4	16.2	15.9	12.6	15.2	1	TSP1
	100	23.5	18.4	29.0	23.7	18.4	2	TSP2
		100	24.2	57.7	26.8	16.8	3	TSP3
			100	30.1	30.5	16.6	4	TSP4
				100	30.0	21.7	5	TSP5
					100	19.3	6	TSP6
						100	7	TSP7

Amino acid sequences were used for the percentage identity analysis of TSP proteins.

(Table 1) were designed to amplify tetraspanin open reading frames (ORFs) and RT-PCR was performed with GoTaq Flexi DNA Polymerase (Promega, USA).

2.11. Data analysis

The protective effect obtained by immunization with each tetraspanin was calculated as the percentage reduction of the number of cyst lesions in vaccinated mice as compared to non-vaccinated ones. Multiple comparison analysis was performed with the Tukey–Kramer method using a computer package (Statcel 2, Japan) to determine differences between the vaccinated groups and control groups, which were considered significant at the 5% level.

3. Results

3.1. Molecular analysis of E. multilocularis tetraspanin

Molecular components analysis of LEL domain using the on-line Clustal W2 program revealed that TSP1 to TSP7, of *E. multilocularis*, share many conserved residues with each other, and with those of *Schistosoma* and *Taenia* (Fig. 1), which has been reported previously [27]. The amino acid sequence of these residues was as follows: E/Q···CCG···D···W/F/Y···P···SCC···C···GC. The percentage conservation of the amino acids CCG, the first C of SCC and the terminal C, of TSP1 to 7, was 100%. Very interestingly, we found that residues F/L at aa 5, G at aa 12 and P/Y at aa 13 are extremely

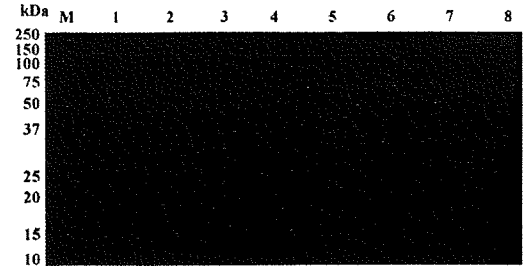

Fig. 4. SDS-PAGE analysis of purified recombinant TSPs. Purified recombinant TSPs were loaded at 5–10 μg/well and separated on a 12% SDS–polyacrylamide gel. CBB staining was performed. Lane M, molecular marker. Lanes 1–7, TSP1 to TSP7. Lane 8, purified thioredoxin (TRX).

conserved in these helminths, while some residues only exist in *Taenia* and *Echinococcus*: A at aa 55, G at aa 69 and aa 81. Percentage identity analysis of the seven TSPs showed that the amino acid sequence identity ranged from 12.6% to 57.7% (Table 2). In addition, the phylogenetic relationship revealed that TSP3 and TSP5 are closely related to T24, a diagnostic antigen of *T. solium*; and TSP2 is related to the protective proteins Sm23, Sh23, Sj23 and TSP2 of *Schistosoma* (Fig. 2).

3.2. Amplified fragments of tetraspanins

Using the primers listed in Table 1, the LELs of the seven tetraspanins from cyst cDNA and the entire ORFs of the seven tetraspanins from oncosphere cDNA were amplified by PCR. The results showed that the amplified fragment sizes of the LELs ranged from 195 to 330 bp (Fig. 3A) and those of the tetraspanin ORFs ranged from 447 to 771 bp as expected (Fig. 3B). Only a very weak band of oncosphere TSP1 ORF could be detected (Fig. 3B).

3.3. SDS-PAGE analysis of recombinant tetraspanins

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified fusion proteins showed the expected molecular masses, which ranged from 24 to 26 kDa (Fig. 4). Purified recombinant proteins were soluble and pure enough for further immunological analysis and vaccine trials in mice.

3.4. Anti-TSPs IgG antibody response

Sera from immunized rabbits were examined for the presence of anti-TSP antibodies against TSPs using immunoblotting. Western blot analysis showed that anti-TSP antibodies had very strong responses to cyst-extracted antigens (Fig. 5). Double bands can be found to TSP3. In contrast, pre-immune serum was not reactive with either recombinant or cyst-extracted proteins. The immunostained bands were approximately 23–29 kDa. Cross-reaction investigation using ELISA showed that TSP1 and TSP3 could induce highly specific antibody responses, while the antibody to TSP2 was strongly cross-reactive with other TSPs including TSP4 to 7. Antisera to TSP4, TSP5, TSP6 and TSP7 showed cross-reactivity with the others to varying extents. According to mean OD value to

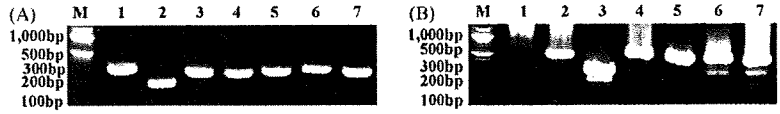

Fig. 3. Amplification of tetraspanins by RT-PCR. (A) LELs of tetraspanins from the cDNA library of *E. multilocularis* metacestodes with sense and antisense primers designed from the large extracellular loop domain. (B) Investigation of tetraspanins from cDNA of oncosphere cDNA with forward and antisense primers designed from ORFs of full-length tetraspanins. In both (A) and (B), Lane M, molecular marker. Lanes 1–7, TSP1 to TSP7.

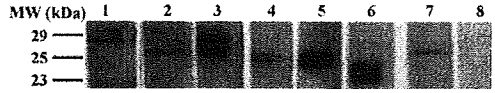

Fig. 5. Immunoblotting analysis of anti-TSP sera (TRX-removed) to cyst-extracted antigens. Serum samples were diluted 500-fold and the anti-rabbit IgG-HRP conjugate was diluted 5000-fold with blocking buffer. Pre-immune serum was used as the negative control. Lane M, molecular marker. Lanes 1–7, reactivity of cyst-extracted antigens to anti-TSP1 to TSP7 antibodies. Lane 8, negative control.

Table 3
Cross-reaction of anti-TSP-sera with TSPs by ELISA.

Antibody[b]	Protein[a]						
	TSP1	TSP2	TSP3	TSP4	TSP5	TSP6	TSP7
Anti-TSP1	**1.903**	**0.573**	**0.237**	**0.172**	**0.224**	**0.154**	**0.154**
Anti-TSP2	**1.878**	**2.345**	**2.115**	**0.311**	**2.011**	**1.730**	**1.649**
Anti-TSP3	**0.182**	**0.293**	**2.301**	**0.147**	**0.731**	**0.149**	**0.155**
Anti-TSP4	**0.656**	**2.713**	**2.257**	**2.559**	**0.720**	**1.393**	**0.887**
Anti-TSP5	**0.568**	**1.023**	**0.594**	**0.531**	**1.367**	**0.432**	**0.440**
Anti-TSP6	**0.524**	**1.717**	**0.848**	**0.268**	**0.543**	**1.908**	**0.181**
Anti-TSP7	**0.463**	**1.265**	**0.801**	**0.344**	**0.375**	**0.359**	**1.293**
PIS[c]	0.147	0.140	0.113	0.143	0.127	0.119	0.122

OD value was measured at A450 nm.
TSP, tetraspanin; TRX, thioredoxin.
[a] Purified recombinant TSPs were used at a concentration of 0.1 μg/ml.
[b] Serum samples were diluted 1:5000 in 3% BSA/PBS.
[c] PIS, pre-immune sera, mean ± SE = 0.130 ± 0.013. This is used as a cut-off point (threshold value) to discriminate between "positive" and "negative". Positive values were presented in bold letters.

pre-immune sera (threshold value), all the OD value to anti-TSPs were determined as "positive" (Table 3).

ELISA results using individual sera collected from vaccinated mice showed that although there were many variations among mice within each group and between groups, relatively strong antibody responses were observed against recombinant tetraspanins and cyst-extracted proteins (Fig. 6).

3.5. Protective efficacy of tetraspanin proteins

The number of counted cyst lesions in the liver and the reduction rate of cyst lesions for the seven TSPs (TSP1 to TSP7) are shown in Table 4. The recombinant proteins TSP1, TSP2, TSP3, TSP4, TSP5 and TSP6 gave statistically significant reductions of more than 65% ($P < 0.05$) in the number of cyst lesions in vaccinated mice as compared to non-vaccinated ones. However, TSP7 protein did not produce a significant reduction in cyst lesions (37.6%) ($P > 0.05$). PBS showed no vaccine efficacy, while TRX displayed a 27.8% reduction

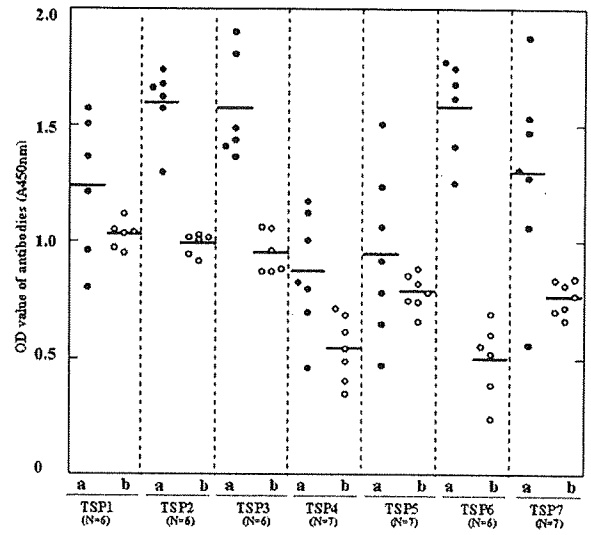

Fig. 6. Reactivity of recombinant TSP-immunized-mice sera (TRX-absorbed) to recombinant TSPs and cyst-extracted antigens measured by ELISA. Serum samples were diluted 5000-fold and the goat anti-mouse-IgG-HRP conjugate was diluted 10,000-fold in blocking buffer. For each group, a and b represent reaction with recombinant TSP (symbol ●) and cyst-extracted antigen respectively (symbol ○). The mean OD value of control reaction where TRX had been used as antigen (range of the mean values among TSP1-TSP7: 0.05–0.12) was subtracted from each group. Vertical bars represent mean OD value of each group. *N*, number of mice in each group.

in cyst lesions ($P > 0.05$). Among the vaccinated groups, two out of six in the TSP1-vaccinated group, one out of six in the TSP2-vaccinated group, two out six in TSP3-vaccinated group, and one out of seven TSP4-vaccinated group showed no cyst lesions in the liver (Table 4). In consequence, vaccination conferred an overall protection rate against infection ranging from 0% (TSP5, 6, 7) to maximally 33% (TSP1, 3).

4. Discussion

Despite high expression levels in the plasma membrane, and intracellular vesicles, tetraspanins remain among the most mysterious transmembrane molecules 20 years after their discovery [28]. To date, several tetraspanins have been identified in helminths, 20 in *Caenorhabditis elegans* and at least 25 in the parasite *Schistosoma* [29]. Although some tetraspanins have been demonstrated to

Table 4
Number of cyst lesions in mouse liver and percentage reduction in cyst lesions in vaccinated groups compared with non-vaccinated control.

Group	Number of cyst lesions			Vaccine efficacy (%)	
	Mean ± S.E	Range	Ratio of mice without lesions	Cyst lesion reduction rate	Protective rate
Non-vaccinated control ($n = 6$)[d]	19.00 ± 3.1	7–25	0/6	–	0
PBS ($n = 7$)	20.28 ± 1.2[c]	16–26	0/7	−6.8	0
TRX ($n = 7$)	13.71 ± 3.5[c]	7–34	0/7	27.8	0
TSP1 ($n = 6$)	2.33 ± 1.1[a]	0–7	2/6	87.9	33.3
TSP2 ($n = 6$)	6.50 ± 2.7[b]	0–18	1/6	65.8	16.7
TSP3 ($n = 6$)	2.83 ± 1.5[a]	0–8	2/6	85.1	33.3
TSP4 ($n = 7$)	6.29 ± 1.7[a]	0–14	1/7	66.9	14.3
TSP5 ($n = 7$)	5.00 ± 0.9[a]	2–8	0/7	73.7	0
TSP6 ($n = 6$)	5.50 ± 1.8[a]	1–13	0/6	72.9	0
TSP7 ($n = 7$)	11.85 ± 2.9[c]	1–27	0/7	37.6	0

[a] Reduction of cyst numbers was significant at $P < 0.01$.
[b] Reduction of cyst numbers was significant at $P < 0.05$.
[c] Reduction of cyst numbers was not significant ($P > 0.05$).
[d] *n* represents the number of mice in each group.

be potential vaccine candidates against different stages of schistosome infection [19–22], nothing is known about them in another important platyhelminth *E. multilocularis*. Recently, many studies have focused on the LEL domain of tetraspanin because of its important functions in mediating protein–protein interactions and homodimerization [30]. In our present study, LEL domain of seven tetraspanins were cloned, expressed and characterized in order to focus on the protective potentials of tetraspanins against *Echinococcus* infection.

Tetraspanins belong to a four transmembrane protein family, nearly all members of which share 26 conserved key residues. These conserved residues have been used as a key criterion to define whether a newly identified membrane protein is a member of tetraspanin family or not [27,31]. Thirteen out of 26 conserved residues are within LEL domain. Molecular component analysis of LEL domain, using the Clustal W2 on-line biological service, indicated that these key residues were conserved in TSP proteins of *E. multilocularis*, *S. mansoni*, *S. japonicum* and *T. solium*. Most importantly, we found that among those proteins the crucial cysteine-cysteine-glycine (CCG) motif has no mutations as previously reported [27]. Three extra conserved sites, F/L at aa 5, G at aa 12 and P/Y at aa 13, were found in tetraspanins of these helminths (Fig. 1), implying the possibly closer relationship of these helminths than others. Moreover, residues A at aa 55, G at aa 69 and aa 81 appear to be more conserved in cestode *Echinococcus* and *Taenia*. The amino acid sequence analysis of the seven TSPs displayed a relatively high percentage identity ranging from 12.6% to 57.7%. This is mainly due to the existence of the largest, conserved domain, known as the large extracellular loop (LEL). Members of the tetraspanin family from different organisms are structurally and functionally related [18,31]. Phylogenetic analysis indicated that TSP2 of *E. multilocularis* is phylogenetically related to TSP2 [15], Sm23 [20] and SJC23 [21,22] of *Schistosoma*, the protective antigens against schistosomiasis, while TSP5 is closely related to the T24 antigen of *T. solium*, a diagnostic antigen for cysticercosis [26]. We suppose that among these proteins, some may be vaccine candidates, while others may be useful as diagnostic antigens.

Cyst-extracted antigens had strong reactivity with TRX-absorbed rabbit anti-TSP antibodies as determined by immunological assays (Figs. 5 and 6) similar to that of recombinant proteins (Fig. 6), suggesting a well antigenicity of TSP proteins. The much closed double bands detected to TSP3 indicated possible differential glycosylation (Fig. 5).

Since tetraspanins in the same family share common crucial residues [27,31], antigenic cross-reactivity would be expected between the different TSP molecules. The ELISA results indicated that recombinant tetraspanin proteins showed varying degrees of cross-reactivity with each other. Although all the OD values to anti-TSPs sera were determined as "positive" referencing to threshold value, anti-TSP1 and anti-TSP3 antibodies exhibited the highest specific reactivities to homologous antigens but had low cross-reactivities with others (Table 3). On the other hand, TSP2 and TSP7 showed a broad range of specificity, while TSP4, TSP5 and TSP6 cross-reacted with other members of the family to varying extents.

Tetraspanin proteins regulate cell morphology, motility, invasion, fusion and signaling, in the brain, immune system, in tumors and elsewhere [32–37], and the most distinctive feature of the tetraspanin family is the ability of its members to form lateral associations with multiple partner proteins, and with each other, in a dynamic assembly, described as the 'tetraspanin web' [34,38]. The fact that some tetraspanin proteins cross-reacted with several others implies that immunization with one tetraspanin antigen could block several tetraspanins functions or disrupt the lateral associations with multiple partner proteins and with each other in the tetraspanin web, thereby providing a more effective protection mechanism.

Primary infection, initiated through the oral *E. multilocularis* egg infection of mice, followed by counting liver cyst lesions four weeks post-infection, revealed that six out of seven TSPs had a cyst lesion reduction rate of over 65% in this study. TSP1 and TSP3 exhibited reduction rates of 87.9% and 85.1%, respectively, while TSP7 had the poorest protective effect of 37.4%. The most encouraging results were found in the groups for TSP1 to 4, in which the number of liver cyst lesions in some mice was found to be zero, indicating that these mice were completely protected against *E. multilocularis*. Compared to the non-vaccinated group, the PBS plus adjuvant group showed no protective efficacy, while TRX plus adjuvant had a 27.8% reduction in cyst lesions.

Much success has been achieved in the vaccination campaign against *E. granulosus* infection using the recombinant protein EG95 [12,14]. In similar studies, Kouguchi et al. [15] reported 74.3% protection against *E. multilocularis* infection in mice using the recombinant protein EMY162, and Gauci et al. [16] and Kouguchi et al. [15] reported 79% and 82.9% protection respectively, against *E. multilocularis* infection in mice using the recombinant protein EM95. In addition, the recombinant protein 14-3-3 was reported to have 97% protection against a primary infection of *E. multilocularis* [17]. It can be seen from the above results that the reduction in liver cysts for EMY162 and EM95 were lower than for TSP1 (87.9%) and TSP3 (85.1%). Moreover, secondary (intraperitoneal) infection has not been performed to evaluate the vaccination efficacy with these proteins. Although the recombinant protein 14-3-3, showed a higher protection against primary infection than those used in this study, showed no protection against secondary infections in mice. As reported, except for erythrocytes, all cells seem to express several tetraspanins [39–41] and individual tetraspanin proteins are often expressed in large copy numbers per cell [33]. Additionally, as mentioned above, tetraspanins have showed their potential in protecting a host against different stages of *Schistosoma* infection [19–22]. We believe that the discovery of one, or more tetraspanins, which are expected to have 'broad-spectrum protection' against different stages of *E. multilocularis* infections, will be achieved in our future studies.

Our RT-PCR results showed that transcripts for these TSPs exist in both the metacestode and oncosphere stages of *E. multilocularis*. Moreover, our study on TSP localization revealed that most of these TSPs are expressed on the germinal layer of *E. multilocularis* metacestodes (in press). Usually, the tegument of *E. multilocularis* metacestodes is thought to be the outer part of the germinal layer [42,43]; therefore, we speculate that TSPs on the *Echinococcus* germinal layer/surface play crucial roles in parasite survival and parasite–host interactions. Proteins expressed on the surface, as membrane-integrated proteins, have a wide range of functions in parasite biology [44]. Loukas et al. [45] emphasized the importance of the schistosome tegument/body wall by describing it as 'the key to its success but also its Achilles heel', because the schistosome tegument has been described as being responsible for parasite survival through immune evasion, while from a vaccine perspective, it constitutes the host–parasite interface. Although we are unsure, in our experiments, whether the antibodies raised by these tetraspanins kill the metacestode or the oncosphere, or even a parasite stage in between, previous studies with schistosomes suggested that once the surface of the parasite is blocked by anti-TSP antibodies, they will lose their survival strategies and render the parasite surface vulnerable to the host defense mechanisms [19].

In this study, we are the first to identify and characterized a new tetraspanin family as vaccine candidates with high protection potential against primary *E. multilocularis* infection in mice. The numerous properties displayed by tetraspanins, and the results from our studies, call for in-depth analyses to explore their vac-

cine potential in protecting intermediate and final hosts against different stages of infections.

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Short communication

Molecular cloning and characterization of a T24-like protein in *Echinococcus multilocularis*

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ABSTRACT

One tetraspanin, designated as E24, was cloned from a full-length enriched vector-capping cDNA library of *Echinococcus multilocularis* metacestode. The amino acid sequence and phylogenetic analysis suggested that E24 is a T24-like protein. The crucial, functional large extracellular loop (LEL) domain of E24 was expressed and characterized using a polyclonal antiserum by Western blot and immunohistochemistry. The results showed that anti-recombinant-E24 (anti-recE24) antibody can specifically recognize approximately 25 kDa recombinant protein and 25 kDa cyst-extracted antigen; the germinal layer of both the protoscolex-free and protoscolex-formed cysts were intensely labeled by immunofluorescent antibody. This study revealed that E24 is an antigenic, germinal layer-located protein of *E. multilocularis* metacestode, implying for its potential in diagnostic and vaccine development.

1. Introduction, results and discussion

Echinococcus metacestode consists of an inner germinal layer (GL), followed with a tegument and if mature, laminated layer (LL) based on the electron microscopic observations [1,2]. However, the tegument was thought to be the most outer part of germinal layer based on light microscopic studies [3,4]. The tegument or germinal layer of Echinococcus is believed to be a very crucial structure for uptake of nutrients, excretion of waste material and formation of laminated layer [5]. Schistosome tegument has been described to be responsible for parasite survival through immune evasion, while from a vaccine perspective, it constitutes the host–parasite interface [6]. It is therefore significant to study proteins located on the germinal layer for the diagnostic and vaccine development against echinococcosis.

Previous studies on Sm-TSP-1, Sm-TSP-2, Sm23, Sm07392 and tetraspanin B of *Schistosoma mansoni* [7–10] and Sj-TSP-2 of *Schistosoma japonicum* [11] indicated that these tetraspanins are located on the outer membrane or tegument of parasites and are involved in protein–protein interplay and parasite immune evasion. Similar studies were conducted by Hancock et al. [12] who characterized T24, a *Taenia solium* tetraspanin, but its location was not elucidated. Among these tetraspanin proteins, some are thought to be diagnostic for cysticercosis and protective against schistosomosis [7,12]. T24 of *T. solium* has been reported to be a diagnostic antigen by Hancock et al. [12]; while TSP-1 and TSP-2 of *S. mansoni* have been reported to be protective proteins by Tran et al. [7].

Although tetraspanin expressed on surface or outer membrane showed importance on many parasitic diseases, little is known about them in Echinococcus, especially regarding their antigenicity and morphological localization in metacestodes. In this paper, we present the first report on molecular cloning and characterization of a tetraspanin protein of *Echinococcus multilocularis*, a homologue of T24.

By blast searching using the sequences from a full-length enriched vector-capping cDNA library of *E. multilocularis* metacestode (Hokkaido isolate), we obtained a gene, the sequence of which well matched with T24 of *T. solium*. The gene was designated as E24. The sequence was submitted to GenBank and was assigned an accession number. E24 peptide sequence showed a significant identity (84%) of ORF (225 aa) to T24 (Supplementary Fig. S1). Transmembrane domain analysis of T24 and E24 indicated that they

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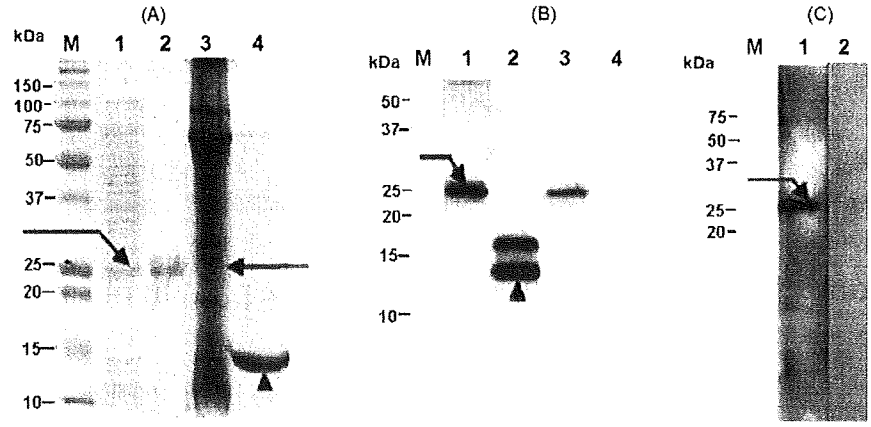

Fig. 1. Characterization of expressed recombinant and cyst-extracted E24 proteins by 12% SDS-PAGE (A) and Western blot (B and C). E24 LEL domain was amplified by PCR and expressed in *E. coli* TOP10 transformed by the pBAD/Thio-TOPO plasmid (Invitrogen). The primers are LEL-F: 5′ cac gac ttc gtt cgt ctt gtt 3′/LEL-R: 5′ cag ggc aga atc ctt tat ctc 3′. Recombinant fusion protein (recE24) was purified with HisTrap affinity column (HisTrap FF crude 1 ml, GE Healthcare). Polyclonal antiserum directed against recE24 was generated in 15-week-old male rabbit. The rabbit was immunized by 3 successive intramuscular injections of purified recE24 protein at a dosage of 200 μg in 1 ml of PBS. For primary immunization, recombinant protein was emulsified in equal volume of Freund's complete adjuvant while those for the second and third immunization were emulsified in Freund's incomplete adjuvant. Immunization was completed within 6 weeks and blood was collected for serum isolation. Cyst blocks were collected from *E. multilocularis* infected cotton rats and lysed by frozen and thawed in liquid nitrogen and then added to PBS containing protease inhibitors and 1% Triton X-100. After homogenized with a glass-homogenizer and subsequently with a teflon-homogenizer, the tissues were further lysed using the ultrasonic disruptor (UR-200P, Tomy Seiko, Tokyo, Japan) and centrifuged. The supernatant was then dialyzed for 48 h in PBS (pH 7.4) and finally filtered through a nitrocotton filter (1 μm pore size). Approximately 2.5 μg of recE24 per well or 15 μg of cyst-extracted crude antigen per well was loaded onto SDS-polyacrylamide gel. Anti-recE24 serum was absorbed using HiTrap affinity columns (HiTrap NHS-activated HP 1 ml, GE Healthcare) coupled with purified thioredoxin (TRX). For Western blot analysis, rabbit anti-recE24 antibody (1:5000) and HRP-conjugated goat-anti-rabbit antibody (1:10,000) were used as primary and secondary antibodies, separately. (A) M, molecular weight marker; Lane 1, recombinant bacterial lysate supernatant (arrow). Lane 2, purified recE24. Lane 3, Cyst-extracted antigen (arrow). Lane 4, purified TRX (arrowhead). (B) Detection of E24 using rabbit anti-recE24 polyclonal serum. M, molecular weight marker. Lane 1, recE24 detected using TRX-pre-absorbed serum. Lane 2, TRX detected using TRX-pre-absorbed serum. Lane 3, recE24 detected using TRX-absorbed serum. Lane 4, TRX detected using TRX-absorbed serum. (C) Detection of cyst-extracted E24 using rabbit anti-recE24 polyclonal serum. M, molecular weight marker. Lane 1, E24 detected using TRX-absorbed serum and Lane 2, negative control (pre-immunized serum).

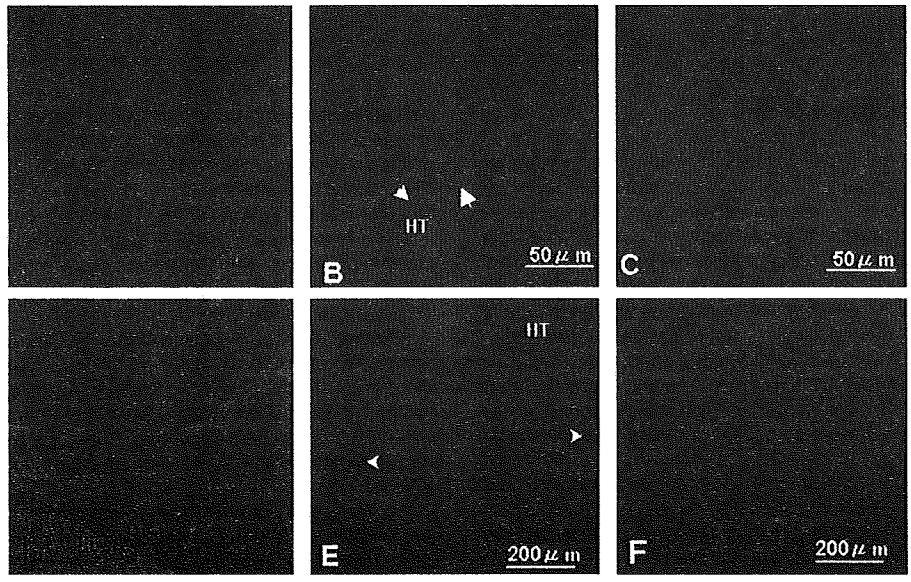

Fig. 2. Immunolocalization of E24 protein on *E. multilocularis* metacestode. Cyst tissues and protoscoleces (Hokkaido isolate) derived from infected Mongolia gerbil and cotton rat were separately fixed in 4% Paraformaldehyde Phosphate Buffer Solution (Wako). Fixed tissues were dehydrated in ethanol solution and embedded in paraffin. The sectioned slices were then deparaffinized in xylene and ethanol subsequently. TRX-absorbed rabbit anti-recE24 serum (1:600) and Alexa Fluor R488 Goat-Anti-Rabbit IgG (H + L) (Invitrogen) (1:1000) were used. Pre-immune serum served as a negative control. Microscopy images of protoscolex-free cyst (A) and protoscolex-formed cyst (D) with H&E stain. Image of protoscolex-free cyst (B) and protoscolex-formed cyst (E) with Alexa fluorescent stain. (C) and (F) are negative controls for cyst and protoscolex. Germinal layer (GL) of cyst and protoscolex was marked with arrowheads; laminated layer (LL) was marked with arrows; the scale of microscopy images is located at lower right of each slice. HT = host tissue.

shared common structures of four transmembrane domains, the key features of which were four or more cysteine residues in the LEL domain (aa position 106–192), with two in a highly conserved 'CCG' motifs at aa position 147–149 and 163–165 (Supplementary Fig. S1). Furthermore, phylogenetic analysis for six transmembrane proteins, E24 (*E. multilocularis*), T24 (*T. solium*), Sm-tsp-1, Sm-tsp-2 (*S. mansoni*), and EM95 and EMY162 (*E. multilocularis*), revealed that E24 is closely related to T24 (Supplementary Fig. S2). Taken together, above analysis theoretically presented the evidence that E24 is a member of yet unknown Echinococcus tetraspanin family and is a T24-like protein, suggesting that E24 is likely to be an antigenic protein inducing a humoral immune response in the host. Moreover, the two molecules are supposed to have similar function based on hydrophobicity analysis [13]. Hydropathy plot analysis of amino acid sequences demonstrated that the hydrophobic domains are within the TM1–TM4 regions of both proteins and the degree of similarity in both hydrophobic and hydrophilic regions between them is significantly high (Supplementary Fig. S3), revealing that they share common ancestor and function [14,15]. On the other hand, the sequence divergence considerably existing in the important, functional LEL domain (27.9%) comprised to that of conserved domains (8.6%) between the two proteins indicated that E24 and T24 probably play some different roles within cells.

The LEL domain was expressed in *E. coli* TOP10 as a fusion form with thioredoxin (TRX). The deduced molecular weight of recombinant and the full-ORF-encoded E24 were approximately 25 kDa and 24 kDa, respectively. SDS-PAGE and Western blot consistently showed that the molecular weight of both the purified recombinant protein and cyst-extracted antigen were approximately 25 kDa as predicted (Fig. 1A–C). Although glycosylation prediction showed that E24 and T24 displayed differences in glycosylation sites – two for E24 and one for T24 (Supplementary Fig. S1), it can be seen from above analysis that they maybe share similar glycosylation pattern. The molecular weight showed by E24 protein on SDS-PAGE and Western blot was quite close to that one predicted, as similarly observed on T24 [12], which implied that the two proteins are possibly lightly glycosylated or not.

Our Western blot and immunohistochemistry results strengthened the above presumption from immunological profile experimentally that E24 should be a highly antigenic protein of *E. multilocularis* metacestodes. Western blot analysis showed that TRX-absorbed anti-recE24 serum had very strong, specific reactivity to purified recE24 and the ~25 kDa cyst-extracted antigen (Fig. 1B and C), while pre-immune serum was not reactive to both proteins and neither was anti-recE24 serum reactive to TRX (Fig. 1B and C). The immunohistochemistry test using anti-recE24 serum revealed that the body wall of the protoscolex-free and protoscolex-formed cysts of *E. multilocularis* metacestode were surrounded intensely by 'rings' (Fig. 2B and E), while there was no obvious label detected on host tissues (Fig. 2B and E). Pre-immunized serum served as a negative control, had no obvious reaction with any slices of fixed tissues (Fig. 2C and F). Microscopy images of H&E-stained metacestodes were presented as a normal reference (Fig. 2A and D). Each cell type in different tissues displays a specific pattern of expression of several tetraspanins [16]. According to reported literatures on the structure of *E. multilocularis* metacestodes [1–5], the intensely stained sections could be confirmed morphologically as 'germinal layer' of cyst or protoscolex, implying that E24 should be a very important protein for the further studies on host–parasite interplay [6,7,11] and for exploitation in diagnosis and prevention of echinococcosis using E24 protein [7,12].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2009.06.009.

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