3. 病因・病態と検査・診断

肝外胆道系に病変を起こす寄生虫について，病態や検査・診断法が類似するものをまとめて以下に記載した．病態や重症度は，寄生虫体の大きさや数によるところが大きいが，胆道系や小腸における寄生部位も重要で，特にVater乳頭付近に浮腫をきたすような場合は，少数寄生であっても急激に閉塞性黄疸を示すことがある．胆汁うっ滞や黄疸の程度は各感染例によってまちまちだが，原虫症で著しい黄疸を示すことは少なく，肝胆道系酵素の中ではalkaline phosphatase（ALP）が早期から上昇し，治療に際しても検査指標として利用できることが多い[5]．胆道系の画像検査については，他の肝胆道系疾患と同様，超音波検査やCT検査がまず利用されるが，経胆道的な処置や外科的な処置に先立ちERCPなどの内視鏡を利用しての検査もよく行われる[6]．回虫や肝吸虫・肝蛭など，比較的大きな寄生蠕虫については，超音波検査では胆道内の異物，造影検査では透亮像として直接検出される場合もある．超音波，CT，MRIなどによる画像検査は，診断のみならず治療後の経過観察にも利用されるが，肝外胆道系寄生虫症にのみ特異的とされる画像変化の所見はほとんどないので，肝外胆道系に病変を起こす寄生虫について，病歴からまずその可能性を認識するのが重要である[6,7]．

a. 肝吸虫，肝蛭

肝吸虫は，胆道系に寄生する体長1–2cmの吸虫で，ヒトに寄生する主な種としては，アジアでは（シナ）肝吸虫*Clonorchis sinensis* ‘the Chinese liver fluke’とタイ肝吸虫*Opisthorchis viverrini*が問題となり，東欧の一部にネコ肝吸虫*Op. felineus*がみられる．第1中間宿主の淡水貝からセルカリアが侵入し，第2中間宿主の淡水魚にメタセルカリアとなって寄生する．第2中間宿主としては，モロコ，タナゴ，ウグイなどコイ科やワカサギなどの淡水魚があげられ，これらの魚の不完全調理や生食により，メタセルカリアを摂取することで，ヒトへの感染が成立する．第1中間宿主はマメタニシで，1970年以前に全国的に行われた調査では，琵琶湖以外では東日本の河川や湖沼を中心に，淡水魚のメタセルカリア陽性率，マメタニシのセルカリア陽性率とも高くなる傾向がみられた[8]．現在，国内感染者は少なくなり相対的に輸入例が増加しているが，現在も，国内でイヌやネコを終宿主として，生活環が維持されていると思われる．

肝蛭も胆道系に寄生する大型の吸虫で，全世界に存在するが，本来の終宿主は，ウシやヒツジなどの草食性の反芻動物である．肝蛭は，発育に第2中間宿主を必要とせず，ヒメモノアラガイなどの淡水貝から出たセルカリアは，清流中の植物に付着してメタセルカリアとなり，偶発的にヒトに感染する．最近，肝蛭は，ヨーロッパ・オーストラリアのものは*Fasciola hepatica*，アジア・アフリカのやや大型のものは*F. gigantica*として区別され，日本産のものでは体長が5cm程度と大きい．

経口的に摂取されたメタセルカリアは，十二指腸内で脱嚢し，肝吸虫の場合は幼虫がVater乳頭から胆道系に侵入していくが，ヒトを本来の終宿主としない肝蛭は，幼虫移行症を起こし，いったん，小腸壁から腹膜を経て肝臓表面から，寄生部位である胆道に入る．したがって肝蛭症の場合は，急性症状として，発熱，じんま疹，腹部症状などがみられ，好酸球増多を示す場合が多い．肝吸虫や肝蛭の成虫は胆道系に寄生し，少数感染の場合症状に乏しいこともあるが，濃厚感染では閉塞性肝胆道系疾患，胆石などを起こす．肝膿瘍や閉塞性膵炎などの原因となることもあり，長期間にわたって感染が継続すると胆汁うっ滞型の肝硬変にまで発展することがある．

肝吸虫，ことにタイ肝吸虫の場合は，慢性的炎症刺激によって生じる胆管上皮の増殖性変化が，胆管癌へと変化することが確実とされる．最近は，タイ肝吸虫症での発癌は，他の原因による胆管上皮の発癌機構とは，かなり異なっていることが，癌遺伝子の研究や一酸化窒素（NO）による内因性ニトロ化に関する研究でも明らかになってきた[9,10]．一方，肝蛭については，慢性炎症が胆道癌と関連するという報告はない．

肝吸虫や肝蛭のメタセルカリア摂取後，胆道系症状の現れるまでの期間は一定しない．肝吸虫では，産卵は1カ月程度で開始され，成虫の寿命は3–10年とされる．濃厚感染の場合や胆道ジスキネジー様症状を示した場合は，比較的早期から症状を示すと思われるが，少数寄生の場合，慢性胆管炎による症状を示すには，かなり長期にわたる感染継続が必要になる．

胆道系の炎症や閉塞を示す血液検査所見以外に，胆道における病変の局在を知るうえで，画像検査が有用であることは，他の胆道病変と同じである．肝内胆管や総胆管の拡張をエコー，CT，ERCPなどで認めることが多い．特に，超音波検査では，胆管の拡張・胆石や胆管壁の肥厚といった所見以外に，肝蛭のように大きな寄生虫では，虫体自体もとらえることができる[11]．肝蛭の場合は，肝臓表面から胆道系に侵入した痕が，肝臓表面の不整な変化として認められることがある．確定診断には，糞便や胆汁，十二指腸液中の虫卵を検出できれば確実である．肝吸虫は産卵数が少ないので，集卵法(ホルマリン・エーテル法やAMS–III法など)を用いて，診断する必要がある．また，ELISAなどの免疫血清検査法が利用されることもある．ヒトを好適な終宿主としない肝蛭では，糞便中や胆汁中に虫卵が検出されることは少なく，免疫血清検査と画像所見で総合的に診断されることが多い．

b. 鉤虫，糞線虫などの消化管寄生線虫

土壌など環境中に生息するフィラリア型(F型)幼虫が，主として経皮的に感染する鉤虫や糞線虫は，虫卵が経口的に感染する回虫や鞭虫とともに，土壌伝播蠕虫(soil transmitted helminths: STHs)と総称される．STH感染症は，熱帯・亜熱帯の途上国においては，一般的な感染症で，世界中では全体で20億人以上の感染者がいると推定されている．ヒトに成虫が寄生する鉤虫としては，アメリカ鉤虫*Necator americanus*・ズビニ鉤虫*Ancylostoma duodenale*の2つが主な種だが，世界的にはアメリカ鉤虫の分布範囲の方が広い．また，経皮的ルート以外に，経口的ルートでも感染することが知られており，特にズビニ鉤虫のF型幼虫では，後者による感染が多い．侵入したF型幼虫は，血行性に肺に到達したあと，気管–食道粘膜から消化管に入り，十二指腸粘膜に吸着して成虫になったあと，宿主の血液を養分にして寄生する．排泄された虫卵は，外界の温暖で湿潤な環境下で孵化し，ラブジチス型(R型)幼虫，F型幼虫と変異して，ヒトへの感染能力を獲得する．糞線虫でも，F型幼虫が経皮感染し，肺に移動した後，小腸で成虫になって寄生生活を送るが，成虫が排泄した虫卵は宿主の消化管内で孵化し，R型幼虫になった段階で糞便中に排泄される．消化管内のR型幼虫は，外界に出ることなくF型幼虫へと脱皮・変異していくことがあり，糞線虫は，自家感染し体内でも増殖できるという点で，蠕虫の中で例外的存在である．

消化管に寄生する蠕虫にあっては，体内に寄生する成虫(特に雌虫)の数は，糞便中に排泄される虫卵数や幼虫数と直接関係する．宿主の小腸内で，消化中の栄養物でなく血球を栄養分とする鉤虫では，特に鉄の摂取が不十分な場合，寄生虫体数は貧血の進行にも大きく関係する．鉤虫や糞線虫に感染しても無症状で経過することはまれではないが，初期症状としてはF型幼虫の侵入に伴って皮膚の発疹がみられる．鉤虫の幼虫が肺を移行する際には，肺炎様の症状(Löffler症候群)を示すこともある．糞線虫症では，大量の幼虫の自家感染により，播種性糞線虫症といわれる致死的ともなる散布性の全身性症状が，初感染後数十年たった後でも，免疫不全を背景に起こることがある．世界的にみると，エイズに合併する糞線虫症が大きな問題だが，日本国内では，沖縄県と鹿児島県を中心に南西部が，成人T細胞性白血病(ATL)の原因であるヒトT細胞白血病ウイルス1型(HTLV–1)の浸淫地として知られており，糞線虫との重複感染がしばしば認められ問題となっている．免疫不全の状態になると，大量に増殖した糞線虫の幼虫(主にF型)や成虫が，腸管内のグラム陰性桿菌を保有して血中に移行し，播種性糞線虫症となり，敗血症，化膿性髄膜炎，細菌性肺炎などの重篤な合併症がみられることがある．胆道系合併症では，糞線虫の移動や増殖に伴って，小

腸内の病変が胆道系にも及ぶ胆道炎が問題となる12)．また，Vater乳頭付近に強い炎症から浮腫が起きると，総胆管起始部が閉塞することになるが，この変化は寄生虫体数よりも寄生部位によるところが大きく，糞線虫以外でも，鉤虫の少数寄生によっても生じることがある13)．鉤虫卵が胆汁中から検出されることもあるが，鉤虫の胆道内寄生による胆道炎を積極的に示唆する報告はない．

画像所見に関しては，糞線虫の多数寄生では，上部小腸の粘膜全体に変化が及び，腹部レントゲンでイレウス像，消化管造影では十二指腸から上部小腸の狭窄，狭小化，浮腫などを認める．内視鏡では粘膜の浮腫，発赤，管腔の狭窄などの所見を呈し，生検で糞線虫成虫を検出できる．このような例では，胆管壁でも全周性の肥厚や浮腫性変化が，超音波検査やCT検査で認められることが多い．

寄生虫学的診断に際しては，鉤虫症は糞便検査（厚層塗抹法，集卵法）で虫卵を検出する．低比重卵である鉤虫の集卵法には，ホルマリン・エーテル遠心沈殿法以外に飽和食塩水浮遊法も用いられる．幼虫感染後に虫卵が産下されるまでには，約2カ月を要する．なお，ズビニ鉤虫とアメリカ鉤虫の鑑別には，糞便内の虫卵を濾紙培養し，発育した幼虫の形態で判断する．糞線虫症の診断は，便から虫体（R型幼虫）を証明することによるが，ホルマリン・エーテル遠心沈殿法を併用しても，少数寄生の場合は検出できないことが多い．感度は，寒天培地の上に少量の糞便を置き，糞線虫幼虫の培地上での移動をみる普通寒天平板培地法が最も優れている14)．また，播種例では，喀痰からF型幼虫が検出されることがある．

c. ジアルジア（ランブル鞭毛虫）とクリプトスポリジウム

ジアルジア症，クリプトスポリジウム症とも，人獣共通感染症であり世界中でみられるが，特に熱帯・亜熱帯の途上国では一般的な感染症で，旅行者下痢症の代表的な病原体としてあげられている．ランブル鞭毛虫の生活環は栄養体と囊子に分かれ，栄養体は4対の鞭毛をもち運動性があり宿主の消化管内で無性生殖により増殖する．囊子には運動性はないが，糞便とともに体外に排出された段階で既に感染性を有しており，この囊子で汚染された水や食品などを介して感染が成立する．クリプトスポリジウム原虫のうちヒトで問題になるのは，主に*C. parvum*と*C. hominis*である．クリプトスポリジウム原虫のヒトへの感染型であるオーシスト（oocysts）は，消化されるとスポロゾイトを放出する．スポロゾイトは，宿主の消化管上皮細胞に侵入し細胞膜に入るが，実際に細胞質までは入らず，無性生殖と有性生殖を行って増殖する．糞便中には排出されるオーシストの数が，下痢便1gあたり100-1,000万と多いうえ，株によってはID_{50}が10程度と極めて感染性が強い15)．ランブル鞭毛虫の囊子やクリプトスポリジウム原虫のオーシストは，排出された段階で既に感染性を有しているので，食品や水，手洗いが不十分であれば手指や食器などから，容易に感染が拡大する．感染のリスクが高いグループとしては，非衛生な途上国からの帰国者，ケア施設などで手指衛生が保てない入所者，男性の同性愛者，動物を取り扱っている者などがあげられる．また，囊子やオーシストに対しては，通常の塩素消毒の効果が弱いので，先進国でも水系感染が問題となり，日本国内でも，クリプトスポリジウムの上水道を介した集団感染の事例は複数報告されている15,16)．

ジアルジア症の潜伏期は一定しないが，2-3週間程度が多い．激しい水様脂肪便から軟便まで程度は様々であり，慢性化する例もある．腹部膨満，上腹部痛，食欲不振，悪心・嘔吐，倦怠感などの急性胃腸炎症状を伴うことも多い．下痢を伴わない無症候性の場合でも，囊子の排出は続くので感染源となる．栄養体は腸管粘膜に組織浸潤することはないので，反応性の場合を除き血便となることはない．慢性化する例や再発を繰り返す例では，消化吸収不良や体重減少が問題となるが，このような例では，免疫不全などの基礎疾患について検索すべきである．クリプトスポリジウム症の潜伏期は5-8日程度で，水様性下痢，腹痛，嘔吐，微熱などの症

状で発症する．下痢の回数は多様だが，免疫能が正常であれば，長くとも2週間程度で自然に回復する．しかし，免疫不全者の場合は，感染しやすいうえ，慢性化し再発を繰り返すことが多い．CD4数が重症度に影響し，重症化した場合は大量の水様便による脱水と電解質異常が補正できず致死的となることもある．

ジアルジア症での胆管・胆嚢炎などの合併は，免疫不全者で多くみられるが，免疫系に異常を認めない例での報告例もある[17]．重症な下痢を示さず慢性の肝胆道系症状で，ジアルジア感染が明らかになった例は日本国内でも報告されている[18]．一方，クリプトスポリジウム症では，免疫不全者の例を除き感染が慢性化することはなく，肝胆道系合併症も，激しい下痢を示す感染者の小腸内で増殖したクリプトスポリジウム原虫が胆管内の上皮にも影響を及ぼすことによる．米国の報告でも，エイズでみられる胆道系病変の病原体として，クリプトスポリジウム原虫は，サイトメガロウイルスと並んで多いとされている[5]．

消化管原虫症の確定診断は，新鮮な下痢便の直接塗抹標本を顕微鏡で観察し，病原体を確認することが基本にある．ジアルジア症では，糞便中に嚢子か栄養体を同定するか，十二指腸や胆汁中に栄養体を同定することでなされる．嚢子は下痢・有形便ともに検出可能で，ホルマリン・酢酸エチル遠心沈殿法(MGL変法)などの集嚢子法にヨード染色法を併用することで検出率が高くなる．嚢子の排出は間欠的なために，数日間，検査を繰り返すのが望ましい．クリプトスポリジウム症の確定診断は糞便中のオーシストを検出することによる．通常の塗抹標本観察では原虫の確認が困難な場合もあり，遠心沈殿法やショ糖浮遊法により集オーシストを行い，蛍光抗体染色や抗酸染色などの染色標本を作製するとよい．ランブル鞭毛虫の嚢子やクリプトスポリジウムのオーシストを検出する際，蛍光抗体染色が最も感度がよい検査法で，海外では簡便な染色用キットも市販されている．また，嚢子やオーシストの内部構造観察には，微分干渉顕微鏡が適している．

その他，日本国内での報告例はほとんどないが，エイズなど免疫不全で胆道系合併症を起こす原虫や類縁生物として，イソスポーラ原虫(*Isospora belli*)やミクロスポリジア(microsporidia)などが知られている．これらの病原体による胆道病変の診断には，分子生物学的手法や免疫組織学的手法を用いた局在診断が必要なこともあり，診断・治療に難渋することが多い[19-21]．

4. 治療と予後

他の原因による胆道閉塞性病変と同じく，著しい閉塞性黄疸に対しては，経皮的・経胆道的なドレナージが行われる．胆道系の広い範囲に赤痢アメーバ性肝膿瘍や包虫性肝嚢胞が穿破した場合は，ショックに陥り重篤な病態になることもあり，時機を逸しない外科的処置が必要になることも多い[3,4]．下痢に対する水分補給，貧血に対する鉄剤投与といった支持的療法も重要だが，基本は，原因となっている寄生虫症の治療である．寄生虫症の治療については，‘寄生虫症薬物治療の手引き改訂(2010年)第7.0版’に詳しく，本稿中で触れたものについては表にしてまとめた[22](**表2，3**)．この‘手引き’には，各寄生虫症の病態や検査・診断についても概略が説明されており，たいへん参考になるが，日本寄生虫学会や厚労科研・ヒューマンサイエンス振興財団政策創薬総合研究事業‘熱帯病治療薬研究班(略称)’(http://www.med.miyazaki-u.ac.jp/parasitology/orphan/index.html)からダウンロードできる．使用の実際にあたっては，上記ホームページなども利用しながら，更に詳細な情報の入手に努めて頂きたい．日本国内での寄生虫症に対する治療薬は，国内承認薬で該当する寄生虫症に保険適応があるもの，国内承認薬だが保険適応がないもの，国内未承認薬だが効果が報告され国際的には評価が定まっているものなど，混乱しているので，実際に使用する際には注意が必要である．また，クリプトスポリジウム症など免疫不全が背景にある場合は，エイズに対するHAARTなど，免疫機能の改善を目指した治療も強化しなければならない．

表２　肝外胆道寄生原虫症に対する治療

	国内で市販されている薬剤の使用例（ただし，保険は未承認）	国内で市販されていない薬剤の使用例（“研究班”に依頼）
赤痢アメーバ症		
アメーバ性大腸炎	メトロニダゾール経口剤（フラジール） 1,500–2,000 mg/日，分3，7日間 チニダゾール経口剤（ハイシジン） 1,200 mg/日，分3，5日間	メトロニダゾール治療に応答しない場合 パロモマイシン（Humatin） 1,500 mg，分3，10日間
アメーバ性肝膿瘍	メトロニダゾール経口剤 1,500–2,000 mg/日，分3，10日間 チニダゾール経口剤 2,000 mg/日，分3，7日間	メトロニダゾール注射剤 500 mg，8時間ごと，7日間 メトロニダゾール注射剤 初回1,000 mg．その後，6時間ごとに500 mg
ジアルジア症	メトロニダゾール経口剤 750 mg/日，分3（小児では15–30 mg/kg/日），5–7日間 チニダゾール経口剤 2 g（小児では50 mg/kg），単回 アルベンダゾール 400 mg/日（22.5 mg/kg/日），分1，5日間	ニタゾキサニド（Alinia） 1 g/日，分2（小児では200–400 mg/日），3日間 パロモマイシン（Humatin） （研究班が保管するが，本疾患は対象外とされる）
クリプトスポリジウム症	アジスロマイシン（ジスロマック） 600 mg/日，分1，14日間 パロモマイシンと併用	ニタゾキサニド（Alinia） （研究班が保管し，免疫不全者のみ対象とする） 1 g/日，分2，14日間 （健常者では3日間で可） パロモマイシン（Humatin） （研究班が保管し，免疫不全者のみ対象とする） 1.5–2.25 g/日（25–35 mg/kg/day），分3，14日間 アジスロマイシンと併用

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表3　肝外胆道寄生蠕虫症に対する治療

	保険適応のある寄生蠕虫症と用量	保険適応のない寄生蠕虫症と用量
パモ酸ピランテル (コンバントリン)	回虫症　10 mg/kg，単回服用 鉤虫症　10 mg/kg，単回服用	
メベンダゾール (メベンダゾール)		回虫症　200 mg/日，分2，3日間 鉤虫症　200 mg/日，分2，3日間 上記を1クールとし陰性化まで
イベルメクチン (ストロメクトール)	糞線虫症　200 μg/kg/日，1日1回，朝食1時間前に服用．2週間後に再度同量を服用． 免疫不全状態や播種性糞線虫症では，陰性化するまで1–2週間隔で4回以上投与する．	
アルベンダゾール (エスカゾール)	エキノコックス症(包虫症) 600 mg/日，分3，28日間服薬した後，14日間休薬のサイクルを繰り返す．	有鉤囊虫症　15 mg/kg/日 (最大800 mg/日)，分2，8–30日間
トリクラベンダゾール (Egaten)		肝蛭症　10 mg/kg 1回服用　食直後 20 mg/kg，分2　食直後(重症例)
プラジカンテル (ビルトリシド)	肝吸虫症　20–40 mg/kg/日，分2，3日間 または　75 mg/kg，分3，1日間	消化管寄生条虫症(成虫寄生の場合) 20 mg/kgを1回服用後，下剤を併用 有鉤囊虫症　50 mg/kg/日，分3，30日間

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熱帯病・寄生虫症に対する研究班保管国内未承認薬

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研究班の目的・沿革●

観光，企業活動，学術調査，途上国援助など種々の形で国際交流が活発化し，大量航空機輸送の発達と相まって，日本からの海外渡航者や海外長期滞在者が増加しつつある．そして，渡航目的国としては熱帯・亜熱帯地域や途上国も増えており，熱帯病・寄生虫症に罹患する日本人も増加しつつある．したがって，国内においてもそれらの治療薬剤の医療上の有用性は高くなっているが，患者数が収益性に見合うほど多くはないので，国内製薬企業は新規薬剤の開発に積極的でない．その問題が1980年当時の厚生省薬務局審査課を中心に検討され，研究班を発足させて国内未承認薬を導入し，熱帯病・寄生虫症患者に対して適切な治療を提供することを目指した．

その結果，1980年に厚生省研究事業「輸入熱帯病の薬物治療法に関する研究班」(代表者：東京大学医科学研究所・田中　寛)が発足し，クロロキン，スルファドキシン/ピリメタミン合剤(当時，国内未承認薬)，キニーネ注，プリマキンなどの抗マラリア薬を含む15種類の国内未承認薬の保管を開始した．また，国立衛生試験所(現：国立医薬品食品衛生研究所)でそれらの薬剤の品質検査を行い，わが国の製剤基準に合致することを確認してから使用することとした．その後の研究班の母体は，厚生省新薬開発研究事業，厚生省オーファンドラッグ研究事業，創薬等ヒューマンサイエンス総合研究事業(代表者：東京慈恵会医科大学・大友弘士，その後，宮崎大学・名和行文)，厚労科研費補助金政策創薬総合研究事業(代表者：名和行文，その後，木村幹男)と変遷を重ね，現在の研究班は，平成22年4月に発足した厚労科研費補助金創薬基盤推進研究事業「国内未承認薬の使用も含めた熱帯病・寄生虫症の最適な診療体制の確立」(代表者：木村幹男)である．なお，本研究班は略称で「熱帯病治療薬研究班」とも呼称される．

研究班の活動●

現在では，抗マラリア薬のアトバコン/プログアニル合剤，アーテメター/ルメファントリン合剤，抗赤痢アメーバ薬のメトロニダゾール注，パロモマイシンその他，種々の疾患に対する治療薬も導入し(**表1, 2**)，わが国で発生する患者に対して，欧米先進国並みのレベルで治療が行える体制の構築を目指している．さらに近年，全国の医療従事者からの診断や治療に関する問い合わせにも対応し，症例の相談においては，血液塗抹顕微鏡像，CT/MRIなどの画像，皮膚症状の写真などを添付した電子メールを通じて症例検討を行ってきた．また，ほぼ3年に一度，「寄生虫症薬物治療の手引き」(現在は改訂7.0版)を出版して，各種学術集会などで広く配付した．さらに，医療従事者に対する有用な情報提供の場として，研究班ホームページ[1)]を更新しているが，そこには「寄生虫症薬物治療の手引き」の電子版を掲載し，随時その電子版の改訂を行うなど，わが国における熱帯病・寄生虫症の総合的ネットワークとしての役割を果たしてきた．

薬剤使用後には，主治医からの治療報告書の提出を求めるが，それらの記載内容を検討し，必要に応じて主治医に詳細を問い合わせ，有効性と安全性に重点をおいた解析を行っている．最近，筆者・木村が中心となり，合併症のない熱帯熱マラリアにおけるアトバコン/プログアニル合剤[2)]，中等症～重症の赤痢アメーバ症におけるメトロニダゾール注[3)]の使用経験をまとめ，雑誌に発表した．また研究報告書レベルでは，マラリアにおけるアーテメター/ルメファントリン合剤，肝蛭症におけるトリクラベンダゾールの有効性と安全性を報告し，リーシュマニア症におけるスチボグル

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- **熱帯病治療薬研究班(略称)は熱帯病・寄生虫症の総合的ネットワークである．**
- **研究班保管薬剤の使用にあたっては，倫理指針を遵守する必要がある．**
- **研究班保管薬剤は，あらかじめ登録された医療機関で使用する．**

表１　研究班が保管する抗マラリア薬

一般名	商品名(含量)	疾患	用法・用量(成人を基本)	備考
クロロキン	Avloclor (250 mg 塩=155 mg 塩基)	三日熱，卵形マラリア(いずれも急性期治療)，四日熱マラリア	クロロキン塩基にして初回 10 mg/kg，6，24，48 時間後にそれぞれ 5 mg/kg	熱帯熱マラリアでは薬剤耐性のため，ほとんど使われない．三日熱マラリアでも耐性が出現している
プリマキン	Primaquine (7.5 mg 塩基)	三日熱，卵形マラリア(休眠原虫に対する根治療法)	プリマキン塩基にして 15 mg/日，14 日間．低感受性が予想される三日熱マラリアでは 30 mg/日，14 日間	G6PD 欠損では禁忌
アトバコン/プログアニル合剤	Malarone (250 mg/100 mg)	熱帯熱マラリア(非重症例)	4 錠を 1 日 1 回，3 日間	欧米では治療のみならず，予防にも評価が高い
アーテメター/ルメファントリン合剤	Riamet (20 mg/120 mg)	熱帯熱マラリア(非重症例)	4 錠を 0，6，24，36，48，60 時間後に投与	欧米では治療薬としての評価が高い
キニーネ注	Quinimax (250 mg 塩基/2 ml)	熱帯熱マラリア(重症例)	キニーネ塩基として 1 回量 8.3 mg/kg を 200～500 ml の 5% ブドウ糖液あるいは生理食塩液に希釈し，4 時間かけての点滴静注を 8～12 時間ごとに繰り返す	重症度が高い場合，初回のみ倍量の負荷投与量 (loading dose) も考慮
アーテスネート坐薬	Plasmotrim Rectocaps (200 mg)	熱帯熱マラリア(重症例)	1 日目 200 mg を 2 回，2～5 日目それぞれ 200 mg/日を直腸内投与	上記のキニーネ注が使用不可能なときに緊急避難的に使用

クロロキン，プリマキン，キニーネでは，塩あるいは塩基としての表示がありうることに注意．治療量については，塩基として示すのが原則．

コン酸ナトリウム，クリプトスポリジウム症におけるニタゾキサニド使用例の解析も行っている．

国内未承認薬とその使用基準●

研究班が保管する国内未承認薬は，わずかな例外を除いて先進国で承認されており，標準的薬剤と位置づけられる．これらの薬剤を使用する基準は患者に対する最大限の利益であり，国際的標準からすると研究的な使用とは考えられない．しかし国内未承認薬であるために，健康被害に対して副作用被害救済制度が適用されない問題がある．そして，臨床研究に際しては厚生労働省「臨床研究に関する倫理指針(平成 20 年 7 月 31 日)」[4]の遵守が求められている．本倫理指針では，「通常の診療を超えた医療行為」は「介入」として扱われ，臨床研究保険に加入することが義務づけられた．研究班保管薬剤を用いることは国際的標準では通常の診療の範囲内と判断されるが，わが国では国内未承認薬であることから，「介入」として扱うべきである．本稿が出版されるころには，本研究班において臨床研究保険の契約がなされているはずである．

研究班保管薬剤の使用が可能となるのは，以下のいずれかの場合である．

1) 当該疾患/病態に対して国内承認薬がなく，研究班保管の国内未承認薬による治療が必要と判断される場合．

2) 当該疾患/病態に対する国内承認薬があるが，効果や副作用を勘案し，国際的標準に照らしても国内未承認薬のほうを選択すべきであると判断される場合．

3) 当該疾患/病態に対して国内承認薬を用いたが，効果あるいは副作用の面から，国内未承認薬

- いくつかの抗マラリア薬では，塩としての表記と塩基としての表記がある．
- メトロニダゾール注は重症赤痢アメーバ症での使用価値が高い．
- ニタゾキサニドは免疫不全者でのクリプトスポリジウム症を対象とする．

表2　研究班が保管する抗マラリア薬以外の主要薬剤

一般名	商品名（含量）	疾患	用法・用量（成人を基本）	備考
メトロニダゾール注	Flagyl Inj.（500 mg/バッグ）	赤痢アメーバ症（経口投与不能例）	500 mg を 8 時間ごと，7 日間	筆者らにより，重症例での優れた効果が示されている
パロモマイシン	Humatin（250 mg）	赤痢アメーバ症（根治療法）	1,500 mg/日・分 3，10 日間	効果の判定が難しい
スルファジアジン	Sulfadiazine（500 mg）	トキソプラズマ症	エイズ患者の脳炎では，スルファジアジン 4～6 g/日・分 4，ピリメタミン初日 200 mg/日・分 2，その後 50～75 mg/日の併用で，症状が軽快してからも 4～6 週間	ロイコボリンを併用
ピリメタミン	Daraprim（25 mg）	トキソプラズマ症		
ニタゾキサニド	Alinia（500 mg）	クリプトスポリジウム症（免疫不全者）	1～2 g/日・分 2，14 日間	難治性のジアルジア症にも使われる
スチボグルコン酸ナトリウム	Pentostam（100 mg/m*l*）	リーシュマニア症	内臓型，粘膜皮膚型では 20 mg/kg を 1 日 1 回静注あるいは筋注，28 日間．皮膚型では 10～20 mg/kg を 1 日 1 回局注，静注，あるいは筋注，10 日間（あるいはそれ以上）	内臓型では薬剤耐性が問題になりつつある
ミルテフォシン	Impavido（50 mg）	リーシュマニア症（内臓型）	100 mg/日・分 2，28 日間	エイズ患者では長期の服用が必要
トリクラベンダゾール	Egaten（250 mg）	肝蛭症	10 mg/kg を食直後に単回服用，重症例では 20 mg/kg・分 2（食直後）	本研究班における使用で，優れた効果がみられている
スラミン	Germanin（1 g/バイアル）	アフリカトリパノソーマ症（別名，睡眠病）（ローデシア型の早期）	初めに 100 mg の試験的静注，その後 20 mg/kg（最大 1 g）を 0，3，7，14，21 日に計 5 回	発熱，発疹，消化器症状などの副作用．ガンビア型の早期ではペンタミジン
メラルソプロール	Arsobal（180 mg/5 m*l* バイアル）	アフリカトリパノソーマ症（ローデシア型の後期）	1 日目 1.2 mg/kg，2 日目 2.4 mg/kg，3 および 4 日目 3.6 mg/kg の静注．7 日間の休薬をおいて 3 回繰り返す（計 26 日間）	毒性は高度で，2～10％に脳症を生じ，うち 50％近くが死亡
エフロールニチン	Ornidyl（200 mg/m*l*）（100 m*l* ボトル）	アフリカトリパノソーマ症（ガンビア型の後期）	100 mg/kg の静注を 6 時間ごと，14 日間	貧血，消化器症状，けいれんなどの副作用
ニフルチモックス	Lampit（120 mg）	アメリカトリパノソーマ症（別名，シャーガス病）	8～10 mg/kg/日・分 4，3～4ヵ月間	小児では 15 mg/kg/日まで増量可能

による再治療が必要と判断される場合．

薬剤使用の実際●

研究班保管薬剤の使用にあたっては，以下の手順に従う．薬剤の使用は登録された機関（薬剤使用機関）（研究班ホームページに掲載）で行う．患者の容態などから薬剤使用機関への搬送が不可能な場合などで，人道的観点から緊急避難的使用として一般の医療機関で薬剤を使用せざるを得ない場合，研究班員（木村幹男，三浦聡之，丸山治彦のいずれか）から許可を得なければならない．

1）薬剤使用機関の責任者は，研究班作成の「薬剤使用説明書」を患者に渡し，それを元に，患者

- 三日熱マラリアのプリマキン療法では，投与量が多くなりつつある．
- 欧米では，合併症のない熱帯熱マラリアの治療に合剤が多く使われる．
- キニーネ注の投与では，心毒性に注意が必要である．

が自由に質問できる状況下で十分な説明を行う．

2)「薬剤使用承諾書」に患者の署名を得る．

3)「薬剤使用登録書」を東京大学医科学研究所の関係者に郵送，ファクス，あるいは電子メール添付で送付する．

4) いわゆる重篤有害事象がみられたら，直ちに「重篤有害事象報告書」を東京大学医科学研究所の関係者にファクス送付する．

5) 治療終了後は一定期間内に，「治療報告書」(マラリア用，非マラリア用)を東京大学医科学研究所の関係者に送付する．

抗マラリア薬治療の概説●

紙面の都合上，マラリアに限って治療の概説を行う．三日熱，卵形マラリアの急性期治療，四日熱マラリアの治療においては，クロロキンを用いるのが世界的標準である．ただし，三日熱マラリアでは軽度であるがクロロキン耐性が出現していることに注意する．また，三日熱，卵形マラリアで急性期治療の後に，再発予防の目的でプリマキンを用いるが，三日熱マラリアではプリマキン低感受性が増えている．そのため，従来のプリマキン塩基15 mg/日・14日間に代わり，倍量にあたる30 mg/日・14日間が多く使われつつある．

合併症のない熱帯熱マラリアで原虫数が多くなければ，国内承認薬のメフロキン，キニーネ末(＋ドキシサイクリン)も選択肢の一つであるが，前者の薬剤では精神神経系副作用が出やすく，耐性も増えており，後者の薬剤では忍容性の問題がある．欧米ではアトバコン/プログアニル合剤，アーテメター/ルメファントリン合剤が多く使われている．

合併症を有する熱帯熱マラリア(重症マラリア)，あるいは原虫数が多い熱帯熱マラリアでは非経口投与を選択し，キニーネ注が第一選択薬である．ただし，心伝導障害などでは禁忌となり，投与中も心電図のモニターが必要である．重症度が高い場合，倍量の負荷投与量(loading dose)も考慮するが，心毒性にはより十分な注意が必要である．キニーネ注が禁忌の場合，入手不可能な場合などでは，緊急避難的なアーテスネート坐薬の使用も考慮するが，注射薬よりも効果の発現は遅いこと，吸収にばらつきが出る可能性にも注意する．これら非経口投与により，赤血球感染率＜1％で経口摂取が可能となれば，それぞれキニーネ経口薬，アーテスネート経口薬(国内での入手は困難)にスイッチし，非経口投与と経口投与とを合わせて7日間用いる5)．最近では，同一薬剤による経口投与の替わりにメフロキン，アトバコン/プログアニル合剤，アーテメター/ルメファントリン合剤のいずれかを投与することも多くなっている．ただし，キニーネ注の後にメフロキンを用いる場合には，前者の投与終了後12時間以上経ってからとする．

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Schistosomicidal and antifecundity effects of oral treatment of synthetic endoperoxide N-89

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ABSTRACT

1,2,6,7-Tetraoxaspiro[7.11]nonadecane (N-89) is a chemically synthesized compound with good efficacy against malaria parasites. We observed strong anti-schistosomal activities of N-89 both *in vitro* and *in vivo*. In a murine model with experimental infection of *Schistosoma mansoni*, orally administered N-89 at the dose of 300 mg/kg resulted in a significant reduction in worm burden (63%) when mice were treated at 2-weeks postinfection. Strong larvicidal effects of N-89 were confirmed *in vitro*; schistosomula of *S. mansoni* were killed by N-89 at an EC50 of 16 nM. In contrast, no significant reduction in worm burden was observed when N-89 was administered at 5 weeks postinfection *in vivo*. However, egg production was markedly suppressed by N-89 treatment at that time point. On microscopic observation, the intestine of N-89-treated female worms seemed to be empty compared with the control group, and the mean body length was significantly shorter than that of controls. Nutritional impairment in the parasite due to N-89 treatment was possible, and therefore quantification of hemozoin was compared between parasites with or without N-89 treatment. We found that the hemozoin content was significantly reduced in N-89 treated parasites compared with controls ($P<0.001$). The surface of adult worms was observed by scanning and transmission electron microscopy, but there were no apparent changes. Taken together, these observations suggested that N-89 has strong antischistosomal effects, probably through a unique mode of drug efficacy. As N-89 is less toxic to mammalian host animals, it is a possible drug candidate against schistosomiasis.

1. Introduction

Schistosomiasis is a parasitic disease caused by trematode flatworms of the genus Schistosoma that is common in many tropical countries and affects more than 200 million people living in conditions of poor sanitation and/or with less developed social infrastructure [1–3]. The World Health Organization (WHO) is leading the global strategy of schistosomiasis control, with a focus on morbidity control through chemotherapy. Praziquantel (PZQ) is a safe and effective drug for schistosomiasis and has been the drug of choice since the late 1970s. This has raised concerns about the development of drug resistance, and suggestive cases of PZQ-resistant parasites have been reported in *Schistosoma mansoni* from African countries [4–6]. Therefore, the development of new antischistosomal drugs is a matter of priority, and new candidate compounds have been reported [7–9].

Artemisinin-derivatives (ADs) are compounds extracted from the plant *Artemisia annua* used in traditional Chinese herbal medicine, which has strong malaricidal effects [10–13]. Recent studies clearly showed that this compound also have strong effects against schistosome parasites [14,15]. The most notable difference between PZQ and ADs is the developmental stages of the parasite at which the drugs show efficacy [16,17]. Adult worms are highly sensitive to PZQ, while the larval stages are less sensitive to the drug [18,19]. On the other hand, ADs are effective mainly against the larval stage parasites, while adult worms are less sensitive to treatment with these drugs. In this sense, PZQ is a therapeutic drug, while ADs are drugs for prophylaxis [20]. Therefore, it is recommended to use a combination of the two drugs [21,22].

Although the mechanism of the efficacy has not yet fully been elucidated, peroxide bridge is necessary for antimalarial activities of ADs [10]. Previously, we reported that synthetic endoperoxide (1,2,6,7-tetraoxaspiro[7.11]nonadecane: N-89) [23] has high antimalarial activity against *Plasmodium falciparum in vitro* and *Plasmodium berghei in vivo*, and it shows low levels of cytotoxicity in mice and rats (LD50: >2000 mg/kg) [23–25]. ADs are structurally complicated and their chemical synthesis is

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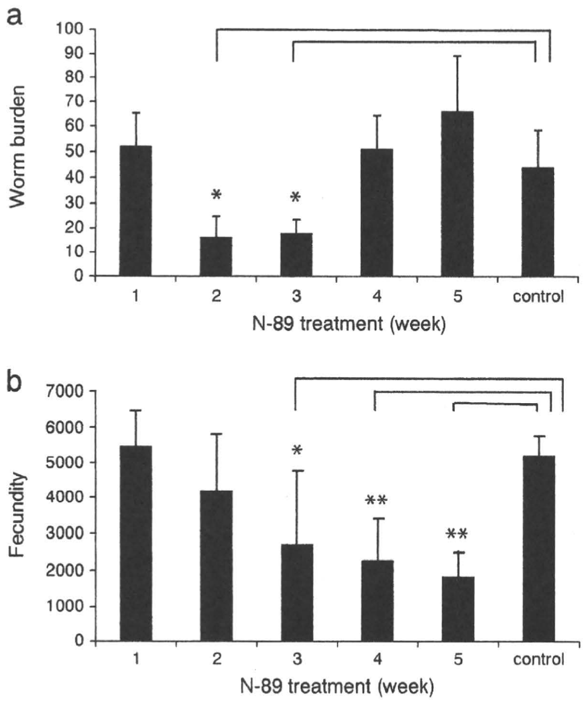

Fig. 1. *In vivo* effects of N-89 to *S.mansoni*. *S. mansoni*-infected mice were orally treated with N-89 from week 1 through week 5 postinfection. (a) Y-axis shows the number of worms that were collected by perfusion 9 weeks postinfection (*$P<0.001$). (b) Y-axis shows the number of eggs produced per female worm. (*$P<0.05$, **$P<0.001$).

not easy. On the other hand, N-89 is a compound with a relatively simple structure and is inexpensive to mass produce [23–25]. If N-89 also has strong effects against schistosome parasites, this will allow a new strategy of schistosomiasis control using a lower cost agent.

In this study, we found strong effects of N-89 against *S. mansoni* both *in vitro* and *in vivo*. The efficacies of N-89 were almost comparable to those of ADs. However, N-89 had additional effects that were not reported in the case of ADs, suggesting that N-89 may be a novel compound with unique antischistosomal activities.

2. Materials and methods

2.1. Parasites and animals

Puerto Rican strain *S. mansoni*, which was kept in our laboratory, was used for the present study. Female 5-week-old BALB/c mice were purchased from CLEA (Tokyo, Japan).

2.1.1. In vivo treatment of S. mansoni-infected mice with N-89

For *in vivo* study, mice were infected with 180 cercariae by the standard method in which mice were percutaneously exposed *via* the tail to cercariae for 1 h at room temperature [14]. BALB/c mice infected with *S. mansoni* were orally treated with N-89 suspended in olive oil at a dose of 300 mg/kg twice a day for two consecutive days. Mice were divided into 6 groups and treated with N-89 at various time points, *i.e.*, from week 1 through week 5 postinfection. To analyze parasite egg burden, eggs were recovered from the liver and intestine by the method reported previously [26]. Briefly, chopped liver and intestine were digested in 4% KOH at 37 °C for 1 h. After incubation, the digested samples were centrifuged at 1500 rpm for 5 min at room temperature, and pellets were resuspended in distilled water. Eggs were counted under a light microscope. Effects on pathological lesions after N-89 treatment were determined by observation of egg granulomas formed in the liver. Liver sections of Azan staining were prepared, and granuloma size was measured by using Image J image processing software (NIH). The mean size of 100 granulomas formed around a single egg in N-89 treated mice was compared to that in control (olive oil-treated mice). In addition, we calculated the body length of the worms using Image J. All *in vivo* experiments were approved by the Committee of Animal Rights and Ethics, Tokyo Medical and Dental University.

2.1.2. In vitro treatment of S. mansoni with N-89

As N-89 seemed to be effective against larval stage parasites, we prepared schistosomula from the lungs of mice and incubated them in

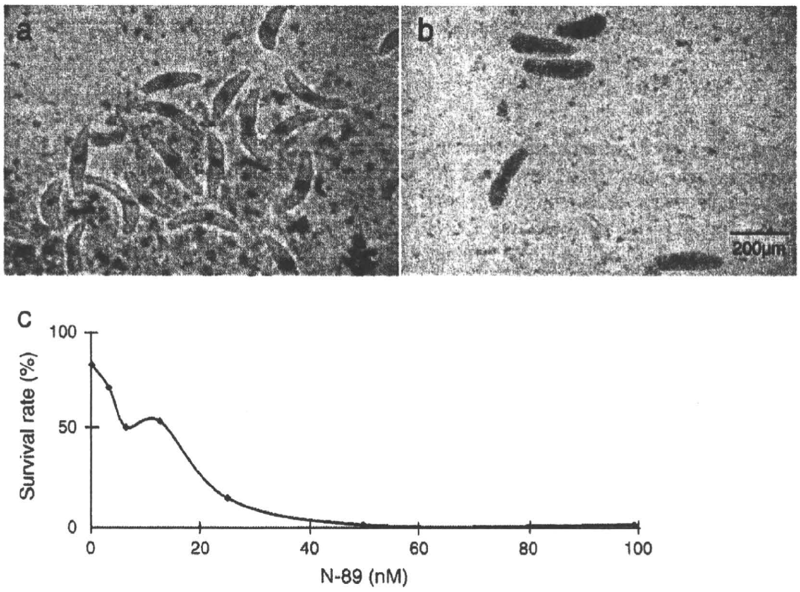

Fig. 2. Schistosomicidal effects of N-89 *in vitro*. (a) 14-day schistosomula were round-shaped and in a state of continuous contraction and extension when they are alive in the medium containing DMSO (2.5%) alone. (b) Schistosomula treated with 50 nM of N-89 were stiff and easily stained with trypan-blue. (c) Y-axis indicates the survival rate of 14-day schistosomula after treatment with serial dilutions of N-89.

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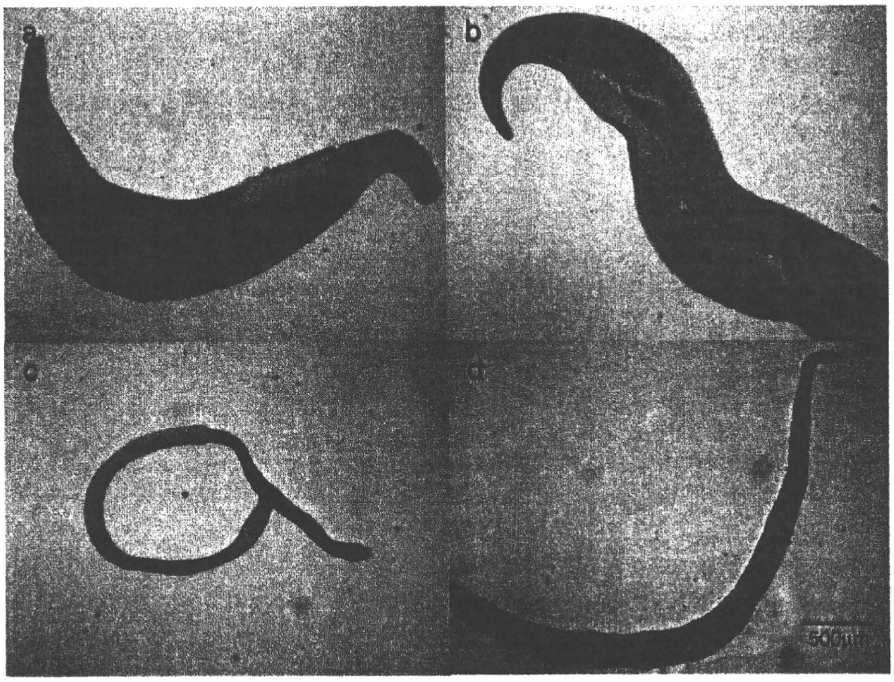

Fig. 3. Light microscopic observation of adult parasites after *in vivo* treatment with N-89. *S. mansoni*-infected mice were treated with or without N-89 5-weeks postinfection. Worms were collected 2 weeks after the treatment. 7-week *S. mansoni* worms were stained with hematoxylin–carmine solution. A male worm from mice treated with N-89 (a), a male worm from control mice (b), a female worm from mice treated with N-89 (c), a female worm from control mice (d).

RPMI-1640 (Wako, Osaka, Japan) supplemented with 10% FBS (JRH Biosciences, Kansas, MO), 150 U/ml of penicillin, and 150 μg/ml of streptomycin (Gibco, Gaithersburg, MD) in 24-well plates (Greiner, Ulm, Germany). N-89 was dissolved in dimethylsulfoxide (DMSO) and added 25 μl to the plates which contains 1 ml of RPMI at various concentrations from 3.12 to 100 nM. Plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 7 days. Survival of the treated schistosomula was determined by trypan blue dye-exclusion test. Based on the observations, we calculated the EC_{50} of N-89 against schistosomula of *S. mansoni in vitro*.

2.2. Morphological observation of adult parasites after treatment with N-89 in vivo

To observe the morphological changes after N-89 treatment, infected BALB/c mice were administered orally with N-89 at 5 weeks post-infection at a dose of 300 mg/kg, and 2 weeks later adult worms were recovered by portal perfusion. Recovered parasites were washed thoroughly with 0.85% NaCl and 0.45% Na-citrate in distilled water, and paired worms were fixed in 70% ethanol and stained with hematoxylin–carmine solution for light microscopic observation. Parasites were observed by scanning electron microscopy and transmission electron microscopy (Hitachi, Tokyo, Japan) according to the method reported previously [27,28].

2.3. Quantification of hemozoin contents of *S. mansoni*

Hemozoin was extracted from *S. mansoni* and quantified by the method reported previously [29–31]. Protein contents of worm homogenates were measured using a protein assay kit (Bio-Rad, Hercules, CA). Infected mice were administered orally with N-89 (300 mg/kg) at 5 weeks postinfection, and 2 weeks later adult parasites were tested for hemozoin contents. The worms used for the tests were paired to compare worms in the same/similar developmental stages. For each experiment, 15 to 30 worms were used from each mouse. Worms were homogenized in 1 ml of PBS (pH 7.2), and centrifuged for 10 min at 10,000 × g. Insoluble pellets were washed with 0.1 M sodium hydrogen carbonate, and then dissolved in 0.1 N NaOH. Hemozoin was converted to heme in this treatment, and we then measured the converted heme as hemozoin in accordance with the reagent manufacturer's protocol (Hemin, Sigma-Aldrich, St. Louis, MO). Heme was quantified spectrophotometrically by measuring absorbance at 405 nm. Hemozoin content in the parasite was expressed as ng heme/mg protein.

2.4. Statistical analysis

Statistical analyses were performed by Student's *t* test. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Schistosomicidal effects of N-89 in vivo

Reduction of worm burden was observed when mice were treated 2 or 3 weeks postinfection, and the maximum effect of N-89 driven reduction in worm burden was observed at 2 weeks postinfection compared with the olive oil control group (Fig. 1a). Schistosomicidal effects became less apparent at 3 weeks postinfection, and there was no detectable reduction in worm burden when mice were treated at 5 weeks postinfection. However, egg production per paired female worm was significantly reduced when mice were treated with N-89 at 5 weeks postinfection. Reduction in egg production per female worm in the N-89-treated group was statistically significant in comparison to the olive oil control group (Fig. 1b). These observations indicated that the larval stage is the target for the killing effect of N-89, while this agent showed inhibitory effects on fecundity of adult worms without killing the parasite.

3.2. In vitro effects of N-89 for schistosomula of *S. mansoni*

To confirm the direct effects of N-89 against the larval stage of *S. mansoni*, schistosomula were treated with serial dilutions of N-89 and

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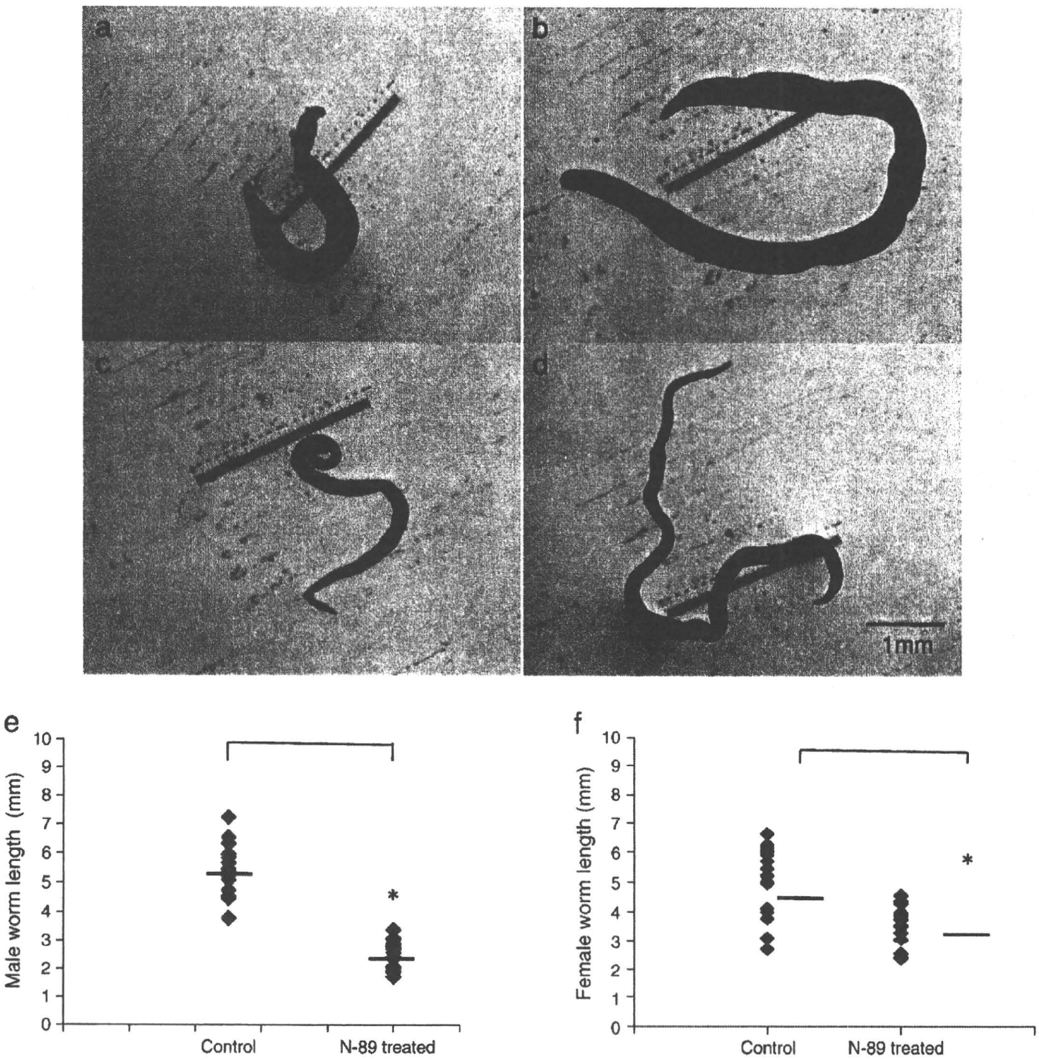

Fig. 4. The mean body length of the worms. All worms used were obtained in the same manner as described in Fig. 3. *Y*-axis indicates the length of male worms (a) (**P*< 0.01) and female worms (b) (**P*<0.01).

cultured for 7 days *in vitro*. The schistosomicidal effects of N-89 were dose-dependent, and the EC_{50} against *S. mansoni* larvae was calculated as 16 nM (Fig. 2a–c). During the observation period, all schistosomula were alive and active under culture conditions containing DMSO alone (data not shown).

3.3. *Pathological changes in the liver in infected mice treated with N-89*

The sizes of granulomas formed around single schistosome eggs in N-89 treated mice was compared to that in control animals. The liver pathology of the mice treated at 5 weeks postinfection showed significantly smaller granulomas compared with controls ($P<0.001$) (data not shown).

3.4. *Morphological changes of N-89 treated adult worms*

To observe morphological changes of the parasite after N-89 treatment *in vivo*, we compared morphological profiles of the adult worms with or without N-89 treatment. The most obvious difference was noted in the intestine of female worms on light microscopic observation. Briefly, the dense substances, probably hemozoin, disappeared in N-89-treated worms (Fig. 3a–d). Furthermore, the mean body length of the treated worms was smaller than that of untreated controls (Fig. 4a–f). On TEM observation, the tegument morphology was compared between parasites with and without N-89 treatment. In both males and females, there were no marked differences between N-89-treated worms and control worms (Fig. 5a–d). In the SEM profiles, we found small surface changes, such as the disappearance of tubercles on the surfaces of males and shortened spines on females, but these changes were not as severe as the findings of previous studies for PZQ and ADs [28,32] (Fig. 5e–h).

3.5. *Heme contents of adult parasites with and without N-89 treatment*

As hemoglobin is the main source of nutrition for adult female worms, we measured hemozoin contents of parasites with and without N-89 treatment to examine whether nutritional impairment occurred in N-89-treated parasites. In the N-89-treated group, the mean heme content was 15 nmol heme/mg protein, while it was 89 nmol heme/mg protein in the untreated controls; this difference in heme content was statistically significant ($P<0.001$) (Fig. 6).

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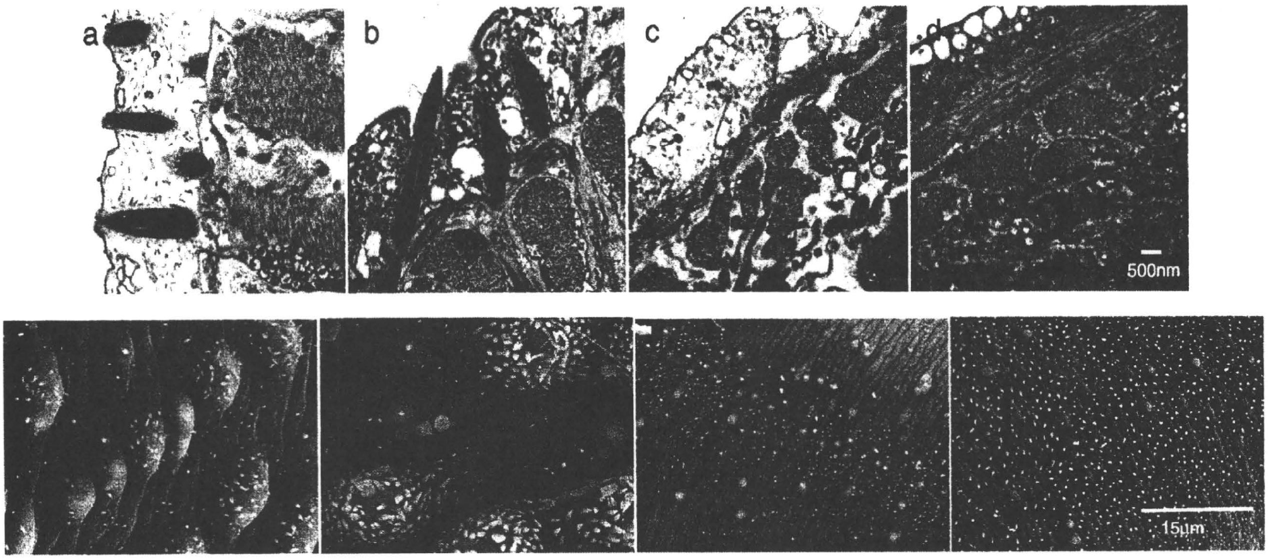

Fig. 5. EM observation of *S. mansoni* adult worms. All worms used were obtained in the same manner as described in Fig. 3. TEM observation of a male worm from mice treated with N-89 (a), a male worm from control mice (b), a female worm from mice treated with N-89 (c), and a female worm from control mice (d). SEM observation of a male worm from mice treated with N-89 (e), a male worm from control mice (f), a female worm from mice treated with N-89 (g), and a female worm from control mice (h).

4. Discussion

Rational drug design should be applied to develop new agents for use against schistosomiasis. As PZQ is the only drug available for controlling disease activity, the appearance of drug-resistant strains is a non-negligible concern. New drug candidates must be developed to address this concern, and ADs are promising candidates for this purpose. However, it should be noted that ADs are used for malaria therapy because of the recent WHO recommendation for use of artemisinin-based combination therapy (ACT). ADs are drugs prepared from plant materials. Due to their structural complexity, these compounds are not easy to chemically synthesize, and the distribution of the product depends on the supply of herbal plant materials. On the other hand, mass production of N-89 is not difficult, and it can be prepared at a much lower cost than ADs. No serious toxicity has been noted for N-89 in animal [23–25]. As N-89 is effective for reducing egg fecundity but not worm burden when it is administered 5 weeks post infection, it can supplement the effect of praziquantel that is effective for reducing worm burden.

The results of the present study suggest that N-89 is a novel antischistosomal compound with a unique mechanism of action compared to other drugs used to combat schistosomiasis, such as PZQ and ADs. Due to the structural similarity, we postulated that N-89 would have both antimalarial and antischistosomal effects in the same manner as observed for ADs. However, reference to previous publications regarding ADs indicated that there were marked differences in its anti-schistosomal effects. That is, N-89 showed two modes of antischistosomal effect — larvicidal effects and antifecundity effects. Previous reports have indicated no such dual modes of drug efficacy for ADs [17]. Thus, it is possible that N-89 has functions distinct from those of ADs.

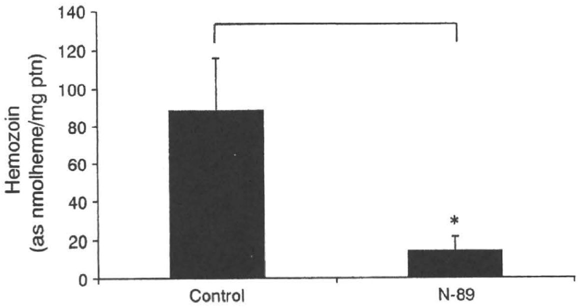

Fig. 6. Heme contents of adult parasites obtained after *in vivo* treatment with N-89.7-week worms collected in the same manner described in Fig. 3 were examined for quantification of hemozoin contents. Y-axis indicates the hemozoin contents (as nmol heme/mg protein) (*$P<0.001$).

It is still necessary to elucidate the detailed mechanisms of action for the two different effects of N-89. Considering the presence of endoperoxide structures in N-89, it is possible that oxygen stress generated by N-89 may be a factor involved in the schistosomicidal effects. Recent studies demonstrated the importance of the redox system for parasite survival [33,34]. However, no direct evidence in support of this possibility is available, nor killing effect of the worms was observed when *Sm*-infected mice were treated with N-89 at 5 week postinfection. In spite of this situation, we observed the reduction of egg fecundity. Morphological observations in the present study suggested that N-89 treatment induce nutritional deficits in the worms, as heme contents in N-89-treated female worms were significantly reduced compared to controls. This may be related to the antifecundity effect of the drug against female worms. It is well discussed that host hemoglobin derived from the host blood is essential for growth, development and reproduction of schistosomes [35,36]. It is possible that N-89 inhibits a process for hemoglobin usage in female worms, and more direct evidence may be obtained by testing the effects of N-89 on the biological pathways involved in hemoglobin uptake. It has been suggested that proteolysis of hemoglobin was important for worm development in male and female, and production of yolk protein in developing egg was also important for female worm [37]. The two modes of drug efficacy in N-89 raise questions regarding why the larval stages were destroyed, while the adult stage was resistant to this drug. In other cases, such as vaccine efficacy, lung stage parasites are the targets for the killing effects [7], although these are immune-mediated mechanisms. Analysis of the direct target molecules for N-89 could provide valuable information for the development of therapeutic strategies. Studies to elucidate these points using other approaches, such as proteomic analysis, are currently underway in our laboratory.

In conclusion, N-89 is a promising compound for use as an antischistosomal drug, which may supplement the effects of PZQ

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through mutually different modes of efficacy. Strategies using N-89 as supplemental effect for praziquantel or ADs would be helpful to avoid the development of drug-resistance. Therefore, N-89 is a good candidate partner for its efficacy, safety, and its low cost of mass production.

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Detection of Early and Single Infections of *Schistosoma japonicum* in the Intermediate Host Snail, *Oncomelania hupensis*, by PCR and Loop-Mediated Isothermal Amplification (LAMP) Assay

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Abstract. Polymerase chain reaction (PCR) with the specific primer set amplifying 28S ribosomal DNA (rDNA) of *Schistosoma japonicum* was able to detect genomic DNA of *S. japonicum*, but not *S. mansoni*, at 100 fg. This procedure enabled us to detect the DNA from a single miracidium and a snail infected with one miracidium at just 1 day after infection. We compared these results with those from loop-mediated isothermal amplification (LAMP) targeting 28S rDNA and found similar results. The LAMP could amplify the specific DNA from a group of 100 normal snails mixed with one infected snail A PCR screening of infected snails from endemic regions in Anhui Province revealed schistosomal DNA even in snails found negative by microscopy. PCR and LAMP show promise for monitoring the early infection rate in snails, and they may be useful for predicting the risk of infection in the endemic places.

INTRODUCTION

Schistosomiasis japonica is a relatively neglected tropical disease, and it is a chronic zoonotic parasitic disease in China, the Philippines, and small pockets of Indonesia.[1] In China, the causative organism, *Schistosoma japonicum*, and its intermediate snail host, *Oncomelania hupensis*, are distributed along the Yangtze River valley and recently, in the hilly and mountainous regions of Sichuan Province.[2] Since the mid-1950s, the People's Republic of China has markedly decreased the prevalence of schistosomiasis through mass-chemotherapeutic treatment and the control of the intermediate snails.[3,4] However, a complete eradication of this disease is difficult in endemic areas. The estimated prevalence in the provinces of Hunan, Hubei, Jiangxi, Anhui, Yunnan, Sichuan, and Jiangsu was 4.2%, 3.8%, 3.1%, 2.2%, 1.7%, 0.9%, and 0.3%, respectively, in 2004.[5] A total of 564, 207, 83, and 57 acute cases of *S. japonicum* infection were reported nationwide in 2005, 2006, 2007, and 2008, respectively.[6] These findings suggest that control measures must be improved among at-risk populations, especially in lake and marshland regions. A new integrated strategy was tested for the control of schistosomiasis in China.[7,8] It involved the reduction of infectious sources by the replacement of water buffaloes with tractors for agricultural work, improved access to clean water and general sanitation, better livestock management through fencing to isolate schistosomal egg sites, and better feces management using newly constructed latrines on-shore. These strategies markedly reduced the infection rate in both humans and intermediate snails in the pilot areas. Remarkably, the prevalence of infected snails reportedly decreased to almost 0% in some areas.[8] To maintain these successes, it may be useful to use new snail-monitoring systems in such areas.

Molecular tools such as conventional polymerase chain reaction (PCR) and improved DNA amplification methods have been shown capable of detecting schistosome DNA in a variety of samples. A highly repetitive, 121-base pair (bp) sequence has been used to detect DNA from *S. mansoni* and *S. haematobium* in stool, serum, urine, and plankton samples.[9–13] Because no similar repetitive sequence has been found in the *S. japonicum* genome, the repetitive non-long terminal repeat (LTR) retrotransposon SjR2[14] was used for DNA detection as a target sequence.[15] In an experimental rabbit model, the SjR2 sequence was detected in serum (1 week after infection) and stool samples using a PCR assay, and the 230-bp band of SjR2 was absent at 10 weeks after treatment with praziquantel,[16] and real-time PCR was applied to the detection of SjR2 gene from cercaria in an environmental water sample.[17] Alternatively, real-time PCR was also applied to the detection of a mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase I gene at low intensity in an infected pig model.[18] Another highly repeated sequence, 28S ribosomal DNA (rDNA), was used for multiplex PCR to detect a distinct *Schistosoma* sp. from human urine samples.[19]

Loop-mediated isothermal amplification (LAMP) is a simple, sensitive, and rapid DNA detection method.[20] The LAMP reaction requires only a single enzyme, *Bst* DNA polymerase, that can synthesize a new strand of DNA while simultaneously displacing the former complementary strand, thereby enabling DNA amplification at a single temperature. The LAMP reaction can be achieved using four primers (FIP, BIP, F3, and B3), two of which (F3 and B3) contribute to the formation of a stem-loop structure, whereas the other two (FIP and BIP), designed complementary to the inner sequence of the stem-loop structure, are used for amplification of the target sequence. This provides a higher specificity to the reaction than conventional PCR methods.[20] The LAMP assay has been widely applied for diagnosis and detection against several infectious diseases, including *Plasmodium*,[21] *Trypanosoma*,[22] *Leishmania*,[23] and *Taenia*.[24] In the present application, LAMP targeting to SjR2 for detecting the DNA from *S. japonicum* was also reported.[25]

In the present study, we evaluated the performance of the PCR method by comparing SjR2 and 28S rDNA from

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S. japonicum. Next, we detected the schistosomal DNA from experimentally infected snails at 1 day after infection and detected schistosomal DNA from wild snails collected from endemic areas of Anhui Province in China. We also applied a LAMP assay to detect infected snails on-site in endemic local areas. Finally, we developed a simple, rapid, and safe screening method for determining the infection rate of snails in endemic areas after implementation of the above-described integrated strategy and detected infections using the LAMP assay with DNA extracted from a large number of snails.

MATERIALS AND METHODS

Parasites and snails. *S. japonicum* was maintained using ICR mice as a final host and *O. hupensis nosophora* from a non-endemic area (Yamanashi strain) as an intermediate host. The livers from infected mice were digested with 1 mg/mL collagenase and 0.5 mg/mL actinase, and then, purified eggs were put into water to hatch the miracidia. The collected miracidia were experimentally infected to each snail in a 96-well plate. Wild snails from endemic areas in China (*O. hupensis hupensis*) were collected from three places in Anhui Province as follows: (1) Shankou-city (30.52° N, 116.93° E) in marshland regions of Anquine county, (2) Shun'an town (30.56° N, 117.54° E) in the sand regions of the Yangtze River in Tongling county, and (3) Guanghui City (30.56° N, 117.45° E) in the marshland regions of the Yangtze River in Tongling county. Figure 1 presents detailed locations about each area. The snails were picked up in Anquine in March 2007 and in Tongling in September 2007. The collected snails were crushed and checked for infection under microscopy before preparation for DNA extraction.

DNA extraction. To detect schistosomal DNA by PCR and LAMP assay, we applied the DNA extraction method using heated NaOH.[26] Briefly, the counted miracidium was put into a 200-µL volume of 50 mM NaOH and heated at 95°C for 30 minutes. After centrifugation, the 50-µL supernatant was recovered and then mixed to an equal volume of 1 M Tris-HCl (pH 8.0). This solution was directly used as a template (1 µL) for the PCR and LAMP methods. For direct extraction from a single infected snail (non-endemic area), each snail was also put into a distinct tube, and 200 µL of 50 mM NaOH solution was added to the tube. After crushing the snail with tweezers, the DNA was extracted using the above procedures. A large-scale DNA extraction from different numbers (100, 50, 25, 10, 5, and 1) of snails from non-endemic area was also performed with 10 mL of 50 mM NaOH in a 50-mL tube that was heated at 95°C for 60 minutes. After neutralization with 1 M Tris-HCL (pH 8.0), 1 µL of the solutions was directly used as a template. Genomic DNA of *S. japonicum* was purified from adult worms using the Get-pure DNA Kit (Dojindo, Kumamoto, Japan), and the concentration of DNA was measured with a spectrometer.

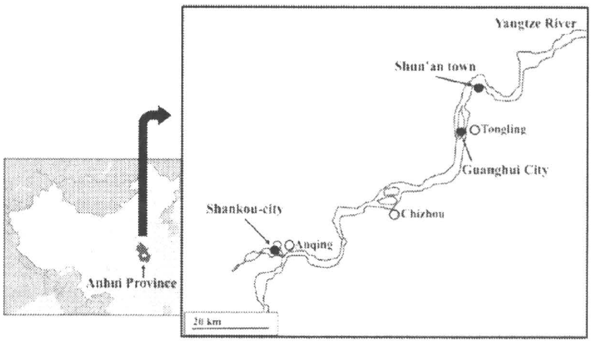

FIGURE 1. Schema of the selective areas for snail sampling in Anhui Province of China. Three points located along the Yangtze River in Anhui Province are shown as closed circles, and the capital of each country is shown as an open circle. Shankou-city in Anquine county and Guanghui City in Tongling county were marshland regions, and Shun'an town in Tongling county was in the sand regions.

Primer sets. To amplify the specific DNA of *S. japonicum*, the 28S rDNA gene (GenBank Accession No. Z46504) was selected as a target sequence. For the conventional PCR and LAMP methods, we designed specific primer sets (Table 1). As in the previous report, SjR2 (GenBank Accession No. AF412221) primers were generated for conventional PCR[16] and the LAMP assay[25] (Table 1). The LAMP primer sets were prepared to be high performance liquid chromatography (HPLC) purification grade.

PCR and LAMP assay. The PCR solution (20 µL) was prepared with a standard procedure using Top polymerase (BIONEER, Daejeon, Korea). The reaction consisted of 35 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. The PCR products were resolved by agarose gel electrophoresis and stained in ethidium bromide. The LAMP method was performed according to the manufacturer's instructions (Eiken Sci, Tokyo, Japan), except for use of the 20-µL total reaction mixture. The LAMP reaction was performed at a constant 65°C. The amplification of the target gene was confirmed based on the turbidity of magnesium pyrophosphate and by gel electrophoresis.

RESULTS

Sensitivity and specificity of PCR and LAMP assay. To determine the sensitivity of the PCR and LAMP methods, we performed the reactions using *S. japonicum* genomic DNA from 10 pg to 10 fg, respectively, by serial dilution. As shown in Figure 2, PCR using specific primers amplified the band of 405 bp from 28S rDNA, and the PCR method was able to detect more than 100 fg of genomic DNA (Figure 2A). The LAMP assay had the same level of sensitivity as the conventional PCR assay (Figure 2B). Furthermore, both methods amplified only DNA from *S. japonicum* and none from *S. mansoni*. Thus, our methods distinguished the *S. japonicum* species from others. However, PCR using SjR2 primers detected DNA at the level of 1 pg (Figure 2A), whereas LAMP did not detect the SjR2 gene at all, contrary to a recent report[25] (data not shown). Taken together with these results, we performed the following experiments using 28S rDNA primers as the appropriate targeting genes because of higher sensitivity.

Detection of the schistosomal DNA from miracidia and infected snails. To confirm whether a single miracidium DNA could be detected by the PCR and LAMP assay using 28S rDNA primers, we extracted DNA using the heated NaOH method from one miracidium and performed both methods with 10 independent samples. The PCR and LAMP detected the DNA from one miracidium in all samples (Figure 3A and B), indicating that the total DNA included in a single miracidium was enough to be amplified by both the PCR and LAMP methods. Furthermore, we performed the infection experiment with the intermediate snail with a different number

TABLE 1

Specific primer sets used in this study

PCR	Sj28S
	Forward primer; 5′-GGTTTGACTATTATTGTTGAGC-3′
	Reverse primer; 5′-TCTCACCTTAGTTCGGACTGA-3′
	SjR2[16]
	Forward primer; 5′-TCTAATGCTATTGGTTTGAGT-3′
	Reverse primer; 5′-TTCCTTATTTTCACAAGGTGA-3′
LAMP	Sj28S
	F3 primer; 5′-GCTTTGTCCTTCGGGCATTA-3′
	B3 primer; 5′-GGTTTCGTAACGCCCAATGA-3′
	FIP primer; 5′-ACGCAACTGCCAACGTGACATACTGGTCGGCTTGTTACTAGC-3′
	BIP primer; 5′-TGGTAGACGATCCACCTGACCCCTCGCGCACATGTTAAACTC-3′
	SjR2[25]
	F3 primer; 5′-GCCGGTTCCTTATTTTCACAAGG-3′
	B3 primer; 5′-CTAACATAATTTTATCGCCTTGCG-3′
	FIP primer; 5′-CTACGACTCTAGAATCCCGCTCCGCGAATGACTGTGCTTGGATC-3′
	BIP primer; 5′-CCTACTTGATATAACGTTCGAACGTATTGGTTTGAGTTCACGAAACGT-3′

of miracidia and extracted total DNA from each snail at 1 day after the infection. As a result, we found four positive samples out of a total of five samples infected with one miracidium, although all samples were positive in the five samples infected with 5 or 10 miracidia, respectively (Figure 3C). We considered that one negative snail was not penetrated by a miracidium, because not all miracidia could enter the snail. These results showed that the PCR detected the schistosome-specific band

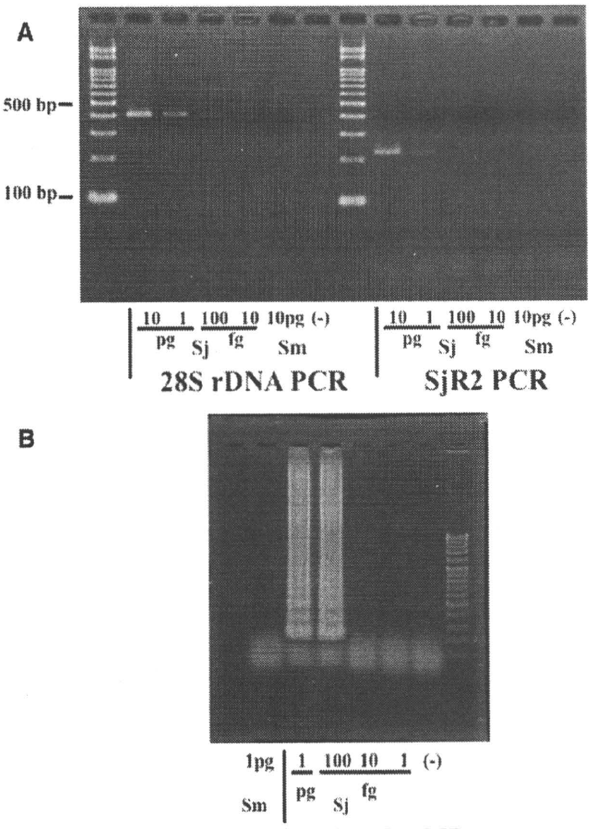

FIGURE 2. Sensitivity of the PCR and LAMP methods using genomic schistosomal DNA comparing 28S rDNA with SjR2 primers. (**A**) PCR was performed with different weights of genomic DNA, and the 28S rDNA primer set was able to detect 100 fg of DNA from *S. japonicum* but none from *S. mansoni*; the SjR2 primer set was able to detect just 1 pg of DNA. (**B**) The LAMP assay method showed the same sensitivity (100 fg) as the PCR method. Neither method reacted to DNA from *S. mansoni*, and no template (-) was the negative control.

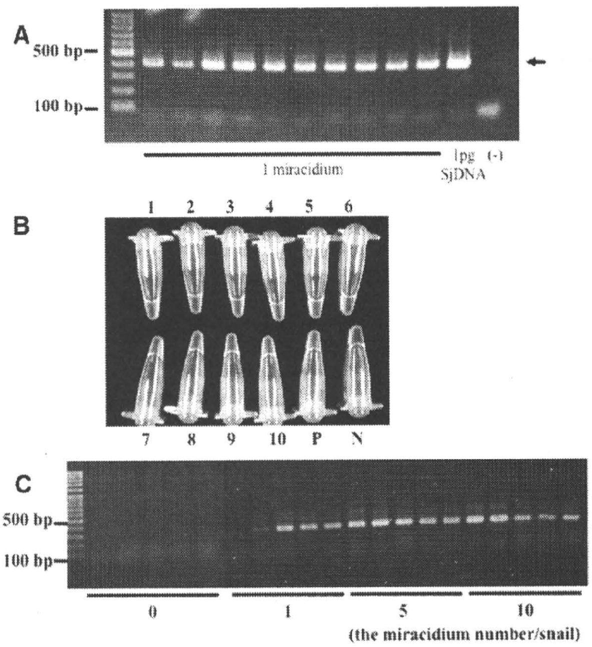

FIGURE 3. Detection of a schistosome-specific band in genomic DNA extracted from naked miracidia and the experimentally infected snail by PCR and LAMP. (**A**) The DNA extracted from one miracidium was amplified by PCR. PCR detected the specific band (arrow) in each of 10 samples extracted distinctly from one miracidium but not the no-template sample (-). Genomic DNA (1 pg) of *S. japonicum* was used for the positive control. (**B**) The DNA extracted from one miracidium was amplified by LAMP assay. LAMP showed the positive results as the white turbidity of magnesium pyrophosphate in all 10 samples extracted distinctly from one miracidium (1–10) and Sj DNA (1 pg) as positive control (P) but not the no-template sample (N). (**C**) Each snail from the non-endemic area was experimentally infected with a different number of miracidia (0, 1, 5, and 10 miracidia/snail), and genomic DNA was extracted from each snail at 1 day after infection. The PCR method detected the schistosome-specific band in DNA from a snail infected with just one miracidium without amplifying DNA from non-infected snails. Each lane represents a distinct snail infected with the same number of miracidia.

TABLE 2
The comparison of detection rate between the PCR assay and microscopy method in wild snails from Anhui Province

	Shankou-city in Anquine		Shun'an town in Tongling		Guanghui city in Tongling	
	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative
PCR positive	10	13	2	0	0	0
PCR negative	0	217	0	72	0	48
Positive rate of microscopic examination	4.2%		2.7%		0%	
PCR positive rate	9.6%		2.7%		0%	

in the DNA extracted from the infected snail with a single miracidium. Furthermore, using the same DNA prepared from the snails infected with a single miracidium of *S. japonicum*, the result of the LAMP method was consistent with that of the PCR method (data not shown). Thus, the PCR and LAMP methods have the high specificity and sensitivity and detect schistosomal DNA immediately after the infection to the snail host.

Detection of the schistosomal DNA in wild snails collected from endemic areas. To evaluate whether the PCR assay could detect schistosomal DNA from the infected snails in the endemic areas, we collected wild snails from three points, Shankou-city, Shun'an town, and Guanghui City of Anhui Province in China (Figure 1), in which the human infection rate is 4%, 0%, and 1.6%, respectively. As shown in Table 2 in snails collected from Shankou-city during the spring, the PCR method detected more positive snails than did the microscopy method with the observation of *S. japonicum* cercaria. Although all positive snails by microscopy were also positive by PCR, PCR also amplified the DNA of *S. japonicum* in the snails negative by microscopy. This indicates that PCR could detect the infection not only in the matured cercaria but also in the early sporocyst. However, in snails from Tongling collected in the autumn, PCR detected DNA only from the snails positive by microscopy.

Screening with large-scale DNA extraction from the infected snail by LAMP assay. The PCR method is difficult to use in the field in endemic areas because of the expense of the thermal cycler and the impracticality of performing gel electrophoresis and staining. To amplify the specific DNA without such problems, we applied the LAMP method, which can be performed at a constant temperature and the result can be determined without gel electrophoresis. The LAMP detected schistosomal DNA from a single miracidium of *S. japonicum* (Figure 2B) and the snail infected with a single miracidium (data not shown). Thus, the LAMP method should be useful for the detection of specific DNA in the field without the need for a thermal cycler or gel electrophoresis. We also screened the rate of infected snails in local areas using large-scale DNA extraction. Different numbers (99, 49, 24, and 4) of non-infected snails from non-endemic areas were prepared, and a single infected snail (1 day after infection with 10 miracidia) was mixed in each group. The snails were crushed together, genomic DNA was extracted in one tube, and each sample was assayed by the LAMP method. LAMP detected 28S rDNA of *S. japonicum* from all infected groups but not non-infected groups (Figure 4), indicating that it is useful for detecting schistosomal DNA from a large number of snails in the field in endemic areas.

DISCUSSION

Schistosomiasis-control activities in China since the mid-1950s have decreased the prevalence of human infection with *S. japonicum* to less than 10%.[27,28] Furthermore, a new integrated strategy was developed and proven effective in endemic areas.[7,8] However, the complete eradication of schistosomiasis japonica and the prevention of its reemergence remain difficult. To monitor the infection rate and distribution of infected snails, we developed molecular detection tools based on the amplification of nucleic acid.

PCR targeting 28S rDNA amplified 100 fg of genomic DNA from only *S. japonicum* and none from *S. mansoni*. The ribosomal DNA was known to have a highly repetitive sequence in the genome,[18,29,30] and each region has been shown to be useful for molecular diagnosis and identification of species

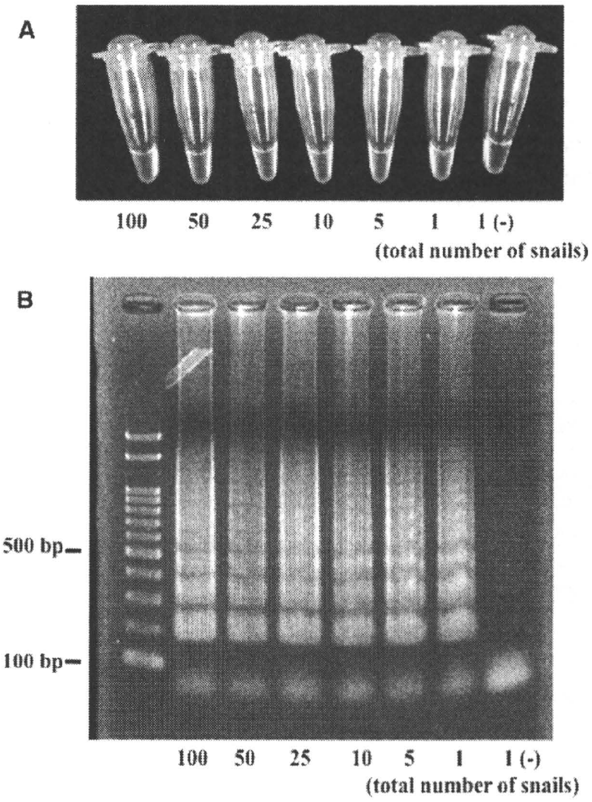

FIGURE 4. Detection of 28S rDNA from *S. japonicum* by LAMP assay in the total DNA from different numbers of non-infected snails artificially contaminated with a single infected snail. The snails infected with 10 miracidium were prepared and mixed with different numbers of snails (99 + 1, 49 + 1, 24 + 1, 9 + 1, 4 + 1, 0 + 1; normal + infected snails). Total DNA was extracted from each group and one non-infected snail (-), and the LAMP assay was performed. The 28S rDNA was amplified from all samples contaminated with the infected snail but not from non-infected snails by the LAMP assay. The results were confirmed based on the white precipitation (**Upper**) and gel electrophoresis (**Lower**).