（6）テスト対象銘柄一覧

分類	銘柄(No.)	商品名	製造者又は販売者名	含有成分	使用方法	うたい文句等
マルチパーパスソリューション	1	コンプリートダブルモイスト	エイエムオー・ジャパン㈱	1mL中、塩酸ポリヘキサニド0.001mg含有 界面活性剤、緩衝剤、安定化剤、等張化剤、粘弾剤 表示指定成分：ユデト酸塩	①洗浄 レンズを眼からはずし手のひらにのせ、本剤を数滴つけて、レンズの両面を各々、20～30回指で軽くこすりながら洗います。 ②すすぎ 洗ったレンズの両面を本剤でよくすすぎます．※こすり洗いとすすぎは必ず正しく行ってください。汚れと細菌を除去します。 ③消毒・保存 専用ケースに本剤を満たし、その中にレンズを完全に浸し、ケースのフタをしっかり締めます。そのまま4時間以上放置して消毒は完了です。レンズは、すすがずに、そのまま装用できます。 使用後の専用ケースは空にして、本剤でよく洗った後、自然乾燥してください。	・1本でうるおい実感&目にやさしい。 ・タンパク汚れも落とす ・目の健康を考えた、目にやさしいうるおいヴェールで、快適な装用感を実現。 ・その秘密は『うるおいヴェール』。レンズ周囲に作られる「うるおいヴェール」によってレンズの乾きを防ぎ、快適なつけ心地が持続します。 ・すっきり！高い洗浄・消毒効果。優れた洗浄・消毒効果を発揮。レンズの汚れをしっかり落とします。 ・視界クリア！優れたタンパク除去効果。タンパク汚れを落とし、クリアな視界を実現します。 ・レンズをしていないみたい！自然の涙に近い。自然の涙に近い性状で目にやさしい。レンズをしていないかのようなナチュラルなつけ心地です。 ・もちろん、これ1本で簡単！1本ケア．「洗浄・すすぎ・消毒・保存」がこれ1本。簡単ケアシステムです。
	2	バイオクレンゼロ	㈱オフテクス	【有効成分】1mL中塩酸ポリヘキサニド0.001mg含有 【配合成分】安定剤、緩衝剤、等張化剤、pH調整剤、界面活性剤、ポリリジン、ヒプロメロース、ヒアルロン酸ナトリウム 【表示指定成分】ホウ酸 本剤はポリオキシエチレンポリオキシプロピレングリコールを含有しています。	使用方法 ◎レンズを取り扱う前には、必ず石鹸等で手をきれいに洗ってください。 1．洗浄 目からレンズをはずし手のひらにのせ、バイオクレン ゼロを数滴つけて、レンズの両面を各々、20～30回指で軽くこすりながら洗ってください。 2．すすぎ 洗ったレンズの両面をバイオクレン ゼロでよくすすいでください。 3．消毒・保存 レンズケースにバイオクレン ゼロを満たし、レンズを完全に液中に沈めてからふたをしっかりしめてください。そのまま4時間以上放置すると消毒が完了します。消毒後、レンズを取り出しそのまま装用してください。 使用後のレンズケースは、水道水でよく洗った後、自然乾燥させてください。	・レンズのうるおい、ずっと続く。 ・ヒアルロン酸ナトリウム配合 ・ヒアルロン酸入りレンズうるおう1本ケア コンタクトレンズにはさまざまな汚れが付着します．レンズを清潔にし、快適な装用感を保つためには毎日の正しいケアが必要です。バイオクレンゼロは1本で洗浄・すすぎ・消毒・保存ができ、さらにタンパク汚れの付着を防止する働きもプラスしたソフトコンタクトレンズ用消毒剤です。 ・レンズうるおう、初めてのヒアルロン酸入り1本ケア ヒアルロン酸ナトリウムを、ソフトコンタクトレンズ用消毒剤で初めて採用。レンズ装着時のクッション性も高く、さらにレンズのうるおい効果が持続します。 ・くもり・ゴロゴロ感の原因、タンパク汚れをブロック ポリリジンがレンズ表面に作用し、レンズのくもり・ゴロゴロ感の原因であるタンパク汚れの付着を防止するため、1日中快適な装用感が得られます。
	3	シードウソフトケア	日油㈱ ㈱シード	有効成分　100g中、20%塩酸ポリヘキサニド液0.5mg含有 配合成分　湿潤剤、等張化剤、緩衝剤、粘弾化剤 表示指定成分　不使用	使用方法 ※レンズを取り扱う前には、手指を必ず石けんでよく洗い、よくすすぎます。 1．こすり洗い 目からはずしたレンズを手のひらの上にのせ、本剤を数滴たらし、レンズの両面を各々20回程度、指の腹で軽くこすり洗いします。 2．すすぎ レンズの両面を充分な量の本剤で、よくすすぎます。 3．消毒 レンズケースに本剤を満たし、レンズを完全に浸します。レンズケースのキャップをしっかりと締め、4時間以上放置すると消毒は完了です。レンズは、すすがずにそのまま装用できます。 ※使用後のレンズケースは液を捨て、本剤でよく洗った後、自然乾燥させてください。	・洗浄力×うるおい力　うるおいバリアで汚れをブロック ・すぐれた洗浄・消毒力とWのうるおい力。しかもうるおい層のバリアで、汚れをよせつけません。 ・レンズをクリアに保つ洗浄・消毒力。　レンズのくもりの原因となる、タンパク質や脂質などの汚れや細菌類を、すっきり除去。高い洗浄・消毒効果でレンズを清潔に保ち、視界をクリアに。 ・Wのうるおいで、乾燥を防ぐ。　「リピジュア」と「HPMC」のダブルのうるおい成分を配合。すぐれた保水性で、レンズ表面にうるおい層をつくり、乾燥から守ります。 ・うるおい層のバリアが、汚れをブロック。　うるおい層のバリア効果で、汚れをシャットアウト。さらに、一度落とした汚れがレンズに再付着するのを防ぐので、快適な装用感をもたらします。 ・高い安全性で、瞳にやさしい。　塩化カリウム配合で涙に近い性状だから、瞳にやさしい使い心地です。表示指定成分である界面活性剤や防腐剤などを一切含まず、高い安全性を実現しました。 ・うるおい成分リピジュア　涙液成分をモデルに開発された、医薬品・化粧品・人工臓器などにも用いられる高分子うるおい成分。レンズをうるおい層でつつみ込みます。 ・高分子うるおい成分「リピジュア」配合！「リピジュア」は、ヒトの細胞膜を構成し、涙液にも含まれる成分（リン脂質）をモデルに開発された、医薬品・化粧品・人工臓器などにも用いられる高分子うるおい成分。乾燥しにくく、快適な装用感を保ちます．また、汚れの付着を抑えてクリアな視界を保ちます。
	4	フレッシュルックケア　10ミニッツ	チバビジョン㈱	＜有効成分＞1mL中に塩酸ポリヘキサニド0.001mg含有 ＜配合成分＞界面活性剤（ポリオキシエチレンポリオキシプロピレングリコール）、安定化剤、緩衝剤、等張化剤、pH調整剤 ＜表示指定成分＞ユデト酸塩	使用方法 ソフトコンタクトレンズを取扱う前に必ず石けんで手をよく洗います。 ステップ1／洗浄 目からはずしたソフトコンタクトレンズを手のひらにのせます。ソフトコンタクトレンズ表面に本剤を3滴以上滴下し、各面20回以上ずつていねいにこすり洗いをします。 ステップ2／すすぎ こすり洗いしたソフトコンタクトレンズの両面を本剤で10秒間以上すすぎます。 ステップ3／消毒・保存 レンズケースに本剤を満たし、レンズを完全にひたし、ケースのフタをしっかりと締めます。そのまま10分以上放置すると消毒が完了します。消毒後のレンズはそのまま装用できます。 ●使用後のレンズケースは本剤ですすぎ、自然乾燥させてください。	・これ1本で洗浄・消毒　10分で完了 ・10分できれいなレンズにリセット ・10分ケアだから…レンズが曇ったり、ゴロゴロしたり、レンズの不快を感じたら、いつでもどこでも快適レンズへスピードリセット。こすり洗い後、10分浸けるだけ。消毒・洗浄が完了します。 ●学校やオフィスの休み時間に ●長時間パソコンを使用したときに。 ●毎日のケアに。もちろん一晩浸けてもOK！ ・フレッシュな装用感 ●ステップ1　汚れをきちんと除去　ポロクサマー・EDTA・リン酸塩の3つの成分が効果的にタンパク汚れを除去し、レンズをきれいにします。 ●ステップ2　レンズに涙を引き寄せる　ポロクサマーが涙を引き寄せ、レンズに涙のベールを作るからうるおいを保ちます。

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マルチパーパスソリューション	5	オプティ・フリープラス	日本アルコン㈱	1mL中塩化ポリドロニウム0.011mg含有、安定化剤（エデト酸塩）、界面活性剤、緩衝剤（ホウ酸）、等張化剤、pH調整剤	ステップ1ー洗浄 あらかじめ石けんなどで手をよく洗っておきます。レンズをはずし、手のひらの上にのせ、本剤を数滴たらし、レンズの両面を各々20秒ほど指でこすり洗いします。 ステップ2ーすすぎ レンズの両面を本剤で20秒以上、または完全に残留物が取り除かれるまで十分にすすぎます。 ステップ3ー消毒・保存 レンズケースに本剤を満たし、レンズを完全に浸し、ケースのふたを完全に締めます。4時間以上放置しておけば消毒が完了し、そのままレンズを装用できます。 <レンズ装用の前に> レンズケースから取り出したレンズの表面に異物などが残っているときは、本剤でレンズを軽くすすいでください。 レンズ装着後、レンズケースは空にして、新しいオプティ・フリープラス（MPS）でよく洗った後、かならず自然乾燥させてください。	・ひとみに安心　レンズをもっと清潔に ・うるおいと洗浄の新成分プラス ・新成分でレンズにうるおい　新成分の作用がレンズ表面に涙の膜をつくりだしうるおい感のあるつけごこちをもたらします。 ・デュアルアクションでレンズを洗浄。 Action 1　アクティブクリーニング　脂質汚れにはたらく新成分をプラスして、レンズの洗浄作用を高めました。 Action 2　パッシブクリーニング　眼に優しいナチュラル成分のクエン酸が、保存中もタンパクの汚れを取り除きます。 ・ポリクォッド（ソフトコンタクトレンズ用消毒成分）でレンズを消毒 眼とレンズに優しい「ポリクォッド」はアルコンだけが使用している成分です。レンズ表面の微生物にはたらきかけ、レンズを消毒します。高分子なので、レンズに入りにくく、蓄積しません。
	6	レニューマルチプラス	ボシュロム・ジャパン㈱	《有効成分》ポリヘキサニド（ダイメッド）1.1ppm含有 《配合成分》緩衝剤、安定化剤、等張化剤、pH調整剤、ポロキサミン、ハイドラネート 《表示指定成分》ホウ酸、エデト酸ナトリウム	【使用方法】 1．洗浄 レンズケースに本剤を満たし、手を石けんでよく洗います。はずしたレンズを手のひらにのせ、本剤を3～5滴落として片面を人差し指で約10秒間ていねいにこすり洗いします。裏面も本剤を3～5滴落として約10秒間こすり洗いします。 2．すすぎ レンズの両面を本剤ですすぎ、表面の残留物を充分に取り除きます。 3．消毒（保存） レンズケースにレンズを入れ、キャップをしっかりしめます。少なくとも4時間この状態で放置します（この間に消毒が完了します）。消毒後、レンズケースから取り出したレンズはそのまま装用できます。 ＊レンズを取り出した後のレンズケースは空にして、水道の流水でケース内をよくこすり洗いしてからすすぎ、自然乾燥させてください。（海外等で使用される場合には、レンズケースは本剤で洗浄し、自然乾燥させてください。）	・タンパク除去もできるスーパー1ボトル ・レンズをきれいに、瞳にやさしく。 ・きもんとタンパク除去　新配合ハイドラネートの作り出すマイナスイオンの力が、レンズに付着したタンパク質の汚れまでも、すっきり取り除きます。だから、これ1本でレンズは毎日きれい。 ・瞳にやさしい　しかも、レンズ装用中にはうるおい成分ポロキサミンが、レンズに涙を引き寄せて涙のクッションをつくるから、瞳にやさしい。
	7	ユビカコールド	㈱メニコン	1mL中、塩酸ポリヘキサニド0.001mg含有 界面活性剤、等張化剤、金属封鎖剤 表示指定成分：エデト酸塩、プロピレングリコール	レンズを取り扱う前には、毎回必ず手を石けんでよく洗い、水道水（流水）でよくすすぎます。 1．洗浄 コンタクトレンズを眼からはずし手のひらにのせ、ユビカコールドを数滴つけて、レンズの両面を各々、20～30回指で軽くこすりながら洗います。 2．すすぎ こすり洗いしたレンズの両面をユビカコールドでよくすすぎます。 3．消毒・保存 ユビカコールドを満たしたレンズケースにレンズを完全に浸し、ケースのキャップをしっかりしめます。そのまま4時間以上放置すると消毒は完了です。消毒後のレンズはすすがずにそのまま装用できます。 使用後のレンズケースは液をすて、ユビカコールドでよく洗った後、自然乾燥させてください。また、より清潔にお使いいただくために、レンズケースは定期的に交換してください。	・レンズの外から中からWのうるおい ・ナチュラルパワーで快適 1．天然系成分フルーツ酸がタンパク汚れを包み込み、レンズへの付着を防止すると共に、付着してしまったタンパク汚れにも効果的にはたらきます。 2．天然系成分フルーツ酸とアミノ酸がレンズの形状を安定させるからクリアな視界を保ちます。 ・しっかり消毒・洗浄 有効成分PHMBと植物原料の界面活性剤のはたらきにより、レンズを優しく、しかも効果的に消毒、洗浄。レンズを清潔に保ちます。 ・瞳とレンズに優しいWのうるおい 植物原料の界面活性剤がレンズ表面にうるおいベールを形成します。さらに、水分子をひきよせた親水成分がレンズ内部にしみ込んでうるおいをキープするため、レンズの外からも中からもうるおいます。
	8	ロートピュアソフトワンモイスティ	ロート製薬㈱	有効成分　1mL中に塩酸ポリヘキサニド0.001mg含有 配合成分　粘調剤、等張化剤、緩衝剤、安定剤、界面活性剤、pH調整剤 表示指定成分：ホウ酸、エデト酸塩 添加物として、ポリオキシエチレンポリオキシプロピレングリコールを含有しています。	◎下記に従い、正しくケアをしてください。レンズを装用する度にこすり洗いを行い、新しい薬液に入れ替えることが必要です。 ソフトコンタクトレンズを取扱う前に、必ず石けんで手をよく洗います。 ステップ1…こすり洗い 目からはずしたソフトコンタクトレンズを手のひらにのせます。ソフトコンタクトレンズ表面に本剤を数滴つけて、レンズの両面を各々、20～30回指で軽くこすり洗いします。 ステップ2…すすぎ こすり洗いしたソフトコンタクトレンズの両面を本剤で1分にすすぎます。 ステップ3…消毒・保存 レンズケースに本剤を満たし、レンズを完全にひたし、ケースのフタをしっかりと閉めます。そのまま4時間以上放置すると消毒が完了します。 ※レンズと瞳のために、本剤ですすいでから装着することをおすすめします。 ※雑菌等が入る恐れがありますので、容器の先がレンズケースや液面、コンタクトレンズや指先等に触れないようご注意ください。 ●使用後のレンズケースは本剤ですすぎ、自然乾燥させてください。 ●レンズケースは定期的に新しい物にお取り替えください。 長期間同じレンズケースを使用していると、ケースについた細菌等が原因で、感染症を起こす場合があります。	・うるおい長持ち ・こすり洗い・すすぎ・消毒・保存がこれ一本で!! ・タンパク汚れもスッキリ　瞳もレンズも快適　抗菌レンズケースつき ・つけた瞬間、クリアな視界　毎日のケアで、いつもおろしたての気持ちよさ ・パワフル洗浄力　タンパク汚れもスッキリ ポロクサマーとポリヘキサニドの2つの働きで、ソフトレンズについたしつこい汚れをしっかり洗浄・消毒。だから、毎日すっきり清潔（※こすり洗いが必要です。） ・瞳もレンズも快適　やさしさを考えたレンズケア ・潤いの膜（HPMC＋ヒアルロン酸Na）がソフトレンズを包み込み、乾燥や汚れから守ってくれる。 ・涙に近いpHで、瞳にやさしいつけ心地。だから、毎日快適。 ・毎日のケアだからこそ、使いやすさにこだわりました 片手で開閉できるワンタッチキャップ&注ぎやすい斜め口　親指で簡単に開けられます。また、注ぎ口がサイドにあり、少し傾けるだけで注げます。 内容量が一目でわかる透明ボトル 小さな手でもすべりにくいフィットボトル　ボトルにくぼみを設けていますので、手にしっかりフィットして片手でも楽に扱えます。

分類		銘柄 (No.)	商品名	製造業又は販売業名	含有成分	使用方法	うたい文句等
参考品32	過酸化水素タイプ	9	コンセプトワンステップ	エイエムオー・ジャパン㈱	〔消毒液〕過酸化水素3.0w/v%、pH調整剤 〔中和錠〕1錠中カタラーゼ4300単位、等張化剤、緩衝剤、滑沢剤、着色剤、コーティング剤	使用方法 レンズを取扱う前には、必ず石けんなどで手を洗い、よくすすいでください。 本剤ご使用の際には、必ず専用ワンステップケース（以下、「専用ケース」とする）を使用してください。 ①レンズをセットする　眼からレンズをはずし、左右のバスケットに入れます。右眼：R（白色）　左眼：L（青色） ②消毒液を入れる　専用ケースのガイドラインまで消毒液を満たします。 ③中和錠を1錠入れる ④フタを締めて3回ゆっくり振る　バスケットに入れたレンズを液に浸し、しっかりフタを締めます。専用ケースを逆さまにし、ゆっくり元に戻す操作を3回繰り返します。 ⑤6時間以上放置　錠剤が溶けて、徐々に専用ケース内の液がうすいピンク色になります。そのまま6時間以上放置します。 ⑥装用前に3回ゆっくり振る　6時間以上放置した上で、専用ケース内の液がうすいピンク色になっている（＝中和が行われている）ことを確認してください。専用ケースを逆さまにし、ゆっくり元に戻す操作を3回繰り返してから、レンズを装用してください。 使用後の専用ケースは空にして、流水でよく洗った後、自然乾燥してください。 レンズ装用前に、「コンセプトすすぎ液」でこするように洗うことにより、レンズをよりきれいに使用できます。（汚れの付着には個人差があります。）	・きちんと消毒、レンズをリフレッシュ。 ・うるおい処方 ・しっかり消毒して、レンズの透明感アップ。H_2O_2（過酸化水素）が目に見えない細菌や雑菌をしっかり消毒。新しいレンズのような快適さを実現します。 ・うるおい処方。だから快適な装用感が続きます。レンズにうるおいを与えて、目になじみやすくします。 ・ワンステップだけのシンプルケア　消毒液と中和錠を一緒に専用ワンステップケースに入れて、6時間以上おくだけのシンプルケア。また、中和が始まるとビタミンB_{12}が溶けて液がうすいピンク色に変わるから、中和忘れの心配がありません。 ・防腐剤が入っていません。瞳に安心です。デリケートな瞳にもやさしい処方です。 ・シリコーン素材のソフトレンズにも使用できます。全てのソフトコンタクトレンズ（グループⅠ～グループⅣ）に安心して使用できます。ただし、虹彩付きソフトコンタクトレンズ（レンズの虹彩部分に着色しているカラーソフトレンズ）には使用できません。
		10	エーオーセプト	チバビジョン㈱	【有効成分】 〔消毒液〕過酸化水素3.42w/v% 〔中和剤ディスク〕1個中、白金1.5mg 【配合成分】 安定化剤、緩衝剤、pH調整剤、等張化剤	【使用方法】 レンズを取り扱う前には必ず手指を石けんでよく洗い、清潔にしてください。 ■ディスポカップはレンズホルダーとレンズカップで組み立てられています。レンズホルダーの先に中和用ディスクが付いていることを必ず確認してください。 中和用ディスクは取りはずしできません。 ■同梱のディスポカップはエーオーセプトを購入するごとに、新しいものと交換してください。 ■使用レンズのケア方法にしたがって、はずしたレンズの洗浄・すすぎを行ってください。 ①レンズホルダーの左右のバスケットにレンズを入れます。（“L”と表示してあるバスケットのレンズホルダーに左のレンズをセットします）バスケットのふちでレンズをはさみ込まないようにフタを閉めます。 ②消毒液をレンズカップの内線まで入れます。 ・この時、消毒液が少なかったり、入れすぎたりしないようにしてください。 ・消毒液を入れすぎると、中和されていない消毒液がフタからもれ出ることがあります。 ③レンズを入れ、6時間以上放置します。 ・常温で消毒・中和を行ってください。低温下（10℃以下）で消毒した場合は、中和完了に6時間以上かかる場合があります。 ・冬場はなるべく暖かい部屋でご使用ください。 ・消毒・中和が6時間以上経過していないレンズは装用しないでください。 ・液がもれるためディスポカップを横にしないでください。 ・消毒・中和後のレンズを24時間以上保存した場合は、装用前に再度消毒・中和を行ってください。 ④レンズを装用前に、ソフトコンタクトレンズ用保存液（ソフトウェアプラスなど）でこすり洗いをしてから目に装用。	・レンズクリア　瞳に優しい　防腐剤フリー ・H_2O_2パワーでしっかり消毒 ・防腐剤フリーで瞳にやさしい ・消毒と中和が同時のかんたんケア ・エーオーセプトはH_2O_2（過酸化水素）パワーでカビや細菌をしっかり消毒。ディスポカップに消毒液をそそぐだけで、消毒と中和が同時に行われ、中和忘れのない安心ケアです。レンズ装用前にソフトコンタクトレンズ保存液でこすり洗いをすることにより、レンズはいつも清潔。だから快適な装用感が一日中続きます。
	ポビドンヨードタイプ	11	バイオクレンエファール	㈱オフテクス	エファールA（消毒顆粒）：（有効成分）ポビドンヨード4.0mg/1包（100mg）、賦形剤、pH調整剤 エファールB（中和錠）：（有効成分）乾燥亜硫酸ナトリウム2.4mg/1錠、洗浄剤、発泡剤、賦形剤、滑沢剤、コーティング剤 エファールC（溶解・すすぎ液）：等張化剤、緩衝剤 （表示指定成分）ホウ酸、エデト酸塩 本剤はポリオキシエチレンポリオキシプロピレングリコールを含有しています。	1. 溶解・すすぎ液を消毒容器の線まで満たし、消毒顆粒1包及び中和錠1錠を入れる。 2. コンタクトレンズを入れ、消毒顆粒が溶けるまで振り混ぜる。 3. そのまま4時間以上又は一晩放置後、コンタクトレンズを取り出し、溶解・すすぎ液でよくすすぐ。	・レンズを交換する日まで使いはじめの快適さがつづきます 消毒成分PVP-I_2ポビドンヨード配合 特長 ①高い消毒効果と安全性 有効成分PVP-I_2の働きで従来のケア用品を凌ぐ*高い消毒効果と安全性の両立を実現。レンズを交換する日まで使いはじめの快適な装用感をお届けします。　※自社従来品との比較 ②パワフルな洗浄力 既存のコールド消毒剤の中で唯一タンパク分解酵素を配合。消毒と同時にゴロゴロ感の原因となるタンパク汚れも強力に除去します。 ③こすり洗い不要のオートマチックケア 色の変化（オレンジ→無色）でケアの進行が一目でわかるユニークなケアシステム。面倒なこすり洗いも不要です。 ④防腐剤フリーの安全設計 エファールの消毒顆粒、中和錠、溶解・すすぎ液は防腐剤を一切含まない、あなたの大切な目とレンズにやさしいケア用品です。

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Identification and differentiation of human schistosomes by polymerase chain reaction

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ABSTRACT

Recent increasing number of travelers, immigrants and foreign workers from schistosomiasis endemic area has thus resulted in the importation of schistosomiasis to non-endemic countries. To avoid ova-induced pathogenicity, sensitive and specific diagnostic means at an early stage of infection are therefore crucial. In this study, we developed polymerase chain reaction (PCR) primers specific for human schistosome species. The PCR products were obtained in a species-specific manner (479 bp, *Schistosoma mansoni*; 365 bp, *S. haematobium*; 614 bp, *S. japonicum*; 303 bp, *S. mekongi*) and were detectable from 0.01 pg of total worm DNA (*S. haematobium*, *S. japonicum*, *S. mekongi*). The primer sets were also available for multiplex use. Although some difficulties were experienced in amplifying the parasite DNA from the infected animals, schistosome DNA could be detected from one day post infection. The PCR method described herein will therefore be beneficial to detect human schistosomiasis, after some improvements in this method.

1. Introduction

Schistosomiasis affects about 200 million people worldwide, and more than 650 million live in endemic areas (WHO, 2005). Moreover, the threat is now spreading globally in non-endemic areas, such as Western countries (Houston et al., 2004; Bierman et al., 2005; Bottieau et al., 2006) and Japan. Recently, increases in the number of travelers, migrants and foreign workers have resulted in the importation of schistosomiasis. Because the main pathogenicity of this disease is severe hypersensitivity (e.g., acute Katayama syndrome and chronic granulomatous diseases) caused by the parasite eggs trapped in the host tissues, the early detection and treatment of this disease are therefore crucial to avoid any subsequent fatalities. The current frequently used diagnostic methods are the detection of parasite eggs and parasite-specific antibodies. The former is distinguishable between species based on the egg morphology and it shows direct evidence of an actual schistosome infection. However, this diagnostic modality is not feasible during the prepatent period when parasite eggs are not detectable. Furthermore, stool examinations such as the Kato–Katz method are insensitive in individuals with only light infections (Doenhoff et al., 2004) and the egg excretion reduced/suppressed with the time course of infection (Cheever et al., 1994) and/or by the host immune status (Hermeto et al., 1994; Montes et al., 2004). The latter has a high sensitivity and it is applicable for mass examinations by means of ELISA. However, it has a rather low specificity due to cross reactions and the existence of antibodies per se shows an unclear state of infection. Namely, according to Hayashi et al. (2000), anti *Schistosoma japonicum* antibody has been detected by ELISA even 33 years after treatment. Moreover, the clue antigens for antibody production tend to be associated with egg deposition (Hillyer and Bruce, 1980; Doenhoff et al., 2004). In this context, there is a need to establish new diagnostic measures that are independent of the pathogenic eggs.

Recently, several investigators have launched new molecular based approaches for detecting schistosomiasis: schistosome DNA in human clinical samples such as feces (Pontes et al., 2002, 2003; Gobert et al., 2005), sera (Pontes et al., 2002) and urine (Sandoval et al., 2006a). The existence of parasite DNA in the host is direct evidence of an actual infection. The data from experimental animals have suggested that potentially effective diagnostic

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tools can be developed for use during the prepatent period (Sandoval et al., 2006b; Suzuki et al., 2006; Xia et al., 2009). Furthermore, the detection of parasite DNA may also be a helpful guideline for selecting the optimal treatment for schistosomiasis.

In this study, we developed new PCR primers for the identification and differentiation of major human schistosomes: *S. mansoni*, *S. haematobium*, *S. japonicum* and *S. mekongi*, using the currently available genetic information and assessed their potentiality/availability.

2. Materials and methods

2.1. Parasite materials and the extraction of parasite DNA

Adult worms of *S. mansoni* (Puerto Rican strain), *S. haematobium* (Kenyan strain), *S. japonicum* (Japanese Yamanashi strain) and *S. mekongi* (Laotian strain) used for template DNA were obtained from either experimentally infected mice or Mongolian gerbils. The infected animals were maintained at the Center for Tropical Medicine and Parasitology, Dokkyo Medical University. The handling and care of all experimental animals in this study strictly complied with the Guidelines for Animal Experiments of Dokkyo Medical University according to the Japanese law. Originally, *S. mekongi* was provided from Mahidol University, Thailand in 2000 and *S. haematobium* was provided from the University of Occupational and Environmental Health, Japan in 2004, and, thereafter, they were maintained in our laboratory. Adult worms of each species were picked up from either the mesenteric or portal veins of all experimental animals, washed with PBS several times and then were stored at −80 °C until use.

The total DNA of adult worms was extracted using commercially available DNA extraction Kits (e.g., Easy-DNA™ Kit, Invitrogen™, USA). The adult worms were lyophilized and minced with scissors, and then were processed according to the manufacturer's instructions.

2.2. Primer design, PCR and sequencing

The complete sequences of the mitochondrial gene of *Schistosoma* spp. were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html): *S. mansoni* (GenBank Accession No. AF216698, Le et al., 2000), *S. haematobium* (GenBank Accession No. DQ157222, Littlewood et al., 2006), *S. japonicum* (GenBank Accession No. AF215860, Le et al., 2000) and *S. mekongi* (GenBank Accession No. AF217449, Le et al., 2000). The sequences of the cytochrome c oxidase subunit 1 (cox1) gene of *Schistosoma spp.* were compared using CLUSTALW (http://align.genome.jp/). Candidate primers were checked not to form primer dimer and then *in silico* PCR was performed by FastPCR (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm). The primer pairs were specific for *S. mansoni* (SmF/CR), *S. haematobium* (ShF/CR), *S. japonicum* (SjF/CR), *S. mekongi* (SmF/CR) and common *Schistosoma* spp. (CF/CR) are shown in Table 1. Basically, PCR was carried out in a final volume of 20 μl with 2 μl of 10× PCR Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 U of Platinum® *Taq* DNA polymerase (Invitrogen™, USA), 0.5 μM of each primer (Tsukuba Oligo Service Co., Ltd., Japan) and 1 μl of template DNA. The reactions were performed initially at 94 °C for 2 min, then 35 cycles, each consisting of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and a final cycle of 72 °C for 7 min.

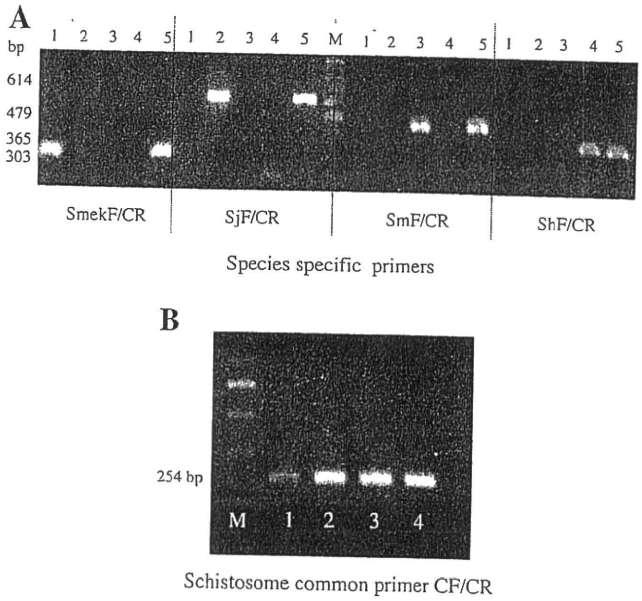

Fig. 1. Specificity tests for the schistosome species-specific primers (A) and common primer (B) using different DNA. Lane M, 100 bp molecular marker; Lane 1, *Schistosoma. mekongi*; Lane 2, *S. japonicum*; Lane 3, *S. mansoni*; Lane 4, *S. haematobium*; Lane 5, mixed four species.

The PCR products were electrophoresed in 2% agarose (Certified™ Low Range Ultra Agarose, BIO-RAD Laboratories, USA) in tris–acetate–EDTA gels with 0.3 μg/ml ethidium bromide, and then were visualized in an UV transilluminator.

The PCR products were purified using the Qiaquick® PCR Purification Kit (Qiagen, USA), and then the samples were prepared to undergo DNA sequencing by BigDye® Terminator FS Cycle Sequencing Kit (Applied Biosystems, USA). DNA sequencing was performed using the ABI Prism 3100 genetic analyzer, and the data were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 1
Primer sets for cox1 gene amplification of *Schistosoma* spp.

Parasite	Primers		PCR Product length (bp)
	Name	Sequence 5′ → 3′	
S. mansoni	SmF	TCCTTTATCAATTTGAGAGG	479
	CR	CCAACCATAAACATATGATG	
S. haematobium	ShF	AGTCGTGTCGATTTTAAGAC	365
	CR	CCAACCATAAACATATGATG	
S. japonicum	SjF	CCGTTTTTTTTGAGTATGAG	614
	CR	CCAACCATAAACATATGATG	
S. mekongi	SmekF	GTTAATATCATTGCCTGTAC	303
	CR	CCAACCATAAACATATGATG	
Schistosoma spp. Four species common	CF	GATCGTAAATTTGGW*ACTGC	254 (*S mansoni*: 253)
	CR	CCAACCATAAACATATGATG	

* W: mixture of A and T.

Table 2
Detection limits of monoplex PCR.

Template DNA	*S. mansoni*		*S. haematobium*		*S. japonicum*		*S. mekongi*	
Primers	SmF/CR	CF/CR	ShF/CR	CF/CR	SjF/CR	CF/CR	SmekF/CR	CF/CR
Detection limit (pg)	0.1	0.01	0.01	0.01	0.01	0.01	0.01	0.01

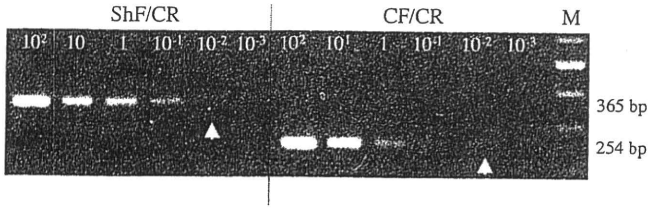

Fig. 2. Representative results of the sensitivity tests for monoplex PCR. The genomic DNA extracted from adult worms of *Schistosoma. haematobium* was serially diluted (10^2 to 10^{-3} pg) and applied *S. haematobium* specific PCR (ShF/CR) or schistosome common PCR (CF/CR). The arrow shows the detection limit of the assay.

2.3. Detection of parasite DNA from biological samples of schistosome infected animals

The biological samples (sera and/or urine) were obtained from the animals experimentally infected with *Schistosoma* spp. These animals were intended to be used either for the maintenance of the parasites or for the other experiments. These samples were collected and stored at −80 °C or in ethanol until use. The DNA was extracted using some commercially available kits (e.g., Nucleo-Spin® Tissue, Macherey–Nagel, Germany; InstaGene™ Whole Blood Kit, Bio-Rad Laboratories, USA, etc.). The PCR reactions were carried out in almost the same manner as described above, with some modification: 2.5 mM of $MgCl_2$, 0.5–1.0 U of *Taq* DNA polymerase, 0.5–2.0 μM of each primer and the number of reactions were increased up to 50 cycles.

3. Results and discussion

The PCR products were successfully amplified in a species-specific manner. The product sizes obtained were 479 bp (*S. mansoni*), 365 bp (*S. haematobium*), 614 bp (*S. japonicum*), 303 bp (*S. mekongi*) and 253/254 bp (schistosome common) (Fig. 1A and B). The sequenced data were analyzed by BLAST and then were confirmed to be identical to each species. The specificity of the primers was checked by PCR combined with primer sets and template DNA from each schistosome species. In the monoplex PCR, no cross amplification was observed to occur between the species (Fig 1A). To evaluate the sensitivity of the species-specific PCR, the total worm DNA ($OD_{260/280} > 1.8$) from each species was serially diluted and applied for PCR. The minimum detection limit of the monoplex PCR (individual species) was 0.01 pg on *S. haematobium*, *S. japonicum* and *S. mekongi* (Table 2/Fig. 2). The low detection limits obtained with a low concentration of the primer (0.5 μM) and Taq polymerase (0.4 U/reaction) demonstrated the high sensitivity of the PCR system.

For multiplex PCR, each species-specific reaction mix was mixed in equal ratios. The conditions were equal to those for the monoplex PCR. Similarly, no cross amplification was observed in multiplex use (Fig. 3).

Table 3 shows the representative data of the PCR positive results from biological samples of the schistosome infected animals. Although some difficulties were encountered in amplifying the parasite DNA from the infected animals (data not shown), schistosome DNA could be detected from 1 DPI (days-post-infection). Because a lower sensitivity may be attributed to the small amount of parasite DNA in comparison to a large amount of host DNA, the

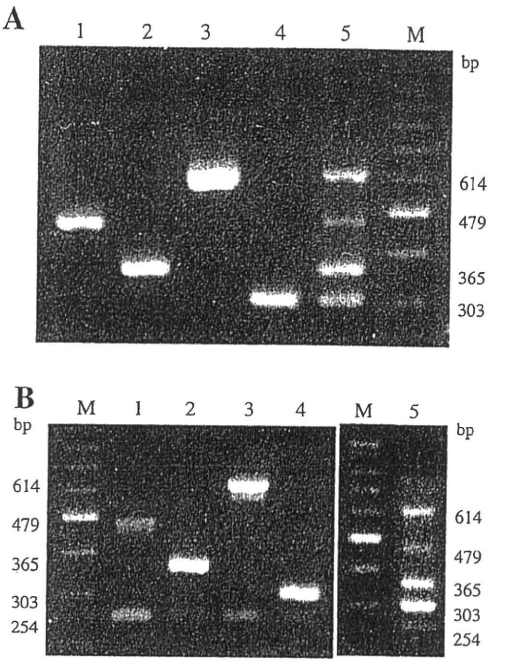

Fig. 3. Specificity tests for multiplex PCR using four sets of schistosome specific primers (A) and multiplex PCR using five sets of schistosome specific primers and common primer (B). Lane M, 100 bp molecular marker; Lane 1, *Schistosoma. mansoni*; Lane 2, *S. haematobium*; Lane 3, *S. japonicum*; Lane 4, *S. mekongi*; Lane 5, mixed four species.

methods available for concentrating and/or purifying parasite DNA are thus required to improve sensitivity.

In the area of infectious diseases, the detection of pathogen derived nucleic acid by PCR or LAMP (loop-mediated isothermal amplification) have thus been introduced as a diagnostic tool (Yam et al., 2003; Thai et al., 2004). Because the main pathology derived by schistosome is triggered by parasite eggs, we therefore need a more sensitive diagnostic means which can be effectively used during the early stage of infection. The detection of schistosome DNA has been reported in the snail host (Jannotti-Passos et al., 1997; Driscoll et al., 2005; Abbasi et al., 2007), in water (Hamburger et al., 1998) and in clinical specimens (Pontes et al., 2002, 2003; Gobert et al., 2005; Sandoval et al., 2006a; ten Hove et al., 2008; Obeng et al., 2008; Allam et al., 2009). It is encouraging that worm DNA was detected in the urine and sera of experimentally infected mice at one and two weeks-post-infection, respectively (Sandoval et al., 2006b; Suzuki et al., 2006). In addition, our cumulative data also showed parasite DNA to be detectable from 1 DPI (Table 3). It is therefore suggested that cell-free circulating DNA of schistosome exists in both the host serum and urine. Circulating schistosome DNA in the host closely reflects the existence live worms and/or eggs. Cell-free circulating nucleic acids in the plasma, serum and urine have been of interest as a clinical diagnostic tool for cancer, for prenatal diagnoses, transplantation and traumatology (Chan et al., 2003). The detection of circulating parasite DNA is therefore expected to become a useful diagnostic tool, not only for identifying the early stage of infection, but also for selecting the optimal treatment regimen in old cases, namely

Table 3
Details of PCR positive results.

Parasite	Host	Course	Sample	PCR		Comments
				Primers	Result (bp)	
S. japonicum	Mice	1 DPI	Pooled urine	CF/CR	254	
		3 DPI	Pooled urine	CF/CR	254	
		1 WPI	Pooled urine	CF/CR	254	
		2 WPI	Pooled urine	CF/CR	254	
		3 WPI	Pooled urine	CF/CR	254	
		4 WPI	Pooled urine	CF/CR	254	
		5 WPI	Pooled urine	CF/CR	254	
	Mice	1 WPI	Pooled urine	SjF/CR	614	
		2 WPI	Pooled urine	SjF/CR	614	
S. mansoni	Mice	1 DPI	Pooled urine	CF/CR	254	
		3 DPI	Pooled urine	CF/CR	254	
		1 WPI	Pooled urine	CF/CR	254	
		2 WPI	Pooled urine	CF/CR	254	
		3 WPI	Pooled urine	CF/CR	254	
		4 WPI	Pooled urine	CF/CR	254	
		5 WPI	Pooled urine	CF/CR	254	
	Mice	4 DPI	Pooled urine	SmF/CR	479	
		5 DPI	Pooled urine	SmF/CR	479	
S. haematobium	Gerbil	34 WPI	Serum	ShF/CR	365	Lesion(−), unisexual
	Gerbil	36 WPI	Serum	ShF/CR	365	Lesion(−), unisexual
	Hamster	~1 year	Serum	ShF/CR	365	Lesion(+)/worm(+)
S. mekongi	Mouse	1 DPI	Serum, urine*	SmekF/CR	303	*2nd PCR
		1 WPI	Serum, urine	SmekF/CR	303	
		2 WPI	Urine	SmekF/CR	303	
		3 WPI	Serum, urine	SmekF/CR	303	
		4 WPI	Serum*, urine	SmekF/CR	303	*2nd PCR
		5 WPI	Serum, urine	SmekF/CR	303	
		6 WPI	Serum	SmekF/CR	303	
	Mouse	3 DPI	Serum	SmekF/CR	303	*2nd PCR
	Mouse	5 DPI	Serum	SmekF/CR	303	
	Mouse	4 WPI	Serum	SmekF/CR	303	
	Mouse	7 WPI	Serum	SmekF/CR	303	
	Mouse	8 WPI	Serum	SmekF/CR	303	
	Mouse	8 WPI	Serum	SmekF/CR	303	
	Dog	>3 years	Serum, urine	SmekF/CR	303	No eggs in feces
	Dog	>3 years	Urine	SmekF/CR	303	No eggs in feces
	Hamster	19 WPI	Serum	SmekF/CR	303	Lesion(+)/worm(+)
	Gerbil	17 WPI	Serum	SmekF/CR	303	Lesion(+)/worm(+)

DPI: days-post-infection.
WPI: weeks-post-infection.
* 2nd PCR: positive result was obtained by the 2nd PCR.

those who have hepatic lesions but no viable worms or eggs, the administration of praziquantel is not required, however, such patients do need supportive measures. As the result, further details about circulating schistosome DNA in the host therefore need to be elucidated.

We have developed simple PCR systems that can differentiate four human schistosome species (*S. mansoni*, *S. haematobium*, *S. japonicum* and *S. mekongi*). PCR can be successfully performed by combining primers for the different species and/or that for a schistosome common region. The use of common reverse primer (CR) allows for simple handling and thus makes this diagnostic modality suitable for performing accurate species differentiation. From our accumulative data obtained using experimental animals, parasite DNA was detected from 1 DPI at the earliest (Table 3). We are presently assessing this usefulness of this PCR diagnostic modality for samples obtained from patients in schistosomiasis endemic areas. The PCR method described herein is therefore considered to be a potentially useful diagnostic tool for human schistosomiasis, independent of the presence of parasite eggs.

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Plasmodium falciparum: Chemically defined medium for continuous intraerythrocytic growth using lipids and recombinant albumin

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ABSTRACT

Dioleoylphosphatidylcholine and other phosphatidylcholines containing different fatty acid moieties were found to increase the ability of nonesterified fatty acids (NEFA) to sustain continuous intraerythrocytic growth of *Plasmodium falciparum* in the presence of specific proteins. Other phospholipids, including phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid, were beneficial to parasite growth. Different combinations and concentrations of NEFA tested in the presence of phospholipids and bovine albumin had variable effects on parasite growth. The most effective combination for promoting parasite growth consisted of 30 μg/ml *cis*-9-octadecenoic acid (oleic acid) plus 15 μg/ml hexadecanoic acid (palmitic acid). Recombinant human albumin could replace bovine or human albumin in culture media enriched with structurally defined lipids. This study therefore established a chemically defined culture medium suitable for sustaining the growth of *P. falciparum*.

1. Introduction

Malaria remains a devastating disease, particularly in the Tropics. The estimated incidence of malaria worldwide is in the order of 300–500 million clinical cases annually. The annual estimates of malaria mortality, particularly those caused by the protozoan *Plasmodium falciparum*, vary from 1.5 to 2.7 million worldwide (World Malaria Report 2005, WHO, http://rbm.who.int/wmr2005/; Snow et al., 2005). Because of emerging resistance to conventional antimalarial drugs and insecticides, there is an increasing need for new drugs with alternative targets (Ridley, 2002). It is therefore necessary to gain a better understanding of malarial parasite biology, and the mechanisms of action of growth-promoting host factors and antimalarial drugs.

It has been suggested that *P. falciparum* requires some factors present in human serum (HS)[1] in order to develop, although the role of HS in the growth of this parasite is still unknown. We previously described a growth-promoting fraction derived from adult bovine plasma (GFS), which supported intraerythrocytic growth of the parasite (Asahi and Kanazawa, 1994). GFS is a 55–70% ammonium sulfate fraction of adult bovine plasma and contains lipid-rich albumin (ALB) as a major component (Asahi and Kanazawa, 1994; Asahi et al., 2005). Similarly, Cranmer et al. (1997) described a commercially available lipid-enriched bovine ALB (Albumax II; Invitrogen Ltd., USA) that could be used to replace HS for in vitro cultivation of *P. falciparum*. Although these serum substitutes have often been used to maintain parasite cultures, data are still insufficient to allow the direct identification of the functional components required for the growth of *P. falciparum*. The replacement of HS or GFS in culture medium with chemically- or functionally-defined substances is not only advantageous for the culture of the parasite, but will also provide critical clues concerning the parasite's requirements for proliferation at the erythrocyte stage. We previously investigated the components of GFS and related substances which have the ability to sustain parasite growth (Asahi et al., 2005). A simple total lipid fraction (GFS-C), which was obtained after lipid extraction of GFS, has been shown to sustain the complete development of the parasite. The importance of, not only GFS-C, but also specific proteins such as bovine and human ALB, has also been indicated (Asahi et al., 2005). GFS-C has been shown to contain phospholipids (Pld), diacylglycerides, cholesterol (CHOL), monoglycerides, nonesterified fatty acids (NEFA) and cholesteryl esters (CE) (Asahi et al., 2005). The components of the NEFA fraction of GFS-C have been shown to contain mainly *cis*-9-octadecenoic acid (C18:1[C18:1-cis-9], 43%),

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[1] *Abbreviations used:* ALB, albumin; ANOVA, multifactorial analysis of variance; BSAF, NEFA-free bovine serum ALB; CE, cholesteryl ester; CHOL, cholesterol; DAG, 1,2-dioleoyl-sn-glycerol; CRPMI, basal medium; GFS, a growth-promoting fraction derived from adult bovine plasma; GFS-C, a total simple lipid fraction obtained from GFS; GFSRPMI, CRPMI containing 10% GFS; HS, human serum; HSAF, NEFA-free human ALB; NEFA, nonesterified fatty acids; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PfLDH, *Plasmodium falciparum* lactate dehydrogenase; PI, phosphatidylinositol; Pld, phospholipids; PRBC, RBC infected with *Plasmodium falciparum*; PS, phosphatidylserine; RBC, red blood cell; recHA, recombinant human ALB.

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hexadecanoic acid (C16:0, 21%), octadecanoic acid (C18:0, 14%), *cis,cis*-9,12-octadecadienoic acid (C18:2), *cis*-9-hexadecenoic acid (C16:1), *cis*-5,8,11,14-eicosatetraenoic acid (C20:4), *cis*-5,8,11,14,17-eicosapentaenoic acid (C20:5), and *cis*-4,7,10,13,16,19-docosahexaenoic acid (C22:6). Each of the NEFA enriched with BSAF has been tested for the ability to promote parasite growth. Mixtures of NEFA, but not individual NEFA, have sustained parasite growth to a low extent (Asahi et al., 2005). However, parasite growth in the presence of several combinations of NEFA is much less than that with GFS-C-, GFS- or HS-containing medium. These results have implied that while the NEFA components of GFS-C are functional factors in promoting parasite growth, other factor(s) must also contribute to the high growth-promoting activity of GFS.

This study was undertaken to determine the ability of structurally defined chemicals to sustain parasite growth and to formulate a chemically defined medium for intraerythrocytic growth of the parasite using chemicals and recombinant human ALB (recHA).

2. Materials and methods

2.1. Parasite and culture

Cultures of the FCR3/FMG (ATCC Catalogue No. 30932, Gambia) strain of *P. falciparum* were used in the experiments. The parasites were routinely maintained by in vitro culture techniques using culture medium devoid of whole serum. It consisted of basal medium (CRPMI) supplemented with 10% GFS (Daigo's GF21; Wako Pure Chemical Industries, Japan), as previously reported (Asahi and Kanazawa, 1994; Asahi et al., 1996). This complete medium was termed GFSRPMI. CRPMI consisted of RPMI1640 containing 2 mM glutamine, 25 mM 4-(2-hydroxylethyl)-piperazine ethanesulfonic acid, 24 mM $NaHCO_3$ (Invitrogen Ltd., USA), 25 μg/ml gentamycin (Sigma–Aldrich Corp., USA), and 150 μM hypoxanthine (Sigma-Aldrich). Briefly, red blood cells (RBC), which had been preserved in Alsever's solution (Asahi et al., 1996) for 3–30 days, were washed, dispensed into 24-well culture plates at a hematocrit of 2% (1 ml of suspension/well), and cultured in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37 °C. For subculture, 4 days after inoculation, infected RBC (PRBC) and uninfected RBC were washed with CRPMI. Parasitemia was adjusted to 0.1% (for subculture) or 0.4% (for growth tests), by adding uninfected RBC, and the hematocrit was adjusted to 2% by adding the appropriate volume of either GFSRPMI or the test medium.

2.2. Growth-promoting activity experiments

The growth experiments were performed by replacing GFSRPMI with CRPMI supplemented with the test substances. The following substances were tested for their growth-promoting activities: CRPMI containing NEFA-free bovine serum-(BSAF) or HS-ALB (HSAF), or recHA (albucult™; Novozymes Delta Ltd., Denmark) at a final concentration of 3 mg/ml, except when otherwise stated, was further supplemented with different concentrations of dodecanoic acid (C12:0), tetradecanoic acid (C14:0), pentadecanoic acid (C15:0), C16:0, C16:1, C18:0, *cis*-6-octadecenoic acid (C18:1-cis-6), C18:1, *cis*-11-octadecenoic acid (C18:1-cis-11), *cis*-13-octadecenoic acid (C18:1-cis-13), *trans*-9-octadecenoic acid (C18:1-trans-9), C18:2, *cis,cis,cis*-6,9,12-octadecatrienoic acid (C18:3), C20:4, C20:5, docosanoic acid (C22:0), C22:6, cholesteryl ester oleoyl (CE-18:1), CHOL, 1,2-dioleoyl-sn-glycerol (DAG), GFS-C; 1,2-dioleoyl phosphatidic acid sodium salt (PA-di18:1), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (PC-di6:0), 1,2-dilauroyl-sn-glycero-3-phosphocholine (PC-di12:0), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (PC-di14:0), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC-di16:0), 1,2-dipalmitoyl-rac-glycero-3-phosphocholine (PC-rac-di16:0), 1,2-distearoyl-sn-glycero-3-phosphocholine (PC-di18:0), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC-di18:1), 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (PC-18:1/16:0), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (PC-di18:2), 1,2-diarachidoyl-sn-glycero-3-phosphocholine (PC-di20:4), 2-arachidonoyl-1-palmitoyl-sn-glycero-3-phosphocholine (PC-20:4/16:0), PC from egg yolk (PC-EY), PC from soybean (PC-SB), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE-di18:1) (Fluka Biochemica, Switzerland), PE from soybean (PE-SB), 1,2-diacyl-sn-glycero-3-phosphoinositol from soybean (PI-SB), and 1,2-dioleoyl-sn-glycero-3-phosphoserine sodium salt (PS-di18:1). Unless otherwise stated, all the compounds were obtained from Sigma. The parasites were cultured for 4 days after inoculation (two cycles of complete growth), except when otherwise stated.

For the reconstitution of lipids, dried lipid precipitates were prepared, added with culture media, and sterilized, as previously described (Asahi et al., 2005).

2.3. Assessment of parasite growth

Samples were taken at the times indicated, and thin smears were made and stained with Giemsa. More than 10,000 RBC were examined to determine the percentages of PRBC (parasitemia). The growth rate was first estimated by dividing the parasitemia of the test sample 4 days after inoculation by the initial parasitemia, except when otherwise stated. Measurement of growth was also performed using the lactate dehydrogenase of *P. falciparum* (PfLDH) assay (Asahi et al., 2005; Makler and Hinrichs, 1993). The Malstat reagent (Flow Inc., USA) was used, and the PfLDH assay was performed according to the manufacturer's instruction. Briefly, PRBC/RBC in cultures was hemolyzed by three freeze-thaw cycles, and a 15-μl aliquot was transferred to each well of a 96-well microtiter plate. Then, 100 μl of the Malstat reagent, 10 μl of 1 mg/ml nitroblue tetrazolium (Wako) and 10 μl of 1 mg/ml diaphorase (Wako) were added to each well. The plate was allowed to stand for 40 min at 37 °C, and the reaction was stopped by the addition of acetic acid. The absorbance at 655 nm was determined and the initial value was subtracted from the final reading. For each experiment, PRBC were divided into identical aliquots, and different treatments were performed simultaneously. To make the results comparable across experiments, untreated control wells, cultured in GFSRPMI, were set up each time. All experiments were repeated two to four times.

2.4. Separation of lipids

Known amounts of GFS were extracted using the method of Bligh and Dyer (Asahi et al., 2005). The GFS-C was evaporated and resuspended in the original volume of culture medium used for the assay.

2.5. Statistical analysis

Statistical significance of differences between means was evaluated using multifactorial analysis of variance (ANOVA). All the calculations were performed using GraphPad PRISM 5 (GraphPad Software, Inc., USA). The *P* value for significance was 0.05, and all pairwise comparisons were made post hoc with Bonferroni's test. For the graphical representation of the data, *y*-axis error bars were added to indicate the standard deviation for each point.

3. Results

3.1. Factors in GFS responsible for amplifying growth-promoting activity of NEFA

For the initial experiments designed to determine the factor(s) responsible for the high growth-promoting activity of GFS,

P. falciparum was cultured with the lipid classes found in GFS-C and with various chemically defined lipids, in the presence of BSAF and different concentrations of a mixture of the two most abundant NEFA found in GFS-C, C18:1 (0–60 μg/ml [212.4 μM]) and C16:0 (0–30 μg/ml [117.0 μM]) at a ratio of 2:1. The growth rate was dependent on the concentrations of the NEFA in the mixture: the maximum effect was obtained with 30 μg/ml C18:1 plus 15 μg/ml C16:0 (mean ± standard deviation, 7.59 ± 1.06), with a decline at 15 μg/ml C18:1 plus 7.5 μg/ml C16:0 (6.53 ± 0.35) and at 60 μg/ml C18:1 plus 30 μg/ml C16:0 (2.32 ± 0.15). These growth rates were, however, much lower than those with GFS-C plus BSAF (22.03 ± 4.50) and GFSRPMI (18.41 ± 1.24).

It was unexpectedly found that, when a lipid mixture containing Pld such as phosphatidylcholine (PC) at a high concentration, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), CHOL, CE, and DAG was added to the culture media containing NEFA, the parasite growth was increased to an extent similar to or greater than that seen with GFS-C + BSAF and GFSRPMI (Table 1). In an attempt to identify the factor(s) involved in this amplified growth-promoting effect on the parasite, each lipid was omitted from the medium in turn. In the absence of PC, parasite growth decreased to a level similar to that seen with NEFA plus BSAF (Table 1). Omission of PE, PS, or PA also resulted in a decrease in parasitemia, but to a lesser extent (Table 1). On the other hand, in the absence of PI, CHOL and CE, the growth rate was significantly higher than that in their presence. These results indicate the critical importance of PC for parasite growth in culture medium. PE, PS, and PA were beneficial to parasite growth, whereas PI, CHOL and CE were detrimental. DAG had no effect on the growth rate of the parasite at the concentration tested.

3.2. Effect of Pld on ability of NEFA to promote parasite growth

Graded concentrations of various PC-containing fatty acid moieties were tested for their abilities to augment the effects of the NEFA mixture on parasite growth, in the presence of other Pld (PE+PS+PA) and BSAF. Among 12 PC tested, PC-di18:1 was found to markedly amplify the growth-promoting ability of the NEFA mixture in a dose-dependent manner and at a wide range of concentrations, to a level similar to that seen with GFSRPMI (Table 2).

Table 1
Effect of various classes of lipid on ability of NEFA to sustain growth of *P. falciparum*

Constituents	Growth rate % of control ± SD
NEFA(C18:1+C16:0)* + BSAF	100.0 ± 10.5
Mixture of all constituents	287.1 ± 3.0*,§
[NEFA(C18:1+C16:0) + Pld(PC+PE+PS+PI+PA) + CHOL + CE + DAG + BSAF]**	
PC (−)***	133.5 ± 11.2*,¶
PE (−)	227.1 ± 13.5*,¶
PS (−)	246.9 ± 6.7*,¶
PI (−)	310.9 ± 10.8*,¶
PA (−)	258.4 ± 0.2*,¶
CHOL (−)	306.9 ± 9.1*,¶
CE (−)	307.8 ± 2.4*,¶
DAG (−)	286.2 ± 1.6*,§
GFS-C + BSAF	261.0 ± 7.6*,¶
GFSRPMI	214.9 ± 7.9*,¶

The culture media contained BSAF, except for GFSRPMI. *,**The concentrations were 30 μg/ml C18:1, 15 μg/ml C16:0, 165 μg/ml PC-di18:1, 20 μg/ml PE-SB, 10 μg/ml PS-di18:1, 10 μg/ml PI-SB, 10 μg/ml PA-di18:1, 10 μg/ml CHOL, 10 μg/ml CE-18:1, 10 μg/ml DAG. ***Each lipid was omitted from the mixture of all constituents. The growth in the presence of BSAF alone (3.7% ± 7.9%) and of a mixture of all constituents depleted of NEFA (33.0% ± 2.9%) was also tested for comparison. #Significant differences ($P < 0.001$) and ##($P < 0.05$) versus the growth in the presence of NEFA (C18:1+C16:0) + BSAF. ¶Significant difference ($P < 0.001$) versus the growth in a mixture of all constituents. §No significant difference.

The addition of PC-di12:0, PC-di16:0, and PC-18:1/16:0 also increased the growth rate to >200% at certain concentrations (Table 2). The addition of PC-di20:4 and PC-20:4/16:0 was also beneficial to parasite growth to a lesser extent. The addition of PC-di6:0, PC-di14:0, PC-rac-di16:0, PC-di18:2, PC-EY, and PC-SB to the medium had no marked effect on, or was detrimental to, parasite growth.

Although Pld other than PC were not critical for optimal growth of the parasite cultured in the presence of high concentrations of PC and NEFA associated with BSAF, PE, PS, and PA were tested for their possible efficacy in augmenting the ability of paired NEFA to promote parasite growth. The addition of PE, PS, or PA failed to increase the growth-promoting efficacy of paired NEFA, indicating that PE, PS, and PA could not substitute for PC (Table 3, Experiment A). However, the addition of mixtures of Pld (PE, PS, and PA) to the medium was beneficial to parasite growth to a lesser extent (Table 3, Experiment B).

3.3. Effects of various types of NEFA on parasite growth in the presence of Pld

NEFA mixtures of C18:1 and C16:0 enriched with BSAF and Pld, were tested for their ability to promote parasite growth. The growth rate was significantly higher than that with corresponding concentrations of NEFA mixtures in the absence of Pld (Fig. 1). The growth rate was dependent on the ratio of the two NEFA, ranging from 1:5 to 5:1 (C18:1 to C16:0), at a total concentration of 45 μg/ml. The highest growth rate was obtained using C18:1 (30 μg/ml, 106.2 μM) plus C16:0 (15 μg/ml, 58.5 μM) (Fig. 1).

The culture media were reconstituted by mixing Pld and BSAF with two NEFA (either C18:1 plus a saturated one or C16:0 plus an unsaturated one). The best combination of NEFA was found to

Table 2
Effect of various types of PC on ability of NEFA to sustain growth of *P. falciparum*

Constituents	Concentration (μg/ml)	Growth rate % of control ± SD
NEFA(C18:1+C16:0)* + BSAF		100 ± 4.9
[NEFA(C18:1+C16:0) + Pld(PE+PS+PA) + BSAF]**		
+PC-di12:0***	160	Hemolyzed
	80	104.4 ± 21.6
	40	245.9 ± 24.7*,§
+PC-di16:0	160	140.8 ± 10.0
	80	270.3 ± 9.4*,§
	40	185.8 ± 2.8*,§
+PC-di18:1	320	242.6 ± 23.8*,§
	160	292.0 ± 15.3*,§
	100	312.7 ± 16.9*,§
	80	273.9 ± 6.9*,§
	40	170.9 ± 8.8*,§
+PC-18:1/16:0	160	215.2 ± 9.7*,§
	80	171.3 ± 9.3*,§
	40	152.9 ± 22.8*,§
+PC-di20:4	160	156.5 ± 23.5*,§
	80	166.7 ± 13.8*,§
	40	157.1 ± 15.3*,§
+PC-20:4/16:0	160	75.4 ± 11.1*,§
	80	157.1 ± 17.2*,§
	40	169.0 ± 16.9*,§
GFSRPMI		294.4 ± 15*,§

The culture media contained BSAF, except for GFSRPMI. *,**The concentrations were 30 μg/ml C18:1, 15 μg/ml C16:0, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, 10 μg/ml PA-di18:1. ***Each PC was added to the mixture of NEFA + Pld (PE+PS+PA) + BSAF. The growth in the presence of BSAF alone (4.7% ± 0.6% and of a mixture of Pld (PC+PE+PS+PA) + BSAF (31.8% ± 0.7% were also tested for comparison. #Significant difference ($P < 0.001$) versus the growth in the presence of NEFA (C18:1+C16:0) + BSAF. §No significant difference. Others are significantly different ($P < 0.001$–0.05) versus the growth in GFSRPMI.

Table 3
Effects of Pld at graded concentrations (A) and combinations of (B) on ability of NEFA to sustain growth of *P. falciparum*

Constituents	Concentration (μg/ml)	Growth rate % of Control ± SD
Experiment A		
NEFA(C18:1+C16:0)*		100.0 ± 8.0
+PE-SB*	80	115.8 ± 8.6
	40	109.2 ± 4.0
	20	102.0 ± 8.0
+PE-di18:1*	80	138.3 ± 7.2**
	40	105.5 ± 5.2
	20	96.3 ± 11.8
+PS-di18:1*	80	111.2 ± 8.3
	40	117.2 ± 6.6
	20	107.5 ± 5.2
+PA-di18:1*	80	108.0 ± 6.0
	40	116.7 ± 13.2
	20	128.4 ± 19.3#
NEFA(C18:1+C16:0)* + Pld(PC+PE+PS+PA)***		272.7 ± 20.7#
GFSRPMI*		258.0 ± 9.8#
Experiment B		
NEFA(C18:1+C16:0)*		100.0 ± 23.9
NEFA(C18:1+C16:0)*		
+Pld(PC)*		229.4 ± 6.4#
+Pld(PC+PE)*		224.5 ± 17.9#
+Pld(PC+PE+PA)*		276.1 ± 13.2#
+Pld(PC+PE+PS)*		230.0 ± 8.8#
+Pld(PC+PE+PS+PA)***		317.9 ± 26.7#,§
GFSRPMI*		294.2 ± 13.5#,§

The culture media contained BSAF, except for GFSRPMI. *,***The concentrations were 30 μg/ml C18:1, 15 μg/ml C16:0, 100 μg/ml PC-di18:1, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, and 10 μg/ml PA-di18:1. **PE-SB and PE-di18:1 in the presence of NEFA + Pld (PS+PA), PS-di18:1 in the presence of NEFA + Pld (PE+PA) and PA-di18:1 in the presence of NEFA + Pld (PE+PS) was tested for the ability to promote growth of the parasite. #Significant differences ($P < 0.001$) and ##($P < 0.01$) versus the growth in the presence of NEFA (C18:1+C16:0) + BSAF. §No significant difference.

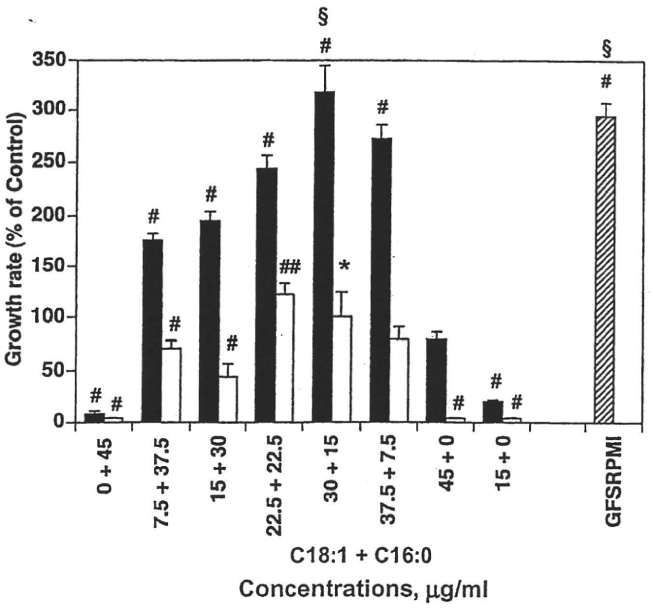

Fig. 1. Growth of *P. falciparum* in the presence of two NEFA at various ratios. The parasite was cultured either in the presence (■) or in the absence (□) of Pld. *The paired NEFA (30 μg/ml C18:1 and 15 μg/ml C16:0) added to media in the absence of Pld served as a control. The Pld concentrations were 100 μg/ml PC-di18:1, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, and 10 μg/ml PA-di18:1. The culture media contained BSAF, except for GFSRPMI. The growth in BSAF alone (4.0% ± 0.5%) and Pld (PC+PE+PS+PA) + BSAF (19.8% ± 3.8%) were also tested for comparison. #Significant differences ($P < 0.001$) and ##($P < 0.05$) versus the growth in the presence of NEFA (•). §No significant difference.

be C18:1 (C18:1-cis-9) plus C16:0, followed by the combinations of C18:1-cis-11 plus C16:0, C18:1 plus C15:0, C18:1 plus C18:0, and C18:1 plus C14:0 (Fig. 2). The combinations of C16:1 plus C16:0, C18:1-cis-6 plus C16:0, C18:1-cis-13 plus C16:0, and C18:2 plus C16:0 had growth-promoting effects at a level similar to that seen with C18:1 plus C16:0 in the absence of Pld. Combinations of C18:1 plus C12:0, C18:1 plus C22:0, C18:3 plus C16:0, C20:4 plus C16:0, C20:5 plus C16:0, and C22:6 plus C16:0 were detrimental to parasite growth. The combination of C18:1-trans-9 plus C16:0 also deterred parasite growth, indicating that the growth-promoting effect of C18:1 on the parasite is specific to the *cis*-form (Fig. 2).

3.4. Chemically defined medium for parasite growth with the use of recHA

To determine if chemically defined proteins could sustain parasite growth, *P. falciparum* was cultured with recHA, paired NEFA, and Pld. Parasite growth in culture medium enriched with recHA was similar to, or better than, that in media supplemented with BSAF or HSAF (Fig. 3). These results indicate that recHA can replace BSAF or HSAF for promoting and sustaining parasite growth in the presence of lipids (NEFA and Pld). These results provide a chemically defined culture medium suitable for sustaining the growth of *P. falciparum*.

3.5. Growth of parasites cultured in chemically defined media containing various growth promoters

PRBC were maintained for 2–5 days in culture media containing recHA or BSAF with various mixtures of lipid growth promoters. Parasite development in the presence of recHA was similar to that in the presence of BSAF and in GFSRPMI (Fig. 4). The parasites could be maintained in medium containing NEFA, Pld (PC+PE+PS+PA) and either recHA or BSAF for ⩾6 weeks (12 subcultures), without any decrease in growth rate.

4. Discussion

The ability of lipids to sustain the growth of *P. falciparum* was determined. It was found that high concentrations of PC-di18:1 was sufficient for the complete augmentation of the poor parasite growth-promoting efficacy of NEFA in the presence of specific proteins. Several other PC, including PC-di12:0, PC-di16:0, PC-18:1/16:0, PC-di20:4, and PC-20:4/16:0, were also beneficial to parasite growth in the presence of NEFA, but to a lesser extent. Although Pld other than PC, such as PE, PS and PA, were not critical for optimal parasite growth in the presence of NEFA and PC, their addition to the medium was beneficial to a small extent. Different combinations and concentrations of paired NEFA had differing effects on parasite growth, with the best combination being C18:1 plus C16:0, in the presence of Pld and BSAF. recHA could replace BSAF or HSAF in culture media enriched with structurally defined lipids, to produce a chemically defined medium suitable for parasite growth.

The maximum efficacy of NEFA mixtures for sustaining parasite growth was much lower than that of GFS-C, and the addition of PC-containing a specific fatty acid moiety amplified the poor growth-promoting efficacy of NEFA to an extent similar to that seen with GFS-C and GFSRPMI. Nevertheless, NEFA could be the dominant factors involved in growth promotion, because Pld plus BSAF alone had no growth-promoting ability. Malarial parasites were long considered to be unable to synthesize fatty acids or Pld via de novo biosynthesis; instead, they were thought to be dependent on fatty acids scavenged from the host plasma and RBC for the synthesis of membrane lipids (Holz, 1977; Vial and Ancelin, 1998). On the basis of our current understanding, however, type II fatty acid synthetic

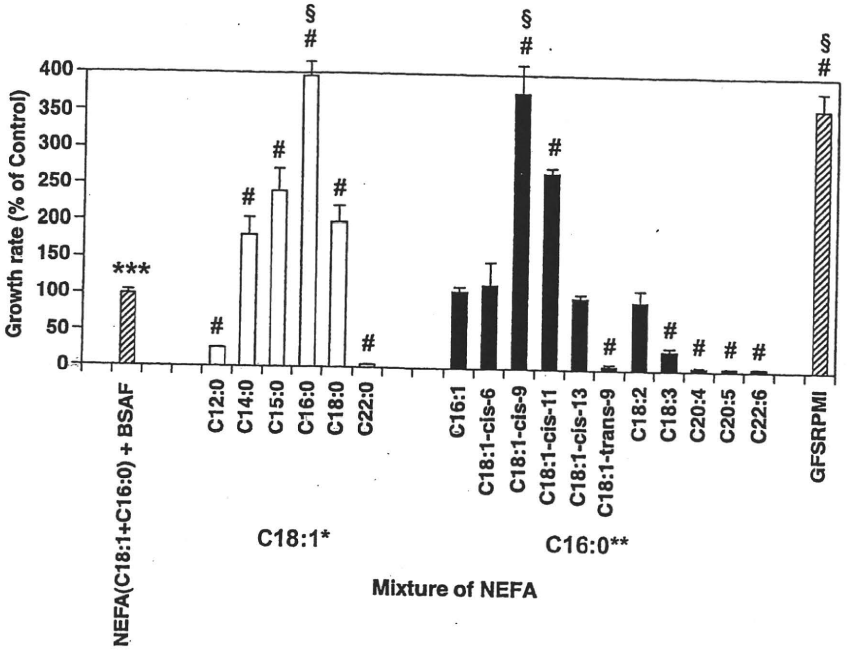

Fig. 2. Growth of *P. falciparum* in the presence of various combinations of paired NEFA. Each saturated NEFA was added at 15 μg/ml in the presence of 30 μg/ml C18:1 (•) and each unsaturated NEFA at 30 μg/ml in the presence of 15 μg/ml C16:0 (••). These culture media contained Pld (100 μg/ml PC-di18:1, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, and 10 μg/ml PA-di18:1) and BSAF. ***NEFA(C18:1+C16:0) + BSAF in the absence of Pld served as a control (100%). The growth in BSAF alone (6.3% ± 0.8%) and Pld (PC+PE+PS+PA) + BSAF (32.8% ± 1.7%) were also tested for comparison. #Significant difference ($P < 0.001$) versus the growth in the presence of NEFA (C18:1+C16:0) + BSAF. §No significant difference.

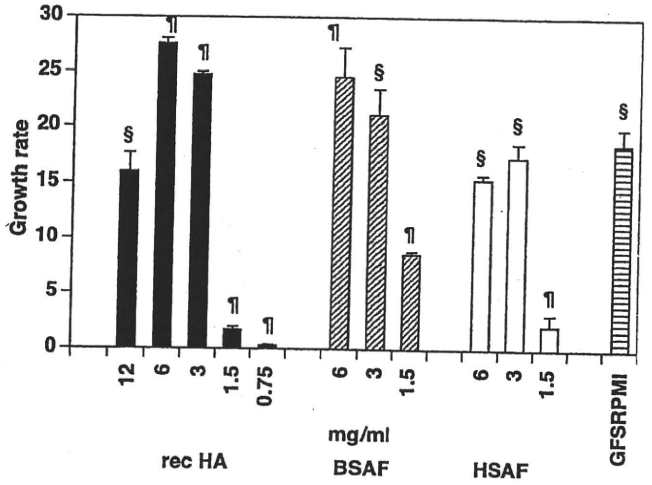

Fig. 3. Effect of various proteins on the ability of a mixture of NEFA and Pld to sustain growth of *P. falciparum*. The culture media contained NEFA (30 μg/ml C18:1 + 15 μg/ml C16:0) and Pld (100 μg/ml PC-di18:1 + 20 μg/ml · PE-di18:1 + 10 μg/ml PS-di18:1 + 10 μg/ml PA-di18:1). recHA alone, BSAF alone, and HSAF alone failed to show a growth-promoting effect on the parasite. ¶Significant difference ($P < 0.001$) versus the growth in GFSRPMI. §No significant difference.

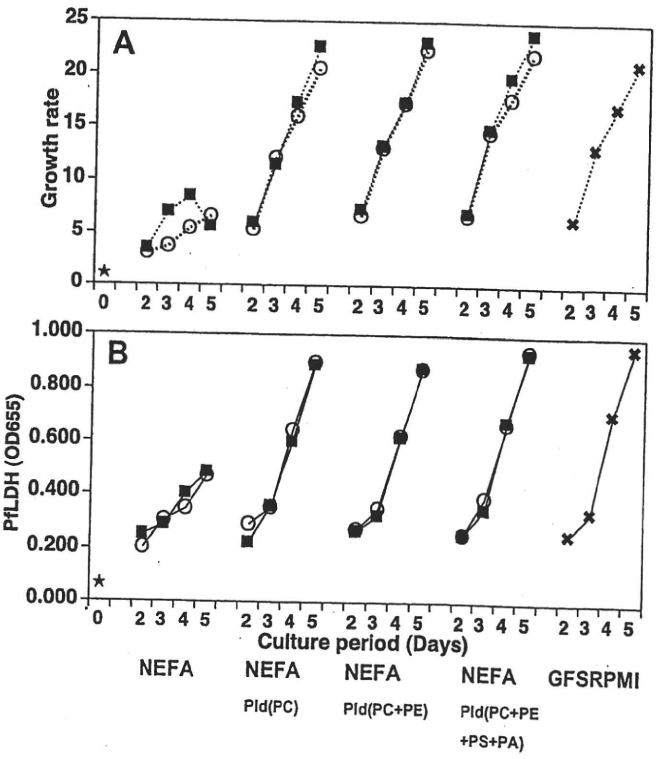

Fig. 4. Growth of *P. falciparum* cultured in the presence of various growth promoters and proteins. The media contained either BSAF (■) at 3 mg/ml or recHA (○) at 4 mg/ml, except for GFSRPMI. Culture media were CRPMI enriched with NEFA alone, NEFA + Pld (PC), NEFA + Pld (PC+PE), or NEFA + Pld (PC+PE+PS+PA). The paired NEFA were added to media at 30 μg/ml C18:1 and 15 μg/ml C16:0. The Pld concentrations were 100 μg/ml PC-di18:1, 20 μg/ml PE-di18:1, 10 μg/ml PS-di18:1, and 10 μg/ml PA-di18:1. The parasite growth was assessed in Giemsa-stained smears (A) and by PfLDH-based Malstat assay (B). The growth of the second subculture is shown, except for the growth of the first subculture seen in CRPMI supplemented with NEFA and either BSAF or recHA. *Start levels of the cultures.

machinery does exist in *P. falciparum* the parasite has been demonstrated to synthesize fatty acids (Surolia and Surolia, 2001; Waller et al., 2003; Yeh and Altman, 2006). Thus, *P. falciparum* may satisfy its fatty acid requirements via two independent mechanisms: (1) by scavenging NEFA from the host plasma or from fatty acids released by the enzymatic action of lipases on the lipids, and (2) by de novo synthesis using the type II synthetase system. The data presented here show that *P. falciparum* predominantly scavenges NEFA from the external milieu for growth promotion, although it is unclear whether the NEFA are modified in any way. In particular, the NEFA involved in the growth promotion of *P. falciparum* have to be in specific pairs. Furthermore, the type and total amount of

NEFA markedly influenced parasite growth in the presence of Pld and BSAF, with the best combination being that of the two most abundant NEFA in GFS and HS. There is increasing evidence showing the involvement of unsaturated NEFA in numerous biological processes, including the activation of protein kinases, cell proliferation, differentiation and cell death (Malhi et al., 2006; Murakami et al., 1986). It has been reported that C16:0 increased oxidative stress, activation of stress-associated protein kinases, and apoptosis of myocyte cells, and that low concentrations of C18:1 completely prevented C16:0-induced cytotoxic stress (Hardy et al., 2000; Miller et al., 2005). Further study is necessary to determine the mechanisms underlying the actions of NEFA, in combination with Pld and proteins, in *P. falciparum*.

Pld metabolism is absent from normal mature human RBC, but in PRBC, the marked increase in membrane content is associated with a considerable increase in the total lipid content (Holz, 1977; Vial and Ancelin, 1998). It has been considered that malarial parasites satisfy their own requirements for nutrition and membrane-building using these Pld (Maguire and Sherman, 1990; Vial and Ancelin, 1998). In addition to the de novo synthesis of Pld, it has been well-demonstrated that RBC infected with *P. falciparum* or *P. knowlesi* readily take up intact Pld from exogenous sources (Grellier et al., 1991; Haldar, 1992; Moll et al., 1988; Simoes et al., 1991, 1992). Among the various PC tested here, PC-di18:1 markedly increased the ability of the NEFA mixture to promote parasite growth, while other PC exerted different effects on parasite growth: the addition of PC-di12:0, PC-di16:0, PC-18:1/16:0, PC-di20:4, and PC20:4/16:0 effectively augmented the growth-promoting effect of the NEFA mixture, but to a lesser extent. The addition of PC-di6:0, PC-di14:0, PC-rac-di16:0, PC-di18:2, PC-EY, and PC-SB either failed to alter the activity of the NEFA mixture significantly, or were detrimental. This suggests that certain structural parameters of not only NEFA, but also PC and proteins, are important for the growth-promoting activity of NEFA. Further studies are necessary to determine the mechanism(s) underlying the actions of Pld in association with NEFA mixtures.

The replacement of HS in culture medium for *P. falciparum* with chemically- or functionally-defined substances is not only advantageous for the culture of the parasite, but will also provide critical clues to the parasite's requirements for proliferation at the erythrocytic stage. Considerable efforts have been made to identify factors and substances with the ability to sustain parasite growth (Asahi and Kanazawa, 1994; Asahi et al., 1996, 2005; Cranmer et al., 1997; Divo and Jensen, 1982; Lingnau et al., 1994; Mi-Ichi et al., 2006; Nivet et al., 1983; Ofulla et al., 1993; Willet and Canfield, 1984). Nevertheless, the inclusion in the culture medium of specific proteins such as bovine and human ALB are essential for parasite growth, indicating that all the serum-free media described so far are only chemically semi-defined. The establishment of a fully-defined culture medium for the parasite still represents a major challenge. Recently, recHA has become commercially available and has been safely used for drug delivery and cell culture applications, with various benefits (Bosse et al., 2005). In this study, recHA could be used for the continuous culture of *P. falciparum*, as a substitute for bovine and human ALB in culture media enriched with structurally defined lipids. This indicates that we have established a chemically defined medium for *P. falciparum*. Further, in the growth-promoting activity experiment, parasites were cultured for 4 days after inoculation, without renewal of the medium, to avoid fluctuation of culture conditions. The rate of parasite growth might, however, be further improved by frequent renewal of the medium, particularly for continuous culture of the parasite.

We are currently attempting to characterize the parasite factors that interact at the molecular level with the growth-promoting agents detected here, with the hope that clarification of the mechanisms underlying the growth promotion of the parasite may lead to the development of novel antimalarial strategies.

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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.exppara.2008.09.009.

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SHORT COMMUNICATION

A case of *Diphyllobothrium nihonkaiense* infection possibly linked to salmon consumption in New Zealand

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Abstract Global increases in salmon consumption and changes in fish-eating habits have meant that *Diphyllobothrium* infections now occur in regions where they were previously absent. In the present paper, a case of diphyllobothriasis through the likely ingestion of raw salmon in New Zealand in a Japanese patient is reported. The causative tapeworm species was identified as *Diphyllobothrium nihonkaiense* based on mitochondrial DNA analysis of proglottid expelled from the patient.

Introduction

Diphyllobothriasis is caused by the infection of adult broad tapeworm (*Diphyllobothrium* spp.). Of the 37 species belonging to the genus, 11, including *Diphyllobothrium nihonkaiense*, *Diphyllobothrium latum*, and *Diphyllobothrium pacificum*, cause diphyllobothriasis in humans (Kamo 1999). Humans are infected by eating raw or undercooked fish containing larval plerocercoids, which develop into adult tapeworms in the small intestine after ingestion. Clinical symptoms are light diarrhea and abdominal discomfort, and, in the case of *D. latum* infection, vitamin-B12-deficienct anemia. Human diphyllobothriasis has been reported in Europe (Dick et al. 2001; Dupouy-Camet and Peduzzi 2004; Chai et al. 2005; Jackson et al. 2007), Asia (Dick et al. 2001; Yamane and Shiwaku 2003; Chai et al. 2005), North America (Dick et al. 2001; Chai et al. 2005), and South America (Torres et al. 1993; Santos and de Faro 2005; Sampaio et al. 2005). Diphyllobothriasis due to *D. nihonkaiense* was previously restricted to Asia, particularly Japan (Yamane et al. 1986; Yoshida et al. 1999; Ando et al. 2001; Yamane and Shiwaku 2003; Yamasaki et al. 2007) and Korea (Kim et al. 2007); however, the *D. nihonkaiense* infections have recently been reported in France (Yera et al. 2006), Switzerland (Wicht et al. 2007; Shimizu et al. 2008), and Canada (Wicht et al. 2008). Here, a Japanese case of *D. nihonkaiense* infection, likely acquired through the ingestion of raw salmon meat in New Zealand, is reported.

Case report

The patient was a 52-year-old Japanese man. On July 21, 2006, he developed abdominal pain and sudden diarrhea, which continued until the next day when his stool became increasingly watery. On the second evening, the patient naturally passed a strobila without a scolex in his watery stool (data not shown). The strobila was preserved in ethanol for molecular identification. On July 23, the patient consulted the Asahikawa Medical College Hospital (Asahikawa, Japan), where he was administered an injection of gastrografin. However, radiological examination did not reveal the presence of any tapeworms and no evidence of tapeworms was observed in stool sample. Repeated treatment with praziquantel (20 mg/kg) on July 29 did not result in purging of the tapeworm. At a

Nucleotide sequence datum reported in the present paper is available in the DDBJ/EMBL/GenBank databases under accession number AB364645.

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retrospective interview, the patient stated that he accidentally ate raw salmon in Christchurch, New Zealand on February 7, 2006, although he disliked salmon and had never eaten it before visiting New Zealand. Based on this history, it appeared that diphyllobothriasis infection occurred through the ingestion of raw salmon in New Zealand.

Materials and methods

To identify the causative tapeworm species, genomic DNA was extracted from the ethanol-fixed proglottid using a DNeasy tissue kit (Qiagen, Germany) and the cytochrome *c* oxidase subunit 1 gene (*cox1*, 1,566 bp) was amplified using a primer pair for the NADH dehydrogenase subunit 3 gene (5′-ATGTTAGCTTTATTTTTGGTGG-3′) and the ribosomal RNA large subunit gene (5′-CTATACACATTTACTTGATCTCCTC-3′) and the following polymerase chain reaction protocol: 94°C for 30 s, 58°C for 30 s, and 72°C for 90 s, repeated for 35 cycles plus one cycle of 72°C for 5 min with *Ex Taq* DNA polymerase (Hot Start version, TaKaRa Bio, Japan). Samples for DNA sequencing were prepared using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA) and sequencing was performed on either ABI PRISM 310 or 3100-Advant Genetic Analyzer (Applied Biosystems). Sequence data were analyzed by Kimura's two parameters (Kimura 1980) and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987).

Results and discussion

The nucleotide sequence (AB364645) of the *cox1* gene of the diphyllobothriid from the patient showed 99.5%, 99.7%, 99.7%, and 100% identities with the *D. nihonkaiense* reference sequences AM412559, AB015755, AB268585, and AM412560, respectively, whereas identities with *D. latum* reference sequences AB269325, AY972071, and DQ985706 were 92.9%, 92.9%, and 92.9%, respectively. Phylogenetic analysis performed using 396-bp *cox1* fragments (nucleotide positions 733–1128) confirmed that the causative tapeworm was *D. nihonkaiense* (Fig. 1).

In order to clarify the probable source of infection, the restaurant where the raw salmon dish was served was contacted for information. Since these requests were unsuccessful, an Internet search on salmons in New Zealand was conducted. The search revealed that chinook salmon (*Oncorhynchus tshawytscha*) is farmed on a significant scale in sea cages in New Zealand and that the

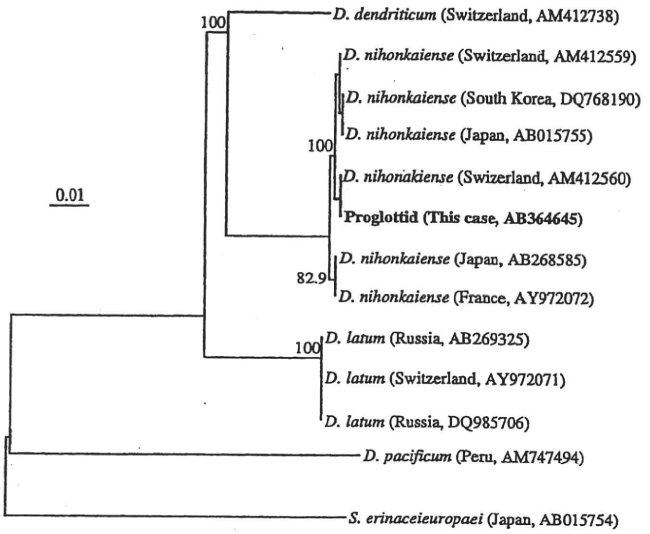

Fig. 1 Phylogenetic tree inferred from 396-bp *cox1* fragments using the neighbor-joining method. Numbers at branches indicate bootstrap values for 1,000 replicates and a scale bar represents the genetic distance based on Kimura's two-parameter model. *Spirometra erinaceieuropaei* is used as an out-group

farmed salmons are produced for both local and export markets (http://www.salmon.org.nz/aboutsalmon.shtml). However, at present, the likelihood of the farmed salmon being an infection source has not been confirmed. According to import–export statistics for the salmon industry in New Zealand, the country imports only canned and smoked/processed pink salmon (*Oncorhynchus gorbuscha*) and sockeye salmon (*Oncorhynchus nerka*) from North America; no fresh/chilled salmon is imported by the country (http://www.alaskaseafood-japan.com/enasmi/data/top.html; http://www.sf.adfg.state.ak.us; http://www.infoexport.gc.ca; http://www.japan.gc.ca). Moreover, according to trade statistics published by the Ministry of Finance, Japan, although any salmons are not exported from Japan to New Zealand on any significant scale (http://www.customs.go.jp/toukei/info/index_e.htm), both wild chum salmon (*Oncorhynchus keta*, frozen condition) and farmed coho salmon (*Oncorhynchus kisutch*, chilled condition) are probably exported to New Zealand on a small scale (personal communication, Fish Information Services, Tokyo, Japan).

Extensive fieldworks on salmonids fishes have revealed that *D. nihonkaiense* infestations in humans occur primarily through masu salmon (*Oncorhynchus masou masou*) and pink salmon (Nagasawa et al. 1987; Kamo 1999). Records of plerocercoid infestation by *Dibothriocephalus latus* (probably *D. nihonkaiense*) in chum salmon caught in the waters off Sakhalin (Eguchi 1929) and *D. latum* (probably *D. nihonkaiense*) in sockeye salmon caught in the rivers in northern Japan (Kato 1931) were published previously. However, despite extensive sampling, any *D. nihonkaiense* plerocercoids were not found in chum salmon going

upstream in the Chitose River, Hokkaido, northern Japan (Urawa 1986), and it was pointed out whether chum and sockeye salmons serve as second intermediate hosts should be reconfirmed (Kamo 1999). It has recently been reported that *D. nihonkaiense* plerocercoids with infection rates as high as 51.1% were found in immature chum salmon commonly referred to as *tokishirazu* (Suzuki et al. 2006). *Tokishirazu* is a salmon originating from the Amur River, the Far East Russia, and caught in the waters off northeastern Japan from April to July, which does not spawn (Okazaki 1986). Most recently, the presence of *Diphyllobothrium klebanovskii*, which is synonymous with *D. nihonkaiense*, has been confirmed in human, brown bear (*Ursus arctos piscator*), and chum salmon from the Kamchatka Peninsula and Sakhalin Island, the Far East Russia (Arizono et al. 2009). Accumulating these evidences (Eguchi 1929; Suzuki et al. 2006; Arizono et al. 2009), it is no doubtful that chum salmon from the Far East Russia is one of the major sources of diphyllobothriasis nihonkaiense in humans. However, given multiple potential sources of the salmon species, the infection source in the case reported here could not be specified. In most cases with *D. nihonkaiense* infections, identification of the salmon species was speculative, being based on the interviews of patients or information provided when the salmons were sold in markets or indicated on the salmon products themselves, and it is difficult to identify salmon species based on fish filets, *sashimi*, *sushi*, and other salmon dishes. Identification of the salmons as sources of infection of the diphyllobothriasis should be done more critically, if possible, and it is necessary not to specify the salmon species based on uncertain information when clinical cases of the diphyllobothriasis are reported. Recently, a human case infected with *D. nihonkaiense* through eating sockeye salmon was reported in Canada (Wicht et al. 2008); however, an involvement of sockeye salmon in the life cycle of *D. nihonkaiense* is still unclear.

Salmon has become increasingly important commodities globally, and exports of fresh/chilled salmons from Japan, USA (Alaska), and Canada to China, the European Union, and other countries have increased markedly year by year with rapid advances of international transport systems (www.seafoodreport.com), resulting in an increase in the occurrence of diphyllobothriasis as an emerging parasitic disease in regions where it was previously absent (Cabello 2007). As mentioned above, the recent reports of *D. nihonkaiense* infections in Europe more likely reflect the advances in the international transport systems of fishes as well as changes in the fish-eating habits of people and preferences for specialties such as *sushi*, *sashimi*, *ceviche*, and *carpaccio* (Yera et al. 2006; Wicht et al. 2007; Shimizu et al. 2008). An outbreak of human diphyllobothriasis in Brazil, probably *D. latum* infection, attributed to the consumption of *sushi* or *sashimi* of aquacultured Atlantic salmon (*Salmo salar*) imported from Chile or an indigenous fish, common snook (*Centropomus undecimalis*; Sampaio et al. 2005).

Given the marked morphological similarities of diphyllobothriid species, attention should be directed at the molecular analysis for identifying *Diphyllobothrium* species (Yera et al. 2006; Nakao et al. 2007), even in cases where diphyllobothriid proglottids are fixed in formalin (Yamasaki et al. 2007).

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Laboratory and Epidemiology Communications

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

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

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

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

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

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

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

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

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