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

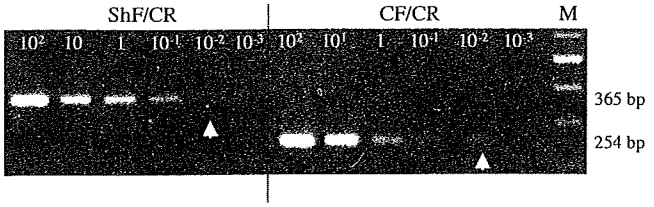

Fig. 2. Representative results of the sensitivity tests for monoplex PCR. The genomic DNA extracted from adult worms of *Schistosoma. haematobium* was serially diluted (10^2 to 10^{-3} pg) and applied *S. haematobium* specific PCR (ShF/CR) or schistosome common PCR (CF/CR). The arrow shows the detection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

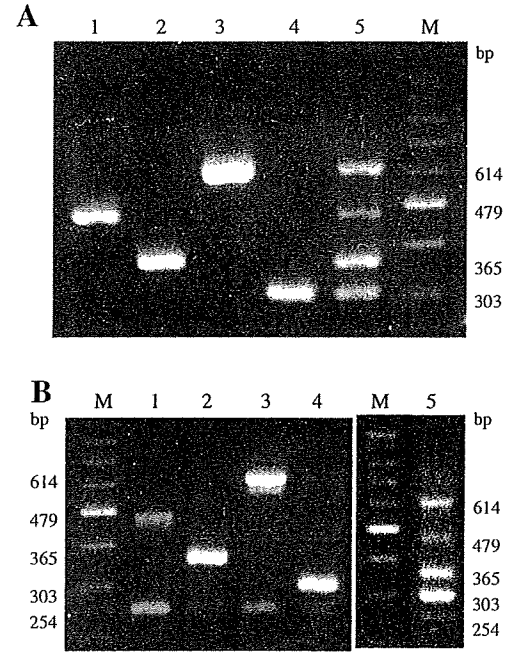

Fig. 3. Specificity tests for multiplex PCR using four sets of schistosome specific primers (A) and multiplex PCR using five sets of schistosome specific primers and common primer (B). Lane M, 100 bp molecular marker; Lane 1, *Schistosoma. mansoni*; Lane 2, *S. haematobium*; Lane 3, *S. japonicum*; Lane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

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 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 ± 13.6#,¶
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 ± 1.9#,§
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	133.3 ± 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 ± 3.2
+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.

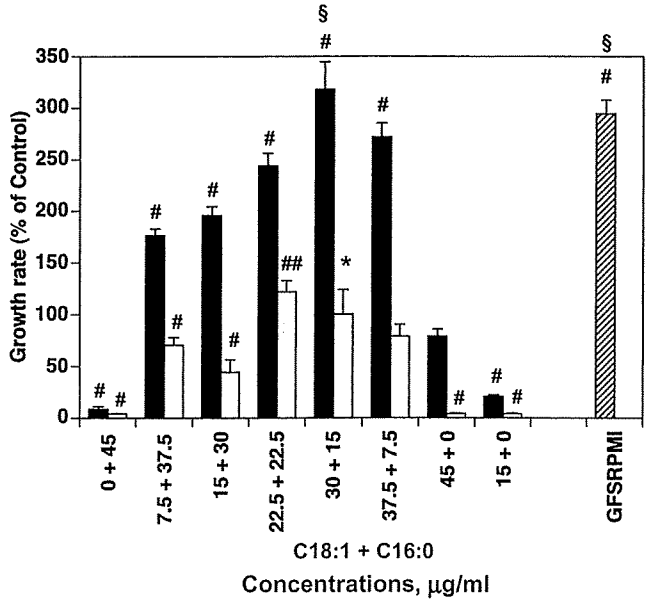

Fig. 1. Growth of *P. falciparum* in the presence of two NEFA at various ratios. The parasite was cultured either in the presence (■) or in the absence (□) of Pld. The paired NEFA (30 μg/ml C18:1 and 15 μg/ml C16:0) added to media in the absence of Pld served as a control. The Pld concentrations were 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

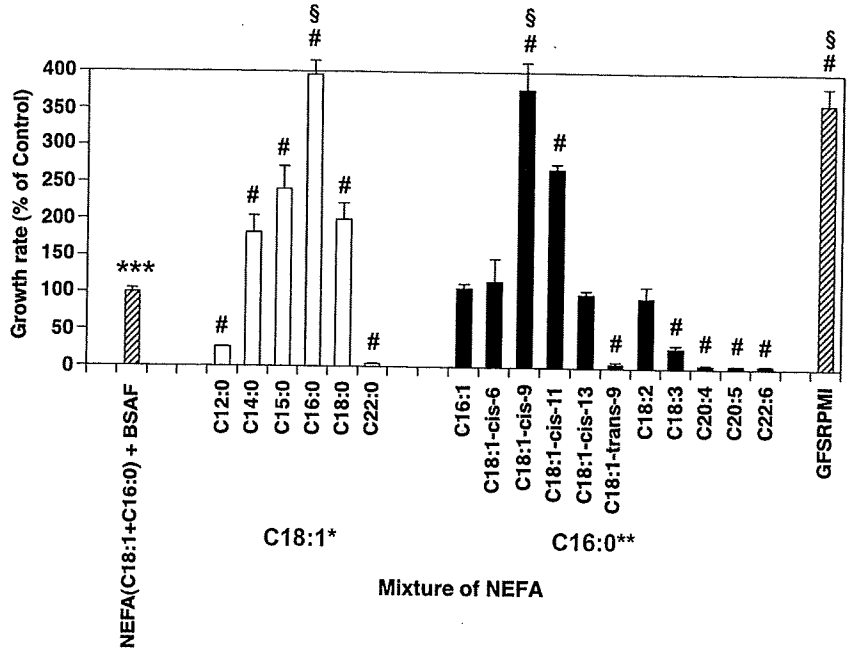

Fig. 2. Growth of *P. falciparum* in the presence of various combinations of paired NEFA. Each saturated NEFA was added at 15 μg/ml in the presence of 30 μg/ml C18:1 (*) and each unsaturated NEFA at 30 μg/ml in the presence of 15 μg/ml C16:0 (**). These culture media contained Pld (100 μg/ml PC-di18:1, 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.

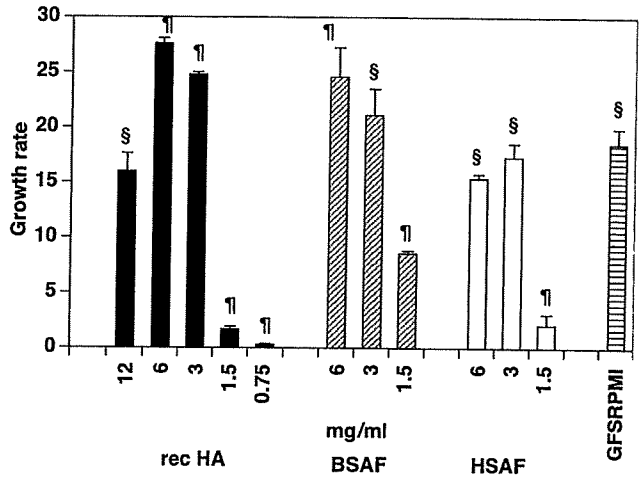

Fig. 3. Effect of various proteins on the ability of a mixture of NEFA and Pld to sustain growth of *P. falciparum*. The culture media contained NEFA (30 μg/ml C18:1 + 15 μg/ml C16:0) and Pld (100 μg/ml PC-di18:1 + 20 μg/ml PE-di18:1 + 10 μg/ml PS-di18:1 + 10 μg/ml PA-di18:1). recHA alone, BSAF alone, 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.

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

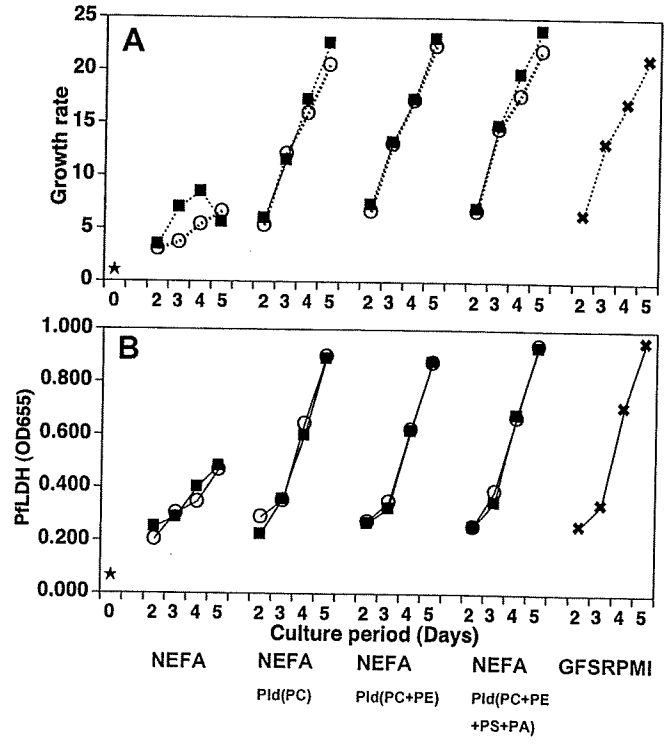

Fig. 4. Growth of *P. falciparum* cultured in the presence of various growth promoters and proteins. The media contained either BSAF (■) at 3 mg/ml or recHA (○) at 4 mg/ml, except for GFSRPMI. Culture media were CRPMI enriched with NEFA alone, NEFA + Pld (PC), NEFA + Pld (PC+PE), or NEFA + Pld (PC+PE+PS+PA). 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.

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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病理解剖用遺体に認められた原虫について

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はじめに

本邦では，太平洋戦争終了直後の時期をピークとして，一般住民間における寄生虫への感染率は減少し続け，1995年以降は0.1%以下になっているとされている[1]。しかしながら，昨今の海外旅行ブーム，国内への海外食品の輸入増加，新鮮魚介類の国内輸送システムの発達により，一般住民における寄生虫感染の増加も現実の問題として懸念されている[2]。

このような状況に鑑み，筆者は，調査対象のリスクグループとして，大学病院に入院し，そこで寄生虫症以外の主因で死亡したとみられる一般住民の患者につき，その遺体にかかわる寄生虫感染の状況を調べる目的で寄生虫相を調査したので報告する。

Pathogenic Protozoa Detected from Pathologic Subjects

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表1　調査遺体に関する主たる死因および死亡前の健康状態等.
(*寄生虫陽性例，調査遺体は全例日本在住の日本人で，特筆すべき渡航歴および施設入所経験なし)

解剖例(No.)	鑑別種	年齢(歳)	性別	主たる死因	死亡前の消化器症状および全身状態
1*	赤痢アメーバ	80	女	肝臓癌	下腹部痛，下痢，嘔吐（血便の記載なし） 細菌性腸炎の診断で抗生剤投与，軽減
2	陰性	52	男	胸腺腫瘍	死亡1週間前に遡っても消化器症状の記載なし，免疫状態低し
3*	赤痢アメーバ， ランブル鞭毛虫	65	男	大腸癌多発転移	腹水貯留．死亡4日前より原因不明の下血 肝内SOLなし
4	陰性	75	男	肺小細胞癌	死亡1週間前に遡っても消化器症状の記載なし，免疫状態低し
5*	赤痢アメーバ	75	男	肝硬変，肝細胞癌	腹水多量．肝不全でモニラック頻用．血便の記載なし
6	陰性	74	女	急性心筋梗塞	死亡1週間前に遡っても消化器症状の記載なし，免疫状態低し
7	陰性	78	女	肺水腫	死亡2日前に吐き気（心因性の診断．下血なし）
8*	赤痢アメーバ	71	男	肝不全	腹部膨満著明．肝内にSOL多数，死亡8日前からタール便
9	陰性	69	男	食道癌	消化器症状の記載なし
10*	赤痢アメーバ	62	男	肺癌・食道癌・ 多形癌	腹腔内転移で腹満・圧痛著明 原疾患によるものとして便検査施行せず
11*	赤痢アメーバ， ランブル鞭毛虫	79	男	慢性閉塞性肺疾患	便潜血陽性が持続．正球性貧血で輸血施行 死亡4日前に水様性下痢
12*	赤痢アメーバ	65	男	肺癌	消化器症状の記載なし
13	陰性	67	男	アルコール性肝硬変 による吐血	急性腎不全．腹水貯留による腹満感あり．下血なし．
14	陰性	78	男	慢性閉塞性肺疾患， 肺気胸	死亡4日前にMRSA腸炎で下痢．バンコマイシンで沈静化しつつあった．
15*	赤痢アメーバ， ランブル鞭毛虫	77	男	不整脈	脳梗塞，糖尿病で寝たきり．長く免疫不全状態で突然死，消化器症状の記載なし
16	陰性	37	女	アルコール性肝硬変， 脳出血	死亡3日前より下血があったが，肝硬変による凝固因子欠乏によるもの．

表2　病理解剖遺体に関する寄生虫検査結果
(*；病原性寄生虫陽性サンプル，nd；抗原検査陰性のため測定せず)

症例番号	便性状	検鏡検査	赤痢アメーバ抗原検査	PCR反応
1*	黒色，軟便	赤痢アメーバ	陽性	陽性
2	褐色泥状便	陰性	陰性	nd
3*	褐色泥水状便，臭い強し	赤痢アメーバ，ランブル鞭毛虫	陽性	陽性
4	褐色泥状便	陰性	陰性	nd
5*	緑色固形便	赤痢アメーバ	陽性	陽性
6	褐色固形便（臭い強し）	陰性	陰性	nd
7	褐色下痢状	陰性	陰性	nd
8*	緑褐色下痢便	赤痢アメーバ	陽性	陽性
9	土色軟便	陰性	陰性	nd
10*	土色軟便	赤痢アメーバ	陽性	陽性
11*	軟便	赤痢アメーバ，ランブル鞭毛虫	陽性	陽性
12*	褐色泥状便	赤痢アメーバ	陽性	陽性
13	土色軟便	陰性	陰性	nd
14	土色下痢便	陰性	陰性	nd
15*	褐色軟便	赤痢アメーバ，ランブル鞭毛虫	陽性	陰性
16	土色軟便	陰性	陰性	nd

方　法

関東地方の某大学病院へ入院し，寄生虫症以外の主因で死亡したと思われる一般入院患者を対象とし，その死亡直後の病理解剖の際に大腸の新鮮便を採取し（死亡確認後12時間以内），その寄生虫相を調査した。病理解剖に際しては，あらかじめ患者遺族から文書にて同意を得た。調査期間は，2007年4月～12月までの9ヵ月間，調査遺体数は16であった。新鮮便は，主に，検鏡検査用に，ホルマリン固定，コーン染色またはMIF液固定を施し，直接塗抹法，ホルマリンエーテル法および簡易蔗糖浮遊法を実施し寄生虫相を調べた。また，最近，その届出数の増加が懸念されている赤痢アメーバ症について

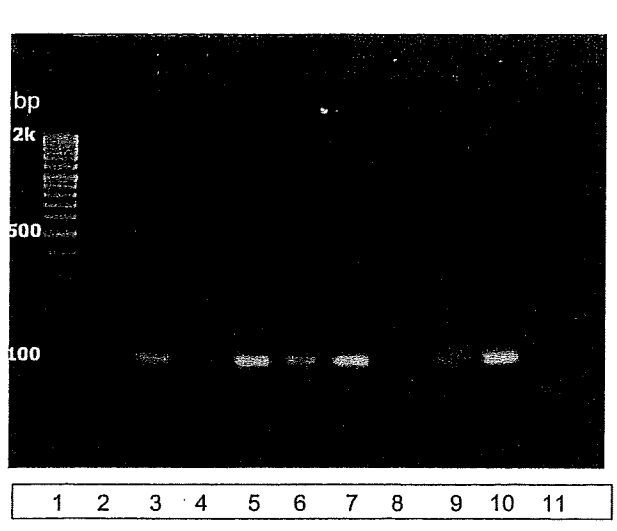

図1 PCR 産物の電気泳動像；PCR 反応の結果（PCR 反応陽性例のみ）
（Ref. 八木田健司博士撮影，レーン8でも，薄いながら陽性コントロール，10，とバンドが一致し，陽性とした，症例15は，陰性を示した。）

は，抗原特異的 ELISA キット（*E. histolytica* II，米国 Wampole（旧 TechLab 社）製）にて新鮮便中の赤痢アメーバ接着因子（抗原）の有無を判定し，抗原陽性サンプルについては，さらに，PCR 検査に供し，特異的 DNA の有無を判定した。

結　果

1）調査年度内に検査できた一般病理遺体の検査結果（全16例，表1，表2および図1）表1に今回の寄生虫調査に供した，一般病理解剖用遺体の一般情報，主たる死因および死亡前の健康状態を示す。

2007年度（2007年4月～12月）に調査し得た遺体は，全部で16例であり，年齢は，37歳～80歳まで（平均69.0歳），男性12名，女性4名であった。

主たる死因（合併例を含む）は，心臓疾患障害が2名，呼吸器障害が3名，脳障害が1名，癌が8名および肝障害が4名であった。また，剖検時所見における，消化管の状態（合併例を含む）としては，腹水貯留が3名，癌状態が3名，目視による異常なしが5名，潰瘍またはポリープ検出が4名，記載なし7名であった。死亡前の消化器症状としては，下痢が4名，下血が2名，記載なしが6名，異常なしが4名であった。また，死亡前における全身症状としては，免疫状態が中程度が1名，低いが15名であった。いずれの寄生虫陽性患者も入院時の血清総タンパク量が基準値（6.5g/ml）未満であり，抗癌剤や抗菌剤持続投与の症例もあり，死亡直前には免疫状態が充分に低い状態であった思われる。消化器症状があっても寄生虫検査をした例はなかった。

各遺体サンプルの寄生虫相調査結果

表2に当教室で確認された2007年度（4～12月）の各遺体のサンプルの検査結果および便性状を示した。

表2に示したように，全16例のサンプルのうち8例が病原性寄生虫（赤痢アメーバまたはランブル鞭毛虫）陽性であった。内訳は，赤痢アメーバ単独感染例が5例，ランブル鞭毛虫との混合感染例が3例であり，寄生虫陽性サンプル全例で赤痢アメーバの感染が認められ，また，ランブル鞭毛虫は赤痢アメーバとの混合感染例のみであった。

赤痢アメーバ陽性サンプルに関するPCR反応の検査結果

表2および図1に示すように，赤痢アメーバの検鏡検査陽性サンプルは，全て *E. histolytica* 抗原陽性を示し，しかも，1例を除いて，PCR反応でも陽性を示していた。この事実は，赤痢アメーバが当該患

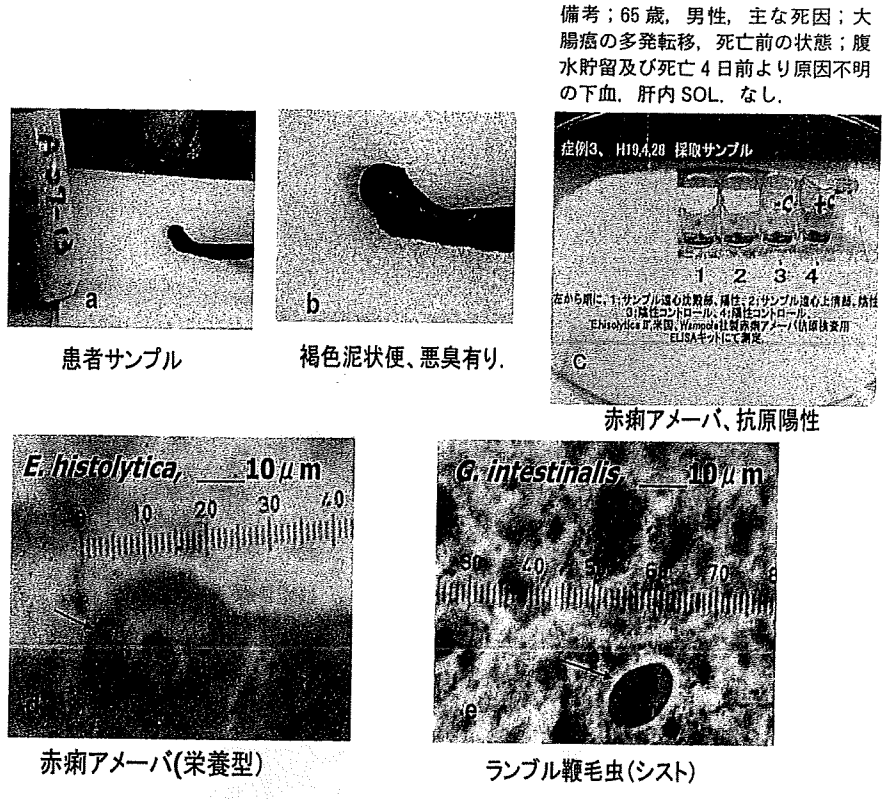

図2（症例3）

者の大腸内に，生前，定着および増殖していたことを示している。また，各調査遺体から得られた大腸内新鮮便の性状については，普通便が2例，軟便が7例，下痢便が7例あった。このうち，寄生虫陽性サンプルでは，普通便が1例，軟便が4例，下痢便が3例で，患者体内での何らかの消化器症状を示唆するものが多かった。

各寄生虫検査陽性症例の紹介

次に，調査遺体のうち，特徴的な例（遺体例3および11）について紹介する。他の寄生虫陽性遺体例については紙面の都合で，検出された寄生虫例（図4a～f）のみ紹介する。

大腸癌の多発転移による死亡例
（症例3，表1，表2，図2）

患者：65歳，男性，無職，横浜市在住。

既往歴：来院1年前に検診にて大腸癌を指摘されるも放置。その後体調不良にて本院へ入院。

入院時の経過：大腸癌の多発転移にて，著明な腹水貯留を認めた。治療するも改善みられなかった。死亡4日前より原因不明の下血を認めた。肝臓内に炎症部位なし。

剖検所見および検査結果：大腸の癌変異部位以外，特別な異常なし。大腸内の便検査では，便性状，黒褐色泥状便，悪臭有り，（図2a，b参照），検鏡検査で，赤痢アメーバ（栄養型，図2d，コーン染色像）およびランブル鞭毛虫（シスト，図2e，コーン染色像）検出および赤痢アメーバ特異的抗原検査（図2c，サンプル遠心沈殿部で陽性；OD値0.72＞0.05＝陰性基準値）とPCR検査で陽性であった。

慢性閉塞性肺疾患による死亡例
（症例11，表1，表2，図3）

患者：79歳男性，無職，東京都在住。

既往歴：来院1年前より慢性閉塞性肺疾患あり。

入院経過：体調不良にて入院。潜血反応持続。入院2週後に，正球性貧血にて輸血施行。死亡4日前（入院1ヵ月後）より，原因不明の水様性下痢出現。剖検所見および検査結果；消化管に肉眼的に異常なし。大腸内の便検査では，便性状，茶褐色軟便（図3a，b参照），検鏡検査で，赤痢アメーバ（栄養型）

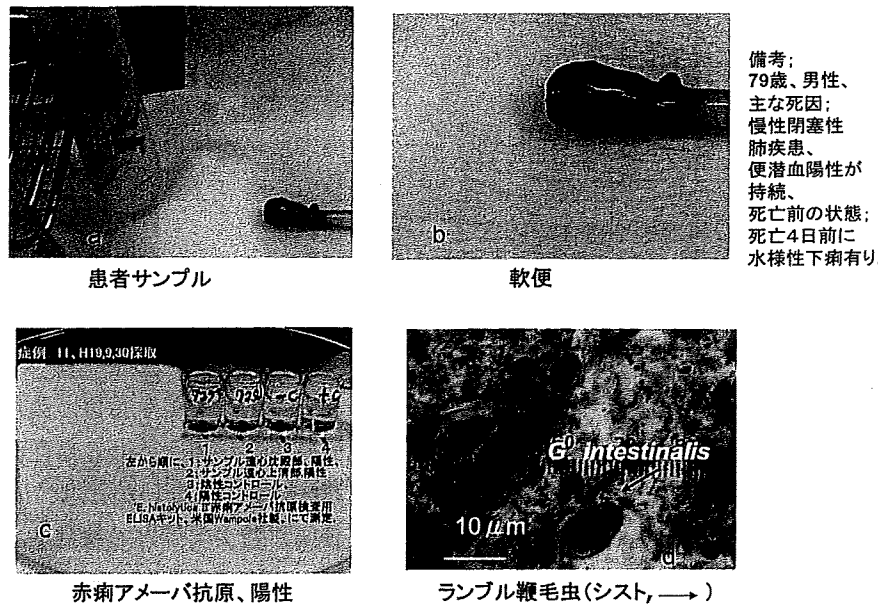

図3（症例11）

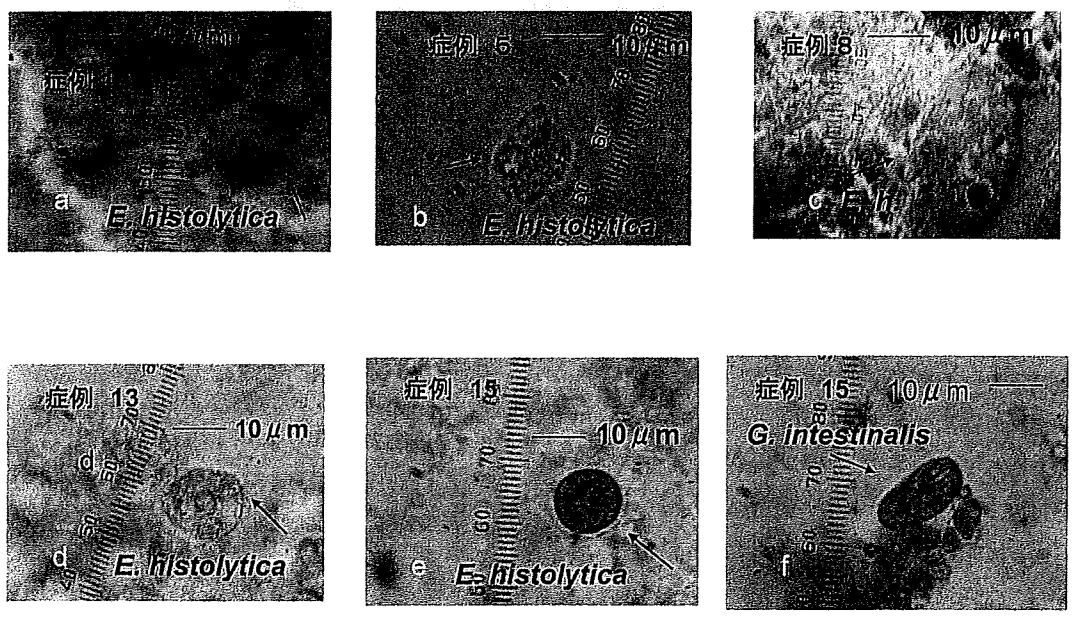

図4　その他の寄生虫陽性症例に認められた原虫（→）

およびランブル鞭毛虫（シスト，図3d，コーン染色像）検出および赤痢アメーバ特異的抗原検査（図3c，サンプル遠心沈殿部（OD値1.32）および上清部（OD値0.91）とPCR検査で陽性であった。

その他の寄生虫陽性例

（症例1，5，8，13，15，図4）

図4に示すように，その他の寄生虫陽性サンプルでも，赤痢アメーバの栄養型（図4a～c；コーン染色，d；蔗糖浮遊法），シスト（図4e；ヨード染色）およびランブル鞭毛虫のシスト（図4f；ヨード染色）が検出された。

考　察

病理解剖の際に寄生虫が発見され，寄生虫症が確定する例は古くから知られている[3]。また，イタリ

アのルネッサンス期の鉤虫研究者の発見に代表されるように，解剖によらずとも，腸管寄生虫を糞便検査により検出することもルネッサンス以来現在まで広く行われてきた[4]。筆者は，このような状況を踏まえ，ヒトの死亡直後における消化管内の寄生虫の潜在的感染を調べる目的で一般病理解剖遺体の調査を行った。

表1に示すように，2007年度（4～12月）に筆者が調査し得た遺体では，死亡原因の主因は多様であったが，解剖時所見も含め，消化管寄生虫が主因の可能性は低かった。

しかしながら，死亡直後の大腸内の新鮮便の寄生虫調査では（表2），赤痢アメーバおよびランブル鞭毛虫に感染していると思われる例が高率（全16例中8例）を占めた。この高率な感染は，一般住民の糞便検査で検出される寄生虫の割合よりもはるかに高く[1]，虫種からいっても軽視できない状況を示すものである。ランブル鞭毛虫に関しては，以前から，不顕性感染が多いとされ，一般の人間ドックでの疫学調査でも，比較的高率の感染が報告されている[5]。赤痢アメーバに関しても，全世界的にみても，推定感染者5億人のうち，多くが無症状の感染者とされている[6]。しかし，今回確認された赤痢アメーバは，いずれも病原性のある種（*E. histolytica*）であり，感染経路が不明であるが[2)7)8)9)]，日和見的に発症をきたす潜在的感染の病原体として重要と思われる。今回の高率な感染の原因は，明らかになっていないが，寄生虫感染者は，いずれも低栄養状態にあり，抗癌剤や抗菌剤を投与されていた例もあり，死亡時の免疫状態は十分に低かったと思われる。感染経路不明のこれらの病原性原虫が，このような一種の免疫抑制状態下で定着し増殖していた可能性が高い。この点，高齢者における栄養管理や寄生虫検査を日頃から徹底し，二次感染による症状の重篤化を防いでゆく必要がある。また，このような病理解剖遺体に関する，疫学的な寄生虫調査は報告例がまだきわめて少なく[9]，今後，調査地域と調査例数を増やし実態を解明し，今後の寄生虫感染予防に役立ててゆく必要がある。

結　語

1) 当教室では，2007年度（4～12月）に計16例の病理解剖遺体の大腸内新鮮便の寄生虫調査を経験し，高率（全16例中8例）な寄生虫の感染を認めた。
2) 寄生虫感染の内訳は，赤痢アメーバ単独感染例が5例，本虫とランブル鞭毛虫との混合感染が3例であり，赤痢アメーバが全例で検出された。
3) これらの病原性寄生虫は，免疫状態の低い患者の健康状態を悪化させる潜在的な感染性病原体として重要ではないかと思われる。

日頃から栄養管理の徹底や寄生虫検査・駆除を行い症状の重篤化を防いでいく必要がある。

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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′-ATGTTAGCTTTATTTTTTGGTGG-3′) and the ribosomal RNA large subunit gene (5′-CTATACACATT-TACTTGATCTCCTC-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

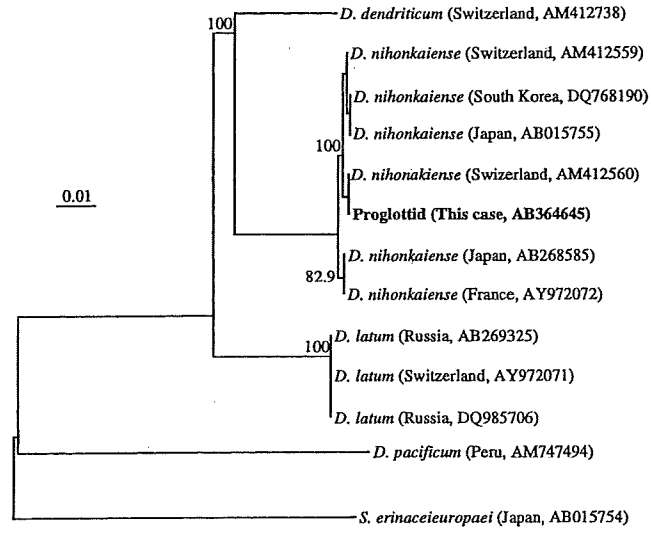

Fig. 1 Phylogenetic tree inferred from 396-bp *cox1* fragments using the neighbor-joining method. Numbers at branches indicate bootstrap values for 1,000 replicates and a scale bar represents the genetic distance based on Kimura's two-parameter model. *Spirometra erinaceieuropaei* is used as an out-group

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.custons.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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