as SDS, and to potential inhibitory substances that may, according to the manufacturer's data sheet, be released from lysed fungi (15).

To reduce the costs of lysing buffer and of the removal of some ALB components, different sets of lysing buffers were used to examine four fungal strains; PCRs were performed after 1 h of lysing with each buffer. Surprisingly, there were no differences among the tested strains, and the only differences seen were in the intensities of the resulting bands (Fig. 1). They differed in accordance to lysing buffers, i.e., the band corresponding to ALB showed the highest intensity. These observations indicated that the role of ALB in our experiment was not crucial, and amplification bands were seen clearly with the buffer lacking SDS and proteinase K. Thus, Ampdirect DCPCR seemed to be applicable to fungi without any lysing treatment.

To enhance our concept and to reduce the number of preparatory steps required for PCR, including DNA template preparation, comparative DCPCR was carried out using both Ampdirect DCPCR and standard PCR. The results indicated a significant difference between the two PCR types, even when the same DNA template and PCR conditions were used (Table 3). These differences in the results may be explained on the basis of variant fungal wall structures and the presence of some Taq inhibitors such as melanin, which is generally produced by fungi and particularly by some *Aspergillus* spp. (16-18). These putative inhibitors were most likely tolerated by the Ampdirect kit but not by the standard PCR reagents (Table 3).

These findings were investigated further by DCPCR with spores and hyphae of four strains separately. Interestingly, we found no differences in the PCR results between spores and hyphae within the same strain (Fig. 3). Thus, the Ampdirect kit is potentially applicable to all forms of fungal DNA template.

In addition, the use of positive and negative controls in all our experiments excluded the possibility of false positives and negatives, which may lead to misinterpretation of the results.

Ampdirect DCPCR reduces the time consumed in DNA template preparation from fungal colonies from about 6 h, the time required to extract DNA by the most convenient methods, to 5 min. It also lowers PCR costs in DNA template preparation by obviating the use of commercial kits or other reagents, such as expensive enzymes.

Moreover, the applicability of Ampdirect® Plus to rapid PCR was not limited to the ITS region; it was tested for the 28S rDNA region of five fungal strains–*Eurotium chevalieri* JCM 1568, *Eurotium herbariorum* JCM 1575, *Eurotium amstelodami* JCM 1565, *Penicillium expansum* TIMM 1293, and *Fusarium moniliforme* TIMM 1294–and corresponding PCR products were produced (data not shown).

The utility of the Ampdirect® Plus kit makes DCPCR possible for fungi in all conditions (spores, hyphae, and directly from fungal cultures) and from a wide range of fungal species. In addition, this kit was obviously superior to standard PCR reagents in DCPCR. Thus, Ampdirect DCPCR is a promising approach to facilitate DCPCR as it reduces PCR cost, labor, and time. The results of the present study may facilitate implementation of Ampdirect® Plus in further studies.

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Original Article

Evaluation of a New Rapid Molecular Diagnostic System for *Plasmodium falciparum* Combined with DNA Filter Paper, Loop-Mediated Isothermal Amplification, and Melting Curve Analysis

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SUMMARY: Falciparum malaria is a fatal infection without immediate diagnosability or treatment. There are shortages of clinicians and examiners skilled in the treatment of malaria in non-endemic countries, including Japan. This study was performed to evaluate a novel rapid molecular diagnostic system consisting of loop-mediated isothermal amplification (LAMP) combined with DNA filter paper (FTA card) and melting curve analysis. Combining LAMP with melting curve analysis enabled diagnosis of *Plasmodium falciparum* more accurately with relative ease. FTA cards could be used to clarify problems regarding storage, infectivity, and transportation. The LAMP assay was carried out at a constant temperature of 63°C for 90 min. The diagnostic system (malaria-LAMP) accurately diagnosed malaria (47 samples from Thailand and 50 from Zimbabwe) with 97.8% sensitivity and 85.7% specificity as compared with microscopic methods, indicating the usefulness of this combined system.

INTRODUCTION

Malaria is one of the most important tropical infectious diseases. From 350 to 500 million clinical episodes of malaria occur each year, and the disease is responsible for more than 1 million deaths annually (1). Especially, *Plasmodium falciparum* causes various complications and may be fatal. The range of areas inhabited by malaria-carrying mosquitoes is currently expanding due to global climate change (2). Therefore, there is a risk of a revival of malaria, not only as a consequence of the increasing number of imported cases with the increase in overseas travelers, but also as a result of the growth of habitats suitable for malaria-carrying mosquitoes. Thus, the rapid diagnosis of this disease is extremely important. Malaria has been diagnosed by microscopic examination using Giemsa stain. However, diagnosis by microscopic examination requires skill, and may be difficult in cases where preventative oral medication was taken before onset, as well as in cases where the level of malaria parasite infection is low (3). In countries where malaria is not endemic, there are shortages of physicians and microscopy-skilled laboratory staff specializing in malaria, and thus the diagnosis of malaria is difficult. In recent years, simple kits have been developed to detect malaria parasite-specific proteins and enzymes as auxiliary diagnostic procedures. However, these kits have a number of problems, such as false positives in patients with rheumatoid factor or in the elderly (4). In addition, these kits are not commercially available in Japan, and are not in common use. Methods for genetic diagnosis of malaria using PCR are also being developed, and there have been reports of cases in which various types of malaria parasites were identified and multiple infections were diagnosed by detecting malaria-specific DNA sequences. However, PCR-based methods require complex procedures, expensive inspections, and several hours before a diagnosis can be made. Therefore, it is necessary to develop a simple, stable, and rapid method for diagnosing malaria. Loop-mediated isothermal amplification (LAMP) (5) meets these requirement and has been put to practical use as a sensitive detection method for many infections (severe acute respiratory syndrome [SARS] coronavirus [6], West Nile virus [7], avian influenza virus [8], norovirus [9], and *Legionella* bacteria [10]). The LAMP method for *P. falciparum* malaria has already been reported by Poon et al. (11) and Han et al. (12) The LAMP method is simple to perform and is less influenced by inhibitors than polymerase chain reaction (PCR) is. To identify LAMP products, it is necessary to develop methods such as restriction enzyme digestion and the confirmation of specific DNA ladder formation by electrophoresis (12). However, to avoid contamination from dispersion of the amplification products during electrophoresis, there has been a demand for simpler and safer technology to identify amplification products. To safely distinguish specific amplification products from non-specific primer-dimers often seen in LAMP, melting temperature curve analysis was used. *P. falciparum* malaria-specific LAMP primers were designed and FTA cards (Whatman, Kent, UK) were used to allow storage, transportation, and extraction of template DNA safely, stably, and easily from blood samples at room temperature. In this study, a new rapid molecular diagnostic system for *P. falciparum* was developed by combining DNA filter paper (FTA cards), LAMP, and melting curve analysis.

The applicability of this system was examined using 97 specimens from Thailand and Zimbabwe where *P. falciparum* malaria is endemic.

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MATERIALS AND METHODS

***P. falciparum* strain and DNA extraction: (i) *P. falciparum* strain**: The malaria parasite used for DNA extraction was a strain of *P. falciparum* (FCN-1/Nigeria [13])

maintained at the Department of Microbiology, School of Medicine, Teikyo University, Tokyo, Japan.

(ii) DNA extraction: The DNA of *P. falciparum* was extracted as follows. The strain was cultured for 3 days in flat-bottomed 96-well microtiter plates. The total volume of medium (RPMI1640 with 10% human serum) in each well was 200 μl, each of which contained 10% erythrocytes and 1% infected blood cells. The supernatant fluid in each well was discarded, and the remaining erythrocytes (including *P. falciparum*) were harvested and frozen at −80°C. The contents were thawed to induce hemolysis, after which distilled water and an equal amount of 1.8% NaCl were added. The mixture was centrifuged at 2,500 rpm for 10 min and the supernatant was discarded. These steps were repeated several times until the supernatant changed from pink to colorless. After heating the remaining pellet at 100°C for 15 min, the DNA was extracted by phenol/chloroform extraction and ethanol precipitation, and used as a template for amplification.

(iii) Preparation of plasmid for sensitivity test: The target sequence for LAMP assay was amplified by PCR using a primer pair (MAL-1, 5′-ACAGATTAAGCCATGCAAGT GA-3′; and MAL-2, 5′-AAACTTCCTTGTGTTAGATACAC-3′) designed according to the 18S rDNA gene of *P. falciparum* (DDDJ/EMBL/GenBank accession no. AL031746). The PCR products were cloned and transformed using an Original TA Cloning Kit® (Invitrogen, Carlsbad, Calif., USA) in accordance with the manufacturer's instructions. The cloning plasmids were extracted from the transformed *Escherichia coli* using an Aurum Plasmid Mini Kit® (Bio-Rad Laboratories, Hercules, Calif., USA) in accordance with the manufacturer's instructions. These plasmids, which were diluted from 10,000 to 5 copies/tube by doubling dilution, were used as control plasmids (c-plasmid) for the sensitivity test to determine the detection limit.

Clinical samples and DNA elution: (i) Clinical samples: Samples of suspected malaria from Thailand (n = 47) and Zimbabwe (n = 50) were prepared by local staff in each area, who took blood samples from patients and directly deposited a few drops of the sample onto FTA cards for molecular diagnosis. As negative controls, normal whole blood samples on FTA cards from 59 individuals with no history of malaria infection (20 healthy individuals and 39 febrile patients, including those with leukopenia) were used. The study protocol was approved by the corresponding Ethical Committees of Teikyo University School of Medicine. All patients gave their informed consent to participate in the study.

(ii) Preparation of blood smears and microscopic examination: For thin blood smears, a single drop of whole blood was placed on a glass slide, immediately spread using a coverglass, and put aside to dry. After methanol fixation, the smears were stained with Giemsa solution.

For thick blood smears, one or two drops of whole blood were placed on a glass slide. The blood was spread to about 1 cm in diameter and put aside to dry. After drying, these smears were stained with Giemsa solution.

Thin and thick blood smears were prepared from 47 patients with suspected malaria infection in Thailand; a microscopist in Thailand provided the results of microscopic examination. Only thick blood smears were prepared from 50 patients with suspected malaria infection in Zimbabwe, and the results of the microscopic examination were unknown. All 97 blood smears were examined for the presence of malaria parasites at the Department of Microbiology, School of Medicine, Teikyo University. To calculate the infection rate in a thin blood smear, the number of infected erythrocytes was counted in increments of 10,000 erythrocytes. To calculate the infection rate in a thick blood smear, the number of infected erythrocytes was counted in increments of several leukocytes, depending on the smear. The results of microscopic examination were compared with those of malaria-LAMP assay using FTA cards.

(iii) DNA extraction from FTA card samples, and transport of FTA card samples: FTA cards with whole blood deposits were dried and stored at room temperature, and were then mailed to Japan. Disks 2.0 mm in diameter were cut from the bloodstained areas with a Harris Micro Punch (2.0 mm; Whatman). Each disk was washed three times with 200 μl of FTA purification reagent and twice with 200 μl of TE-1 buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The disks were dried and used directly as the DNA template.

Oligonucleotide primers for LAMP reaction: The 18S rRNA gene nucleotide sequences were obtained for eight *Plasmodium* spp., including four species of human malaria parasite (*P. falciparum*, accession no. AL031746, NC004325; *P. ovale*, AB182489, AB182493; *P. vivax*, U03079, AY579418; *P. malariae*, AF145336; *P. knowlesi*, AY327557) from DDDJ/EMBL/GenBank Database, and by using DNA analysis software (GENETYX®-Mac ver.13; GENETYX, Tokyo, Japan). Six sites of specific nucleotide sequences of *P. falciparum* were determined as the primer sets. One pair of primers (F3 and B3) was designed based on the sequences at two sites in the outermost region, while another pair of primers (FIP and BIP) was designed to connect the sequences at two sites in the inner region. Thus, four primers were prepared for LAMP assay to detect *P. falciparum* genes. In addition, one pair of loop primers corresponding to this malaria-LAMP system was also designed. The location and nucleotide sequence of each primer are shown in Table 1.

The results of BLAST (Basic Local Alignment Search Tool) (14) analysis against DDBJ/EMBL/GenBank databases indicated that the sequences of malaria-LAMP primers were specific only for *P. falciparum*. Species-specific LAMP primer sets for *P. falciparum* (11) and *P. vivax* (12) were also used. The specificity of malaria-LAMP primers was tested with each of four species of human malaria DNA. Template DNAs for three malaria parasites, *P. vivax*, *P. malariae*, and *P. ovale*, were provided by Dr. Tsuboi, Ehime University, Japan (12).

Malaria-LAMP condition and melting curve analysis: The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The reaction mixtures (25 μl) contained 40 pmol of primers FIP and BIP, 40 pmol of primers loopF and loopB, and 10 pmol of primers F3 and B3, 2 × reaction mixture (12.5 μl), 1 μl *Bst* DNA polymerase, 0.5 μl of YO-PRO®-1 iodide (Molecular Probes, Eugene, Oreg., USA), 1 to 3 μl of DNA sample, and distilled water (made up to a final volume of 25 μl/tube). The LAMP reaction was performed at 63°C for 90 min. As negative controls, several tubes containing distilled water supplied with the kit were also prepared per the reaction. The c-plasmid was used as a positive control at 10^7 copies/reaction. LAMP products were identified by melting curve analysis. Melting temperature curves were obtained using the default settings recommended by the manufacturer in the presence of a fluorescent interchelator (YO-PRO®-1 iodide). Amplification and melting curve analysis were carried out using a Genopattern Analyzer GP1000 (Yamato Scientific Co., Ltd., Tokyo, Japan).

Table 1. Malaria-LAMP primers

A	F3	gaggtgaaattctaagattttct	
	FIP(F1c+F2)	cacctagtcggtatagtttatggtgcctaatctatttccattaat	
		F1c F2	
	B3c	ttccgtcaattcttttaactttc	
	BIP(B1+B2c)	gtagcatttcttagggaatgttggccccagaacccaaagactttga	
		B1 B2c	
	Loop F	ggtatctgatcgtcttcactccc	
	Loop B	gaat tgcttccttc agtacctta	
B	1001	tcagaggtgaaattctaagattttctggagacggactactgcgaaagcat	1050
		F3	
	1051	ttgcctaatctatttccattaatcaagaacgaaagttaagggagtgaaga	1100
		FIP(F2) **loop F**	
	1101	cgatcagataccgtcgtaatcttaaccataaactataccgactaggtgtt	1150
		FIP(F1)	
	1151	ggatgaatataaaaaatatataaatatgtagcatttcttagggaatgttg	1200
		BIP(B1)	
	1201	attttatattagaattgcttccttcagtaccttatgagaaatcaaagtct	1250
		loop B **BIP(B2)**	
	1251	ttgggttctggggcgagtattcgcgcaagcgagaaagttaaaagaattga	1300
		B3	
	1301	cggaagggcaccacc	1315

A: Sequences of malaria-LAMP primers FIP, BIP, F3, B3, LoopF, and LoopB are used in this study.
B: Locations and sequences of malaria-LAMP target sequence and primer binding sites of *P. falciparum*. The locations of the primer binding site in the reference sequence (18SrRNA, GenBank accession no. AL031746) are underlined. Base numbers in the reference sequence are indicated at both ends.

RESULTS

Sensitivity and specificity: The malaria-LAMP system was able to detect c-plasmid at 10 copies/tube, but not at 5 copies/tube (Fig. 1). All of the melting curve peaks ($10 - 10^4$ copies/tube) obtained in this sensitivity test with c-plasmid were consistent (Fig. 2). The time required to amplify 10^4 copies/tube was 35 min, and that to amplify 10 copies/tube, which was decided as the detection limit, was 60 - 80 min.

The malaria-LAMP system was specific only for *P. falciparum* genomic DNA and c-plasmid, not for the three other human pathogenic malaria parasites. The peaks of the melting temperature curves were identical in genomic DNA and c-plasmid products. However, no melting temperature curves were obtained from other species of malaria parasite, because no products were amplified from their template DNAs (Fig. 3).

Application of malaria-LAMP to clinical samples: (i) Results of microscopic examination: Thailand (Table 2): A local microscopist examined all 47 samples of clinically suspected malaria infection, and found that 29 samples indicated the presence of *P. falciparum* parasites, while 4 samples

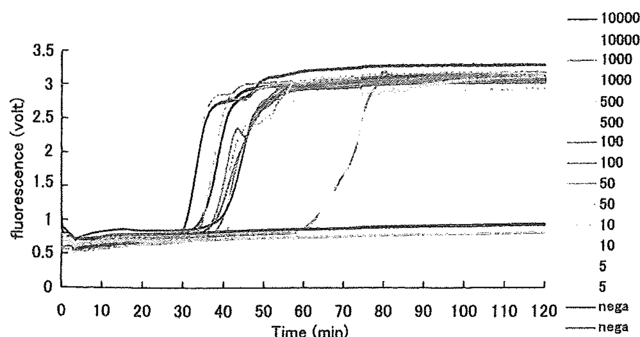

Fig. 1. Sensitivity of malaria-LAMP with control plasmid. The lower limit of detection of malaria-LAMP was 10 copies/tube, diluted from 10,000 to 5 copies/tube by doubling dilution.

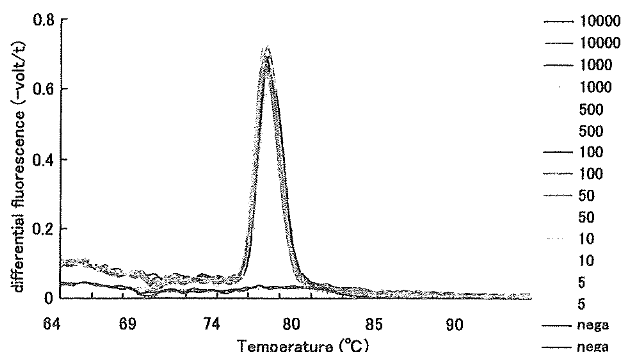

Fig. 2. Melting temperature curves of malaria-LAMP with control plasmid. The peaks of melting temperature curves of malaria-LAMP products amplified from control plasmid coincided with those from clinical specimens.

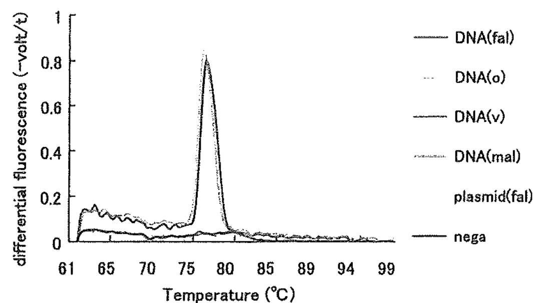

Fig. 3. Melting curve analysis for malaria-LAMP products. Malaria-LAMP amplified only *P. falciparum* genomic DNA and control plasmid, and their peaks of melting temperature curve were identical. DNA(fal), *P. falciparum* genomic DNA; DNA(o), *P. ovale* genomic DNA; DNA(v), *P. vivax* genomic DNA; DNA(mal), *P. malariae* genomic DNA.

Table 2. Comparison of results of malaria-LAMP and microscopy for 47 patients from Thailand

Patient no.	Diagnosis	Malaria-LAMP	Parasitemia of thin blood smears (%erythrocyte)	
			Microscopist (Teikyo University)	Microscopist (Thailand)
1	*P. falciparum*	+	6.12	13.4
2	*P. falciparum*	+	5.04	−
3	*P. falciparum*	+	4.98	1.8
4	*P. falciparum*	+	1.77	2.2
5	*P. falciparum*	+	1.71	0.9
6	*P. falciparum*	+	1.62	2
7	*P. falciparum*	+	1.5	1.5
8	*P. falciparum*	+	1.26	0.5
9	*P. falciparum*	+	0.87	−
10	*P. falciparum*	+	0.78	0.9
11	*P. falciparum*	+	0.74	0.7
12	*P. falciparum*	+	0.63	1
13	*P. falciparum*	+	0.63	1.1
14	*P. falciparum*	+	0.58	1.4
15	*P. falciparum*	+	0.51	0.3
16	*P. falciparum*	+	0.51	0.6
17	*P. falciparum*	+	0.39	0.3
18	*P. falciparum*	+	0.33	0.4
19	*P. falciparum*	+	0.32	0.5
20	*P. falciparum*	+	0.25	0.6
21	*P. falciparum*	+	0.21	0.6
22	*P. falciparum*	+	0.2	0.4
23	*P. falciparum*	+	0.16	0.2
24	*P. falciparum*	+	0.15	0.3
25	*P. falciparum*	+	0.12	0.4
26	*P. falciparum*	+	0.09	0.1
27	*P. falciparum*	+	0.08	0.1
28	*P. falciparum*	+	0.07	−
29	*P. falciparum*	+	0.06	1.3
30	*P. falciparum*	+	0.05	−
31	*P. falciparum*	+	0.04	−
32	*P. falciparum*	+	0.03	−
33	*P. falciparum*	+	0.02	−
34	*P. falciparum*	+	0.02	0.1
35	*P. falciparum*	+	0.02	−
36	*P. falciparum*	+	0.01	−
37	*P. falciparum*	+	0.01	−
38	*P. falciparum*	+	0.009	0.1
39	*P. falciparum*	+	0.007	0.1
40	*P. falciparum*	+	0.007	−
41	*P. falciparum*	+	−	−
42	*P. falciparum*	+	ND	−
43	*P. falciparum*	−	0.0000654	−
44	*P. vivax*	−	0.03	0.2
45	*P. vivax*	−	0.21	0.5
46	*P. vivax*	−	0.06	0.2
47	*P. vivax*	−	0.06	0.3

ND: Microscopist was not able to count because the condition of the specimen was poor.
−: Result of LAMP reaction was negative, or indicated absence of malaria parasites.
+: Result of LAMP reaction was positive.

Table 3. Comparison of results of malaria-LAMP and microscopy for 50 patients from Zimbabwe

Patient no.	Diagnosis	Malaria-LAMP	Parasitemia of thick blood smears (/leukocyte)
			Microscopist (Teikyo University)
48	*P. falciparum*	+	many
49	*P. falciparum*	+	many
50	*P. falciparum*	+	many
51	*P. falciparum*	+	many
52	*P. falciparum*	+	many
53	*P. falciparum*	+	many
54	*P. falciparum*	+	many
55	*P. falciparum*	+	74.8/leukocyte
56	*P. falciparum*	+	51.3/leukocyte
57	*P. falciparum*	+	40.3/leukocyte
58	*P. falciparum*	+	35.5/leukocyte
59	*P. falciparum*	+	32.5/leukocyte
60	*P. falciparum*	+	20.5/leukocyte
61	*P. falciparum*	+	18.3/leukocyte
62	*P. falciparum*	+	13/leukocyte
63	*P. falciparum*	+	11.2/leukocyte
64	*P. falciparum*	+	11/leukocyte
65	*P. falciparum*	+	10.5/leukocyte
66	*P. falciparum*	+	9.7/leukocyte
67	*P. falciparum*	+	9.7/leukocyte
68	*P. falciparum*	+	8.7/leukocyte
69	*P. falciparum*	−	8.3/leukocyte
70	*P. falciparum*	+	8/leukocyte
71	*P. falciparum*	+	6.7/leukocyte
72	*P. falciparum*	+	6.2/leukocyte
73	*P. falciparum*	+	6/leukocyte
74	*P. falciparum*	+	6/leukocyte
75	*P. falciparum*	+	6/leukocyte
76	*P. falciparum*	+	4.7/leukocyte
77	*P. falciparum*	+	3.7/leukocyte
78	*P. falciparum*	+	3.2/leukocyte
79	*P. falciparum*	+	2.9/leukocyte
80	*P. falciparum*	+	2.6/leukocyte
81	*P. falciparum*	+	2.4/leukocyte
82	*P. falciparum*	+	2.4/leukocyte
83	*P. falciparum*	+	2.4/leukocyte
84	*P. falciparum*	+	2.4/leukocyte
85	*P. falciparum*	+	2/leukocyte
86	*P. falciparum*	+	2/leukocyte
87	*P. falciparum*	+	1.7/leukocyte
88	*P. falciparum*	+	1.5/leukocyte
89	*P. falciparum*	+	1.2/leukocyte
90	*P. falciparum*	+	1/leukocyte
91	*P. falciparum*	+	0.9/leukocyte
92	*P. falciparum*	+	0.6/leukocyte
93	*P. falciparum*	+	0.14/leukocyte
94	*P. falciparum*	+	few
95	−	−	−
96	−	−	−
97	−	+	−

Many: Large number of infected erythrocyte.
Few: A few infected erythrocyte per field.
−: Result of LAMP reaction was negative, or indicated absence of malaria parasites.
+: Result of LAMP reaction was positive.

showed the presence of *P. vivax* parasites in both thick and thin blood smears. Twelve samples (#9, #28, #30-32, #35-37, #40-43) showed the presence of *P. falciparum* parasites only in thick blood smears (i.e., they were absent in thin blood smears). The local microscopist was not able to detect parasites in 2 samples (#2, #33), but a microscopist at Teikyo University detected *P. falciparum* parasites in both of these samples. The diagnoses of the malaria parasite species were the same between Thailand and Teikyo University, although the infection rate in thin blood smears differed between the microscopists.

Zimbabwe (Table 3): A microscopist at Teikyo University examined 50 samples of clinically suspected malaria infection, and found that 47 samples were positive for the presence of *P. falciparum* parasites in thick blood smears, whereas 3 samples (#95-97) did not have any parasites.

Results of malaria-LAMP of clinical samples: Malaria-LAMP was performed using template DNA extracted from FTA card specimens of thin and thick blood smear samples from which *P. falciparum* was detected (90 cases; 43 from Thailand, 47 from Zimbabwe). *P. vivax* was detected in samples from 4 cases. Howerer, remaining 3 cases were negative on

microscopic examination. Melting temperature curves were used to identify the amplification product. The samples were defined as negative if the peaks of the melting temperature curves did not reach 2/5 the height of the peaks of the positive control using c-plasmid. All 90 cases of falciparum malaria showed gene amplification, but the peaks of the melting temperature curves of 2 cases (#43, #69) differed from those of *P. falciparum*, and the sensitivity of malaria-LAMP was 97.8%. Case #43 showed a very low infection rate of 0.0000654% on the thin blood smear sample.

In addition, 1 (#47) of the 4 cases of vivax malaria showed gene amplification, but the peaks on the melting temperature curves differed from those of *P. falciparum*, and thus could be distinguished from falciparum malaria. *P. vivax* DNA was amplified from this sample with *P. vivax*-specific LAMP reaction (12). Of the 3 cases that were negative on microscopic examination, 1 case (#97) showed gene amplification, while the remaining 2 (#95, 96) did not. *P. falciparum* DNA was also amplified from #97 with another *P. falciparum*-specific LAMP reaction (11). Assuming that the 3 cases that were negative on microscopic examination as diseases other than falciparum malaria, the specificity compared to microscopic examination was 85.7%.

The results of the microscopic examinations, as well as those of malaria-LAMP for specimens from Thailand and Zimbabwe, are shown in Tables 2 and 3, respectively.

The time required to confirm the reaction in clinical specimens using FTA cards was 33 - 85 min (average, 49 min).

Nonspecific amplification and characteristics of melting temperature curves: When this reaction was performed for FTA cards from 59 non-malaria cases, amplification was confirmed in 7 cases and in the negative control in one reaction. However, none of the melting temperature curves matched that for *P. falciparum*, so they were classified as nonspecific reactions. These nonspecific amplifications had at least one of the following characteristics: (i) no reproducibility of amplification; (ii) no reproducibility of peak positions of the melting temperature curves.

DISCUSSION

A number of reports have indicated that PCR's minimal detection level of PCR is from 1 to 10 copies/reaction (15-18). Therefore, the sensitivity of the system presented here is equivalent to that of PCR. The levels of microscopic examination by skilled laboratory staff are 10 - 30 parasites/μl in thick blood smear samples under optimal conditions (15), and theoretically the present method is equivalent to or better than microscopic examination. When samples of 125 μl of blood, a feasible sample volume according to the manufacturer's literature, are applied to FTA cards, the expected erythrocyte number on the disc is 3.6×10^6, and the detectable infection rate of malaria is theoretically as low as 0.000278% when the detection limit in the present system is 10 copies. The sensitivity for the clinical sample compared to the microscopic examination was 97.8%. The LAMP method is superior to PCR-based methods in that the procedures involved are less complex. The LAMP method is also superior to the microscopic method, which is affected by the skill of the individual microscopist, in its reproducibility. The present method could detect parasites in cases where the infection was undetectable on microscopic examination in thin blood smears. The specificity of this system compared to microscopic examination was 85.7%, because only 1 sample (#97) was shown to be negative on microscopic examination but was positive in malaria-LAMP. However, sample #97 was positive in another *P. falciparum*-specific LAMP reaction reported by Poon et al. (11). Therefore, considering #97 to be from a falciparum malaria patient, the specificity of the present method would be 100%.

Turbidimetry to visual observation (11,12), with which the malaria-LAMP products are identified, is both simple and accurate, but identification is expected to be difficult in some clinical samples. In addition, as the present paper indicated, there is a possibility of nonspecific amplification even for the LAMP method, and therefore the amplification products should always be checked. Melting curve analysis can automatically differentiate between specific and nonspecific amplification using commercially available real-time PCR analyzers. As reported previously (19), the process of agarose gel electrophoresis tends to cause severe contamination by dispersion of LAMP products, so melting-curve analysis was used rather than electrophoresis to confirm specific malaria-LAMP products. Melting temperature curves have been used to identify PCR products in diagnosing malaria or in identifying the species of malaria parasite (15,16). Although the application of melting curve analysis for LAMP products has already been reported to identify pneumocystis (19), this is the first report to identify the LAMP products of malaria using melting curves.

The cells in a sample are destroyed after they are dropped onto the FTA cards (specially processed filter paper) with DNA fixation. The cards can therefore be stored at room temperature and mailed. Indeed, the cards were obtained by mail from two countries in the present study (Thailand and Zimbabwe). In hospitals with no examination apparatus or skill for identifying malaria, mailing the FTA card presents a useful diagnostic opportunity. Zhong et al. (20) reported the usefulness of the FTA card in detecting malaria parasite DNA by PCR. Mas et al. (21) reported that the DNA recovery rate in samples of FTA cards was better in the presence of ethylenediaminetetraacetic acid (EDTA), and tubes containing EDTA were used to collect blood samples. The DNA synthetic enzyme in the LAMP method is unaffected by the presence of hemoglobin or excessive salt concentration, etc., while Taq DNA polymerase used in PCR (22) is affected by these conditions. Poon et al. (11) reported that they were able to use heat-treated whole-blood specimens directly as a DNA template for LAMP assay. However, in a preliminary experiment, we could not obtain stable results through DNA extraction by heat treatment, unlike the case using FTA cards. If pretreatment of FTA cards can be performed in 30 min, it would take approximately 2 h from blood sampling to obtain the results. Due to its rapidity and ease of use, as well as the lack of a requirement for specialized skill, this system will be useful in countries where malaria is not endemic, such as Japan. Using this system in combination with microscopic examination, it will be possible to obtain a more accurate diagnosis of malaria in clinical practice.

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ORIGINAL ARTICLE

Comparative evaluation of *Trichosporon asahii* susceptibility using ASTY colorimetric microdilution and CLSI M27-A2 broth microdilution reference methods

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ABSTRACT

The *in vitro* activity of AMPH-B, 5-FC, FLCZ, MCZ, ITCZ, and VRCZ against 50 isolates of *T. asahii* was determined using CLSI M27-A2 microdilution and ASTY colorimetric methods. Observed agreement ranged from 96 to 100% according to the drug. Overall, the agreement between two methods was 97.7%. The ASTY colorimetric method was thus determined to be comparable to the CLSI reference method when testing the susceptibility of *T. asahii* to a variety of antifungal agents.

Key words ASTY, CLSI, susceptibility, *Trichosporon asahii*.

Deep-seated infection with *Trichosporon* species is an emerging mycosis in neutropenic patients, particularly those who are being treated for hematological malignancies with cytotoxic and immunosuppressive therapy. The prognosis for deep-seated trichosporonosis with persistent neutropenia is very poor, with a high mortality rate (1, 2). This infection is caused mainly by *T. asahii*. In recent years, trichosporonosis has also been recognized as a breakthrough infection after treatment with candin derivatives, such as micafungin or caspofungin (3, 4). Therefore, the frequency of deep-seated trichosporonosis may be gradually increasing, although data on the antifungal susceptibilities of *T. asahii* are limited. In addition, azole-resistant *T. asahii* have been isolated from patients (5, 6).

Commercial colorimetric methods are available to test antifungal agents *in vitro*. Comparative evaluation studies of the Sensititre YeastOne Panel (Trek Diagnostic Systems, Westlake, OH, USA) have found close agreement between the methods for both yeasts and molds (7–9). A second commercial colorimetric panel is ASTY (Kyokuto Pharmaceutical Industry, Tokyo, Japan), which uses an oxidation–reduction colorimetric indicator to aid in determining the antifungal MIC endpoint. However, only one comparative study testing *Candida* spp. has been reported using this method (10), and only a few studies have included MIC data for antifungal agents against *Candida* spp. and *Geotrichum capitatum* using the ASTY panel (11–13).

To the best of our knowledge, no study has examined the performance of these colorimetric microdilution panels for testing the *in vitro* susceptibility of *T. asahii* isolates. Therefore, we evaluated the ASTY method with AMPH-B, 5-FC, FLCZ, MCZ, ITCZ, and VRCZ for 50 isolates of *T. asahii*, the major causative agent of deep-seated trichosporonosis. MIC obtained using ASTY were compared

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List of Abbreviations: AMPH-B, amphotericin B; CLSI, Clinical Laboratory Standards Institute; 5-FC, 5-flucytosine; FLCZ, fluconazole; *G. capitatum*, *Geotrichum capitatum*; IGS region, intergenic spacer; ITCZ, itraconazole; MCFG, micafungin; MCZ, miconazole; MIC, minimal inhibitory concentration; *T. asahii*, *Trichosporon asahii*; *T. mucoides*, *Trichosporon mucoides*; VRCZ, voriconazole

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to those obtained using the CLSI (formerly National Committee for Clinical Laboratory Standards) M27-A2 method.

MATERIALS AND METHODS

Fungal isolates

We evaluated 50 *T. asahii* strains. These were isolated from clinical specimens (blood, urine, and lung) from immunocompromised hosts in Thailand and Japan between 1996 and 2006. The isolates were identified using the DNA sequence from the IGS region of 26S rDNA according to the method of Sugita *et al.* (14).

Antifungal susceptibility

We assessed the antifungal susceptibility of *T. asahii* strains in two ways. First, we performed susceptibility testing with 96-well plates, as described in the M27-A2 procedure for yeast susceptibility testing (15). AMPH-B (Wako Pure Chemical, Tokyo, Japan), 5-FC (Wako Pure Chemical), FLCZ (ICN Biochemical, Cleveland, Ohio, USA), ITCZ (Janssen Pharmaceutical, Tokyo, Japan), MCZ (Wako Pure Chemical) and VRCZ (Pfizer Japan, Tokyo, Japan) were used. Briefly, the final concentrations of the antifungal agents were 0.03–16 μg/ml for AMPH-B, 0.12–64 μg/ml for 5-FC, 0.12–64 μg/ml for FLCZ, 0.03–16 μg/ml for MCZ, 0.01–8 μg/ml for ITCZ, and 0.01–8 μg/ml for VRCZ. The plates were incubated at 35°C for 48 hr and then read visually. For AMPH-B, the MIC endpoint was defined as the lowest concentration with complete (100%) growth inhibition. For the azoles and 5-FC, 50% inhibition in growth compared to that of the drug-free growth control was used as the MIC endpoint.

Second, we used ASTY colorimetric panels containing AMPH-B, 5-FC, FLCZ, MCZ, MCFG, ITCZ, and VRCZ according to the manufacturers' instructions. The final concentrations of the antifungal agents were the same as those used in the CLSI reference method (15). Growth in each well was indicated by a color change from blue (no growth) to red (growth). For all drugs, the MIC was defined as the lowest concentration of antifungal agent that prevented the development of a red color.

Data analysis and definitions

We applied standard antimicrobial susceptibility testing definitions (16) and compared the resulting MIC using ASTY versus CLSI methods. First, we determined the distribution of differences in MIC results and calculated the agreement between MIC as follows: if the MIC obtained using the reference method and tested ASTY method were identical, the difference was 0; if the tested method gave a MIC one dilution step larger than the reference method, the difference was +1, and so on; for lower MIC, the differences were negative (17). The results were considered to essentially be in agreement when the test result was within two dilutions of the reference value, that is, the percentage of isolates that gave identical results were between plus and minus one dilution step (18). The percent essential agreement was calculated by using the number of test results in essential agreement as the numerator and the total number of organisms tested as the denominator. The results could not be compared as being in categorical agreement (*i.e.*, susceptible, susceptible dose-dependent, intermediate, or resistant) because of the no interpretative limits or MIC breakpoints stipulated in CLSI documents for *Trichosporon* species.

RESULTS

We used the MIC of six antifungal agents against 50 *T. asahii* strains to evaluate the accuracy of the ASTY method compared to the recommended CLSI broth microdilution method. Micafungin MIC were obtained only with the ASTY panel; the CLSI method was not used because *Trichosporon* species are resistant to echinocandins. Table 1 shows the distribution of differences in MIC using the ASTY method versus the CLSI method. The data in this table show that the growth of almost all *T. asahii* isolates was inhibited by low concentrations of MCZ, ITCZ, and VRCZ, but only by very high concentrations of 5-FC (4–64 μg/ml) and FLCZ (4–64 μg/ml). Moreover, 42 and 40% of the isolates showed MIC greater than 64 μg/ml for 5-FC when using the ASTY and CLSI methods, respectively. The most effective antifungal agent was VRCZ, whereas the least active drug against the *T. asahii* isolates was 5-FC.

Table 2 shows the number of isolates with $\log_2$ MIC differences and the overall essential agreement for each antifungal agent. Of the 50 isolates used in this study, seven had more than two dilution differences. The overall essential agreement ($\pm 1 \log_2$ dilution) was 97.7%, and the essential agreement was 96% for AMPH-B, 98% for 5-FC, 100% for FLCZ, 96% for MCZ, 98% for ITCZ, and 98% for VRCZ. For these drugs, the ASTY method showed good agreement with the reference method, the best essential agreement being observed for FLCZ. The MIC of the six antifungal agents tended to be slightly lower using the ASTY method.

DISCUSSION

The most important requirement for a mycology laboratory for susceptibility testing is the ability to produce accurate results quickly in order to expedite clinical

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Table 1 MIC distribution of 50 isolates of *T. asahii* against six antifungal drugs using the CLSI microdilution reference method and the ASTY colorimetric test.

	AMPH-B		5-FC		FLCZ		MCZ		ITCZ		VRCZ	
MIC (μg/mL)	ASTY	CLSI	ASTY	CLSI	ASTY	CLSI	ASTY	CLSI	ASTY	CLSI	ASTY	CLSI
0.01												
0.03											5 (10)	1 (2)
0.06											20 (40)	18 (36)
0.12	1 (2)[a]	3 (6)									15 (30)	18 (36)
0.25	4 (8)	3 (6)					2 (4)		1 (2)	1 (2)	6 (12)	9 (18)
0.5	12 (24)	11 (22)					19 (38)	18 (36)	16 (32)	11 (22)	1 (2)	1 (2)
1	22 (44)	17 (34)					21 (42)	18 (36)	30 (60)	38 (76)	1 (2)	
2	8 (16)	12 (24)					6 (12)	11 (22)	3 (6)		2 (4)	3 (6)
4		1 (2)	1 (2)	1 (2)	8 (16)	5 (10)	2 (4)	3 (6)				
8			5 (19)	8 (16)	29 (58)	28 (56)						
16	3 (6)	1 (2)	14 (28)	10 (20)	9 (18)	13 (26)						
32			7 (14)	6 (12)	1 (2)	2 (4)						
64			2 (4)	5 (10)	3 (6)	2 (4)						
>8												
>16		2 (4)										
>64			21 (42)	20 (40)								

[a]Percentage

Table 2 Distribution of differences in MIC determined using the CLSI microdilution reference method and ASTY colorimetric test.

	n (%) of isolates with $\log_2$ MIC differences of							
	>+2	2	1	0	−1	−2	<−2	% Agreement
AMPH-B		2 (4)	6 (12)	27 (54)	15 (30)			96
5-FC		1 (2)	8 (16)	32 (64)	9 (18)			98
FLCZ			4 (8)	36 (72)	10 (20)			100
MCZ		1 (2)	7 (14)	26 (52)	15 (30)	1 (2)		96
ITCZ		1 (2)	7 (14)	31 (62)	11 (22)			98
VRCZ			2 (4)	33 (66)	14 (28)	1 (2)		98

decisions (19). Before an alternative antifungal susceptibility test device can safely be used for clinical testing in place of the CLSI M27-A2 reference standard, a detailed comparative evaluation of its performance has be made.

The ASTY method, another colorimetric microdilution method, has not been evaluated as often as the YeastOne Panel. In general, CLSI and colorimetric tests give similar MIC ranges for antifungal agents (7–9). Our results indicate that, for susceptibility of *T. asahii* isolates, the ASTY method shows good concordance with the reference microdilution method. The obtained MIC ranges for MCZ, ITCZ, and VRCZ were narrow and low; in contrast, the MIC ranges for FLCZ were narrow and high. For AMPH-B and 5-FC, the MIC were higher and broader than those for other antifungal agents.

To our knowledge, no other study has examined the antifungal susceptibility of *T. asahii* isolates using the ASTY colorimetric method. Therefore, our study provides important comparisons between the reference method and colorimetric panels.

A few studies have evaluated the ASTY panel. A multicenter evaluation study compared the susceptibility of 802 *Candida* species using ASTY and reference microdilution methods (10). In that study, the MIC of ITCZ, 5-FC, and AMPH-B were compared and the overall agreement was 93% at 24 hr and 96% at 48 hr. Agreement ranged from 90% with ITCZ and 5-FC to 96% with AMPH-B at 24 hr, and from 92% with ITCZ to 99% with AMPH-B and 5FC at 48 hr. Thus the ASTY colorimetric microdilution panel method appeared to be comparable to the CLSI reference method for testing the susceptibility of *Candida* spp. to some antifungal agents. Other studies have reported antifungal susceptibility results obtained using the ASTY method. These studies used ASTY to determine the MIC

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of *Candida* spp. and *G. capitatum* clinical isolates without comparison to the reference method (11–13). Therefore, we could not compare our results with these studies. We believe that the ASTY panel has certain advantages in a clinical setting. Namely, standardized antifungal dilutions, media, and inoculum concentrations minimize the requirement for an expert in antifungal susceptibility testing and ASTY can be performed easily, even by inexperienced laboratory personnel.

Our antifungal susceptibility results should be compared to those with similar research methodology. For example, Metin *et al.* (18) compared the Etest and disk diffusion methods with the CLSI M27-A2 reference method for 27 clinical isolates of *Trichosporon* spp. (14 *T. mucoides*, 13 *T. asahii*). The observed agreement was 81–100% for AMPH-B, FLCZ, ITCZ, and VRCZ. With microdilution they found relatively high MIC for FLCZ and relatively low MIC for VRCZ. However, they found lower AMPH-B and ITCZ MIC than our MIC results (18). In another study, Arikan *et al.* (17) investigated the *in vitro* activity of AMPH-B, FLCZ, and ITCZ against 43 *T. asahii* isolates by comparing the Etest and CLSI method. They found microdilution ranges following 48-hr incubations of 1 to 8, 0.25 to 16, and 0.06 to 4 μg/ml for AMPH-B, FLCZ, and ITCZ, respectively, and reported relatively lower MIC than in our study (17).

In this study, some *T. asahii* isolates showed high MIC values for AMPH-B (>16 μg/mL), FLCZ (>64 μg/mL), and VRCZ (>1 μg/mL). Thirty strains showed MIC values of >32 μg/mL for 5-FC. ASTY is a valuable method for detecting resistant strains in a clinical setting, because both ASTY and CLSI methods show good agreement regarding the high MIC of antifungal agents.

Using a commercial product does not guarantee product performance in an individual laboratory under actual testing conditions. Therefore, it is still necessary to evaluate the colorimetric panels for relatively uncommon pathogens, such as *Trichosporon* spp., in individual laboratories.

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ORIGINAL ARTICLE

Protective effects of farnesol against oral candidiasis in mice

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ABSTRACT

Farnesol is known as a quorum-sensing molecule for *Candida albicans* and is recognized to play pathogenic roles in *Candida* infection. To assess the possible role of farnesol in mucosal *C. albicans* infection, the effects of farnesol treatment against experimental oral candidiasis in mice were examined. Prednisolone-pretreated ICR mice were orally infected with *C. albicans* and 3, 24 and 30 hr later the animals were orally given farnesol. Forty-eight hr later they were killed for observation. Farnesol treatment in a dose ranging between 1.125 and 9 μmol/mouse showed a protective effect against oral candidiasis in a dose-dependent manner, at least as estimated by symptom scores of tongues. At 9 μmol/mouse it decreased bodyweight loss. Histological studies of 2.25 μmol/mouse farnesol-treated animals indicated that farnesol suppressed mycelial growth of *C. albicans* on the surface of tongues, but microbiological study did not prevent the change of CFU of *C. albicans* cells not only on tongues but also in feces, kidneys and livers. These results suggest that farnesol has very characteristic roles in protection against mucosal candidiasis.

Key words *Candida* mycelial growth, dimorphic switch, mucosal infection, tongue lesion.

As described by Odds (1), *Candida albicans* is one of the fungi most often found in the intestinal tracts of vertebrates including humans, which suggests that it survives in humans for a long biological period. This fungus frequently causes superficial infections of mucosa and skin under certain conditions, for example, when the normal microbial flora is unbalanced due to extensive antibacterial treatment. In immunocompromised patients, *C. albicans* may invade deeper tissues, penetrate the blood vessel system, and cause life-threatening systemic infections.

Candida albicans has the capacity to switch from a yeast form to a hyphal form (dimorphic switching), one of its major virulence determinants (2). This dimorphic switching can be controlled by numerous chemical and environmental factors, including temperature, pH, glucose levels, nitrogen source, carbon dioxide levels, transition metals, chelating agents, and inoculum size or initial cell density (3).

Farnesol is known as a quorum-sensing molecule produced by *C. albicans* (4). The micromolar presence of farnesol in culture medium of *C. albicans* inhibits both the morphological change of the fungus from yeast to hyphae without influencing growth rate (5) and biofilm formation (6). Recently, Navarathna *et al.* (7) reported that farnesol had pathogenic activity to systemic candidiasis in mice. They also reported that farnesol suppressed lymphoid cell functions such as Th1 cytokine production and their activities (8). But the effects of farnesol on mucocutaneous candidiasis have not been identified.

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List of Abbreviations: CFU, colony forming unit; FCS, fetal calf serum; PAS, periodic acid–Schiff; SDA, Sabouraud dextrose agar.

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We speculate that long-term interaction between hosts and *Candida* in evolutionary history must have given farnesol an important regulatory role in the host–parasite interaction. The purpose of the present study was to investigate the effects of farnesol using a murine oral candidiasis model in order to ascertain its importance to *Candida* mycelial growth.

MATERIALS AND METHODS

Organisms

Candida albicans strain TIMM1768, isolated from a typical case of human venous blood, was maintained in our laboratory and was stored at −80 °C in Sabouraud dextrose broth (Becton Dickinson, Sparks, MD, USA) containing 0.5% yeast extract (Becton Dickinson) and 10% glycerol until the experiment was carried out.

For *in vitro* study, *C. albicans* was grown on SDA (Becton Dickinson) at 37 °C for 24 hr, and the cell suspension was prepared in RPMI-1640 medium containing 2.5% FCS (complete medium) at 5×10^3 cells/mL.

For *in vivo* study, *Candida* cells were pre-cultivated by the same method as the *in vitro* study. Then, the yeast cells were harvested and suspended in complete medium for oral inoculation.

Mice

All animal experiments were performed according to the guidelines for the care and use of animals approved by Teikyo University. Seventy-five 6-week-old female ICR mice (Charles River Japan, Inc., Kanagawa, Japan) were used. The photoperiods were adjusted to 12 hr of light and 12 hr of darkness daily, and the environmental temperature was constantly maintained at 21 °C. The mice were kept in cages housing five to six animals and were given *ad libitum* access to food and water.

In vitro activity of farnesol against *Candida* mycelial growth

Farnesol (Nacalai Tesque, Inc., Kyoto, Japan) was dissolved in dimethyl sulfoxide to be 10%, then further diluted in complete medium. Farnesol solution (100 μL) was poured into the wells of 96-well flat-bottom culture plates, followed by 100 μL *Candida* cell suspension. After mixing, the mixtures were incubated for 18 hr at 37 °C in a 5% CO_2 incubator.

To determine the extent of mycelial growth of *C. albicans*, crystal violet staining assay was performed as described previously (9).

Oral candidiasis in mice

The experimental procedure of the oral candidiasis model was described previously (10). Briefly, immunosuppressed mice were induced by subcutaneous treatment with a dose of 100 mg/kg prednisolone (Mitaka Pharmaceutical Co., Tokyo, Japan) 1 day prior to oral infection. Tetracycline hydrochloride (Takeda Schering Purau Animal Health Co., Tokyo, Japan) in drinking water at a dose of 0.08% was also given to the mice beginning 1 day before infection. The animals were anesthetized by intramuscular injection in each hind limb with 50 μL of 0.2% chlorpromazine chloride (12 mg/kg; Wako Pure Chemical Industries, Osaka, Japan). They were orally infected by means of a swab (baby cotton buds; Johnson & Johnson, Co., Tokyo, Japan) which had been immersed in 2.0×10^8 cells/mL viable cells of *C. albicans* TIMM 1768 in complete medium. The cell number of *Candida* inoculated in the oral cavity was calculated to be approximately 1×10^6 cells/mouse (11).

Farnesol was suspended to 0.5, 1 and 4% by 1% Tween 80 (Kanto Chemical Co. Inc., Tokyo, Japan) solution. Farnesol suspension (50 μL) was given orally three times, 3, 24 and 30 hr after *Candida* inoculation. Control mice received the same volume of 1% Tween 80. The doses of 0.5, 1 and 4% solution corresponded to 1.125, 2.25 and 9 μmol/mouse.

Bodyweights were measured before and 48 hr after *Candida* inoculation. The mice from each group were killed 48 hr after inoculation for the further experiments described below.

Macroscopic observation of the tongues

Macroscopic effect of farnesol treatment was indicated by a lesion score (10) from 0 to 4 on the basis of the extent and severity of whitish, curd-like patches on the tongue surface as follows: 0, normal; 1, white patches over less than 20%; 2, white patches over less than 90% but more than 21%; 3, white patches over more than 91%; 4, thick white patches like pseudomembranes over more than 91% of the tongue.

Measurement of CFU of *C. albicans*

For microbiological evaluation, oral cavities of the mice were swabbed with a cotton bud and *Candida* cells collected by the bud were suspended in 3 mL saline. The resected tongues were then immersed in 2 mL saline and homogenized by homogenizer (Polytron PT 1200; Kinematica AG, Littau, Switzerland). The CFU of swabbed suspension and homogenates were counted by 24 hr culture on a *Candida* GS plate (Eiken Chemical Co., Tokyo, Japan) at 37 °C, and the total per mouse was calculated.

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To evaluate the adherence ability of *Candida* cells to the tongue surface, the tongues were resected and washed gently by hand in 2 mL saline for 5 s, then immersed in 2 mL saline and homogenized by homogenizer. The CFU of the washing fluid and homogenates of the tongue were counted as above.

The feces were collected from the descending colon, and the liver and kidneys were resected. Three or four pieces of collected feces in 1 mL saline were suspended with a vortex mixer. The liver and kidneys were immersed in 2 mL saline, and then homogenized. Their CFU were counted and the CFU/0.1 g feces, liver and kidney were calculated.

Histological observation

For morphological study, the washing fluids and homogenates of the tongue prepared for CFU counting were gently smeared onto a microscope slide, stained by the PAS stain method, and mounted in Entellan Neu (Merck KGaA, Darmstadt, Germany).

For histological study, the resected tongues were fixed with 4% paraformaldehyde (pH 7.4) at 4 °C, and embedded in paraffin. Sections on the slide were sectioned postfixed in 4% paraformaldehyde for 15 min, and stained with PAS.

Statistical analysis

Data were expressed as mean ± SD and evaluated by Kruskal Wallis H-test and Mann-Whitney *U*-test with Bonferroni correction. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Inhibitory effect of farnesol on *Candida* mycelial growth

In our culture condition, *C. albicans* grew almost completely in form after 18 hr of culture. Figure 1a shows that 224 μM farnesol almost inhibited the *Candida* mycelial growth, but induced vigorous yeast growth. The effect of farnesol on mycelial growth of *C. albicans* was examined *in vitro* and, as shown in Figure 1b, the molecule dose-dependently inhibited this growth. The farnesol concentration of 50% inhibition (IC_{50}) of *Candida* mycelial growth was approximately 150 μM, although even at a lower concentration (14 μM), enhanced yeast-form growth of *C. albicans* was observed (data not shown).

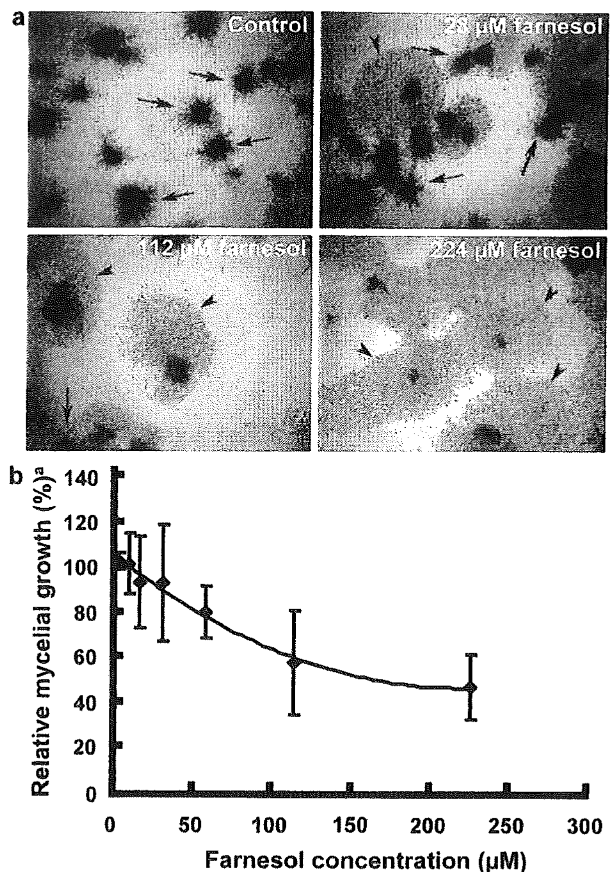

Fig. 1. Effects of farnesol on *Candida* growth *in vitro*. (a) Upper left panel, control; upper right panel, 28 μM farnesol; lower left panel 112 μM farnesol; lower right panel 224 μM farnesol. Change of growth form of *C. albicans* cultured in the presence of farnesol. *Candida* mycelial growth (arrows) was seen in the control group, but vigorous growth of yeasts (arrowheads) was observed in the 112 and 224 μM farnesol groups. (b) Inhibitory effects of farnesol on *Candida* mycelial growth. a, relative mycelial growth of *C. albicans* measured by crystal violet staining as described in Materials and Methods.

Effects of farnesol treatment against oral candidiasis

The protective effects of farnesol were examined using the murine oral candidiasis model described previously (10), in which lesions of candidiasis can be estimated by lesion scores of the tongues. As shown in Figure 2a, oral administration of farnesol at 1.125 μmol or above significantly improved the score 48 hr after infection dose dependently (control: 3.58 ± 0.67; 1.125 μmol: 2.22 ± 0.44; 2.25 μmol: 1.91 ± 0.70; 9 μmol: 0.20 ± 0.42, respectively). Figure 2b shows the macroscopic appearance of a typical lesion of the tongues 48 hr after *Candida* infection. The tongues of control mice were covered with thick white patches, whereas the tongues treated with 2.25 μmol farnesol looked healthy and displayed fewer patches.

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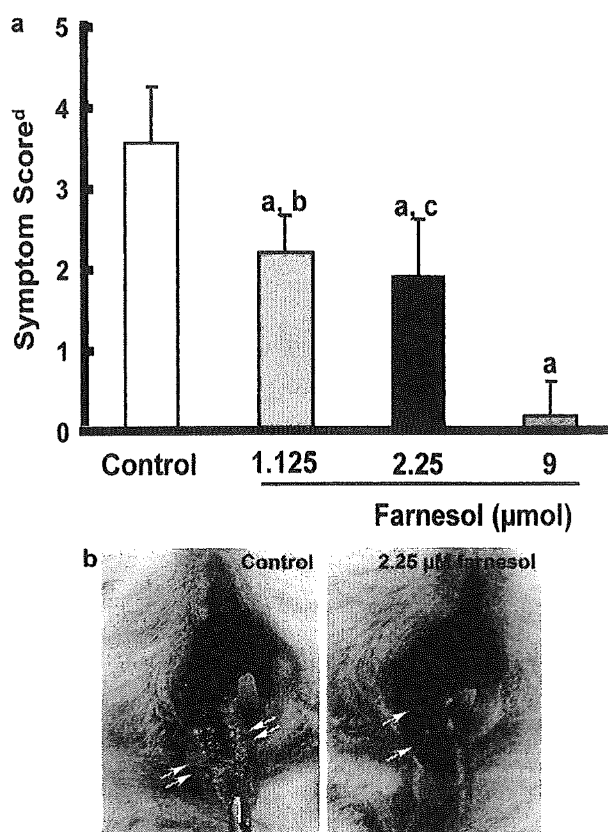

Fig. 2. Macroscopic effects of farnesol on oral candidiasis. (a) Symptoms of oral candidiasis after oral treatment of farnesol. Mice were orally inoculated with *C. albicans* 48 hr earlier. They were treated with various doses of farnesol 3, 24 and 30 hr after infection. □, control group ($n = 12$); ▨, 1.125 μmol farnesol group ($n = 9$); ■, 2.25 μmol farnesol group ($n = 11$); ▨, 9 μmol farnesol group ($n = 10$). a, $p < 0.01$ compared to control; b, $p < 0.05$ compared to 9 μmol farnesol group; c, $p < 0.01$ compared to 9 μmol farnesol group (means ± SD); d, symptom scores estimated by tongue white patches. (b) Macroscopic appearance of a typical tongue lesion after farnesol treatment. Left panel, control (score 4); right panel, 2.25 μmol farnesol (score 1). White patches of the control group covered thickly like pseudomembranes (double arrows) on the tongue surface whereas the patches on tongues of the farnesol group were thin (single arrows).

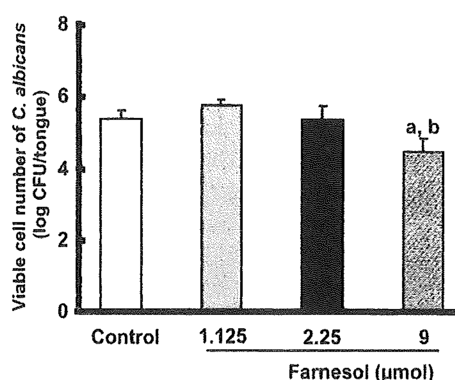

Fig. 3. Effects of farnesol on the viable cell number of *C. albicans* recovered from the oral cavity. See legend to Figure 2b. *Candida* cells were recovered by swabbing and tongue homogenates. □, control ($n = 9$); ▨, 1.125 μmol farnesol ($n = 7$); ■, 2.25 μmol farnesol ($n = 8$); ▨, 9 μmol farnesol ($n = 8$). a, $p < 0.01$ compared to control; b, $p < 0.01$ compared to 2.25 μmol farnesol (means ± SD).

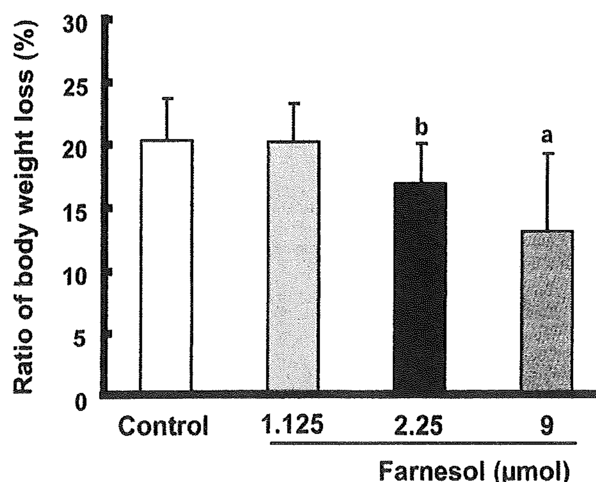

Fig. 4. Effects of farnesol treatment on bodyweight loss in *Candida*-infected mice. The loss after infection was calculated by the following formula: $[BW_{day0} - BW_{day2}]$. □, control group ($n = 11$); ▨, 1.125 μmol farnesol group ($n = 9$); ■, 2.25 μmol farnesol group ($n = 11$); ▨, 9 μmol farnesol ($n = 10$). This figure represents total results obtained from two independent experiments. a, $p < 0.01$ compared to control (means ± SD); b, NS, but *P*-value calculated without Bonferroni correction was 0.02.

We then examined the effects of farnesol on the viable cell number of *C. albicans* recovered by the swab from the infected tongue tissues by counting CFU. Treatment with only 9 μmol farnesol significantly decreased the cell numbers compared with control (4.50 ± 0.40, 5.40 ± 0.23 log CFU/tongue, respectively; Fig. 3).

As a parameter of systemic physical deterioration of the infected mice, bodyweight loss after *Candida* infection was calculated. Treatment with farnesol at doses of 9 μmol suppressed the weight loss observed 48 hr after infection (Fig. 4). Treatment with 2.25 μmol of the molecule seemed to inhibit the weight loss, even though there was no statistical significance.

In this experiment, we noticed that the mice given 9 μmol farnesol exhibited an unusual behavior (sedated condition with loss of normal movement) after the first administration and one of them died within 24 hr. As we were afraid that 9 μmol farnesol might accidentally cause a toxic reaction, we evaluated its effect using 2.25 μmol solution in further experiments.

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Analysis of *Candida* infections in farnesol-treated mice

As known in the case of clinical oral candidiasis, the white patches including viable *Candida* cells on murine tongues appeared to be easily detached from the tongue surface by physical perturbation (12). So, in order to assess the adherence conditions of *Candida* cells to the tongue surface of control and 2.25 μmol farnesol-treated mice, the tongues were washed with saline, and the number of viable *Candida* cells in washing fluid and homogenates of residual tongues was measured. As shown in Figure 5a, the CFU recovered from washing fluid and homogenate and the total CFU were not significantly different between the 2.25 μmol farnesol group (2.58 ± 1.20, 5.61 ± 0.20, 5.61 ± 0.20 log CFU/tongue, respectively) and control (2.39 ± 0.76, 5.54 ± 0.29, 5.61 ± 0.30 log CFU/tongue, respectively). This suggested that the CFU, as a parameter of *Candida* cell number, did not reflect the different adherent conditions of the cells between these groups.

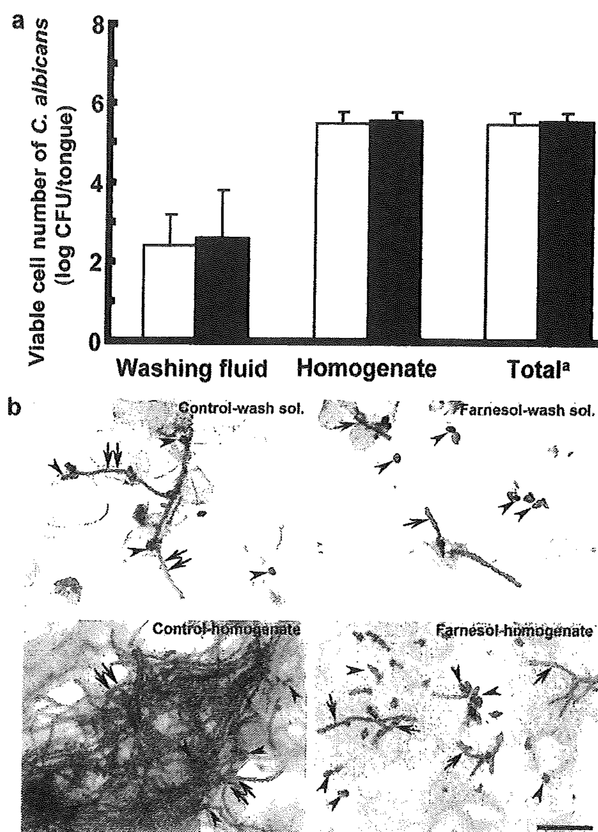

Fig. 5. *Candida* cells recovered from tongues of mice with or without farnesol treatment. (a) Viable *Candida* cell number recovered by washing or homogenization of infected tongues. □, control (n = 19); ■, 2.25 μmol farnesol (n = 16). a, total counts were calculated by the following formula: [washing fluid] + [homogenates]. (b) Micrograph of washing fluids and homogenates of tongues. Left panels, control; right panels, 2.25 μmol farnesol; upper panels, smears of saline – washing fluids; lower panels, smears of homogenates. Mycelia of the elongated type (double arrows) and the dispersed type (arrows) were observed by the control and farnesol group, respectively. Yeast cells (arrowheads) were seen in both the control and farnesol group. Bar = 50 μm.

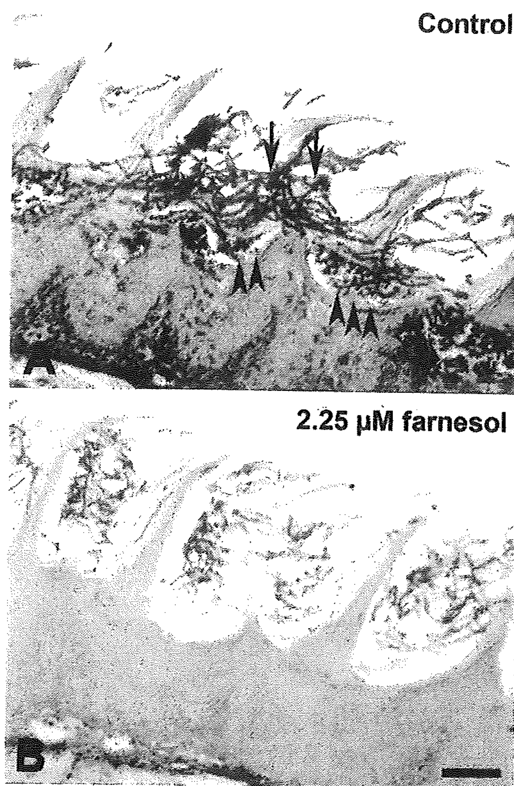

Fig. 6. *Candida* mycelial growth of tongues in longitudinal section. (a) Control group; (b) 2.25 μmol farnesol group. Vigorous mycelial growth (arrows) was seen on the dorsal surface of tongues of control mice. Inflammatory cells stained with hematoxylin (arrowheads) accumulated in the submucosal layer under the *Candida*-colonized area. There was poor mycelial growth and almost no inflammatory cells in the farnesol group. Bar = 50 μm.

We observed morphologically the smears of cell suspension in the washing fluids and homogenates (Fig. 5b). The elongated mycelia and yeast cells were observed in the smear from both washing fluids and homogenates of the control whereas dispersed mycelia and yeast cells were seen in the 2.25 μmol farnesol group.

Histological sections of tongues showed that there was vigorous mycelial growth on the dorsal surface of the tongues of control mice (Fig. 6a). Inflammatory cells also accumulated in the subcutaneous area under the *Candida*-colonized lesions. In contrast, there was little mycelial growth or inflammatory cells on the tongues of 2.25 μmol farnesol-treated mice (Fig. 6b).

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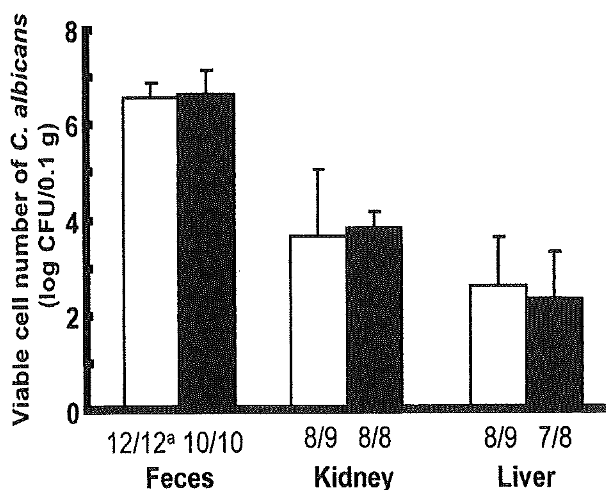

Fig. 7. *Candida* cells recovered from feces, kidneys and livers of infected mice. □, control; ■, 2.25 μmol farnesol. a, [mice with detectable number of *C. albicans*]/[number of mice tested].

Because there were no differences in CFU between control and 2.25 μmol farnesol in spite of their morphological and histological differences, we can assume that the CFU did not correspond with the lesions score.

Effects of the oral application of 2.25 μmol farnesol on *Candida* infection in tissues other than the oral cavity were checked. As shown in Figure 7, the number of viable *C. albicans* cells in the feces obtained from farnesol-treated mice reached the level of 10^6 CFU/0.1 g feces, which was equal to that of control mice. This suggests that *Candida* cells colonized in the intestinal tracts of the mice regardless of farnesol treatment. The dissemination to kidneys and livers was observed in more than 85% of control and 2.25 μmol farnesol-treated mice. There were no differences in the *Candida* cell numbers of kidney (3.58 ± 1.38, 3.76 ± 0.31 log CFU/0.1 g kidney, respectively) and liver (2.56 ± 0.98, 2.26 ± 0.97 log CFU/0.1g liver, respectively) between the two groups (Fig. 7).

DISCUSSION

The present study showed that giving oral farnesol protected mice from oral candidiasis, as estimated by the inhibition of the formation of white patches on the tongue surface, and inhibition of bodyweight loss of infected mice. As far as we know, this is the first report that farnesol elicits a protective effect against mucosal candidiasis.

In spite of the diminished lesions of the tongues, the number of CFU of *Candida* associated with tongues was not decreased by the treatment with 2.25 μmol farnesol. This discrepancy between lesions and cell numbers might be explainable by the method of counting CFU measured; that is, a relatively large mycelium is counted as one CFU like a single yeast cell. It is our opinion that, to examine the microbiological effects of farnesol in further studies, some quantitative assay parameters other than CFU are required for discrimination of the growth form of *C. albicans*. Actually, as shown in Figure 5, washing fluids and homogenates of tongues of farnesol-treated mice contained many yeast cells. At the present time, we can assume that farnesol may inhibit hyphal growth of the fungus which is necessary for invasion into tongues, because more than 150 μM farnesol clearly inhibited the mycelial growth of this fungus as shown in Figure 1. As recently described by Ramage *et al.* (6), farnesol controls germination of the adherent yeast cells in concentration dependence *in vitro*, such as true hyphae (3 μM farnesol), pseudohyphae (30 μM farnesol). The agent (300 μM) prevented successful germination of the *Candida* cells.

We must note that the possible interaction of immunological activities of farnesol may affect this pathological change of oral candidiasis, as many inflammatory cells were accumulated in the subepithelial area of control *Candida*-infected tongues but not in those of farnesol-treated ones. Our recent observation (unpublished data) suggests that farnesol may suppress the function of inflammatory leukocytes. Furthermore, Navarathna *et al.* (8) reported that farnesol suppressed immunocyte functions, such as Th1 cytokine (γ-interferon and interleukin-12) production, which mediated local accumulation of inflammatory cells.

Systemic effects of the application of farnesol on *Candida* infection in mice remain to be clarified. We observed that oral farnesol administration did not affect the *Candida* growth in the intestinal tracts, kidneys or livers. Therefore, we believe it is possible that the beneficial effects of farnesol on oral candidiasis may be limited locally. In this regard, we can note that oral or pharyngeal infection by *Candida* is easily influenced by local treatments with immunosuppressive agents, as our previous report indicated that oral inhalation of beclomethasone dipropionate degenerated the lesions of experimental pharyngeal candidiasis in mice (13).

The results shown in Figure 4 indicated that giving 9 μmol/mouse of farnesol appeared to give the mice systemically protective effects, such as moderated bodyweight loss and a lower cell number of *C. albicans* in feces (data not shown). However, in this group of mice, one mouse died. The cause of death is unknown but we speculate that accidental inhalation of the farnesol solution may have induced mortal toxicity, because these mice received the solution orally while in a sedated state. Very recently, Navarathna *et al.* reported that intraperitoneal administration of 20 μmol/mouse of farnesol was not lethal but facilitated systemic *Candida* infection to kill the mice rapidly (7). These differences in the effects of farnesol,

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which appear contradictory, can be explained by the different conditions of the administered dose or route of farnesol, immunosuppressed conditions of mice and in its local and systemic impacts. In order to clarify the reasons causing these differences, further studies on the immunological activities of farnesol *in vivo* are needed.

Finally, we wish to discuss the role of farnesol production by *C. albicans* on mucosal membranes in the oral cavity. As reported by Hornby *et al.* (14), a growing mass of *C. albicans* produces a considerable quantity of farnesol. Here, we presented evidence indicating that farnesol prevented invasion of the fungus to the mucosal membrane which then induced host inflammatory reactions with tissue degradation and host defense cellular responses. Therefore, we wish to propose a hypothesis that the role of farnesol may be to maintain the stable condition of *C. albicans* on the mucosal surface. Of course, this hypothesis should be verified by further experiments elucidating the condition under which farnesol is produced and its functions *in vivo*. Elucidation of the pharmacological action of the molecule against leukocytes *in vivo* may promote our understanding of the mechanisms of the stable existence of *C. albicans* as normal microbial flora of vertebrates.

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Short Communication

A new genotype of *Arthroderma benhamiae*

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Nucleotide sequence analysis of chitin synthase 1 (*CHS1*) indicated 90% sequence similarities among human and animal isolates of *Arthroderma benhamiae.* In particular, greater than 99% similarity was noted in the nucleotide sequence among Americano-European race isolates, African race isolates, and five isolates from four-toed hedgehogs (*Atelerix albiventris*). The phylogenetic analysis indicated that the five isolates from hedgehogs were included in the same cluster and distinct from the Americano-European and the African race standard strains of *A. benhamiae.* These results were confirmed by ITS analysis. Therefore, it was proposed that genotypes could be established for the isolates of *A. benhamiae* in association with the mating behavior.

Keywords *Arthroderma benhamiae,* chitin synthase 1 gene, genotype

Introduction

Trichophyton mentagrophytes, which frequently causes human and animal dermatophytoses, has been proven to be a complex consisting of more than three teleomorphs, i.e., *Arthroderma benhamiae, A. simii* and *A. vanbreuseghemii* [1–3]. Hironaga and Watanabe reported in 1980 that none of the isolates of *T. mentagrophytes* recovered in Japan from human and animal dermatophytoses had ever been identified as *A. benhamiae* [4]. However, in 1998 we reported the first isolation of *A. benhamiae* in Japan from a case involving a rabbit [5]. More clinical isolates of *A. benhamiae* have since been recovered from humans, rabbits, guinea pig and hedgehogs in different areas of Japan. The isolates have been reported to divide on the basis of mating behavior into two races, i.e., the Americano-European race and the African race [3]. Strains of Americano-European race were frequently isolated from rabbits and guinea pigs, while African race strains were recovered from western European hedgehogs (*Erinaceus europaeus*) [6,7]. In recent years, the number of cases of dermatophytosis in Japan due to *A. benhamiae* in the four-toed hedgehog (*Atelerix albiventris*) has increased [6]. The hedgehogs are popular household pets and this particular fungus spreads easily to humans. Mating experiments have been conducted with clinical isolates from hedgehogs with the (+) and (−) standard strains of the Americano-European and African races of *A. benhamiae* (Table 1) [6]. The (+) and (−) isolates of the hedgehogs produced many more gymnothecia when crossed with themselves than with the standard strains of the indicated races, suggesting the establishment of the new race [6]. However, the hedgehog isolates have not been molecularly well characterized from an epidemologic perspective.

In order to understand phylogenetic relationships of *A. benhamiae,* we sequenced and analyzed partial DNA fragments of the chitin synthase 1 (*CHS1*) gene of several dermatophytes [8–10]. The results of these studies proved to be very useful in better understanding the evolution of the dermatophyte species, as well as in

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