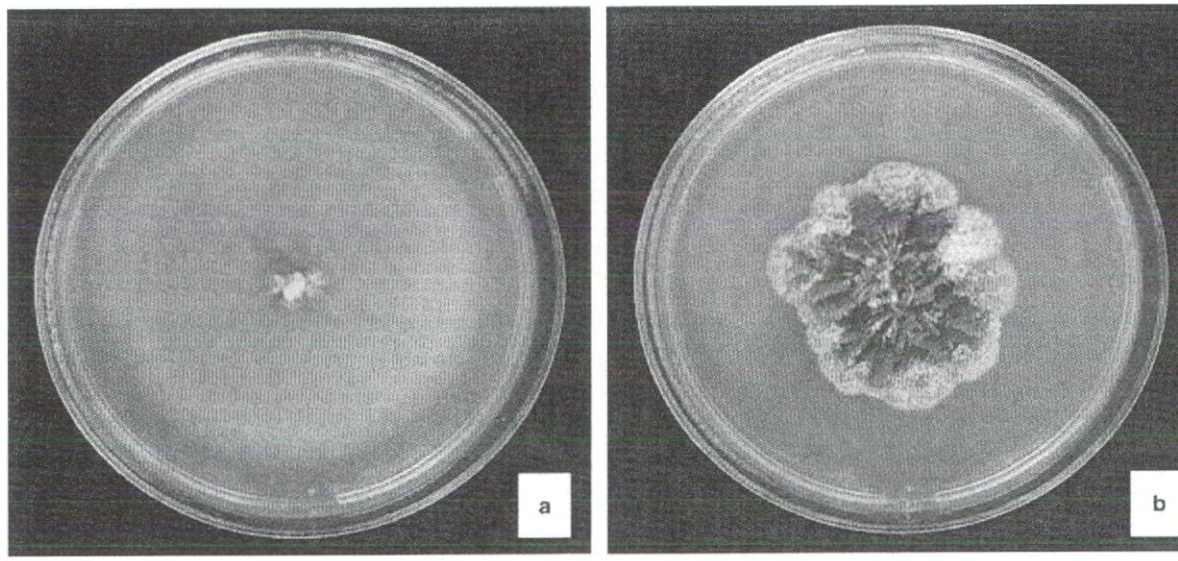

Fig. 3 Colonies on SDA (a) and PDA (b) plates cultured at 25°C for 1 month.

Discussion

This constitutes the first case of *L. hoffmannii* osteomyelitis leading to the development of systemic infection in an animal in Japan. The diagnosis was based on clinical, mycological and molecular data. A tonic convulsion before the euthanasia indicated an invasion to the central nervous system (CNS) following lymphangial dissemination. Unfortunately, we were not permitted to conduct a postmortem examination on the dog to confirm CNS involvement.

It was impossible to detect the infection route as the dog had no history of injuries or problem with nutrients. The housing conditions and dog's environment were unremarkable but it is possible that we might have overlooked an injury caused by a small thorn or abrasion. *L. hoffmannii* is distributed in natural and indoor environments [1–3], and the fact that the fungal species is a food contaminant suggests that the dog might have acquired its infection by contact within its environment.

Infections caused by *L. hoffmannii* have been reported in immunocompromised hosts [5,7] but no such relationship was established in the present case. The fatal outcome might be due to the strong virulence of this species or to this particular isolate.

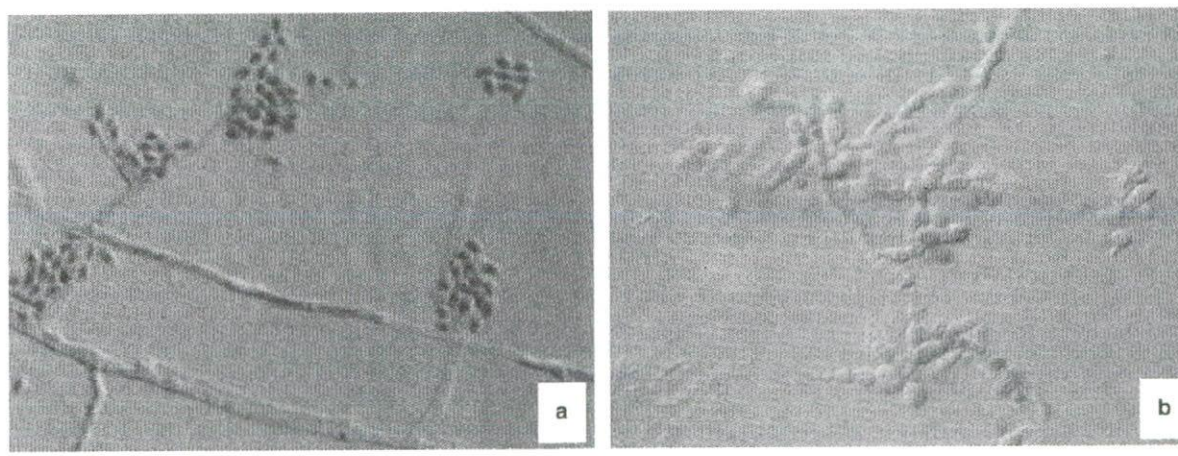

Fig. 4 Micro-culture on PDA block cultured at 25°C for 3 weeks stained with lactophenol cotton blue (a), ×400, and direct mount from a culture on PDA slant at 25°C for 2 months fixed with lactophenol (b), ×400.

The fungal species is known to be susceptible to antifungal drugs [19,20] but the present and tested isolates were apparently exceptions. This suggests the difficulty of treating a disseminated infection caused by *L. hoffmannii*. Even if the fungus could have been isolated at the first visit, a fatal outcome would not have been avoidable.

Lecythophora mutabilis is a species closely related to *L. hoffmannii* which is also known as an emerging lethal fungal pathogen causing peritonitis [9] and endocarditis [10,11]. *Lecythophora* spp. might be more highly virulent than originally estimated, although they were classified as biosafety level 1 by de Hoog *et al*. [19].

In addition, *Coniochaeta ligniaria* has been described as the teleomorph of *L. hoffmannii* [19]. *C. ligniaria* is known as an industrially detoxification agent but has not, as of yet, been reported associated with infections in man or animals [21].

In conclusion, emerging fungal diseases caused by environmental fungal species are increasing in number [22–25], suggesting that fungal isolates in clinical laboratories that look like environmental contaminants should be recognized as potential true pathogens.

Acknowledgements

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Pathogenicity of *Ochroconis gallopava* isolated from hot springs in Japan and a review of published reports

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Abstract Four strains of *Ochroconis gallopava* from 3 out of 15 Japanese hot springs were isolated. Colonies of the hot spring isolates were uniformly floccose and dark olive green on the surface and dark brown on their reverse side on potato dextrose agar (PDA) plates, however, they became felty, flat, and brownish-black, and produced a reddish-brown pigment after several times of subculture at room temperature. Shapes and sizes of conidia of the four strains were individual, while the D1/D2 domain of the large subunit ribosomal RNA gene sequences showed 99.7% identity in the GenBank database. The DNA pattern of the hot spring isolates amplified by species specific loop mediated isothermal amplification method were as the same pattern as that of a clinical isolate. The minimum inhibitory concentrations of antifungal agents to *O. gallopava* isolated from the hot springs were ranged from 0.5 to 1 μg/ml in amphotericin B, 1 to 16 μg/ml in flucytosine, 0.125 to 0.25 μg/ml in itraconazole, 1 to 4 μg/ml in miconazole, 16 to 64 μg/ml in fluconazole and 0.03 to 0.5 μg/ml in micafungin. The isolates had fatal outcome in experimentally infected mice intravenously with severe invasiveness to brains and kidneys. These findings suggested that *O. gallopava* habitats in hot springs could be one of sources for infection.

Keywords Hot spring · *Ochroconis gallopava* · Pathogenicity

Abbreviations

MOPS	3-(N-Morpholino) propanesulfonic acid
DDBJ	Center for Information Biology and DNA Data Bank of Japan
LAMP method	Loop mediated isothermal amplification (LAMP) method
MIC	Minimal inhibitory concentration
PDA	Potato dextrose agar
D1/D2 LSU rDNA	The D1/D2 domain of the large subunit ribosomal RNA gene

Introduction

Ochroconis gallopava is a species of dematiaceous fungi recognized as a causative agent of emerging and zoonotic fungal infections [1]. Upto date, 39 human cases including 3 from Japan have been reported worldwide both immunocompromised and healthy subjects [2–25] (Table 1). The pathogen also caused outbreaks in poultry and birds grown in zoos

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Table 1 Human cases of *Ochroconis gallopava* infection

Case	Authors, published year and reference number	Country	Sex	Age	Affected organ	Remarks[a]	Outcome
1	Dixon and Salkin 1986 [2]	USA	N.D	N.D.	Lung	N.D.	N.D.
2	Fukushiro et al. 1986 [3]	Japan	F	58	Subcutaneous	Leukemia	Survived
3	Terreni et al. 1990 [4]	USA	M	62	Systemic	Leukemia, diabetic	Died
4	Sides et al. 1991 [5]	N.D.	M	N.D.	Lung	Immunocompromised	N.D.
5	Sides et al. 1991 [5]	South Africa	N.D.	N.D.	Lung	Coal mine worker	N.D.
6	Sides et al. 1991 [5]	South Africa	N.D.	N.D.	Lung	Coal mine worker	N.D.
7	Sides et al. 1991 [5]	USA	N.D.	N.D.	Lung	Immunocompromised	Died
8	Sides et al. 1991 [5]	USA	N.D.	N.D.	Brain	Immunocompromised	N.D.
9	Sides et al. 1991 [5]	USA	M	47	Lung	Cardiovascular disease	N.D.
10	Sides et al. 1991 [5]	USA	M	60	Brain	Lymphoma, nocardiosis	Died
11	Mancini and McGinnis 1992 [6]	USA	M	30	Lung	Heart transplant recipient	Survived
12	Prevost-Smith et al. 1993 [7]	N.D.	M	46	Systemic	Heart transplant recipient	Died
13	Vukmir et al. 1994 [8]	USA	M	68	Brain	Liver transplant recipient	Survived
14	Kralovic et al. 1995 [9]	USA	M	63	Brain	Liver transplant recipient, diabetic	Died
15	Rossmann et al. 1996 [10]	USA	M	59	Brain	Liver transplant recipient, nocardiosis	Died
16	Bonham et al. 1996 [11]	USA	N.D.	N.D.	Brain	Liver transplant recipient	Survived
17	Jenney et al. 1998 [13]	Australia	M	58	Lung	Heart transplant recipient, diabetic	Survived
18	Horré and de Hoog 1999 [14]	UK	N.D.	N.D.	Systemic	AIDS	N.D.
19	Horré and de Hoog 1999 [14]	Australia	N.D.	N.D.	Systemic	N.D.	N.D.
20	Horré and de Hoog 1999 [14]	USA	N.D.	N.D.	Brain	Diabetes mellitus	N.D.
21	Horré and de Hoog et al. 1999 [14, 15]	N.D.	M	48	Lung	HIV-positive transplant recipient	N.D.
22	Horré and de Hoog et al. 1999 [14, 15]	Australia	N.D.	N.D.	Lung	N.D.	N.D.
23	Horré and de Hoog 1999 [14]	USA	N.D.	N.D.	Lung	Transplant recipient	N.D.
24	Burns et al. 2000 [16]	Canada	F	58	Lung	Lung transplant recipient with pulmonary nodule	Survived
25	Odell et al. 2000 [17]	USA	M	38	Lung	Wood pulp worker with pulmonary abscess	Survived
26	Bowyer et al. 2000 [18]	UK	M	69	Eye	Chronic lymphocytic lymphoma	Died
27	Mazur et al. 2001 [19]	USA	F	32	Subcutaneous	Lung transplant recipient with diabetic	Survived
28	Malani et al. 2001 [20]	USA	M	32	Lung	Renal transplant recipient with diabetic	Died
29	Zhao et al. 2002 [21]	China	M	68	Lung	Pemphigus	Survived
30	Wang et al. 2003 [22]	China	M	13	Systemic	Renal transplant recipient	Died
31	Fukushima et al. 2005 [23]	Japan	F	66	Systemic	Chronic lymphocytic lymphoma	Died
32	Ohori et al. 2006 [24]	USA	M	54	Systemic	Heart transplant recipient	Died
33	Ohori et al. 2006 [24]	Japan	M	79	Lung	Pneumoconiosis	N.D.
34	Ohori et al. 2006 [24]	Canada	F	68	Lung	N.D.	N.D.
35	Ohori et al. 2006 [24]	USA	N.D.	N.D.	Lung	N.D.	N.D.
36	Ohori et al. 2006 [24]	New Zealand	M	83	Lung	N.D.	N.D.

Table 1 continued

Case	Authors, published year and reference number	Country	Sex	Age	Affected organ	Remarks[a]	Outcome
37	Boggild et al. 2006 [25]	Canada	M	28	Systemic	AIDS	Died
38	Database of CBS	Netherlands	N.D.	N.D.	CSF	Spondylodiscitis	N.D.
39	Database of CBS	Germany	M	50	Lung	Bronchial carcinoma	Died

N.D.—No data; CSF—Cerebrospinal fluid; [a] Underlying disease or occupation; CBS—Centraalbureau voor Schimmelcultures, The Netherlands

[26–36] and a few cases in domestic animals [2, 35, 36] (Table 2) has raised a serious problem for differentiation from SARS and highly pathogenic avian influenza [24]. The natural habitat of the fungal species was thought to be places with very low pH and extreme temperatures such as in thermal soils [37–41], hot spring effluents [39, 40], sewage from nuclear power plants [42, 43], a pulp sample [24] and broiler-house litters [30, 31] (Table 3), however there is no report on isolation of this fungal species from any natural environment in Japan.

It is very famous that Japan has many active volcanoes and hot springs. Japanese people have customs of hot spring bathing and drinking for refreshments, health promoting and treatments for chronic diseases, sometimes nakedly. There are several types of bathtubs in hot spring facilities about their location and materials; outdoors or indoors, and made of stone, ceramic tile or wood. Interestingly, customers prefer to use bathtubs placed outdoors as a recent trend. Then we thought such hot spring environments might be a dangerous place for *O. gallopava* infection via direct contacts to skin and inhalations. In the present study, we tried to isolate the fungal species from hot spring water, and to estimate a risk of *O. gallopava* infection by contacts with hot springs.

Materials and methods

Isolation

Fifteen hot spring water samples were collected from all 14 hot spring areas in all Japan and kept at 4°C within 2 days. Sample number 2 was taken from a hot

Table 2 Animal cases of *Ochroconis gallopava* infection

Authors, published year and reference number	Country	Animal species	Affected organ(s)
Georg et al. 1964 [26]	USA	Turkey[a]	Brain
Connole 1967 [27]	Australia	Chick[a]	Brain
Blalock et al. 1973 [28]	USA	Turkey[a]	Brain
Ranck et al. 1973 [29]	USA	Chick[a]	Brain
Waldrip et al. 1974 [30]	USA	Chick, 6 cases[a]	Brain
Randall and Owen 1981 [31]	UK	Chick, 2 cases[a]	Brain and lung
Shane et al. 1985 [32]	USA	Japanese quail chicks[a]	Brain and lung
Dixon and Salkin 1986 [2]	USA	Cat	Lung
Karesh et al. 1987 [33]	USA	Gray-winged trumpeter chicks	Brain
Salkin et al. 1990 [34]	USA	Snowy owl chick	Brain
Padhye et al. 1994 [35]	USA	Cat	Systemic
Horré and de Hoog 1999 [14]	India	Chick[a]	Brain
Ohori et al. 2006 [24]	New Zealand	Antipodean parakeet	Lung
Singh et al. 2006 [36]	USA	Dog	Systemic

[a] Epidemic outbreaks at poultry farms

Table 3 Isolation of *Ochroconis gallopava* from environments

Authors, published year and reference number	Country	Origin
Evans 1971 [37, 38]	UK	Coal tip
Tansey and Brock 1973 [39]	USA	Hot spring effluents
Tansey and Brock 1973 [39]	USA	Geothermal soils
Tansey and Brock 1973 [39]	USA	Self-heated coal waste piles
Waldrip et al. 1974 [30]	USA	Broiler-house litter
Tansey et al. 1979 [42]	USA	Aerosol from a nuclear reactor station
Rippon et al. 1980 [43]	USA	Warm effluent from a nuclear reactor station
Randall and Owen 1981 [31]	UK	Broiler-house litter
Weitzman et al. 1985 [40]	USA	Soil near a hot spring
Weitzman et al. 1985 [40]	USA	Hot spring effluents
Weitzman et al. 1985 [40]	USA	Elk droppings near a hot spring
Horré and de Hoog 1999 [14]	New Zealand	Unknown
Horré and de Hoog 1999 [14]	France	Unknown
Redman et al. 1999 [41]	USA	Geothermal soil
Ohori et al. 2006 [24]	Canada	Pulp sample with pink slime

river and others from bathtub. The bathtubs of sample number 6 and 14 were located outdoors and others indoors. The bathtubs of sample number 13 and 14 were made of wood and others were of stone or tile. The temperatures of samples were 41–42°C except for sample number 4 at the times of collection, however sample of number 6, 7, 13, 14, and 15 were warmed cold mineral springs. PH values of samples except one were weak acidic and sample number 12 was basic. Samples from number 4–15 emitted odor of hypochlorite (Table 4).

Five hundred milliliters of each hot spring water samples was filtrated with a 0.22-micrometer-pore-sized filter. The filters used for the filtration were put PDA plates and cultured at 42°C for 2 weeks. Olive-green to brownish green and/or black-brown colonies were picked up, and maintained on PDA slants at room temperature until the experiments.

Mycological studies

The purified isolates were used for the mycological studies. Colonies on PDA plates at 25°C, 37°C, and 42°C, micro culture on 1/10 diluted Sabouraud dextrose agar at 25°C for 3 weeks, and maximum growth temperature on PDA slants up to 50°C were examined. Conidia size was measured under light microscope. The mean length and the maximum widths of apical cells calculated from 30 conidia.

Molecular biological identification

The D1/D2 domain of the large subunit ribosomal RNA gene (D1/D2 LSU rDNA) sequences confirmed by a routine method [44] was compared with the GenBank database [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD = search&DB = nucleotide]. In addition, species-specific primer set for loop mediated isothermal amplification (LAMP) method based on D1/D2 LSU rDNA reported by Ohori et al. [24] was tested on the hot spring isolates. The patterns were compared with a clinical isolate IFM41473 originated from NHL 2917 [3].

Antifungal susceptibility testing

Antifungal susceptibility tests on conidia of *O. gallopava* isolates from hot spring water samples cultured on PDA slants at 25°C for 2 weeks were performed according to the broth microdilution modified method of the CLSI M38-A [45] approved standard using RPMI 1640 medium (Sigma, Poole, UK) buffered to pH 7.0 with 3-(N-Morpholino) propanesulfonic acid (MOPS) (Sigma) using a kit

Table 4 Characteristics of hot spring water samples and numbers of *Ochroconis gallopava* isolates

Hot spring water samples								*Ochroconis gallopava* isolates[d]	
No.	Hot spring	Source[a]	Location	Date	Indoor/ outdoor	Temperature (°C)	pH	Number of colonies	IFM number
1	Kanagawa	Hot spring bath	Kanto	Mar. 2004	Indoor	41–42	5.6–5.8	2	254734, 54735
2	Kanagawa	Hot spring river	Kanto	Mar. 2004	Indoor	41–42	5.6–5.8	1	54736
3	Yamanashi-1	Hot spring bath	Koshin-etsu	Sept. 2004	Indoor	41–42	5.6–5.8	1	54737
4	Yamanashi-2	Mineral spring bath	Koshin-etsu	Sept. 2004	Indoor	27	5.8	0	
5	Tochigi	Hot spring bath	Kanto	Oct. 2004	Outdoor	41–42	5.6–5.8	0	
6	Nagasaki	Hot spring bath[b]	Kyushu	Nov. 2004	Indoor	41–42	5.6–5.8	0	
7	Hyogo-1	Hot spring bath[b]	Kinki	Nov. 2004	Indoor	41–42	5.6–5.8	0	
8	Hyogo-2	Hot spring bath	Kinki	Nov. 2004	Indoor	41–42	5.6–5.8	0	
9	Hyogo-3	Hot spring bath	Kinki	Nov. 2004	Indoor	41–42	5.6–5.8	0	
10	Miyazaki	Hot spring bath	Kyushu	Dec. 2004	Indoor	41–42	5.6–5.8	0	
11	Hokkaido	Hot spring bath	Hokkaido	Dec. 2004	Indoor	41–42	5.6–5.8	0	
12	Yamanashi-3	Hot spring bath	Koshin-etsu	Sept. 2005	Indoor	41–42	10.1	0	
13	Chiba-1[c]	Hot spring bath[b]	Kanto	Mar. 2006	Indoor	41–42	5.8	0	
14	Chiba-2[c]	Hot spring bath[b]	Kanto	Mar. 2006	Outdoor	41–42	6.4	0	
15	Ibaraki	Hot spring bath[b]	Kanto	Mar. 2006	Indoor	41–42	6.8	0	

[a] Sample number from 4 to 15 emitting odor of hypochlorite

[b] Warmed mineral spring

[c] Wooden bathtub, others tiled or stone-built

[d] *O. gallopava* isolates being collected from 500 ml of water sample

(Dryplate, Eiken, Tokyo Japan) against antifungal substances: amphotericin B, flucytosine, itraconazole, miconazole, fIconazole, and micafungin. Isolate of *Candida albicans* IFM 40213 equal to ATCC 90028 was included as quality control strains for susceptibility testing. The microdilution plates were incubated in air. Readings were made after 48 h of incubation at 37°C (the *Candida* control strain was examined at 24 h) in RPMI medium. The minimal inhibitory concentration (MIC) endpoints for amphotericin B and itraconazole were read visually as the lowest drug concentration that prevented any discernible growth. The MIC endpoints for other antifungal drugs were read visually and taken as that which reduced growth by 80% compared with the drug-free control.

Virulence to experimentally infected mice

The virulence of 4 *O. gallopava* isolates from hot spring water samples in mice was examined. Ten 5-week-old male ddY mice (Nihon SLC, Shizuoka, Japan) of one strain were used. They were divided into four groups of five that were housed at 25 ± 1°C, with 55 ± 5% humidity. Mice were provided with clean drinking water ad libitum and fed a commercial chow (Nihon CLEA, Tokyo). They received 5×10^5 conidia/10 g of body weight suspended in sterilized physiological saline intravenously at 6 weeks of age. The conidial suspension was made in the same manner as the antifungal susceptibility tests under sterile conditions. The behavioral changes and survival rates were recorded up to 28 days after the inoculation of the fungal conidia.

The mortal mice during the observation period and the surviving ones killed by inhalation of ether anesthesia at day 28 after the inoculation of conidia were examined. The livers, kidneys, spleens, hearts, lungs, and brains of the mice were examined macroscopically. Organs were cut into pieces approximately $5 \times 5 \times 5$ mm^3, placed onto PDA plates, and cultured at 37°C for 2 weeks. Fungal sprouts from each organ were noted. Pathogenicity scores for each organ ware shown as number of mice with

fungal-positive organ per total number of mice [46] in percent.

The remaining organs were fixed with buffered 10% formalin and processed by routine histopathological methods, stained using hematoxylin and eosin, and the periodic acid Schiff technique (PAS), then observed under light microscopy. The animal experiment complied with all relevant guidelines and policies of the Animal Welfare Committee of the Faculty of Medicine of Chiba University, Japan.

Results

Fifteen hot spring water samples were collected from 14 hot spring areas in all Japan. Four isolates of *O. gallopava* were obtained from each 500 ml of 3 water samples, 2 hot springs and a hot water river. These springs were located in Kanto-Koshin-etsu area, which is a metropolitan area of Japan. One of the hot springs and its flowing river were located in Kanagawa Prefecture at latitude 35°20′ north and longitude 139°06′. The other hot spring was in Yamanashi Prefecture at latitude 35°39′ north and longitude 138°34′. The isolates were deposited in our center with accession numbers IFM 54734, IFM 54735, IFM 54736, and IFM 54737 (Table 4).

Colonies cultured from the pure culture of the four isolates were floccose, and dark-olive green on the surface and dark brown on the reverse side on PDA plates. The colonies became felty, dry, flat, and brownish-black and produced reddish brown pigment on PDA slants after receiving four times of passage with a 6-month interval.

The isolates uniformly showed an excellent growth at 42°C rather than at 37°C and 25°C (Fig. 1). All isolates could grow up to 48°C showing tiny colonies, however showing tinny colonies but no growth at 50°C.

Hyphae were brown consisted of rather thick walls. Isolates IFM 54734, IFM 54735, and IFM 54737 produced two-celled clavate conidia attached to dark-brown colored, and cylyndrical conidiophores with denticles, while IFM 54736 isolated from the hot water river had a few conidia without conidionenous cells (Fig. 2). Conidia of isolate IFM 54734 were wider apical cells than the basal cells or equal sized one. Isolate IFM 54735 produced elongated conidia. Isolate IFM 54736 produced poor conidia showing

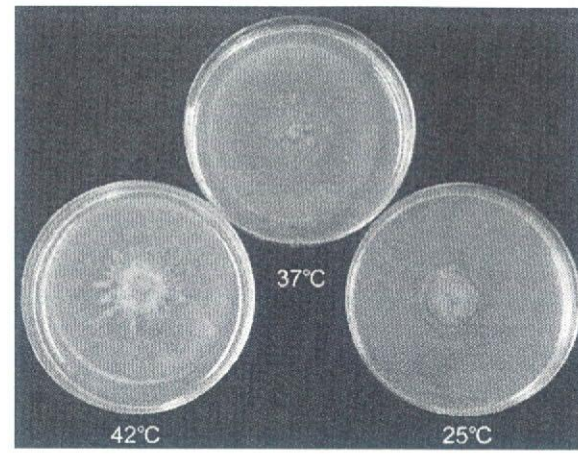

Fig. 1 *Ochroconis gallopava* isolate IFM 54737 cultured on PDA at 25, 37 and 42°C for 7 days

normal morphology on 1/10 diluted Sabouraud dextrose agar, however, produced abundant conidia on PDA slant. Isolate IFM 54737 had peanut-shaped conidia without difference of the cell size in apical and basal cells. The conidia size of isolates IFM 54734, IFM 54735, IFM 54736 and IFM 54737 were 10.4 (ranged from 7.5 to 12.0) × 3.9 (3.0–5.0) μm, 10.4 (9.5–12.5) × 2.3 (1.5–3.0) μm, 10.5 (9.0–12.0) × 2.8 (2.0–3.5) μm and 8.0 (5.5–10.5) × 4.6 (3.0–5.0) μm, respectively.

The sequences of D1/D2 LSU rDNA were 99.7% identical to those in the GenBank database with serial numbers from AB125280 to AB125286 and from AB161047 to AB161062. All the hot spring isolates were registered in Center for Information Biology and DNA Data Bank of Japan (DDBJ, Mishima, Shizuoka Japan). The accession numbers of IFM 54734, IFM 54735, IFM 54736, and IFM 54737 were AB272162, AB272163, AB272161, and AB272164, respectively. A phylogenetic tree based on the distance tree of results *via* BLAST analysis showed *O. gallopava* as an independent species involving the present sequences. DNA band patterns of the hot spring isolates amplified by the LAMP method were the same as those of a clinical isolate (Fig. 3).

Susceptibilities to antifungal drugs are shown in Table 5. The MIC values ranged from 0.5 to 1 μg/ml in amphotericin B, 1 to 16 μg/ml in flucytosine, 0.125 to 0.25 μg/ml in itraconazole, 1 to 4 μg/ml in miconazole, 16 to 64 μg/ml in fIconazole and 0.03 to 0.5 μg/ml in micafungin.

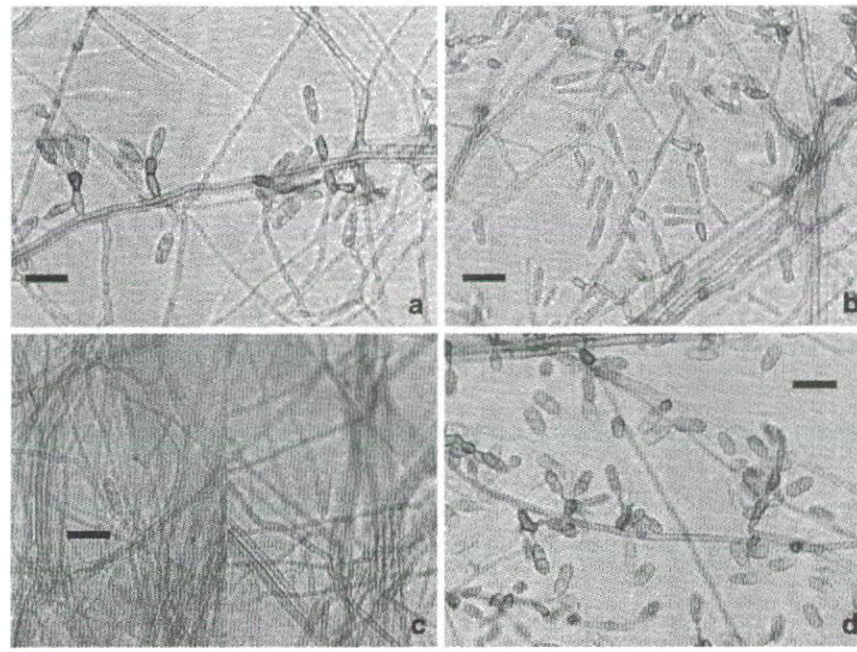

Fig. 2 Conidia of *Ochroconis gallopava* (**a**; IFM IFM 54734, **b**; IFM 54735, **c**; IFM 54736 and **d**; IFM 54737) isolated from hot springs cultured on PDA at 25°C for 3 weeks, lactophenol fixation, ×400. The bar indicates 10 μm

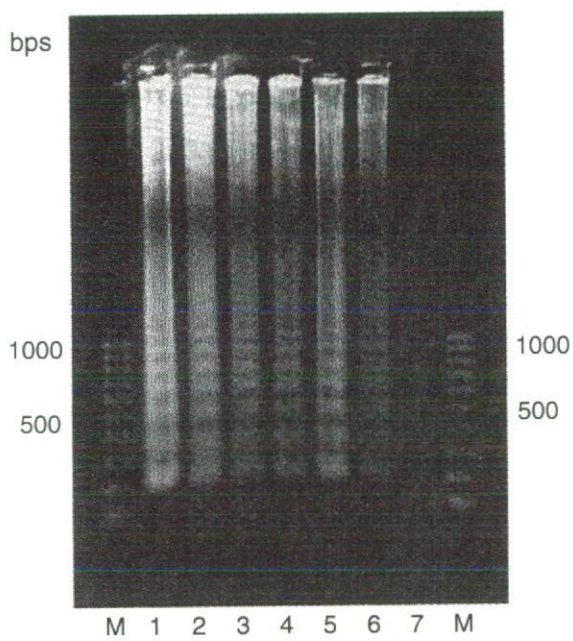

Fig. 3 DNA pattern by loop madiated isothermal amplification method (LAMP) specific for *Ochroconis gallopava* using 20 pg of fungal DNA. Lanes 1 and 6; a clinical isolate (IFM 41473), 2–5; hot spring isolates (IFM 54734, 54735, 54736 and 54737), 7; a negative control using distilled water for a template, and M; marker

In the experimental infection study, all of the infected mice showed rotating movement and sometimes in sedation 4 days after inoculation, irrespective of the isolates. Mice inoculated with the hot spring isolates showed mortal outcome, followed to the death of mice inoculated with IFM 54734, IFM 54735, and IFM 54737, at 5 days after the inoculation. The survival rates in mice were 60–0% (Fig. 4). The recovery ratios of fungal cells from six organs were as follows. It ranged from 2 out of 5 (40%) to 5 out of 5 (100%) mice in the liver, 60–100% in the heart and lung, and 80–100% in the kidney, spleen and brain (Table 6).

The marked macroscopic alterations were hemorrhage of the brains in the mice died within 7 days after the inoculation, and retractions of the surface of kidneys both in dead and survived mice regardless of the strain. Spleens were swollen at the end of observation period in the most of mice. The livers, lungs and hearts had no marked change.

Brains in the mice that died within 7 days were filled with spreading hyphae surrounded by a large number of polymorphonuclear leucocytes (Fig. 5a). Surviving mice more than 2 weeks showed small numbers of lesions consisting of polymorphonuclear leukocytes and macrophages with or without fungal elements.

Proximal renal tubules, distal ones, and renal pelvis were filled with fungal masses surrounded by polymorphonuclear leukocytes both in died and survived mice up to the end of the experimental period (Fig. 5b). Severe damages of distal tubules

Table 5 Susceptibility of *Ochroconis gallopava* to antifungal agents on micro dilution method

Isolate	Mean of MIC values (μg/ml)					
	AMPH	5-FC	FCZ	ITZ	MCZ	MCFG
IFM 54734	1	16	32	0.125	1	0.125
IFM 54735	0.5	1	16	0.125	1	0.03
IFM 54736	1	2	32	0.125	1	0.03
IFM 54737	1	4	64	0.25	4	0.5
Candida albicans (IFM 40213 = ATCC 90028)	0.5	2	1	0.125	0.25	0.03

O.gallopava were inoculated to 10^4 conidia/ml RPMI 1640 medium, and incubated for 48 h at 37°C. *C. albicans* were inoculated to 10^4 yeast cells/ml RPMI 1640 medium, and incubated for 24 h at 37°C. AMPH; amphotericin B, 5-FC; flucytosine, FCZ; fluconazole, ITZ; itraconazole, MCZ; miconazole, MCFG; micafungin

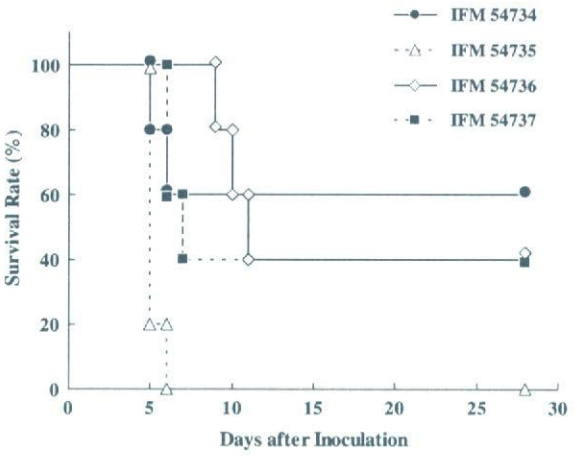

Fig. 4 Survival curve of experimentally infected mice with hot-spring isolates of *Ochroconis gallopava*. The mice received 5×10^5 conidia/10 g of body weight suspended in sterilized normal saline intravenously at 6 weeks of age and were observed up to 28 days after the inoculation

were observed in mice died within 7 days. Some of tubules were filled with decidual epithelial cells, polymorphonuclear leukocytes and mycelia. Granulomatous lesions with or without fungal elements were observed in survived mice. In addition, fungal masses surrounded by granulomatous cells at the renal pelvis were observed in mice survived more than 2 weeks.

Livers of mice died within 7 days had cystic lesions (Fig. 5c) or small inflammatory lesions with fungal elements, and those of survived ones during the observation period showed small inflammatory lesions with or without fungal elements although the culture were positives.

Damage in spleens, lungs, and hearts were mild. Small granulomatous lesions containing a few mycelial cells were detected in the hearts of mice died within 7 days (Fig. 5d), while survived mice showed migrations of inflammatory cells without fungal element.

Discussion

Four strains of *O. gallopava* were isolated from 3 out of 15 hot spring water samples from natural environments in Japan, and caused fatal systemic infection in experimental conidia injection in mice. Our data showed a possibility that hot spring bath water may be one of the sources of *O. gallopava* infections, fortunately such infection is not recorded up to now.

On the other hand, the hygienic control of hot springs and public baths in Japan became severe because of outbreaks of *Legionella pneumophila* [47]. In fact, all hot spring water samples negative of fungal isolation smelled of hypochlorite. It may explain the reason of low rate of isolation of *O. gallopava* from hot springs. Artificial waterfall systems were also prohibited after the outbreaks because of making aerosol of pathogens [47], however, small sized and natural ones still exist in some of hot springs. *O. gallopava* infection caused by inhalation might not be denied completely, because the fungal species was isolated from hot spring bath water directly.

The hot spring isolates were identified by morphological, physiological and molecular biological techniques. The colonies of the present hot spring isolates were atypical until subculture. According to the textbook, the characteristics of colonies are

Table 6 Pathogenicity of *Ochroconis gallopava* isolated from hot springs

Isolate	Mortality at 28 days (%)	Pathogenicity scores (%)[a]					
		Liver	Kidney	Spleen	Heart	Lung	Brain
IFM 54734	40	40	100	80	80	60	80
IFM 54735	100	100	100	100	100	100	100
IFM 54736	60	80	100	80	80	80	100
IFM 54737	60	60	80	80	60	60	100

The mice received 5×10^5 conidia/10 g of body weight suspended in sterilized normal saline intravenously at 6 weeks of age and were observed up to 28 days after the inoculation

[a] Pathogenicity scores are calculated as number of mice with fungal-positive organ per total number of mice

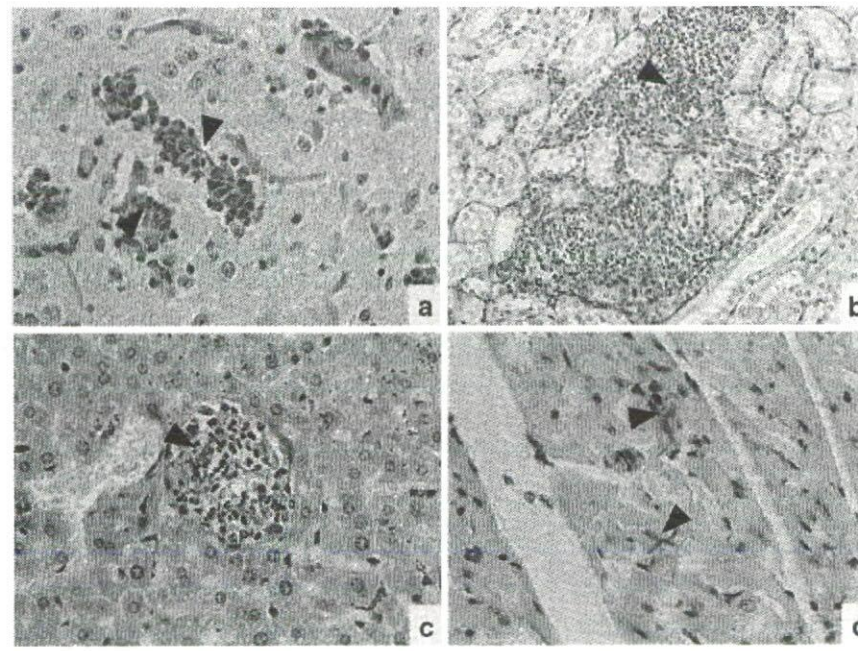

Fig. 5 Representative histopathological remarks in a mouse infected with IFM 54735 died at day 5. Abundant small lesions containing filamentous fungal cells in the brain (**a**; PAS, ×400), renal tubules filled with epithelial cells, polymorpho-nuclear leukocytes and mycelia in the kidney (**b**; PAS, ×200), microabscesses with mycelial cells in the liver (**c**; PAS, ×400), small lesions containing a few mycelial cells with mild inflammatory reactions in the heart (**d**; PAS, ×400). Arrows indicate fungal elements

represented as being felty, flat, and brownish-black, and producing a reddish-brown pigment [1]. The colonies of present isolates were uniformly floccose and dark olive green on the surface, and dark brown in the reverse on PDA plates, however, they gained the same appearance as the clinical isolates after receiving several times of subculture. It suggested that the fresh and wild or environmental isolates of *O. gallopava* might form floccose and dark olive green colonies.

The morphology of conidia varied depending on isolate. Typical conidia size of *O. gallopava* shown in the textbook ranged as 11–18 × 2.5–4.5 μm having wide apical cells [1]. The length of all present isolates was slightly shorter than normal one. The shape of isolate IFM 54735 was slender form, and isolates IFM 54734 and IFM 54737 were stout one. According to the morphological studies on *O. gallopava* by Dixson and Salkin [2], the conidia sizes of 6 strains of this fungul species were ranged 3.3–16.6 μm in the length and from 2.1 to 5.8 μm in the width. Therefore, the variations of conidia size at the present isolates were one of error ranges.

Growth ability at 48°C might be one of the important physiological characteristics of *O. gallopava*. The present hot spring isolates could grow up to 48°C. The physiological characteristic of thermotolerance was coincident to the description [1].

According to Fukushiro et al. [3], the maximum growth temperature of *O. gallopava* was 50°C, while the related species such as *O. constricta*, *O. humicola*, and *O. tshawytschae* could not grow at 37°C [1, 2]. Therefore thermotolerance test seemed to be important to differentiate *O. gallopava* from related species forming clavate conidia. In addition, avian outbreaks that have been reported in foreign countries [26–34] might be due to higher body temperature in birds at approximately 42 °C, which was the best growth temperature for *O. gallopava*.

The morphological differentiation between *O. gallopava* and related *Ochroconis* spp. is also important. *O. constricta* and *O. tshawytschae* produced conidia with verrucose surfaces, and the conidium-poor without a capsule-like structure and with a smooth surface observed by scanning electronic microscopy [1]. Furthermore, *O. gallopava* and *O. humicola* produced bicellurar conidia. The differentiation between *O. gallopava* and *O. humicola* should be done by morphological observations to detect apical cells of conidia; the former are clavate, while the latter are from cylindrical to slightly clavate. However, conidia of strains IFM 54734 and IFM 54737 showed slightly rounded forms and obfuscate clavate forms. These isolates also produced conidia with obvious septa resembles to conidia of *O. constricta*. A thermotolerance test might help a differentiation *O. constricta* and *O. gallopava*

The strains IFM 54734 and IFM 54735 were isolated from the same sample water. However, the conidia of these two strains were morphologically different from each other indicated that these isolates were independent.

O. gallopava had a very homolytic genotype in the D1/D2 domain of the LSU rRNA gene. The sequences of D1/D2 domain of the hot spring isolates were 99.7% identical to all of 23 clinical and environmental isolates from around the world in the GenBank database.

The species specific LAMP method for *O. gallopava* [24] was also useful for identification of hot spring isolates. Although we did not tested the method to the sample water, the LAMP method might be worth for direct detection of *O. gallopava* gene from hot spring samples, because of its excellent sensitivity at 100 fg of DNA.

Except for the MIC of IFM 54737 for fluconazole, MICs of antifungal agents to *O. gallopava* isolated from hot springs were almost equivalent to the clinical isolates. According to some reports, MICs for *O. gallopava* were relatively low; MIC of amphotericin B ranged from 0.015 μg/ml to 2.0 μg/ml [8, 9, 18–20, 30, 48–50], that of flucytosine ranged from 0.5 μg/ml to 80.7 μg/ml [1, 8, 9, 19, 20], that of fluconazole was 8 μg/ml to 32 μg/ml [1, 8, 20], that of itraconazole ranged from 0.02 μg/ml to 0.5 μg/ml [8, 9, 18–20, 30, 48–50], that of miconazole ranged from 0.5(μg/ml to 2.0 μg/ml [52], that of voriconazole ranged from 0.03 μg/ml to 1.0 μg/ml [30, 48–50], that of ketoconazole was 0.25 μg/ml to 3.2 μg/ml [1, 8], and that of terbinafine ranged from 0.03 μg/ml to 0.06 μg/ml [1], respectively. Both amphotericin B and itraconazole showed lower MICs to clinical and wild isolates. The data indicated that amphotericin B, with or without itraconazole has been recommended as the first choice drug for *O. gallopava* infection [8, 13, 17] seemed to be agreeable.

The behavioral change showing circulating movements in mice was correspondent that one of the target organs of *O. gallopava* was brain. The hemorrhage and marked lesions in brains supported this phenomenon. In fact, human cases [4, 5, 7–12, 14, 22, 23, 25] and the outbreaks of encephalitis in poultry [14, 26–34] reported cerebral involvements. These findings suggested that the central nerves system should be carefully examined in *O. gallopava* infected patients.

According to Dixon et al., clinical and animal isolates of the fungal species were also fatal in experimentally infected mice intravenously [51, 52]. Furthermore, Blalock and Derieux also tried experimental infection using environmental isolates of *O. gallopava* on 1-day old turkey chicks intratracheally, and could isolate the fungus from the brain of fatal chicks [39]. The present studies on the virulence of *O. gallopava* isolated from hot springs showed that these isolates were also fatal in mammals. The numbers of inoculated conidia and the mortalities at the present study were equivalent at their report [39, 51, 52] indicated that *O. gallopava* isolated from hot springs might have as the same pathogenicities as clinical, animal, and other environmental isolates.

The mortal outcomes in mice also suggested that clinical and hot spring isolates of *O. gallopava* might cause fatal infection in immnocompetent individuals. In contrast, a clinical isolate reported by Ohori et al. [24] showed much lower virulence. According

to their experiment, in spite of treatment with corticosteroid and inoculation, and almost as the same numbers of conidia as present study, there was no mortal mouse. The difference in virulence of *O. gallopava* strains might depend on histories of storage and/or condition of maintenance [53].

The fungal cells overcame the host defense mechanism in mice, although the grades of migrations of polymorphonuclear leukocytes and macrophages to mycelial cells in host tissues were dependent on isolate in the present experimental infections. Fresh and wild strains of *O. gallopava* might contain strong virulence even in healthy hosts. In fact, a case of *O. gallopava* infection without underlying disease in a wood pulp worker who was exposed to the pathogen that might have been contaminated in composts, inhaled day-after-day, and caused multiple lung abscesses suggested that constant inhalation of a huge number of spores could attack a healthy subject [5, 17].

Gardening was listed as one of risk factors for infection to in immunocompromised hosts [23]. A warm and humid environment is conductive to growth of *O. gallopava*. Composts for horticultures processed by fermentation might be an excellent selective material to separate *O. gallopava* from other microbes. Some indoor and outdoor materials such as composts, silage, nighsvil treatments, dusts of foods, and stock risings have been receiving fermentation or decomposition processes, might be hotbeds for contamination by *O. gallopava* [30, 31].

The hot spring facilities have bathtubs located indoors and/or outdoors. As the recent trends of hot spring bathing, customers prefer to use the latter type for enjoying natural atmosphere. In fact, there are many outdoor bathtubs bounded by flowerbeds and plantations. Therefore outdoor bathtubs might have a higher risk of contamination from soil and environments than those indoors. It seemed to be impossible to avoid contamination of *O. gallopava* from such environments. However, it is difficult to conclude which type of hot spring bathing is better hotbed for *O. gallopava* because of 2 outdoor samples being negative for isolation of this species.

Further study should include a survey of the neighboring environments of hot springs, such as bathtubs and washing places made of rock, stone and/or wood. There are many hot and humid interspaces among these materials which are not exposed to sterilized bath water constantly. Such environments might allow *O. gallopava* to colonize densely.

In conclusion *O. gallopava* habitats in hot springs might be one of sources for infection.

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Reactivity of Paracoccidioidomycosis Patients Serum with *Arthrographis kalrae* Antigens

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Summary

Arthrographis kalrae has been considered opportunistic fungi and it has been isolated from skin, nails, and respiratory sites. The present study investigate if *A. kalrae* antigens (Ags) cross react with paracoccidioidomycosis (PCM) patients serum by immunoenzyme assay (ELISA), using *A. kalrae* cell free antigen (CFA). Additionally positives samples were analyzed by western blotting (WB) by using CFA and sonicated Ag (SA). The positive reaction was observed in 11 of 28 serum samples from PCM patients by ELISA and ~70kDa antigen was detected by WB mainly with SA preparation.

Introduction

Arthrographis kalrae is a cosmopolitan filamentous fungus isolated from soil and compost. It has been reported as an etiologic agent of mycetoma, photophobia in a contact-lens wearer, sinusitis and meningitis in an AIDS patient, and sinusitis and ophthalmitis in a healthy individual following trauma to the eye[1-5].

Paracoccidioidomycosis (PCM), caused by the dimorphic fungus *Paracoccidioides brasiliensis*, is a granulomatous disease, one of the most important systemic mycoses in Latin America[6]. Diagnosis can be accomplished most rapidly by serological procedures[7]. The methods frequently used are immunodiffusion (ID) and enzyme immunoassay; however, they show a certain degree of cross-reaction with other mycotic infections such as histoplasmosis, lobomycosis, candidiasis, cryptococcosis and sporotrichosis[8,9]. The

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present study investigated whether antigens of *A. kalrae* cross-react with serum from PCM patients.

Materials and Methods

Serum samples were obtained from 28 patients with the chronic form of PCM and from 12 healthy control subjects (30 -59 years). Cell-free antigen (CFA) was obtained according to Camargo *et al.*[7], modified by the addition of phenylmethanesulfonyl fluoride protease inhibitor to the supernatant. For sonicated antigen (SA), the fungal mass was initially triturated and then sonicated 15 cycles (50 voltz) of 2 minutes each. For ELISA, immunoplates coated with *A. kalrae* CFA (25µg/ml) were incubated for 1h with sera (1/200). The threshold was previously determined by titration of positive and negative serum samples. The plates were incubated with mouse anti-human IgG labelled with peroxidase and the absorbance was read at 492 nm. For CFA and SA analysis by immunoblotting (IB), samples in reducing sample buffer were fractionated by SDS-PAGE (10-20%) and transferred to a nitrocellulose membrane (NCM). The NCM was incubated with serum samples (1:40), followed by the addition of goat anti-human IgG-peroxidase conjugate diluted 1:2000 and then of DAB. Protein standards with the following molecular masses were used: 180, 116, 84, 58, 48.5 and 36.5 kDa.

Results and Discussion

The positive reaction was observed in 11 of 28 serum samples from PCM patients by ELISA and the results expressed as optical densities were higher in PCM patients' serum (0.185±0.20) than in normal serum (0.102±0.016),

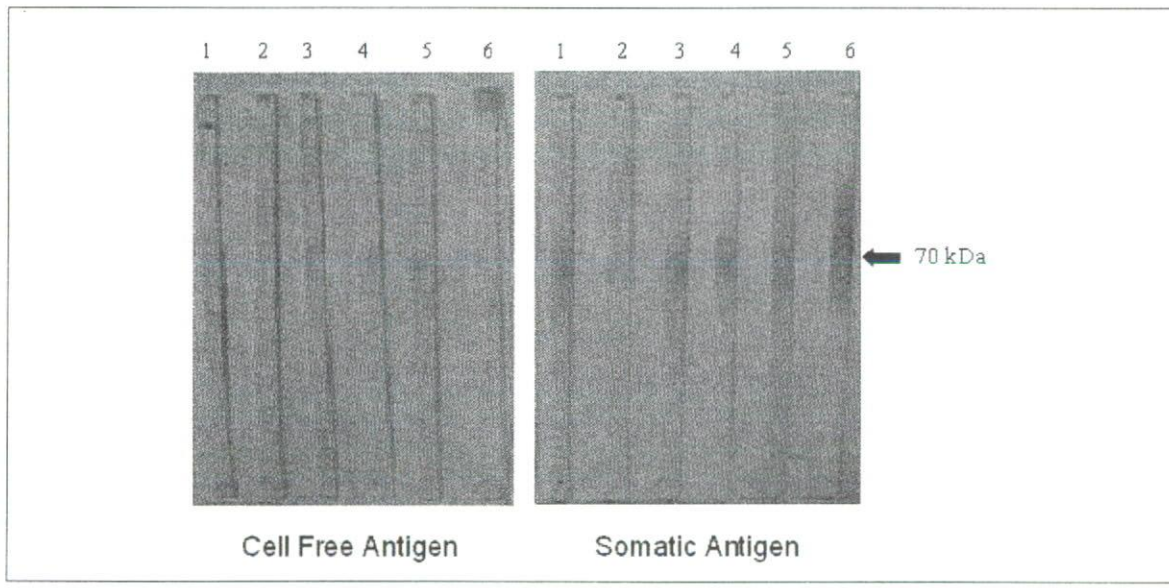

Figure 1 - Results of A. kalrae *cell-free antigen (CFA) and sonicated antigen (SA) immunoblotting with serum from chronic PCM patients and mouse anti-human IgG conjugated with peroxidase. The CFA and SA SDS-PAGE were performed in 10-20% gradient acrylamide gel.*

$p<0.05$. Considering that ELISA is used as a diagnostic method as well as for epidemiological studies of PCM, it is important to investigate agents that may cause a false-positive reaction, because this is a very sensitive method. Cross-reactions with several fungi have been described previously[8,9].

In order to better characterize the antigens of *A. kalrae* that participate in this reactivity, most reactive samples were submitted to IB, and ~70 kDa band was observed in both CFA or SA antigens, but more evident with SA antigens (Figure 1).

This study provided the first data on cross-reactivity between the fungus *P. brasiliensis* and the fungus *A. kalrae*. Further investigation is necessary to establish whether this reactivity can interfere with the diagnosis or epidemiological study of PCM.

Conclusions

A. kalrae antigens cross react with PCM patients serum and possibly it occurs due to ~70 kDa antigens more evident in SA than CFA preparations.

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Partial Characterization of Soluble Components of *Arthrographis kalrae*

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Summary

The present study was carried out to partially characterize the soluble components of *Arthrographis kalrae*, an opportunistic human pathogen. The antigens obtained from *A. kalrae* (isolated from thermal waters) were analyzed by immunodiffusion and the enzyme linked immunosorbent assay (ELISA) and western blotting (WB) were performed with antigens fractions obtained from Sephadex G-100 and G-200 chromatography. Additionally, the hemolytic activity was investigated. From this study, we concluded that the fungus *A. kalrae* shows at least two main components of molecular mass above 200 kDa and another below 100 kDa with antigenic and immunogenic activity. The profiles of the antigenic components differed in respect to the antigen preparation and *A. kalrae* releases a component with hemolytic activity.

Introduction

The microorganism *Arthrographis kalrae*, which can be isolated from soil, is a dimorphic fungus (temperature- and nutrition-dependent) that grows in the mycelial phase at room temperature and in the yeast phase at 37°C[1]. This fungus has been described as a pathogen in eumycetoma of dorsal hand[2]; sinusitis and meningitis in a patient with AIDS[3]; panophthalmitis and invasive sinusitis[4], and keratitis[5,6].

The pathogenicity of the fungus and its neurologic effects were demonstrated in infected mice, which showed a complex neurologic syndrome and lesions mainly in the kidney and brain[1]. The mechanism of infection of *A. kalrae* is unknown. The ability to form mycelia and the existence of proteolytic activity in the fungus seem to be important factors in its pathogenesis[1,7].

Considering that virulence factors of this organism are few known, especially when *A. kalrae* is isolated from thermal waters, this study was carried out to partially characterize the soluble components and to investigate the hemolytic activity of *A. kalrae*.

Materials and methods

Antigens preparation: yeast culture of *A. kalrae* isolated from recreation baths of natural thermal waters in Japan were used to prepare the antigens Cell Free Antigen (CFA) and sonicated antigen (SA). CFA was obtained according to Camargo *et al.*[8], modified by the addition of phenylmethanesulfonyl fluoride protease inhibitor to the supernatant. To obtain SA, the fungal mass was initially triturated and then sonicated 15 cycles (50 voltz) of 2 minutes each.

Polyclonal antibodies anti-*A. kalrae*: 200µg of CFA was subcutaneously injected into rabbit. Immunization was performed in 3 doses with intervals of 15 days. Ten days after the last dose, rabbit serum was collected.

Chromatography in Sephadex: CFA or SA samples were applied to Sephadex G-100 or G-200 columns, using PBS as elutent. Fractions of 2 mL were collected and read at 280 nm.

ELISA for chromatography fractions: Immunoplates were coated with the fractions of Sephadex columns. Plates were incubated with polyclonal antibodies anti-*A. kalrae*, followed by anti-rabbit IgG peroxidase conjugate and ortho-phenylenediamine substrate. The absorbance was read at 492 nm.

Western blotting: CFA, SA and Sephadex G-100 and G-200 chromatography peaks were subjected to SDS-PAGE gradient gel (5-15%) and transferred to a nitrocellulose membrane. The membrane was incubated with polyclonal antibodies anti-*A. kalrae* followed by anti-rabbit IgG peroxidase conjugate and diaminobenzidine substrate solution.

Double radial immunodiffusion: glass slides were covered with agar 1%. The central well was filled with anti-*A. kalrae* rabbit serum and the others, with CFA or SA samples (pure and diluted 1:2, 1:4, 1:8, 1:16 e 1:32).

Hemolytic activity: CFA and SA samples were diluted in series from pure to 1:32 and then incubated with a 1% sheep red blood cells suspension for 1 h at 37°C and 24 h at 4°C.

Results

The results demonstrated two well-defined peaks in Sephadex G-100 and G-200 chromatography for both CFA and SA. ELISA tests demonstrated one peak of reactivity in fractions of Sephadex G-100 and two peaks of reactivity